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The Role of TRPC3 Channels in Molecular and Cellular Events Associated to Atherogenesis

by

Kathryn B. Smedlund

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Sciences

__________________________________________
Dr. Guillermo Vazquez, Major Advisor

__________________________________________
Dr. David Giovannucci, Committee Member

__________________________________________
Dr. Sonia Najjar, Committee Member

__________________________________________
Dr. Sandrine Pierre, Committee Member

__________________________________________
Dr. Edwin Sanchez, Committee Member

__________________________________________
Dr. Patricia Komuniecki, Dean
College of Graduate Studies

The University of Toledo

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An Abstract of

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Atherosclerosis is a chronic inflammatory vascular disease that constitutes the major cause of death in western societies and one of the main vascular complications of diabetes, obesity and metabolic syndrome. Recruitment of circulating monocytes to the endothelium and their migration into the subintima is a fundamental event in both initiation as well as progression of the atherosclerotic lesion. Endothelial vascular cell adhesion molecule-1 (VCAM-1), which is upregulated in response to pro-atherogenic stimuli, is critical in mediating monocyte tethering and firm adhesion to the endothelium. Whereas Ca$^{2+}$ signaling has been recognized to play a role in regulation of VCAM-1 expression and atherogenesis. An understanding of the molecular identity of the channels involved and/or the mechanism by which they contribute to the atherogenic process is still lacking.

Transient Receptor Potential Canonical (TRPC) channels are among the most important non-voltage gated Ca$^{2+}$-permeable non-selective cation channels in vascular
endothelium where they participate in the modulation of various vascular functions. Recent observations from our laboratory have revealed new aspects within the mechanism underlying regulated expression of VCAM-1 and monocyte adhesion, two of the earliest events in atherogenesis. While studying the role of Ca\(^{2+}\) influx in ATP-dependent expression of VCAM-1 in human coronary artery endothelial cells (HCAECs) we found that native TRPC3 proteins form, or are part of, Ca\(^{2+}\)-permeable channels that participate in the Ca\(^{2+}\) influx that follows P2Y\(_2\) receptor activation, and that TRPC3 expression is required for ATP-induced VCAM-1 and monocyte adhesion. Notably, ATP also induces TRPC3 expression and this is paralleled by augmented constitutive, non-regulated TRPC3-mediated cation influx. Interestingly, tumor necrosis factor-alpha (TNF\(\alpha\)), a cytokine involved in the pathogenesis of atherosclerosis but not associated with regulated Ca\(^{2+}\) influx, induces VCAM-1 expression in HCAECs through a mechanism that also requires, at least in part, Ca\(^{2+}\) entry into the cell. Based on these findings and considering that high constitutive, non-regulated activity is an intrinsic property of TRPC3 channels, we hypothesized that upregulated expression of constitutively active TRPC3 in coronary endothelium might represent a novel contributing factor to the molecular/cellular events associated to initiation and progression of atherosclerotic lesions.

Additionally, the balance between apoptosis and survival is crucial in maintaining endothelial cell integrity during vascular damage and alterations in these processes has been associated to atherosclerosis. It has recently been shown that stimulation of non-neuronal nicotinic acetylcholine receptors (nAChR) is involved in a cholinergic anti-inflammatory reflex signaling circuit, with anti-inflammatory actions at the macrophage
level that are suggestive of potential anti-atherogenic role for nAChRs. Furthermore, many studies on endothelial cells from different vascular beds have revealed anti-apoptotic actions of nAChR stimulation; however, whether this action involves participation of downstream survival signaling has not been explored. Our lab has revealed that in HCAECs cholinergic receptors promote activation of three typical survival signaling pathways: the phosphatidyl-inositol-3 kinase (PI3K)/AKT axis, activated downstream muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs, respectively); the JAK2/STAT3 axis, activated downstream nAChR; and ERK1/2 MAP kinases, activated by both mAChR and nAChR. In addition, we have identified nAChR-dependent regulation of JAK2/STAT3 and ERK1/2 downstream α7-nAChR. Altogether, these findings suggest that stimulation of cholinergic receptors in HCAECs may act in a concerted way to induce an efficient survival signaling response when endothelial cells are exposed to pro-apoptotic stimuli.
This work is dedicated to my wonderful parents Eugene and Wavalynne Smedlund.
Thank you for all your love, encouragement and support. I would not have been able to accomplish such an achievement without your loving support.
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Literature review

1.1. Atherosclerosis

In the United States, one in five annual deaths are due to coronary artery disease (CAD), a manifestation of atherosclerosis in the coronary arteries, in both male and females, with an estimated annual health care cost of more than one hundred fifty billion dollars (Association, 2008). The disease results from a complex interplay between mechanisms of the vascular endothelium (e.g., endothelial dysfunction, oxidative stress, and inflammation), genetic and environmental risk factors. Atherosclerotic lesions occur primarily in large and medium sized elastic and muscular arteries and can lead to numerous cardiovascular events including ischemia of the heart, brain or extremities and eventually to infarction, stroke and/or other complications derived from ischemic or thromboembolic events (Glass and Witztum, 2001; Mizuno, 2011; Ross, 1999).

The early phase of atherosclerosis involves the expression of specific cell adhesion molecules (CAMs) on the activated endothelium and the subsequent recruitment and infiltration of inflammatory cells (predominantly monocytes) into the subintima. Indeed, monocytes are attracted to activated endothelium at lesion prone sites; monocyte
rolling and tethering occurs along the endothelial surface with the subsequent firm adhesion with simultaneous activation, and migration into the subintima (figure 1). This sequence of events is mediated by the selectin family of CAMs and is followed by the more firm attachment of the monocyte by means of integrins expressed on their surface with CAMs that belong to the immunoglobulin superfamily, including intercellular adhesion molecules-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Quehenberger, 2005). There is consensus that the formation of the atherosclerotic lesion begins with the subendothelial retention of apolipoprotein (apo)B-containing lipoproteins in the arterial subendothelium. After these lipoproteins become modified either through oxidation or acetylation a series of maladaptive inflammatory responses are triggered, including the release of phospholipids that can activate endothelium and the subsequent attraction of monocytes. Subintimal activated monocytes differentiate into macrophages which begin to express scavenger receptors that bind and facilitate the uptake of modified lipoproteins. Overtime the macrophages internalize the lipoproteins leading to the formation of large distinct foam cells (Mizuno, 2011; Tabas, 2010). As the lesion progresses these and other cells in the vascular wall release multiple cytokines and growth factors that stimulate smooth muscle cell (SMC) migration and proliferation. The SMCs can also uptake the oxidized lipoproteins, and thus contribute to foam cell formation, and synthesize extracellular matrix proteins that leads to the development of the fibrous cap. Eventually, inflammatory mediators induce the expression of a potent pro-coagulant and the matrix degrading proteinase known as platelet tissue factor that leads to the weakening of the fibrous cap. In addition, the fibrous cap can also become destabilized by activated macrophages, T cells and mast cells at the sites of plaque, due to
their production of several types of molecules, including inflammatory cytokines, proteases, radicals and vasoactive molecules, which degrade the collagen in the cap, and initiate thrombus formation which can lead to various cardiovascular events, including thrombus, ischemia, myocardial infraction, etc (Hansson, 2005; Mizuno, 2011) (figure 2).

Figure 1. Monocyte recruitment to the endothelium. This cartoon illustrates the general role of CAMs in the process of monocyte recruitment, and highlights the participation of VCAM-1 throughout all phases.
1.1.1. Endothelial Dysfunction

The vascular endothelium provides the cellular lining to all the blood vessels in the circulatory system and forms a permeable barrier between the vascular space and tissues. The endothelium plays a key role in the regulation of the cardiovascular system, and is the source of many factors that influence vascular tone and remodeling, blood coagulation and angiogenesis (Sagach V, 2006). Under normal physiological function the endothelium is involved in the regulation of cholesterol and lipid homeostasis, signal transduction, immunity and inflammation. The endothelium also maintains the balance...
between vasodilatation and vasoconstriction, inhibition and stimulation of smooth muscle cell proliferation and migration, and thrombogenesis and fibrinolysis (Davignon and Ganz, 2004). The maintenance of vascular tone is achieved by the release of numerous dilator and constrictor molecules. The major vasodilator substance released in the endothelium is nitric oxide (NO). NO is synthesized from the substrate L-arginine via endothelial nitric oxide synthase (eNOS) and plays a critical role in regulating a wide spectrum of functions in the cardiovascular system, including inhibition of leukocyte-endothelial adhesion, vascular smooth muscle cell (SMC) migration and proliferation, among many other functions. Some endothelium derived molecules, including bradykinin and acetylcholine stimulate the release of NO (Davignon and Ganz, 2004; Giannotti and Landmesser, 2007), whereas, prostacyclin, another vasodilator, acts together with NO to inhibit platelet aggregation and smooth smooth muscle cell relaxation (Davignon and Ganz, 2004).

Common vasoconstrictors produced in the endothelium include endothelin-1 (the most potent known to date) and angiotensin II. Endothelin-1 and angiotensin II promote proliferation of smooth muscle cells and thus contribute to atherosclerotic lesion formation (Davignon and Ganz, 2004). However, NO inhibits both the release and the actions of endothelin-1.

When the endothelium becomes altered the balance between vasoconstriction and vasodilatation becomes disrupted and a number of events are initiated that exacerbate the development of atherosclerotic plaques and other various clinical manifestations associated to vascular diseases (Sima et al., 2009), including increased endothelial permeability, platelet aggregation, leukocyte adhesion and cytokine production.
(Endemann and Schiffrin, 2004; Ross, 1999) leading to endothelial dysfunction. These events are caused by the decreased production and bioactivity of vasodilators, such as NO, prostacyclin and endothelium derived hyperpolarizing factor and the increased production of vasoconstrictors, i.e. endothelin and thromboxanes (Kofler et al., 2005). Moreover, endothelial cell dysfunction is now recognized as a major component in the pathogenesis of various cardiovascular diseases, including hypertension, atherosclerosis, coronary syndrome, heart failure and other cardiovascular diseases that can also present as a complication of the endocrine and metabolic diseases such as diabetes, obesity and metabolic syndrome (Kwan, 2007).

1.1.2. Cytokines

Cytokines are low-molecular-weight protein mediators usually acting within short range between neighboring cells in lymphoid organs or inflamed tissues. The cytokine group consists of more than one hundred secreted factors, clustered into several classes, including interleukins (IL), tumor necrosis factor (TNF), interferons (IFN), colony-stimulating factors (CSF), transforming growth factors (TGF), and chemokines (Ait-Oufella et al., 2011). All cells involved in atherosclerotic lesion development are able to produce and respond to cytokines and a plethora of cytokines can be found in plaques.

In early stages of atherosclerosis, cytokines are known to alter and activate endothelial cell function. For example, TNFα and IFN-γ can alter the distribution of vascular endothelial cadherin-catenin complexes and prevent the formation of F-actin stress fibers. Therefore, this can result in restructuring of the intercellular junctions
leading to the loss of endothelial permeability (Tedgui and Mallat, 2006). Cytokines also play a major role in the activation of adhesion molecules and chemokine expression involved in leukocyte recruitment, adhesion, and migration into the inflamed vascular wall. Once in the intima, the leukocytes can be activated by the locally generated cytokines, which can also accelerate the transformation of macrophages into foam cells by stimulating the expression of scavenger receptors and enhancing cell-mediated oxidation. Furthermore, at a more advanced stage of the disease, proinflammatory cytokines destabilize atherosclerotic plaques by promoting cell apoptosis and matrix degradation. Indeed, macrophage apoptosis has been shown to contribute to the enlargement of the lipid core of the plaque whereas smooth muscle cell (SMC) apoptosis can induce the thinning of the fibrous cap, favoring plaque rupture. Several proinflammatory cytokines, including IL-1, TNFα and IFN-γ are known to induce SMC and macrophage apoptosis (Tedgui, 2010).

1.1.3. Adhesion molecules

As described above, one of the earliest phases in development of atherosclerotic lesions is the recruitment of inflammatory cells from the circulation (predominantly monocytes) to the endothelium and their migration through activated endothelial cells (transendothelial migration). This process is mostly mediated by CAMs. CAMs are expressed on the vascular endothelium and on circulating leukocytes either constitutively or in response to several inflammatory stimuli depending on the type of CAM (Blankenberg et al., 2003). Monocytes are initially attracted to lesion-prone sites by CAMs expressed on activated endothelial cells –indeed; upregulated expression of CAMs
on activated endothelium is pathognomonic of atherosclerotic lesion-prone sites.

Recruitment and infiltration of circulating monocytes into the subendothelial milieu involves a sequence of events that includes monocyte rolling and tethering along the activated endothelial surface, firm adhesion with simultaneous activation, and migration to the subintima (Figure 1). The initial monocyte rolling and tethering is mostly mediated by the selectin family of CAMs and is followed by firmer attachment by means of integrins expressed on the monocyte surface with endothelial CAMs that belong to the immunoglobulin superfamily, including intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Li, 2002; Quehenberger, 2005).

Compelling evidence accumulated over the last decade has revealed that VCAM-1 has a prominent role in mediating attachment and migration of monocytes.

1.1.4. VCAM-1 expression in atherosclerotic lesions

As stated in the previous section, in addition to VCAM-1 there are other adhesion molecules that contribute to attachment and migration of monocytes, including ICAM-1 and E-selectin. However, VCAM-1 is unique regarding regulation of its expression pattern. While other CAMs are constitutively expressed in non activated endothelium, VCAM-1 is nearly absent, but its expression increases dramatically when the endothelium is exposed to pro-inflammatory stimuli (Galkina, 2007). For example, it has been demonstrated that in hypercholesterolemic animals, VCAM-1 expression is largely restricted to lesions, or to regions prone to lesion formation, whereas ICAM-1 expression also extends to uninvolved aorta and lesion-protected regions (Cybulsky et al., 2001). This variation of expression strongly suggests different functions for VCAM-1 and
ICAM-1 in lesion formation. In addition, the generation of mice homozygous for the VCAM-1 molecule lacking the Ig-like extracellular domain 4 (Vcam1^{D4D/D4D}), which partially overcomes the embryonic lethality of global VCAM-1 deficiency (Vcam-1^{−/−} mice), further identifies VCAM-1 has a dominant role in early lesion formation (Cybulsky et al., 2001).

In most endothelial cells, VCAM-1 expression is regulated by the transcription factor Nuclear Factor kappa B (NFkB) (Zhang, 2008). NFkB activation involves the release of the inhibitory protein IkBα and then the subsequent translocation of NFkB to the nucleus where it modulates the transcription rate of target genes. Release of NFkB from IkBα is preceded by phosphorylation of the IkBα by IkBα kinase (IKK)β, ubiquitination and proteasomal degradation of the phosphorylated IkBα protein (Tergaonkar, 2006). We have shown that in HCAECs, as is the case in most endothelial cells derived from other atherosclerosis prone vascular beds, VCAM-1 expression is regulated by NFkB (Smedlund et al., 2010).

1.1.5. VCAM-1 structure and Function

VCAM-1 is a member of the immunoglobulin superfamily. It binds to the α4β1-integrin on leukocytes. In humans, VCAM-1 comprises two isoforms: the full length form that contains seven immunoglobulin-like extracellular domains (1-7) and thought to be predominantly expressed on the cell surface, and a form with just domains 1-3 and 5-7. Domains 1 and 4 conforms the ligand binding domain of VCAM-1 which is directly responsible of monocyte adhesion. However, binding is primarily mediated by residues within the NH2-terminal domains in the intact full length VCAM-1 (Woodside, 2006).
VCAM-1 is involved in many different physiological and pathological processes. It has been reported that VCAM-1 stimulates the endothelial cell NADPH oxidase with the subsequent generation of reactive oxygen species (ROS, such as superoxide anion and its catabolic product, hydrogen peroxide) and that their function is required for VCAM-1 dependent lymphocyte migration (Matheny, 2000). Other processes include leukocyte retention in tissues and cellular activation by interaction with $\alpha_4\beta_1$-integrin (Woodside, 2006).

1.1.6. Role of calcium signaling in VCAM-1 expression

Calcium ($Ca^{2+}$) homeostasis is crucial to maintaining normal vascular function. It is one of the principal intracellular signaling mechanisms by which cells of the vascular wall, such as endothelial cells and smooth muscle cells, respond to external stimuli such as fluid stress, hormones and vasoactive substances (Plank et al., 2006). $Ca^{2+}$ is a key intracellular second messenger in almost all eukaryotic cells. In non-excitable cells, including T lymphocytes, hepatocytes, mast cells, and endothelial and epithelial cells, the major pathway for $Ca^{2+}$ entry is through store-operated $Ca^{2+}$ channels (SOC) in the plasma membrane. This process is initiated when phospholipase C (PLC) becomes activated by the binding of a stimulus (i.e. hormones or growth factors) to its receptor either the G-coupled protein rectors (GPCRs) or receptor tyrosine kinases (RTKs) and converts phosphatidylinositol-4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). DAG can then directly activate channels, whereas IP$_3$ binds to the IP$_3$ receptor (IP$_3$R) in the endoplasmic reticulum (ER) membrane resulting in the release of intracellular $Ca^{2+}$. This subsequent decrease in the ER $Ca^{2+}$ concentration
then signals to the plasma membrane and activates the SOCs. These changes in intracellular Ca\(^{2+}\) levels exert a significant impact on many endothelial functions. For example, immediate consequences of Ca\(^{2+}\) influx include short and long term cellular responses, such as activation of kinases, synthesis and or release of vasoreactive molecules, activation of pro-inflammatory signaling and gene transcription (Tano, 2010). Moreover, it has become well established that regions of disrupted blood flow and low shear stress at arterial bifurcations are predisposed to lesion development. Importantly, at these regions endothelial Ca\(^{2+}\) is found to be altered, and this alteration has been suggested to be a contributing factor to the onset of atherosclerosis (Plank et al., 2006; Yuan et al., 2009).

Ca\(^{2+}\) signaling has also been identified as an important factor in the mechanism by which a number of inflammatory mediators induce VCAM-1 expression. For example, changes in intracellular Ca\(^{2+}\) in relation to Ca\(^{2+}\) release from internal stores have been linked to the capability of Substance P to induce VCAM-1 expression subsequent to Ca\(^{2+}\)-dependent activation of NFAT and NFkB in microvascular endothelium (Quinlan et al., 1999) and to the ability of \(\beta\)2-microglobulin to induce VCAM-1 in synovial fibroblasts (Chen et al., 2002). Furthermore, in HCAECs, Ca\(^{2+}\) mobilization contributes to lipoprotein-A and ATP-dependent VCAM-1 expression (Allen et al., 1998; Seye et al., 2003). Nevertheless, the specific role of Ca\(^{2+}\) influx in VCAM-1 expression was not directly examined. One of the major objectives of our studies was to examine if and how Ca\(^{2+}\) influx through members of the TRPC family of Ca\(^{2+}\)-permeable cation channels (see section 1.2. on TRPC channels) exert a role in VCAM-1 expression and function.
Recent work from our laboratory, examined the specific role of Ca\(^{2+}\) influx in the expression of VCAM-1 in HCAECs (Smedlund, 2008). Our studies demonstrate that HCAECs express all members of the TRPC family, however only TRPC3 forms, or is part of, endogenous Ca\(^{2+}\) permeable channels that contribute to ATP stimulated Ca\(^{2+}\) influx ATP actions occur through ATP-dependent activation of the purinergic P2Y\(_{2}\) receptors, and result in upregulation of the total and plasma membrane levels of VCAM-1 with the subsequent increase in monocyte adhesion (Smedlund, 2008). These findings represented the first direct evidence indicating that Ca\(^{2+}\) influx plays a role in the signaling driving VCAM-1 expression. In addition, this is the first time that an endothelial TRPC channel is shown to be fundamental within the signaling underlying VCAM-1 expression and monocyte recruitment.

1.2. Transient Receptor Potential Canonical (TRPC) proteins: structure and function

TRPC proteins are among the most important channel forming proteins in the vascular endothelium. TRPCs participate in Ca\(^{2+}\) influx that is required for the modulation of diverse vascular functions (Vazquez, 2004; Yao, 2005). TRPC proteins are part of a larger TRP superfamily of channel forming proteins and most closely related to the original *Drosophila melanogaster*. TRPC proteins can be subdivided into four different subfamilies based on structural and functional similarities: TRPC1, TRPC2 (a pseudogene in humans, old world monkeys and apes), TRPC3, 6, and 7 and TRPC4 and 5 (Vazquez, 2004).
All members of the TRPC family share common topology. They are described to have N- and C- termini separated by six transmembrane domains (TM1-TM6), including a putative pore region between TM5 and TM6. The N-terminus is composed of three to four ankyrin repeats, a predicted coiled-coil region and putative caveolin binding region. The cytoplasmic C-terminus includes the invariant TRP box sequence (EWKFAR), a highly conserved proline rich motif, the CIRB (calmodulin/IP$_3$ receptor binding) domain and also a predicted coiled-coil region (Pedersen et al., 2005; Vazquez, 2004) (Figure 3).

All TRPC proteins form non-selective, non-voltage gated, Ca$^{2+}$ permeable cation channels that are activated by stimulation of receptors that activate different isoforms of phospholipase C (PLC) (Venkatachalam, 2007). For example, stimulation of G-protein coupled receptors (GPCRs) activates PLC$\beta$ and activation of receptor tyrosine kinases (RTKs) activates PLC$\gamma$. Under physiological conditions, members of the closely related TRPC3, 6 and 7 subgroup are presumably activated by diacylglycerol (DAG) generated upon PLC stimulation. Endogenous candidate molecules that may account for activation of TRPC1, 4 or 5 channels remain elusive, although their activation, under physiological situations, requires PLC activity (Vazquez et al., 2004; Trebak et al., 2003).
1.2.1. TRPC proteins in cardiovascular disease

Endothelial cells generate a variety of factors that regulate vascular tone, vascular permeability, angiogenesis, and inflammatory responses and endothelial dysfunction is now recognized as a major component in the pathogenesis of various cardiovascular diseases, including hypertension, atherosclerosis, heart failure, coronary syndrome, thrombosis, and cardiovascular disease that presents as a complication of endocrine and metabolic diseases such as diabetes, obesity and metabolic syndrome (Kwan, 2007).

Endothelial cells express multiple TRP channels and malfunction or dysregulation of these channels has been associated with endothelial dysfunction (Kwan, 2007). Over the last several years many studies have made it evident that TRPC-mediated Ca\(^{2+}\) influx
plays a role in cardiovascular disease. For example, regulated Ca\(^{2+}\) entry through TRPC1, 3 and 6 activates the nuclear factor of activated T cells (NFAT) signaling pathway that underlies development of cardiac hypertrophy (Guinamard, 2007). TRPC5 also promotes cardiomyocyte hypertrophy through the activation of calcineurin and its downstream effector NFAT (Nelson et al., 2010). TRPC1 and 6 have also been shown to participate in regulation of vascular tone and hence play a role in hypertension (Lin, 2004). More recently, it has been reported that mice with global deletion of TRPC1 gene exhibit less ventricular hypertrophy and chamber dysfunction in response to pressure overload or neurohormonal stimulation than TRPC1\(^{+/+}\) animals (Rowell et al., 2010). Thilo et al. has recently shown a correlation between enhanced expression of pro-inflammatory cytokines (e.g., TNF\(\alpha\)) and TRPC3 and TRPC5 protein levels in circulating monocytes from patients with essential hypertension (Thilo, 2007). TRPC3 levels have also been shown to rise in spontaneous hypertensive rats and in pressure overloaded rats (Liu et al., 2009). In addition, TRPC3 overexpression in transgenic mice results in cardiomyopathy and cardiac hypertrophy leading to premature death (Nakayama, 2006). Moreover, lack of TRPC4 expression has been related to the impairment of endothelium-dependent vasorelaxation and endothelial barrier function in mice (Nilius, 2007). In addition, the knockout of TRPC6 in mice results in elevated aortic and cerebral arteries contractility with a hypertensive phenotype, due to compensatory upregulation of TRPC3 channels (Dietrich, 2003).
1.3. The cholinergic anti-inflammatory reflex pathway

Inflammatory responses to different infections or tissue damage are distinguished by the complex interactions between pro- and anti-inflammatory cytokines and other inflammatory mediators directed toward abolishing invaders and promoting health. The release of both pro-inflammatory (TNFα and IL-1β) and anti-inflammatory (IL-4, IL-10 and IL-13) cytokines is partially regulated through the autonomic nervous system. The parasympathetic division of the autonomic nervous system is associated with regulation of inflammatory responses. Recently, it has been discovered that efferent vagus nerve signals suppress pro-inflammatory cytokine release from macrophages within the arterial wall and inhibit inflammation: this has been termed the “the cholinergic anti-inflammatory reflex pathway” (CAIR) (Pavlov and Tracey, 2005). Because the CAIR seems to be related to the activation of non-neuronal nicotinic cholinergic receptors (nAChR) on the macrophage, a potential anti-inflammatory function of nAChRs, at least at the macrophage level, has been proposed.

Indeed, macrophages and other cytokine producing cells express nAChR, which transduces an intracellular signal that inhibits the synthesis of cytokines. Among all the nicotinic cholinergic receptors potentially involved, the most likely candidate is the alpha seven nicotinic AChR (α7-nAChR) (Tracey, 2007). Interestingly, recent studies have shown that the absence of α7-nAChR in mice promotes inflammation, with a phenotype characterized by increased markers of inflammation, increased oxidative stress and enhanced uptake of lipoproteins in macrophages – which leads to cholesterol
accumulation and reduced macrophage anti-oxidant capacity- (Wilund et al., 2009).

Altogether, these findings suggest an anti-inflammatory role for macrophage α7-nAChR.

More recently, nAChRs have also emerged as critical players in the modulation of endothelial function. For example, several studies have shown that nAChR stimulation and function plays a role in the pro-inflammatory and pro-apoptotic actions of cytokines on endothelial cells (Hakki et al., 2002), prevent leukocyte migration through the endothelium (Saeed et al., 2005), and inhibit TNFα-induced endothelial barrier dysfunction, particularly through the α7-nAChR (Li et al., 2010). Furthermore, many studies on endothelial cells from different vascular beds have revealed anti-apoptotic actions of nicotinic stimulation; however, whether this action involves participation of downstream survival signaling has not been explored. Our lab has recently demonstrated that in human coronary endothelial cells (HCAECs) cholinergic receptors promote activation of three typical survival signaling pathways: the phosphatidyl-inositol-3 kinase (PI3K)/AKT axis, activated downstream muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs, respectively); the JAK2/STAT3 axis, activated downstream nAChR; and ERK1/2 MAP kinases, activated by both mAChR and nAChR. In addition, we have identified nicotinic regulation of JAK2/STAT3 and ERK1/2 downstream α7-nAChR, at least based on their sensitivity to α-bungarotoxin, a selective antagonist of the α7-nAChR (Smedlund et al., 2011). Therefore, these findings represents a novel contribution to the understanding of molecular events for cholinergic-dependent modulation of coronary endothelial cell survival under inflammatory conditions, and add up to the evidence described above that nAChRs may play an anti-inflammatory and/or anti-atherogenic role.
Feng et al., demonstrated that in *Caenorhabditis elegans* (*C. elegans*) TRPC channels regulate nicotine dependent behavior by functionally modulating nicotine-induced cellular responses in the locomotion circuitry (Feng et al., 2006). In addition, when using transgenic animals expressing human TRPC genes (hTRPC1, 3, and 4) on the TRP-2 mutant background in *C. elegans*, there was no response to hTRPC1 or hTRPC4; notably, expression of hTRPC3 restored the responses of the TRP-2 mutant animals to nicotine, revealing functional conservation of TRPC channels in regulating nicotine dependent behavior. Altogether, these observations suggest that TRPCs, more specifically TRPC3, play an unexpected role in nervous system function and, most importantly, reveal a functional interplay between TRPC3 channels and nAChR signaling (Feng et al., 2006).

These and our observations regarding the roles of TRPC3 and nAChRs in inflammatory and survival signaling in HCAECs, immediately prompted us to ask the question whether a potential crosstalk exists between TRPC3 channels and nAChRs in the coronary endothelium. Future studies will be designed in order to examine this possibility, and how such interplay may impact the role of endothelial inflammatory signaling in the context of atherogenesis.
Chapter 2.

Mouse Models of Atherosclerosis

Introduction

Animals have been widely used to study the pathogenic steps in atherosclerosis and have played a crucial role in understanding the cellular and molecular events underlying atherogenesis, as well as in studies aimed at testing pharmacological and molecular therapeutic strategies for this disease. As far back as 1908, a Russian scientist, Alexander Ignatowski, published a study demonstrating that atherosclerosis can be induced in rabbits by feeding them milk and egg yolks (Ignatowski, 1908). Since then, different animal models, including mice, rats, guinea pigs, hamsters, dogs, avian, swine and non-human primates, have been used to study atherosclerosis, particularly the events occurring at the arterial wall throughout the course of the disease, providing valuable information about diagnostic, prognostic and therapeutic strategies (Xiangdong et al., 2011). However, most animal species are expensive to house and maintain or available reagents such as antibodies, vectors and/or delivery systems are limited, not allowing for full characterization of the lesions. To date, rodents and rabbits are the most commonly used animals for atherosclerosis. Some of the most popular mouse models are briefly discussed in the following sections.
2.1. Current mouse models of atherosclerosis

In general, mice are resistant to diet induced atherosclerosis. This is likely due to the fact that approximately 70% of the total plasma cholesterol is high density lipoprotein (HDL), probably a consequence of the lack of plasma cholesteryl ester transferase protein (CETP). However, the ability and ease of manipulating the mouse genome and the availability to hundreds of well established inbred and genetic strains, have made the mouse the most widely used animal model to study atherosclerosis despite its non-atherogenic phenotype (Xiangdong et al., 2011).

Genetic manipulations in mice used for atherosclerosis research depends on the disruption of normal lipoprotein regulation and metabolism. All dietary and genetic manipulations for promoting atherosclerosis involve a change in the balance of lipoproteins, those containing apoprotein B (apoB) proteins being dominant. Among mouse inbred strains, C57BL/6 mice are the most susceptible to development of diet induced atherosclerosis and therefore is the most widely used genetic background (Getz, 2006).

2.1.1. C57BL/6 Mice

The initiation of atherosclerosis in C57BL/6 requires dietary manipulation. Beverly Paigen and colleagues developed a special high fat diet known as the Paigen’s diet to induce lesion formation in C57BL/6 mice. The diet composes of 15% fat, 1.25% cholesterol, and 0.5% sodium cholate (Paigen, 1995). The addition of cholate increases the cholesterol loading and hence hypercholesterolemia that is induced by the cholesterol
and the high fat content of the diet. This is caused from facilitating fat and cholesterol absorption and the inhibition of cholesterol-7α-hydroxylase, which is the rate limiting enzyme in the conversion of cholesterol to bile acid (Getz, 2006).

However, C57BL/6 mice only develop lesions with features of the earliest stages of atherosclerosis, where the lesions are usually confined to the aortic root, associated with the cusps of the aortic valve. Furthermore, prolonged feeding of the C57BL/6 on the cholesterol enriched, high fat diet has been shown to promote lesion development that extends out to the most proximal portion of the aortic arch, but the lesions are very small and again, only representative of those in the earliest stages of atherosclerosis (Whitman, 2004). Based on these findings and that C57BL/6 have an inherent genetic susceptibility to atherosclerosis, it has become common practice to backcross genetically modified mice onto the C57BL/6 strain. Moreover, there have been several different mouse models of atherosclerosis generated using the C57BL/6 background. Discussed in the sections below are the two most widely used models to study atherosclerotic lesion formation and progression.

2.1.2. Apolipoprotein E deficient (ApoE\(^{-/-}\)) mice

ApoE\(^{-/-}\) mice were introduced in the early 1990s, and since then have been used as a popular model for studying spontaneous hypercholesterolemia and the subsequent development of atherosclerotic lesions when on chow diet, similar to that found in humans. The ApoE\(^{-/-}\) mice were created from C57BL/6 mice using targeted disruption of the apoE gene. ApoE is synthesized in liver and macrophages and is a ligand for cell surface lipoprotein receptors and mediates the uptake of several different lipoproteins,
including atherogenic particles, low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (O'Neill, 1997).

In these mice the atherosclerotic plaque develops throughout the arterial tree, with the heaviest plaque formation seen in the aortic arch. The aortic arch is prone to atherosclerosis because of its unique anatomical location and arched shape, its proximity to the heart, contractile pulsatility and highest blood pressure (Coleman et al., 2006). Furthermore, three major arteries, including the right and left carotid, and left subclavian arteries, are also sites of plaque formation and originate from the aortic arch. The aortic arch also experiences shear-flow disturbances due to its shape and frequent branching, which is thought to promote endothelial cell dysfunction, the initial step in atherosclerotic lesion formation (Coleman et al., 2006). Over time, the lesions can become very complex, progressing past the fatty streak phase. When ApoE\(^{-/-}\) are fed a diet higher in fat and cholesterol content (21% fat and 0.15% cholesterol) known as the western diet, it accelerates the development of lesion from all stages, from the foam cell to the advanced fibrous plaque (Getz GS, 2006).

ApoE\(^{-/-}\) mice have been extensively studied as a mouse model of atherosclerosis. A wealth of published work has identified ApoE\(^{-/-}\) mice as an excellent experimental model for studying the histopathological development of atherosclerosis and the use of dietary therapies to delay the formation of atherosclerotic lesions (Coleman et al., 2006; Getz GS, 2006).

2.1.3. Low Density Lipoprotein Receptor deficient (LDLR\(^{-/-}\)) mice

LDLR\(^{-/-}\) mice are another commonly used mouse model of atherosclerosis. The
LDLR plays a critical role in the regulation of plasma LDL levels by mediating the clearance of LDL. Loss of the LDLR leads to decreased LDL catabolism and elevated LDL levels. The LDLR can be affected by diet, hormones, and mutations in the LDLR locus, leading to familial hypercholesterolemia (Twisk et al., 2000). When mice lack the LDLR they display a modest elevated level of plasma cholesterol when on regular chow diet and develop atherosclerosis slowly in comparison to ApoE\(^{-/-}\) mice on chow diet. Conversely, when these mice are put on a high fat diet they exhibit highly elevated plasma levels of cholesterol. The morphology of the lesions in LDLR\(^{-/-}\) is similar to those found in ApoE\(^{-/-}\) mice, with the lesions developing in the proximal aorta in a time dependent manner. Overall, these mice are a more moderate model of atherosclerosis in comparison to ApoE\(^{-/-}\) mice, mainly due to the milder degree of hyperlipidemia (Zadelaar et al., 2007).

2.2. Development of novel mouse models to study the role of TRPC3 in atherosclerosis

Recent work from our lab (Smedlund et al., 2010; Smedlund, 2008) demonstrated that pro-inflammatory/pro-atherogenic stimuli (i.e., ATP and TNF\(\alpha\)) increase endogenous levels of constitutively active TRPC3 in HCAECs and that this correlates with augmented expression of VCAM-1 and monocyte adhesion, two key events in vascular inflammation underlying atherosclerosis (Calin, 2007; Szekanecz, 2008). In addition, we showed (Smedlund et al., 2010) that expression of TRPC3 is increased in lesions of atherosclerotic ApoE\(^{-/-}\) mice compared to wild-type mice, as examined by immunohistochemistry (IHC) on aortic root sections. These findings, although
suggestive, do not establish a causal link between increased TRPC3 expression and atherogenesis. Therefore, to determine whether TRPC3 plays a role in atherosclerotic lesion development in vivo, it is necessary to examine the contribution of TRPC3 expression/function to atherogenesis within the context of the multiple mechanisms that take place in vivo in intact vessels. To specifically address this, we generated mice that overexpress TRPC3 in the endothelium and mice that lack endothelial expression of TRPC3 (endothelialTRPC3−/−) which will allow us to examine the characteristics of vascular lesion development when maintained on an atherogenic diet.

2.2.1. Generation of TRPC3 transgenic mice:

We used human Flt-1 promoter (InVivoGen) to drive endothelial specific expression of wild-type human Trpc3 (Trpc3). Trpc3 cDNA with C-terminal HA epitope (from Dr. L. Birnbaumer, NIEHS, NC) was cloned into pDRIVE (InVivoGen) between Flt-1 promoter and SV40 polyA. TRPC3-HA was shown to be functional in mice with cardiomyocyte overexpression of TRPC3-HA (Nakayama et al., 2006) and in HEK293 cells (Vazquez et al., 2004). The Flt-1/Trpc3-HA minigene (5,178 bp) was sequenced, excised, purified and injected into C57BL/6 oocytes (in collaboration with Dr. Philbrick, Yale University). Genotyping was performed by PCR of tail gDNA from founders (primers TgF: 5’-AAGACAAGAGGCAAGCACTG; TgR: 5’-CTCCCCCTGAACCTGAAACA); this primers rendered a 334 bp fragment (last 92 bp of Trpc3+HA+212 bp of polyA) when transgene insertion occured. Founders (F0, 2 males, 3 females) represented 50% inbred F0 lines on C57BL/6 background. Once transferred to our facility F0 mice were mated with B6 breeders; all them were fertile and
passed the transgene to progeny; no gross phenotypes were observed. At F4 we identified high (line C) and low (line E) expressors by Western blot analysis of TRPC3-HA in lung lysates (figure 1). Lungs were chosen as these are highly vascularized and the ratio of endothelium to other cell types is higher than in other tissues/organs. We kept these lines on B6 background and we are currently also breeding them on ApoE⁻/⁻ background (ApoE⁻/⁻ mice on B6 background were obtained from Jackson Labs) to generate TgEST3ApoE⁻/⁻ mice (figure 1C). No aberrant TRPC3-HA expression was found in macrophages from these mice (figure 1) which is important, as most endothelial cell specific promoters leak into the hematopoietic lineage. Having identified a high and a low expressor is an important strategical advantage as it will ensure that the resulting phenotype is replicated in independent transgenic lines and is therefore not the result of genomic disruption or integration site; in addition, this will also allow us to verify if the severity of the phenotype (effect on lesion size and complexity) correlates with the degree of expression.
Figure 1. A.) Western blot of TRPC3-HA. B.) PCR of macrophage gDNA from TgEST3 mice. C.) PCR of tail gDNA from TgEST3ApoE−/−.
2.2.2. Generation of endothelial-specific TRPC3<sup>−/−</sup> mice:

A Cre/loxP strategy was used to generate endothelial cell (EC)-specific TRPC3<sup>−/−</sup> mice. Floxed TRPC3 mice (TRPC3<sup>lox/lox</sup>, from Dr. L. Birnbaumer, NIEHS, NC) have loxP sites into introns flanking exon 7 of Trpc3 which codes for pore and 6th transmembrane regions of the channel (Hartmann et al., 2008). Homozygous TRPC3<sup>lox/lox</sup> were backcrossed on B6, then crossed with homozygous Cdh5Cre mice (B6 background, Jackson Labs; endothelial expression of Cre is driven by cadherin 5 promoter (Jackelyn et al., 2006) to generate Cdh5Cre<sup>+/−</sup>/TRPC3<sup>lox/+</sup> which were bred again with TRPC3<sup>lox/lox</sup> to obtain Cdh5Cre<sup>+/−</sup>/TRPC3<sup>lox/lox</sup> mice. Cre recombinase cDNA was inserted downstream the VE-Cadherin (Cdh5) mouse promoter, and thus provides highly specific endothelial expression. PCR of tail gDNA was used to test for loss of exon 7 with primers A (5′-GAATCCACCTGCTTACAACCATGTG) and B (5′-GGTGAGGTAACACACAGCTAAGCC; as in (Hartmann et al., 2008)); Cdh5Cre<sup>+/−</sup>/TRPC3<sup>lox/lox</sup> mice show a 300 bp fragment (recombined allele) +, a 925 bp fragment (floxed allele from non-ECs) in tail biopsies (figure 2). Cdh5Cre<sup>+/−</sup>/TRPC3<sup>lox/lox</sup>
showed no gross phenotypes. Controls are TRPC3\textsuperscript{lox/lox} littermates negative for Cre. Cdh5Cre \textsuperscript{+/−}/TRPC3\textsuperscript{lox/lox} and TRPC3\textsuperscript{lox/lox} are currently being bred into ApoE\textsuperscript{−/−} background to generate control TRPC3\textsuperscript{lox/lox}ApoE\textsuperscript{−/−} (non-ko littermates) and Cdh5Cre\textsuperscript{+/−}/TRPC3\textsuperscript{lox/lox}ApoE\textsuperscript{−/−} mice.

**Figure 2.** PCR of tail gDNA in Cdh5Cre\textsuperscript{+/−}/TRPC3\textsuperscript{lox/lox} (ECTrp3-KO)
Chapter 3.

Involvement of Native TRPC3 Proteins in ATP-dependent Expression of VCAM-1 and Monocyte Adherence in Coronary Artery Endothelial Cells


Kathryn Smedlund and Guillermo Vazquez

Department of Physiology and Pharmacology and the Center for Diabetes and Endocrine Research at the University of Toledo College of Medicine, Health Science Campus, 3000 Arlington Av, Toledo, Ohio 43614 USA

*To whom correspondence should be addressed at: Dept. of Physiology and Pharmacology, UTHSC Mail stop 1008, Toledo OH 43614 USA.

E-mail: Guillermo_Vazquez@utoledo.edu

Tel: 419-383 5301

Fax: 419-383 2871

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**Background** - Vascular cell adhesion molecule-1 (VCAM-1) is critical in monocyte recruitment to the endothelium, a key event in development of atherosclerotic lesions. Stimulation of human coronary artery endothelial cells (HCAEC) with ATP positively modulates VCAM-1 expression and function through a mechanism involving Ca$^{2+}$ signaling. We here examined the role of Ca$^{2+}$ influx and native TRPC3 channels in that mechanism. **Methods and Results** – Omission of extracellular Ca$^{2+}$ or pre-treatment of cells with channel blockers markedly reduced ATP-induced VCAM-1 and monocyte adhesion. Using a siRNA strategy and real-time fluorescence, we found that native TRPC3 proteins contribute to constitutive and ATP-regulated Ca$^{2+}$ influx. ATP-dependent upregulation of VCAM-1 was accompanied by an increase in basal cation entry and TRPC3 expression. Notably, TRPC3 knock-down resulted in a dramatic reduction of ATP-induced VCAM-1 and monocyte adhesion. **Conclusions** – These findings indicate that in HCAEC native TRPC3 proteins form channels that contribute to constitutive and ATP-dependent Ca$^{2+}$ influx, and that TRPC3 expression and function are fundamental to support VCAM-1 expression and monocyte binding. This is the first evidence to date relating native TRPC3 proteins with regulated expression of cell adhesion molecules in coronary endothelium, and suggests a potential pathophysiological role of TRPC3 in coronary artery disease.

**Condensed Abstract:**

We examined the role of Ca$^{2+}$ influx and native TRPC3 proteins in regulated expression of VCAM-1 in coronary artery endothelial cells. The evidence shows that TRPC3 forms Ca$^{2+}$-permeable channels whose expression and function are fundamental to VCAM-1
expression and monocyte adhesion, suggesting a potential pathophysiological role of TRPC3 in atherogenesis.
Recruitment of circulating monocytes to the arterial intima is a crucial event in initiation, progression and fate of the atherosclerotic lesion. Indeed, monocyte infiltration in the subintima is observed early in atherogenesis, and also at more advanced stages, when plaque infiltration and neovascularization may occur\textsuperscript{1,2}. At the molecular level, monocyte adhesion to the vascular wall is secured by the interaction between the integrin \(\alpha_4\beta_1\) (Very Late Antigen 4; CD49d/CD29) expressed on the monocyte and vascular cell adhesion molecule-1 (VCAM-1, CD106) on the endothelial cell\textsuperscript{3}. Next, VCAM-1-dependent signaling drives transendothelial migration of the bound monocyte. VCAM-1 is virtually absent in resting endothelium, but its expression is rapidly upregulated in response to pro-inflammatory and pro-atherogenic stimuli, particularly in vascular areas prone to lesion development\textsuperscript{3}. In vascular endothelium, nucleotides (i.e., ATP, UTP) released to the extracellular milieu in response to ischemia, hypoxia, chemical or mechanical stress are known to exert a strong pro-inflammatory effect (reviewed in\textsuperscript{4}). For instance, ATP stimulates adhesion of neutrophils to pulmonary artery endothelium\textsuperscript{5}, and promotes release of inflammatory mediators such as interleukin-6 and monocyte chemoattractant protein-1 in dermal microvascular endothelium\textsuperscript{6}. In human coronary artery endothelial cells (HCAEC) ATP induces expression of VCAM-1 and monocyte adhesion through stimulation of P2Y\textsubscript{2} receptors\textsuperscript{7}, in line with its effect in an in vivo model of neointima hyperplasia\textsuperscript{8}. This effect is specific for VCAM-1, as other cell adhesion molecules such as intercellular cell adhesion molecule-1 are not affected\textsuperscript{7}. The underlying signaling, although not fully defined, is known to involve transactivation of VEGF receptor (VEGFR) type 2 and stimulation of the small GTPase Rac1\textsuperscript{9}. As is the case for several inflammatory mediators acting on vascular beds other than the coronary
circulation\textsuperscript{10,11}, changes in intracellular Ca\textsuperscript{2+} levels associated to Ca\textsuperscript{2+} release from internal stores also seem to contribute to ATP-induced VCAM-1 in HCAEC\textsuperscript{7,12}. However, despite that in these cells stimulation of P2Y\textsubscript{2} receptors promotes a robust Ca\textsuperscript{2+} influx, the specific role of Ca\textsuperscript{2+} entry in regulation of VCAM-1 has not been examined. In pilot studies we found that HCAEC express all members of the Canonical Transient Receptor Potential (TRPC) family of channel forming proteins (TRPC1-7, except TRPC2, a pseudogene in humans\textsuperscript{13}) and that TRPC3 forms, or is part of, endogenous Ca\textsuperscript{2+}-permeable channels that contribute to ATP-stimulated Ca\textsuperscript{2+} influx\textsuperscript{14}. Based on this, in the present work we examined if Ca\textsuperscript{2+} influx and TRPC3 contribute to the actions of ATP on VCAM-1 expression and monocyte adhesion in HCAEC. Our findings suggest that in these cells those two events depend, to a significant extent, on Ca\textsuperscript{2+} influx, and that native TRPC3 plays a prominent role in the underlying signaling mechanism. These findings underscore a potential novel function of TRPC3 within the context of development and progression of atherosclerotic lesions in coronary artery disease.

**Methods**

**Cells and transfections:** HCAEC (Lonza, CA) were grown in endothelial basal medium (EBM-2) supplemented with endothelial growth factors and 5% fetal bovine serum (FBS) at 37°C under humidified air (5% CO\textsubscript{2}), and used between passages 2-10. U937 human monocytic cells (ATCC, VA) were grown in RPMI containing 10% FBS. TRPC3 siRNA (100 nM; Dharmaco) or non-specific oligonucleotides were delivered to cells with Lipofectamine2000 (Invitrogen, CA) and cells used 48 hours after transfection.
**Cell lysis and immunoblotting:** Cells (~80% confluence) were made quiescent by replacing growth medium with EBM-2 (10 mM glucose, no serum or growth factors) during 24 hours and processed for SDS-PAGE and immunoblotting as in 15. Proteins were separated in 10% acrylamide gels, transferred to nitrocellulose membranes and immunoblotted with anti-VCAM-1 (clone E-10, Santa Cruz, CA), antibodies against TRPC1, 3-6 (Alomone Labs, Israel), anti-TRPC7 (kindly provided by Dr. W. Schilling, Case Western University School of Medicine, Cleveland OH) or anti-beta actin (Millipore, MA). After incubation with secondary antibodies, immunoreactive bands were visualized by ECL (Amersham, PA), quantified by densitometry within the linear range of the film, and their values normalized against those for β-actin.

**Cell ELISA:** HCAEC grown to confluence in 96-well plates were made quiescent as described above. After the indicated treatments cells were fixed in 0.5% glutaraldehyde, non-specific sites blocked with 0.5% bovine serum albumin, and then incubated (1 h, 37°C) with VCAM-1 monoclonal antibody (R&D Systems, MN) and peroxidase-conjugated anti-mouse antibody (Amershan). Peroxidase reaction was performed with 3,3′,5,5′-Tetramethylbenzidine (Sigma) and stopped with 2 N HCl within the linear range of color development (10-15 min). Cell surface VCAM-1 was estimated as optical density at 450 nm after background subtraction (O.D. in the absence of primary antibody).

**Monocyte adhesion:** HCAEC grown to confluence in 24-well plates were made quiescent as described above. After indicated treatments calcein-loaded U937 cells were added (50,000/well) and incubation proceeded for 45 min at 37°C. Following washes with PBS, bound monocytes were counted (3 fields/well, triplicates/condition). In siRNA
experiments, transfected HCAEC were plated onto 24-well plates for 48 hours and then processed as above.

**Real-time Fluorescence:** Coverslip-plated cells loaded with the Ca\(^{2+}\)-sensitive dye Fura-2 were used to monitor real-time fluorescence changes of intracellular Ca\(^{2+}\) or Ba\(^{2+}\) on multiple cells with a CCD camera-based imaging system (Intracellular Imaging Inc., Cincinnati OH) as previously described\(^{15}\). Measurements were performed at room temperature and treatment conditions were in HEPES-buffered saline solution (HBSS) containing (in mM): 140 NaCl, 4.7 KCl, 1 MgCl\(_2\), 10 glucose, 10 HEPES pH 7.4, 2 CaCl\(_2\). “Nominally Ca\(^{2+}\)-free medium” means HBSS with no Ca\(^{2+}\) added (free Ca\(^{2+}\) ~5 µM). In transfection experiments GFP was used as a marker and measurements were performed on GFP\(^+\) cells selected by their green fluorescence (excitation, 485 nm; emission, 520 nm).

**Statistical analysis:** Means of cytosolic Ca\(^{2+}\), rates of Ca\(^{2+}\)/Ba\(^{2+}\) entry, or densitometric values were compared using a two-tailed \(t\) test for two means, using Graph Pad InStat version 3.00 for Windows 95 (Graph Pad Software, San Diego CA, [www.graphpad.com](http://www.graphpad.com)). Averaged results are from 3-5 independent experiments. P<0.05 was considered significant.

**Results**

Treatment of HCAEC with ATP (100 µM) or TNF\(\alpha\) (10 ng/ml) induced a significant increase in the amount of plasma membrane, or pathophysiologically relevant VCAM-1, as evaluated by cell ELISA (Fig. 1A). VCAM-1 levels increased as early as 3 hours after treatment and started to decline by 16-24 hours (not shown). The effects of ATP and
TNFα were also evident in total VCAM-1 protein levels, as evaluated by immunoblot analysis of whole-cell lysates (7 and not shown) and were translated into augmented monocyte adhesion (Fig. 1B). Pre-incubation of HCAEC with an antibody that recognizes the extracellular domain of VCAM-1 (clone E-10, Santa Cruz, CA) markedly reduced the binding of U937 monocytes, evidencing the contribution of VCAM-1 to the adhesion process (Fig. 1B). Blocking VEGFR1 (Flt-1), expressed in HCAEC but not involved in adhesion 9, did not affect monocyte binding (Fig. 1B). To examine if Ca^{2+} influx played a role in regulated expression of VCAM-1, we treated cells with ATP or TNFα in the presence or absence (nominally Ca^{2+}-free) of extracellular added Ca^{2+}. As shown in figure 1A, VCAM-1 levels were markedly reduced when Ca^{2+} was omitted in the bath. While TNFα effect was partially reduced (~30-40%), that of ATP was completely abolished. Alternatively, we tested the effect of various Ca^{2+} channel blockers on ATP-induced VCAM-1. The inorganic pore channel blocker gadolinium, the non-selective cation channel blockers SKF96365 and flufenamic acid, and the non-dihydropyridine verapamil, all caused a significant reduction of VCAM-1 expression (Fig. 1A) at concentrations that markedly reduced ATP-dependent Ca^{2+} influx (inhibition of peak Ca^{2+} influx was: 95 ± 3% with 10 µM gadolinium or 30 µM SKF96365; 85 ± 6% with 50 µM flufenamic acid; 45 ± 15% with 50 µM verapamil; all reductions had at least P<0.05 respect to control, n=3-4). These chemically unrelated blockers were chosen on the basis of their ability to block a broad spectrum of Ca^{2+}-permeable channels, which includes store-operated and non-store-operated channels with different degrees of selectivity for Ca^{2+} (16 and references therein). Importantly, neither treatment with channel blockers nor transfection with siRNA oligonucleotides (see below) altered
expression of P2Y<sub>2</sub> receptor (not shown). In HCAEC ATP induces a typical biphasic Ca<sup>2+</sup> response composed by a transient increase in cytosolic Ca<sup>2+</sup> due to IP<sub>3</sub>-induced Ca<sup>2+</sup> release from internal stores which is followed by a robust Ca<sup>2+</sup> influx phase (14 and Fig. 2A). Both phases operate simultaneously, as indicated by experiments in which cells were challenged with ATP in the presence of extracellular Ca<sup>2+</sup> (Fig. 2A, dotted trace). Neither Ca<sup>2+</sup> release nor influx were altered by NF279 or MRS2179, P2X and P2Y<sub>1</sub> antagonists, respectively (peak Ca<sup>2+</sup> release and influx were, respectively, 185 ± 20 and 138 ± 10 nM, regardless of the absence or presence of NF279 or MRS2179; n=15-22 cells) suggesting that the Ca<sup>2+</sup> response was mediated by P2Y<sub>2</sub> receptors, as is the case for ATP-induced VCAM-1 and monocyte adhesion. Under basal conditions, i.e., in the absence of ATP stimulation, Ca<sup>2+</sup> influx was not detectable (Fig. 2A, open circles). However, the use of Ba<sup>2+</sup> (10 mM) as a surrogate for Ca<sup>2+</sup> unmasked the existence of constitutive or non-regulated cation influx (Fig. 2B, control basal). The inability to detect constitutive cation influx with 2 mM Ca<sup>2+</sup> in the bath likely reflects operation of a highly efficient Ca<sup>2+</sup> buffering system. In line with this, when cells were exposed to higher Ca<sup>2+</sup> gradients (10 mM in the bath) a significant, yet transient Ca<sup>2+</sup> influx was observed (not shown). Ba<sup>2+</sup> is not subject to the counteracting actions of such buffering systems, and enters the cell unidirectionally, magnifying any existing basal influx (discussed in 18).

HCAEC express message for all members of the TRPC family, namely, TRPC1, 3-7 (TRPC2, a pseudogene in humans, is not present) and we confirmed expression at the protein level by immunoblot analysis of cell lysates (14 and Fig. 2C). Among all TRPC proteins, TRPC3 forms channels endowed with significant constitutive activity. Using a siRNA approach we examined if native TRPC3 contributed to constitutive cation influx.
in HCAEC. The results shown in figure 2B (+TRPC3 siRNA, basal) indicate that indeed that is the case, as non-regulated Ba$^{2+}$ influx was completely suppressed in cells transfected with siRNA oligonucleotides specific for TRPC3. Knock-down of TRPC3 also caused a significant reduction in both initial rate (2-3 fold decrease) and magnitude (~50% reduction at peak) of ATP-induced Ca$^{2+}$ influx (Fig. 2A, open triangles) suggesting that TRPC3 is also an important component of receptor-regulated cation entry. Notably, ATP-dependent upregulation of VCAM-1 was accompanied by a gain in basal cation entry, as evidenced by a more than two fold increase in the rate of constitutive Ba$^{2+}$ influx (Fig. 2B, control+ATP). This Ba$^{2+}$ influx remained unchanged in the presence of the phospholipase C inhibitor U73122 (not shown), indicating it was genuine non-regulated, receptor independent cation influx. Remarkably, this was correlated with a significant increase in TRPC3 protein levels after 3 hour treatment with ATP (Fig. 3). Again, constitutive influx was absent if TRPC3 was knocked-down before treatment with ATP (Fig. 2B, ATP +TRPC3 siRNA). No change was observed under these conditions in any of the other TRPC proteins expressed in HCAEC (not shown). In addition, the siRNA protocol targeted TRPC3 in an effective and specific manner, as protein expression levels of TRPC7, a structurally close relative of TRPC3, or the more distantly related member TRPC1, were not altered (Fig. 2D).

Because Ca$^{2+}$ influx was necessary for ATP-induced VCAM-1 and TRPC3 contributed to ATP-regulated Ca$^{2+}$ influx, we next examined if TRPC3 was part of the mechanism underlying ATP-regulated VCAM-1 expression and function. The experiments in Figure 4A show that knock-down of TRPC3 completely reduced ATP-induced VCAM-1. Of importance, VCAM-1 is not the sole cell adhesion molecule mediating monocyte
adhesion, whereas Ca^{2+} influx is a critical component of the signaling associated to monocyte adhesion and migration \(^{20-22}\), regardless of the adhesion molecules involved \(^{23-26}\). Thus, we examined to what extent Ca^{2+} influx and/or TRPC3 were required for monocyte adhesion to HCAEC. Cells were exposed to ATP in the presence or absence of extracellular added Ca^{2+}, or pre-treated with channel blockers, and monocyte adhesion was evaluated as described in Methods. Alternatively, HCAEC were transfected with TRPC3 siRNA (100 nM) and 48 hours later processed for monocyte binding. In any case, during the incubation with monocytes, Ca^{2+} in the bath was kept at 2 mM, as Ca^{2+} is required for proper interaction between VLA-4 and VCAM-1 \(^{27}\). As shown in figure 4B, omission of Ca^{2+} in the bath, or adding channel blockers during treatment with ATP, markedly reduced adhesion of U937 monocyctic cells. Notably, TRPC3 knock-down reduced monocyte adhesion to almost the same extent as nominally Ca^{2+}-free conditions.

**Discussion**

The importance of Ca^{2+} signaling in regulated expression of VCAM-1 has been appreciated in previous studies. For example, changes in intracellular Ca^{2+} associated to Ca^{2+} release from internal stores have been linked to the ability of Substance P and \(\beta_2\)-microglobulin to induce VCAM-1 in microvascular endothelium \(^{10}\) and synovial fibroblasts \(^{11}\), respectively. In HCAEC, Ca^{2+} mobilization has been related to the mechanism by which lipoprotein A and ATP promote VCAM-1 expression \(^{7,12}\). Nevertheless, the specific role of Ca^{2+} influx has not been directly examined. Besides, in most instances VCAM-1 expression was evaluated under conditions of strong cytosolic Ca^{2+} buffering, which may prevent a contribution from Ca^{2+} entry if Ca^{2+} microdomains
at the channel mouth are perturbed. Here we addressed the role of Ca\(^{2+}\) influx in ATP-dependent regulation of VCAM-1 in HCAEC. Several important conclusions can be derived from the present findings. That Ca\(^{2+}\) influx contributes to the signaling underlying ATP-induced VCAM-1 was first suggested by the observation that maneuvers that prevent Ca\(^{2+}\) entry into HCAEC significantly impaired VCAM-1 expression. This was evident not only on the amount of total cellular VCAM-1 protein, but most importantly on the levels of plasma membrane resident VCAM-1, which is the pathophysiologically relevant form in terms of its role in monocyte recruitment to the endothelium. This not only indicated that Ca\(^{2+}\) influx was necessary, but that Ca\(^{2+}\) release from internal stores was not sufficient. This is particularly important when we consider the action of agonists that induce biphasic Ca\(^{2+}\) responses, such as ATP (see for instance Fig. 2A). ATP-induced VCAM-1 occurs even if cells are exposed shortly (few minutes) to ATP; it was interpreted that the early signaling triggered by ATP, which includes Ca\(^{2+}\) release, is sufficient to drive the pathway controlling VCAM-1 expression. If Ca\(^{2+}\) release were sufficient, we would have expected that under our conditions ATP would induce full expression of VCAM-1 regardless of Ca\(^{2+}\) influx; clearly, this was not the case. Although the extent of contribution from Ca\(^{2+}\) release versus Ca\(^{2+}\) influx to VCAM-1 expression was not evaluated here, it is possible that different events within the underlying signaling may differentially depend upon those two different sources of Ca\(^{2+}\). Because both phases of the Ca\(^{2+}\) response occur simultaneously (dotted trace in Fig. 2A), as they would under physiological conditions, it is reasonable to speculate that both Ca\(^{2+}\) release and influx may be necessary, at least early in the signaling, to trigger a fully operational mechanism leading to transcriptional regulation of VCAM-1 expression.
Using a siRNA approach, we showed for the first time that native TRPC3 forms, or is part of, Ca\(^{2+}\)-permeable channels that contribute not only to ATP-regulated, but also to constitutive cation influx in HCAEC. Notably, besides its effect on VCAM-1, ATP treatment also increased expression of TRPC3 protein, which was correlated with augmented TRPC3-dependent constitutive cation influx. It should be noted here that after three hours of treatment with ATP acute channel stimulation by the nucleotide (Ca\(^{2+}\) entry phase in Fig. 2A) subsides—in fact, Ca\(^{2+}\) levels are back to basal-. Thus, at this point Ba\(^{2+}\) influx reflects constitutive, non-regulated channel function only. This favors the existence of a scenario where augmented expression of TRPC3 protein seems to be translated into more functional channels in the plasma membrane. Strikingly, knock-down of TRPC3 completely suppressed ATP-dependent VCAM-1 expression, in agreement with a clear decrease of ATP-induced monocyte adhesion. TRPC3 has been shown to be sensitive to diverse channel blockers, with relative sensitivities varying considerably depending upon expression conditions. In most instances, TRPC3 constitutive and regulated functions are inhibited by micromolar concentrations of gadolinium, SKF96365, flufenamic acid or verapamil\(^{13,18,30}\). Although none of these blockers can be claimed as specific for TRPC3, the observation that all of them markedly reduced both VCAM-1 expression and monocyte binding is in agreement with the view of TRPC3 contributing to those events. Altogether, these findings strongly support the notion that in HCAEC native TRPC3 proteins form, or are part of, endogenous channels that contribute to Ca\(^{2+}\) influx following stimulation of purinergic P2Y\(_2\) receptors, and that TRPC3 is fundamental within the signaling underlying ATP-induced VCAM-1. Studies are underway to determine the nature of the Ca\(^{2+}\)-dependent events activated downstream.
TRPC3-mediated Ca\(^{2+}\) influx that participate in regulated expression of VCAM-1. The observation that ATP-induced TRPC3 protein expression is paralleled by augmented constitutive cation influx, raises an important question: does TRPC3 contribute to ATP-induced VCAM-1 through regulated activity, constitutive activity or both? The reduction in TNFα-induced VCAM-1 when external Ca\(^{2+}\) is omitted (Fig. 1A) suggests that, besides regulated Ca\(^{2+}\) influx, constitutive activity may also play a role, as TNFα does not stimulate Ca\(^{2+}\) influx in HCAEC \(^a\). Additional studies are required to determine the extent of contribution, if any at all, of TRPC3 constitutive function into such mechanism. Interestingly, increased constitutive activity derived from upregulated expression of TRPC3 in vascular smooth muscle has been shown to account for the augmented vasoconstriction in TRPC6 knock-out mice \(^{31}\). Besides TRPC3, other TRPC members are also expressed in HCAEC (\(^{19}\) and not shown). Functional TRPC channels are thought to be formed by either homo- or hetero-tetrameric arrangements of four TRPC proteins (discussed in \(^{13}\)). Thus, the possibility exists that native channels in HCAEC are formed by either TRPC3 alone, or in association with other TRPCs. Our observations favor the notion that if other TRPCs are part of the native channels contributing to VCAM-1 expression and function, it must be in combination with TRPC3. Otherwise, homo-tetramers made of TRPC proteins other than TRPC3 would be expected to behave independently and their contribution to VCAM-1 expression should remain, even after knocking-down TRPC3; nevertheless, TRPC3 siRNA completely abrogated ATP-induced VCAM-1 and monocyte binding. TRPC channels are now recognized among the most important Ca\(^{2+}\)-permeable cation channels in vascular endothelium physiology \(^{32,33}\). In addition, it is becoming evident that
they are critical players in cardiovascular disease. For instance, TRPC1, 4 and 6 participate in regulation of vascular tone and thus play a role in hypertension \(^{34-37}\).

TRPC1 and 6 modulate proliferation of vascular smooth muscle cells and may have implications in the pathogenesis of intima hyperplasia \(^{38,39}\). \(Ca^{2+}\) entry through TRPC3 and 6 promotes cardiac hypertrophy \(^{40,41}\). TRPC3 and 5, by yet to be known mechanisms, are upregulated in monocytes from patients with essential hypertension \(^{42}\). Our studies represent the first evidence to date suggesting a link between native TRPC3 proteins expressed in coronary artery endothelial cells and cellular and molecular events that are crucial in development of the atherosclerotic lesion. Advances on elucidating molecular and cellular components involved in lesion formation and progression, such as VCAM-1 and its role in monocyte recruitment, have been enthusiastically received in the field as promising new opportunities to develop anti-inflammatory therapies for atherosclerosis \(^{43-45}\). Therefore, identifying new players within the signaling underlying VCAM-1 expression and function is imperative to develop alternative therapeutic targets for effective treatment of this disease. Within that context, our studies warrant further in vitro and in vivo studies to determine the relevance of TRPC3 in development and progression of coronary artery disease.

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**Footnotes**

\(a\) Smedlund, K. and Vazquez, G., manuscript in preparation.
**Figure Legends**

**Figure 1.**

**A)** HCAEC treated with ATP (100 µM) or TNFα (10 ng/ml) for 3 hours in the presence (+Ca$^{2+}$) or absence (-Ca$^{2+}$) of extracellular Ca$^{2+}$, or ATP only (100 µM, 3 h) in the presence or absence of channel blockers (Gd: Gd$^{3+}$, 10 µM; FFA: flufenamic acid, 50 µM; SKF: SKF96365, 30 µM; verapamil, 50 µM) were processed for ELISA detection of surface VCAM-1. *P<0.01; **P<0.02; ***P<0.056, not quite significant; ns: not significantly different. Neither cell viability nor pH of the medium was altered by the channel blockers at these concentrations. **B)** HCAEC were treated with ATP (100 µM) or TNFα (10 ng/ml) for 3 hours before evaluation of monocyte adhesion. When indicated, cells were incubated with 10 µg/ml anti-VCAM-1 (VCAM-1-Ab, clone E-10, Santa Cruz) or 10 µg/ml anti-VEGFR1 (VEGFR1-Ab, clone RR9S, Santa Cruz) antibodies 45 min before addition of monocytes. *P<0.03 respect to control; **P<0.05 respect to control and TNFα alone; ***P<0.06, not quite significant respect to control, and P<0.04 respect to ATP alone.

**Figure 2.**

**A)** Fura-2 loaded HCAEC were exposed to 100 µM ATP (● control cells; Δ transfected with TRPC3 siRNA) to evaluate the Ca$^{2+}$ response. ○ cells not exposed to ATP. The dotted trace shows ATP-dependent Ca$^{2+}$ response when Ca$^{2+}$ (2 mM) is present in the bath. Traces are averages of 15-22 cells; n=3. **B)** Fura-2 loaded HCAEC transfected with TRPC3 siRNA or non-specific oligonucleotides (Control) were kept in nominally Ca$^{2+}$-
free medium for 5 min and then Ba\textsuperscript{2+} (10 mM) was added to the bath to evaluate constitutive influx. The rate of Ba\textsuperscript{2+} entry was assessed within 2 min following Ba\textsuperscript{2+} addition. *P<0.01. C) Representative blots showing expression of TRPC members in HCAEC; molecular weights: ~97-105 kDa. D) Protein expression level for TRPC1, 3 and 7 in control or TRPC3 siRNA transfected HCAEC. For comparison, normalized densitometric values are expressed as percent of control. *P<0.0001; ns: not significantly different; n=3.

**Figure 3.**
HCAEC were treated with ATP (100 µM, 3 h) and processed for immunodetection of TRPC3. Bars show average normalized values of densitometric analysis of 5 experiments, expressed as fold induction over control (vehicle treated cells). *P<0.04.

**Figure 4.**
A) HCAEC transfected with non-specific oligonucleotides (control) or TRPC3 siRNA were treated with ATP (100 µM, 3 h) and processed for ELISA detection of surface VCAM-1. *P<0.01. B) HCAEC were treated with ATP (100 µM, 3 h) in the presence or absence of extracellular Ca\textsuperscript{2+}, or channel blockers (see legend to Fig. 1A for details) or transfected with non-specific oligonucleotides (nso) or TRPC3 siRNA prior to ATP treatment, and then monocyte adhesion was evaluated. *P<0.03 respect to control; **P<0.02 respect to ATP treatment in normal conditions, P<0.05 respect to their corresponding control; ns: not significantly different.
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Figure 1

A

Cell surface VCAM (fold induction over control)

- +Ca\(^{2+}\)
- -Ca\(^{2+}\)
- Channel blockers

ns

B

Monocyte adherence (fold over control)
Figure 2

A

B

Control
+ TRPC3 siRNA

Rate of Ba$^{2+}$ influx (arbitrary units/second)

Basal
ATP (5 h)
Basal
ATP (5 h)
Figure 2 continued

C

D
Figure 3

TRPC3

β-actin

Control

ATP

TRPC3 protein/β-actin (fold increase over control)

0

0.5

1

1.5

2

Control

ATP

*
Figure 4

A

![Bar graph showing cell surface VCAM-1 levels between control and +TRPC3siRNA conditions.](image)

B

![Bar graph showing monocyte adherence.](image)
Chapter 4.

The Constitutive Function of Native TRPC3 Channels Modulates VCAM-1 Expression in Coronary Endothelial Cells through NFκB Signaling

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Smedlund, TRPC3 channels and atherogenesis

Kathryn Smedlund¹, Jean-Yves Tano¹, and Guillermo Vazquez, Ph.D. ¹,²,¶

¹Department of Physiology and Pharmacology and ²Center for Diabetes and Endocrine Research at the University of Toledo College of Medicine, Health Science Campus, 3000 Arlington Av, Toledo, Ohio 43614 USA

¶To whom correspondence should be addressed at: Dept. of Physiology and Pharmacology, UTHSC Mail stop 1008, Toledo OH 43614 USA.

E-mail: Guillermo.Vazquez@utoledo.edu

Tel: 419-383 5301

Fax: 419-383 2871

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Rationale- Upregulation of endothelial VCAM-1 and the subsequent increase in monocyte recruitment constitute critical events in atherogenesis. We have recently shown that in human coronary artery endothelial cells (HCAECs) regulated expression of VCAM-1 depends, to a significant extent, on expression and function of the Ca$^{2+}$-permeable channel TRPC3, regardless of the ability of the stimulatory signal to induce regulated Ca$^{2+}$ influx, leading to the hypothesis that TRPC3 constitutive, rather than regulated function, contributes to the underlying signaling mechanism. Objective- The present studies addressed this important question and gathered mechanistic insight on the signaling coupling constitutive TRPC3 function to VCAM-1 expression. Methods and Results – In HCAECs, maneuvers that prevent Ca$^{2+}$ influx or knockdown of TRPC3 markedly reduced TNFα-induced VCAM-1 and monocyte adhesion. TNFα also induced TRPC3 expression and TRPC3-mediated constitutive cation influx and currents. Stable (HEK293 cells) or transient (HCAECs) overexpression of TRPC3 enhanced TNFα-induced VCAM-1 compared to wild-type cells. IκBα phosphorylation/degradation was reduced by TRPC3 knockdown and increased by channel overexpression. Inhibition of calmodulin (CAM) completely prevented NFκB activation, while blocking calmodulin-dependent kinases (CAMK) or NADPH oxidases, rendered partial inhibition. Conclusions – Our findings indicate that in HCAECs expression of VCAM-1 and monocyte adhesion depend, to a significant extent, on TRPC3 constitutive function through a signaling mechanism that requires constitutive TRPC3-mediated Ca$^{2+}$ influx.
for proper activation of NFκB, presumably through Ca\(^{2+}\)-dependent activation of the CAM/CAMK axis.

**Key words:** TRPC3; VCAM-1; constitutive Ca\(^{2+}\) influx; NFκB signaling; atherogenesis

“Non-standard Abbreviations and Acronyms.”

VCAM-1, vascular cell adhesion molecule-1; HCAECs, human coronary artery endothelial cells; TRPC, transient receptor potential canonical; TNFα, tumor necrosis factor alpha; NFκB, nuclear factor kappa B; HUVECs, human umbilical vein endothelial cells; HPAECs, human pulmonary artery endothelial cells.
Atherosclerosis is a chronic inflammatory disease of the vascular wall that constitutes a major cause of cardiovascular morbidity/mortality in western societies. Over the last two decades basic and clinical research underscored the critical role of vascular inflammation not only in initiation, but also in progression of the disease and extent of its complications. Recruitment of circulating monocytes to the endothelium and their migration into the subintima is a fundamental event in early and advanced stages of atherosclerosis. Monocyte adhesion to endothelium requires interaction of the integrin α4β1 on the monocyte with vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cell. Expression of VCAM-1 is upregulated in response to pro-inflammatory/pro-atherogenic stimuli, such as nucleotides, interleukin-1β, and oxidized low density lipoproteins, among others. In human coronary artery endothelial cells (HCAECs) nucleotides activate P2Y2 receptors to induce VCAM-1 through a mechanism that in part requires Ca2+ signaling. Recently we showed that in HCAECs native TRPC3, a member of the Transient Receptor Potential Canonical (TRPC) family of non-selective cation channels, contributes to the Ca2+ influx that follows stimulation of P2Y2 receptors, and that TRPC3 expression is fundamental in the signaling underlying ATP-induced VCAM-1 and monocyte adhesion. Treatment with ATP also promotes augmented TRPC3 expression, and an increase in constitutive cation influx is detectable even after the acute phase of channel stimulation has subsided. Tumor necrosis factor α (TNFα) also induces VCAM-1 in endothelium from most vascular beds including HCAECs. Despite the fact that TNFα signaling is not associated to regulation of Ca2+ influx, omission of extracellular Ca2+ impairs the ability of TNFα to induce VCAM-1 in HCAECs. These observations altogether with the fact that in native and heterologous
expression systems TRPC3 forms channels endowed with high constitutive, non-regulated activity \cite{12,15}, led us to hypothesize that TRPC3 constitutive rather than regulated function, contributes to the mechanism driving VCAM-1 expression and monocyte adhesion in these cells. In the present work we show that those two events depend, to a significant extent, on upregulated expression of native TRPC3 and the consequent gain in constitutive channel function. Mechanistic insight is provided suggesting a role for the CAM/CAMKII axis in coupling constitutive TRPC3 function to activation of NFκB. We discuss our findings within the context of the potential impact of upregulated expression of a channel endowed with high constitutive activity in atherogenesis.

**Materials and Methods**

Cell culture and transfections, immunoblotting, cell ELISA, monocyte adhesion, Ca\(^{2+}\) imaging and electrophysiological measurements were performed essentially as previously described \cite{11,16,17}. Protocols, composition of solutions and buffers, sequence of siRNA oligonucleotides and source of antibodies are provided in the online supplementary material.

**Results**

Treatment of HCAECs with TNFα (10 ng/ml) resulted in robust expression of VCAM-1 already after 3 hours of treatment, remaining elevated for up to 16 hours (Fig. 1; also \cite{11,18}). TNFα effect was manifest in total (whole lysate, Fig. 1A, +Ca\(^{2+}\) lanes) and plasma membrane (Fig. 1B) VCAM-1, with increased adhesion of U937 monocytes to
HCAECs (Fig. 1C). When studying the role of regulated Ca$^{2+}$ influx on ATP-induced VCAM-1\textsuperscript{11}, we found that omission of Ca$^{2+}$ in the extracellular bath (nominally Ca$^{2+}$-free medium) also impaired the ability of TNFα to induce plasma membrane VCAM-1. The experiments in figure 1 show that not only surface VCAM-1 (Fig. 1B, and 1\textsuperscript{11}) but also total VCAM-1 protein (Fig. 1A, -Ca$^{2+}$ lanes; normalized densitometric values in the absence of Ca$^{2+}$: 0.081 ± 0.010 0.096 ± 0.015 VCAM-1/GAPDH, for control vs. TNFα-treated cells, respectively, not statistically different, n=3) and monocyte adhesion (Fig. 1C) were reduced when Ca$^{2+}$ was omitted in the bath. This was unexpected considering that TNFα, unlike ATP, is not associated to regulated Ca$^{2+}$ influx in endothelium. In fact, acutely challenging HCAECs with TNFα did not result in noticeable Ca$^{2+}$ release or influx (Fig. 2A) or changes in whole-cell currents (inward currents at -60 mV: -1.10 ± 0.14 vs. -1.40 ± 0.10 pA/pF, outward currents at +60 mV: 2.10 ± 0.20 vs. 1.90 ± 0.11 pA/pF, for control vs. TNFα-treated cells, respectively; n=6-8 cells/condition, P<0.07, not statistically significant; see also Fig. IIA). Because TRPC3 is a non-selective cation channel that can permeate Na$^+$ under physiological conditions (see \textsuperscript{12} and references therein), we tested the possibility that membrane depolarization subsequent to TRPC3-mediated Na$^+$ influx could be masking any potential effect of TNFα on Ca$^{2+}$ entry in the Fura-2 measurements, as in those experiments membrane potential is not clamped and thus subject to changes depending on ion movements across the membrane. Cells were challenged with TNFα but in the presence of N-methyl-D-glucamine as a substitute for Na$^+$ in the bath -thus preventing or minimizing membrane depolarization--; still, no changes in Ca$^{2+}$ influx were observed (not shown). Altogether, these findings strongly suggested that if Ca$^{2+}$ influx contributed to TNFα-dependent expression of VCAM-1, it
was likely through constitutive, non-regulated channel function. To more directly explore this, HCAECs were exposed to TNFα (10 ng/ml, 3 h) but in the presence of the Ca\(^{2+}\) channel blockers Gd\(^{3+}\) or SKF96365. As shown in figures 2B and C, those blockers, at concentrations that abolish Ca\(^{2+}\) influx in HCAECs\(^{11}\) (regulated and constitutive Ca\(^{2+}\) influx were inhibited by 94 ± 2% with 10 µM Gd\(^{3+}\) or 30 µM SKF96365; see supplemental Fig. III, panel B) markedly reduced TNFα-induced VCAM-1 and monocyte adhesion.

Combining real-time fluorescence measurements of Ba\(^{2+}\) influx with a siRNA approach we demonstrated that in HCAECs constitutive cation (Ca\(^{2+}\)/Ba\(^{2+}\)) entry is mediated by native TRPC3 channels\(^{11}\) (and Fig. III, panel C). Using this strategy, we examined if TRPC3-mediated non-regulated cation influx contributed to TNFα actions in these cells. HCAECs were transfected with TRPC3 siRNA (see supplemental information and Fig. IV) and forty eight hours later surface VCAM-1 and monocyte adhesion were evaluated. Figures 2B and C show that both events were reduced when TRPC3 was knocked down\(^8\); importantly, knockdown of the close TRPC3 relatives TRPC6 and 7 had no effect (Fig. 2B and C; also Fig. V). Moreover, co-transfecting HCAECs with TRPC3 siRNA and a TRPC3 encoding plasmid, rescued the siRNA effect (normalized densitometric values for total VCAM-1: 0.433 ± 0.070 vs. 0.395 ± 0.080 VCAM-1/GAPDH, for control TNFα-treated vs. “rescued” TNFα-treated cells, respectively; not statistically different, n=3; see also Fig. VI). ICAM-1, but not E-selectin, was also upregulated after 3 hour treatment of HCAECs with TNFα (Fig. VII). Knockdown of TRPC3 drastically reduced TNFα-induced ICAM-1 total protein (Fig. VII) but not surface ICAM-1 (not shown). To examine if TRPC3 also contributed to the action of
other pro-inflammatory/pro-atherogenic stimuli in HCAECs, we tested the effect of IL-1β and LPS on VCAM-1 expression. After 3 hours of treatment, IL-1β (10 ng/ml) but not LPS (from *E. coli*, 1 µg/ml), induced VCAM-1 expression to a level comparable to that of TNFα (Fig. VIII). TRPC3 knockdown reduced IL-1β effect by ~41% (normalized densitometric values for IL-1β-induced VCAM-1: 0.352 ± 0.040 vs. 0.156 ± 0.030, for cells transfected with non-specific oligos vs. TRPC3 siRNA, respectively; P<0.05, n=3). Treatment with TNFα also resulted in a gain in basal cation entry (~two fold increase in rate of constitutive Ba\(^{2+}\) influx, Fig. 3A and 3B) which correlated with a prominent increase in whole-cell current density (Fig. 3C and Fig. IIB) and amount of TRPC3 protein (Fig. 3D). Remarkably, those effects were absent when TRPC3 was knocked down before TNFα treatment (Fig. 3A-C). Constitutive Ba\(^{2+}\) influx remained unaffected by knockdown of TRPC6 or 7 (Fig. IIIC) supporting the notion that constitutive cation influx in HCAECs is mostly mediated by TRPC3. TRPC4 and 7 proteins were also increased by TNFα while TRPC1 was reduced to almost undetectable levels (Fig. IX). TRPC5 and 6 remained unaltered (not shown). To gather preliminary insight regarding expression of native TRPC3 *in vivo*, we examined TRPC3 by immunohistochemistry in aortic root sections from ApoE\(^{-/-}\) and wild-type (C57BL/6) mice (Fig. X; see Methods in supplemental information). As expected, ApoE\(^{-/-}\) mice showed unequivocal lesions which stained positive for neutral lipids and exhibited significant macrophage infiltration (Fig. X, A-C). TRPC3 immunoreactivity in sections from wild-type animals, when present, was barely detectable or only manifested as few positive cells. TRPC3 staining, although diffuse, was more notorious in sections from ApoE\(^{-/-}\) mice (Fig. X, D-F).
Constitutive Ca\(^{2+}\) influx in HCAECs is masked by an efficient Ca\(^{2+}\) buffering system. Despite that acute stimulation of these cells with TNF\(\alpha\) did not induce any noticeable Ca\(^{2+}\) influx, the possibility remained that if TNF\(\alpha\) were to promote weak activation of native TRPC3, the subsequent Ca\(^{2+}\) entry might be rapidly coped by the buffering apparatus thus going undetectable under our experimental conditions. However, even when Ba\(^{2+}\) was used as a surrogate for Ca\(^{2+}\) - Ba\(^{2+}\) is not substrate for buffering systems, enters cells unidirectionally and magnifies the otherwise unnoticeable basal cation influx; see\(^{15}\), acutely challenging HCAECs with TNF\(\alpha\) did not result in detectable Ba\(^{2+}\) entry (rate of Ba\(^{2+}\) influx: 0.015 ± 0.002 vs. 0.011 ± 0.003 ratio units/min, for non-treated vs. TNF\(\alpha\)-treated cells, respectively; P<0.08, not statistically different; n=25-30 cells). To more directly assess if TNF\(\alpha\) had any effect on TRPC3, we used HEK293 cells stably overexpressing human TRPC3 (T3-HEK293\(^{19}\); Fig. XI, panel B). As expected, T3-HEK293 cells responded to the diacylglycerol analogue 1-oleyl-2-acetyl-sn-glycerol – OAG, a potent TRPC3 activator in overexpression systems\(^{15}\) - with robust regulated Ba\(^{2+}\) influx, a manifestation of overexpressed TRPC3\(^{16,20}\) (not shown); nevertheless, acute treatment with TNF\(\alpha\) did not affect basal cation influx (rate of constitutive Ba\(^{2+}\) influx: 0.030 ± 0.002 vs. 0.027 ± 0.001 ratio units/min, for non-treated vs. acutely TNF\(\alpha\)-treated cells, respectively; P<0.07, not statistically different, n=45-60 cells). We next asked the question: is the increase in TRPC3 expression, and thus in constitutive Ca\(^{2+}\) influx, sufficient to promote VCAM-1 expression? To address this, two alternative approaches were undertaken. First, we examined VCAM-1 expression in T3-HEK293 cells. Due to the high levels of TRPC3, T3-HEK293 cells exhibit robust constitutive cation entry compared to wild-type cells (rate of Ba\(^{2+}\) influx: 0.030 ± 0.002 vs. 0.013 ± 0.002 ratio
units/min, for T3-HEK293 vs. wild-type HEK293 cells, respectively; P<0.001, n=45-55 cells; see also 16, 20). As shown in Figure 4A, VCAM-1 expression in wild-type HEK293 cells was significantly augmented (~1.5 fold over basal, P<0.05) after 16 h of treatment with TNFα (10 ng/ml). Notably, whereas basal VCAM-1 was not quite different from wild-type cells, the time course for TNFα-induced VCAM-1 in T3-HEK293 cells was left-shifted, with a trend to be significantly augmented already at 3 h (1.5 fold over basal) and a marked increase respect to wild-type cells at 16 h (~2.2 fold over corresponding basal, P<0.05). Notably, in wild-type and T3-HEK293 cells TNFα-induced VCAM-1 was decreased in the presence of 10 µM Gd$^{3+}$ (Fig. 4A). Second, we examined basal and TNFα-induced VCAM-1 in HCAECs transiently overexpressing TRPC3. Cells were transfected with a hemagglutinin epitope (HA) tagged version of human TRPC3 (T3-HA, 1 µg; see also Fig. XI, panel C). In mammalian cells T3-HA trafficking, function and regulation are indistinguishable from the non-tagged channel14, 21. T3-HA-transfected HCAECs, but not mock-transfected cells, exhibited a robust increase in constitutive and OAG-induced Ba$^{2+}$ influx (rate of constitutive Ba$^{2+}$ influx: 0.026 ± 0.02 vs. 0.012 ± 0.002 ratio units/sec; rate of OAG-induced Ba$^{2+}$ influx: 0.052 ± 0.002 vs. 0.015 ± 0.003 ratio units/sec, for T3-HA-transfected vs. mock-transfected cells, respectively; P<0.01, n=30-45 cells per group)b. Whereas no differences were observed in basal VCAM-1 expression, TNFα-induced VCAM-1 was significantly higher in T3-HA-transfected HCAECs compared to mock transfected cells (Fig. 4B).

In most endothelial cells, regulated expression of VCAM-1 – and ICAM-1- is driven by Nuclear Factor kappa B (NFκB) 21. NFκB activation involves its release from the inhibitory IκBa protein, which requires phosphorylation of IκBa by IκB kinase β (IKKβ)
followed by IkBα degradation \(^{22}\). Treatment of HCAECs with TNFα (10 ng/ml) resulted in rapid (peaking within 5 min) and sustained NFκB activation, as indicated by the extent of IkBα phosphorylation/degradation (Fig. XII); both events were suppressed by the IKK inhibitor hypoestoxide (50 µM, not shown) which also abolished TNFα-induced VCAM-1 (normalized densitometric values: 0.420 ± 0.060 vs. 0.050 ± 0.010 VCAM-1/GAPDH, in the absence or presence of 50 µM hypoestoxide, respectively). Because our data suggested a contribution of TRPC3 constitutive function to the mechanism driving VCAM-1 expression and considering that NFκB activation depends, directly or indirectly, on Ca\(^{2+}\) influx \(^{23}\), we speculated that TRPC3 might be required for activation of NFκB in HCAECs. As shown in figure 5, knockdown of TRPC3 markedly decreased TNFα-induced IkBα phosphorylation (1.8 vs. 5.1 fold increase over control, for “TRPC3siRNA” vs. “NSsiRNA” transfected cells, respectively; n=3, P<0.05) and degradation (1.6 vs. 2.8 fold reduction of control, for “TRPC3siRNA” vs. “NSsiRNA” transfected cells, respectively; n=3, P<0.05). Conversely, transient overexpression of TRPC3-HA in HCAECs enhanced TNFα-induced IkBα phosphorylation (+1.3 fold) and degradation (+1.4) respect to mock-transfected cells (n=3, P<0.05; Figure 6). Based on studies indicating that in several cell types, including endothelium (see for instance \(^{24}\)), Ca\(^{2+}\)-influx dependent activation of NFκB is often associated to calmodulin (CAM) and CAM-dependent kinases – CAMKs, particularly CAMKII, CAMKIV- we undertook a pharmacological approach to gather preliminary insight on their potential participation in mediating the role of constitutive Ca\(^{2+}\) influx in activation of NFκB by TNFα in HCAECs. We focused on two of the earliest events associated to TNFα-dependent activation of the canonical NFκB route: phosphorylation of IkBα and its upstream
regulator, IKKβ. As shown in Figure 7A, TNFα-induced phosphorylation of IKKβ was suppressed by the CAM inhibitor W-7 (50µM), the general CAMK antagonist KN-62 (10 µM) and SKF96365—which prevents constitutive Ca^{2+} influx in HCAECs; Fig. IIIB and 11-. Importantly, knockdown of TRPC3 also prevented TNFα-induced IKKβ phosphorylation. Similarly to IKKβ, phosphorylation of IκBα was completely abrogated by W-7 and SKF96365, and partially reduced by KN-62 (Fig. 7B). Inhibition of NADPH oxidases with apocynin (0.5 mM) resulted in partial decrease of IκBα phosphorylation (Fig. 7B). Neither basal nor TNFα stimulated phosphorylation of IκBα were affected by Gö6976 (1 µM), a selective inhibitor of Ca^{2+}-dependent PKCs, or the phorbol ester PMA (0.1 mM) (Fig. XIII).

Discussion

The findings in the present work show that in HCAECs TNFα-induced VCAM-1 and monocyte adhesion depend, to a considerable extent, on non-regulated Ca^{2+} influx through a mechanism that requires the constitutive function of TRPC3 channels. This is supported by several lines of evidence. First, maneuvers that prevent constitutive Ca^{2+} entry into cells or knockdown of native TRPC3 protein markedly impaired TNFα-induced VCAM-1 and monocyte adhesion. Second, regulated expression of VCAM-1 was enhanced in two different TRPC3-overexpression systems in the absence of receptor-dependent channel activation. Third, knockdown of TRPC3 drastically reduced activation of NFκB, which controls VCAM-1 expression in HCAECs.

Constitutive function of TRPC3 is inhibited by micromolar concentrations of the non-selective channel blockers SKF96365 and Gd^{3+}. Whereas none of these compounds is
specific for TRPC3, they reduced VCAM-1 expression and monocyte adhesion to the
same extent as knockdown of TRPC3 did, supporting the notion that TRPC3 constitutive
function contributes to those events. The partial reduction of surface VCAM-1 and
monocyte adhesion when TRPC3 is knocked down can be attributed to incomplete
downregulation of TRPC3 under our experimental conditions. However, constitutive
Ca\(^{2+}\) influx, but not VCAM-1 or monocyte binding, was completely suppressed by Gd\(^{3+}\)
and SKF96365, suggesting that in HCAECs those processes are also regulated by
alternative Ca\(^{2+}\)-independent mechanisms (see also \(^{11}\)). Importantly, our studies also show
that in HCAECs TNFα-dependent activation of NFκB requires constitutive Ca\(^{2+}\) influx,
presumably through TRPC3, which in HCAECs accounts for most of the constitutive
Ca\(^{2+}\) entry -Figs. 3B, 3C, III-C and \(^{11}\)-. Indeed, TRPC3 knockdown significantly reduced
TNFα-induced phosphorylation of IκBα and its upstream activator, IKKβ.
Pharmacological inhibition of CAM or CAMKs drastically reduced TNFα-dependent
phosphorylation of IKKβ and its target IκBα, pointing to the CAM/CAMK axis as a
likely candidate in the mechanism coupling TRPC3-mediated constitutive Ca\(^{2+}\) influx to
IKKβ phosphorylation and NFκB activation. Whereas KN-62 is equally efficient in
inhibiting CAMKII or CAMKIV \(^{25}\), the predominant nuclear vs. cytosolic localization of
CAMKIV \(^{25}\) suggests that is presumably CAMKII the one linking constitutive Ca\(^{2+}\) influx
to NFκB. Interestingly, CAMKII is emerging as a critical CAMK in endothelial
dysfunction within the context of cardiovascular disease \(^{26}\). The partial reduction of
TNFα-induced IκBα phosphorylation by apocynin indicates that NADPH oxidase-
mediated production of ROS –see footnote “c”- is necessary for full activation of NFκB;
however, ROS are not sufficient, as in the presence of W-7 –which blocks CAM activity
without interfering with the constitutive Ca\textsuperscript{2+} entry that might be necessary for proper ROS generation- phosphorylation of IκB\(\alpha\) was totally abrogated. SKF96365, similarly to W-7, also prevented TNF\(\alpha\)-induced phosphorylation of IKK\(\beta\) and IκB\(\alpha\), suggesting a mandatory requirement for constitutive Ca\textsuperscript{2+} entry-mediated activation of CAM at least early in the signaling (see below). A molecular approach will be required to unequivocally identify the CAMK isoform involved and the putative downstream targets that, acting in concert with ROS, lead to IKK\(\beta\) phosphorylation and activation of NFκB. Recent work showed that CAM couples receptor-regulated activity of TRPC1 and TRPC4 to thrombin-induced NFκB stimulation in human and mouse endothelial cells\textsuperscript{24}, whereas TNF\(\alpha\) signaling was Ca\textsuperscript{2+}-independent. Our findings represent the first evidence for a role of the Ca\textsuperscript{2+}/CAM/CAMK axis in TNF\(\alpha\)-dependent activation of NFκB in endothelial cells, and the first suggesting a requirement for TRPC3 constitutive function in that process. Knockdown of TRPC3 also reduced TNF\(\alpha\)-induced ICAM-1 – also driven by NFkB\textsuperscript{21} and IL-1\(\beta\)-induced VCAM-1, indicating that TRPC3 may exert a more general role in NFκB-dependent regulated-expression of these adhesion molecules by different pro-atherogenic stimuli, rather than a specific function in TNF\(\alpha\)-induced VCAM-1. Unlike VCAM-1, TRPC3 knockdown reduced total ICAM-1 protein levels but not those in the cell surface, indicating that ICAM-1 trafficking might be slower than that of VCAM-1, precluding for changes in surface ICAM-1 to be seen after only three hours of TNF\(\alpha\) treatment.

The increase in TRPC3-mediated constitutive cation influx, whole-cell ionic currents and TRPC3 protein that follows TNF\(\alpha\) treatment indicates that in HCAECs augmented expression of TRPC3 in response to pro-atherogenic factors translates into more
functional channels in the membrane. Because basal cytosolic Ca$^{2+}$ levels were not affected by the increase in constitutively active TRPC3 (96 ± 7 nM, with or without TNFα treatment) we speculate that any effect of TRPC3-mediated constitutive Ca$^{2+}$ influx should occur within the immediate vicinity of the channel; such localized changes may suffice to modulate signaling components in close proximity to TRPC3 without manifesting into changes in bulk cytosolic Ca$^{2+}$ - subject to rapid Ca$^{2+}$ buffering-. In line with this, it should be noted that experiments involving inhibition of CAM and CAMKs were performed under constitutive Ca$^{2+}$ influx conditions –TNFα does not stimulate Ca$^{2+}$ entry-, suggesting that highly localized elevations in submembranous Ca$^{2+}$ under basal conditions might be responsible for signaling to NFκB, as shown in other cell types.

Other groups have also observed TNFα-dependent modulation of Ca$^{2+}$ influx in relation to TRPC function. In airway smooth muscle cells TNFα increased constitutive Ca$^{2+}$ entry that was abrogated by knockdown of TRPC3. In those studies TNFα augmented TRPC3, while TRPC1, 4, 5 and 6 remained unaltered. TNFα-induced TRPC1 expression was observed in human endothelial cells from dermal microvessels, umbilical vein (HUVECs) and pulmonary artery (HPAECs). In HUVECs and HPAECs that was accompanied by augmented receptor-regulated Ca$^{2+}$ influx but the impact of TNFα on constitutive influx was not examined. Our studies in HCAECs show a differential effect of TNFα on individual TRPC members. While TRPC3, 4 and 7 proteins were increased by the cytokine, TRPC1 was drastically reduced, an observation that contrasts with the studies above (cf. 29, 30). Although differences in experimental conditions may account for such disparate results, it is likely that responsiveness of
individual TRPCs to TNFα actions vary, not only in different cell types but also throughout the vascular tree.

Altogether, our findings suggest that a gain in constitutively active TRPC3 may result in pathological Ca\textsuperscript{2+}-dependent signaling, even in the absence of receptor stimulation. This prompted us to ask whether a mere increase in the number of TRPC3 channels would be \textit{sufficient} to promote VCAM-1 expression. Our experiments using two different TRPC3 overexpression systems indicate that whereas under basal conditions high constitutive channel function did not induced VCAM-1, regulated expression was increased. In line with this, TRPC3 overexpression enhanced TNFα-induced IκBα phosphorylation/degradation without altering basal activity. The simplest interpretation is that TRPC3 constitutive function is \textit{necessary} but not \textit{sufficient} and that a priming signal, such as TNFα, might be needed. However, two aspects of the overexpression systems used here should be noted. First, HEK293 cells (non-endothelial) do not express VCAM-1 as robustly as HCAECs and they always require priming with TNFα. Second, in transient TRPC3 overexpression in HCAECs a low plasmid concentration was used; because increasing DNA copy number through plasmid copy number leads to increases in TRPC3 protein\textsuperscript{31}, the possibility remains that if higher expression levels are attained a threshold is reached at which constitutive TRPC3 function \textit{per se} will be sufficient to alter basal VCAM-1 levels even in the absence of a priming signal. Are there \textit{in vivo} examples of biological consequences of a gain in TRPC3 constitutive activity? Studies on TRPC6 knockout mice show that compensatory upregulated expression of TRPC3 resulted in elevated contractility of aorta and cerebral arteries\textsuperscript{32}. In mouse skeletal myocytes TRPC3 is increased by neuromuscular activity and this correlates with
enhanced constitutive NFAT activity. Acting as Ca\textsuperscript{2+} entry gates is not the only way TRPCs can influence intracellular Ca\textsuperscript{2+}. As non-selective cation channels TRPCs can alter membrane potential by mediating Na\textsuperscript{+} influx thus modulating the driving force for Ca\textsuperscript{2+} entry and/or affecting the activity of voltage-gated Ca\textsuperscript{2+} channels. Also, TRPC3 is involved in modulation of IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release from endoplasmic reticulum. Although the latter has not been yet demonstrated to operate in endothelial cells, it may be of particular relevance in the action of stimuli endowed with Ca\textsuperscript{2+} mobilizing properties in endothelium, such as ATP.

TRPC channels participate in a myriad of Ca\textsuperscript{2+}-dependent events that are part of the endothelial cell physiology, and are emerging as critical players in signaling events associated to cardiovascular disease. A review of existing literature shows that the role of TRPC3 constitutive activity in cardiovascular pathophysiology has been scarcely examined or, in some instances, overlooked. The present findings underscore the potential impact of upregulated expression of constitutively active TRPC3 within the context of molecular/cellular events that are critical in the pathogenesis of coronary artery disease. Aortic root sections from atherosclerotic ApoE\textsuperscript{-/-} mice showed more notorious immunoreactivity for TRPC3 than sections from wild-type animals. Although suggestive, a multitude of inflammatory mediators underlie lesion development in vivo and thus a causal relationship between increased expression of native TRPC3 and atherogenesis cannot be established at this time. Generation of mouse models of atherosclerosis in which TRPC3 expression can be manipulated – i.e., conditional transgenic or knockout animals for TRPC3- would be required to properly appreciate the contribution of TRPC3 to lesion development in vivo.
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Disclosure

None.

Footnotes:

a Expression of TNFα receptor type-1 (TNFR1), evaluated by immunoblot, did not change under the different experimental conditions (normalized densitometric mean ±SEM value for TNFR1/GAPDH was 1.032 ± 0.29, for conditions including omission of Ca\textsuperscript{2+} in the bath, channel blockers or siRNA transfections).

b Despite expressing TRPC3/6/7 proteins\textsuperscript{11} HCAECs do not exhibit OAG-induced Ca\textsuperscript{2+} influx, likely because native TRPC3/6/7 proteins are inhibited by PKC\textsuperscript{37-39} which is massively activated by the diacylglycerol analogue. We have shown that native TRPC7 in lymphocytes\textsuperscript{40,41} and native TRPC3 in HCAECs\textsuperscript{42} respond to OAG only after PKC inhibition. Thus, under the present conditions (no PKC inhibition) OAG-induced Ba\textsuperscript{2+} influx is considered a manifestation of overexpressed TRPC3.

c In HCAECs membrane associated, apocynin-sensitive NADPH oxidases (Nox2/Nox4A) are the major sources of TNFα-induced ROS\textsuperscript{43}.

References


Figure Legends

Figure 1.

HCAECs were treated with TNFα (10 ng/ml, 3 h) in the presence (+Ca\(^{2+}\)) or absence (-Ca\(^{2+}\)) of extracellular Ca\(^{2+}\) and processed for: A) immunodetection of VCAM-1 in whole cell lysates. The doublet (~90-105 kDa) represents different extents of glycosylation of VCAM-1 (see for instance\(^{44}\)). The position of the 76 Kda and 102 Kda molecular weight markers is indicated; B) ELISA detection of surface VCAM-1, *P<0.01; **P<0.02. C) monocyte adhesion; *P<0.01; **P<0.02; ***P<0.03. When indicated, cells were incubated with anti-VCAM-1 (10 µg/ml) or anti-VEGFR1 (10 µg/ml; negative control) antibodies for 45 min before monocyte addition, as in\(^{11}\).

Figure 2.

A) Fura-2 loaded HCAECs were acutely exposed to TNFα (10 ng/ml) to evaluate changes in intracellular Ca\(^{2+}\). Shown is the average response of 12 cells, representative of 51 cells from four independent experiments. Before ending the recording, cells were challenged with ATP (100 µM) to test for responsiveness (see also\(^{10, 24}\)). B) HCAECs were treated with TNFα (10 ng/ml, 3 h) in the presence or absence of the channel blockers Gd\(^{3+}\) (Gd3+, 10 µM) or SKF96365 (SKF, 30 µM) and processed for detection of surface VCAM-1. Alternatively, cells were transfected with non-specific oligonucleotides (NSsiRNA), siRNA specific for TRPC3 (TRPC3siRNA), TRPC6 (TRPC6siRNA) or TRPC7 (TRPC7siRNA), or co-transfected with TRPC3 siRNA plus TRPC3-HA cDNA (“TRPC3 Rescue”; see Methods in supplemental information) and 48 hours later treated with TNFα (10 ng/ml, 3 h) and surface VCAM-1 detected by ELISA. *P<0.01;
**P<0.02. C) HCAECs were treated as in B) and then processed for evaluation of monocyte adhesion. *P<0.01; **P<0.02. Shown are results from three independent experiments.

**Figure 3.**

A) HCAECs were transfected with TRPC3 siRNA (gray squares trace) or non-specific oligonucleotides (empty and filled square traces) and 48 hours later treated with TNFα (10 ng/ml, 3 h; “+TNF” and “+TNF+TRPC3siRNA traces”) or vehicle (“Control” trace). After Fura-2 loading, the rate of constitutive Ba^{2+} (10 mM) influx was determined by calculating the slope of fluorescence ratio increase (F340/F380 per min) assessed within 2 min following Ba^{2+} addition. B) Averaged data (n=5) of rate of Ba^{2+} influx for conditions described in A). Absolute values for rate of fluorescence ratio are shown. *P<0.01; ns: not quite different from fluctuations in basal fluorescence (i.e., before Ba^{2+} addition. C) Transfections and treatment conditions of HCAECs were as in A). Whole-cell currents were measured as described in Methods (see supplemental information) and normalized by cell capacitance (pA/pF). Shown are means ± SEM of current densities at +60 mV and -60 mV for at least 8 cells per condition. *P<0.01 respect to control; **P<0.01 respect to the “+TNF” condition, P<0.01 respect to control and not quite different from leak currents. D) HCAECs were treated with TNFα (10 ng/ml, 3 h) and processed for immunodetection of TRPC3. Inset: representative blot. Bar graph: average results of TNFα-induced TRPC3 protein derived from densitometric analysis; *P<0.01, n=5.
Figure 4.

A) Wild-type HEK293 cells, or T3-HEK293 cells were treated with TNFα (10 ng/ml) for the indicated times (or 16 h when in the presence of 10 µM Gd<sup>3+</sup>, “TNF+Gd<sup>3+</sup>”) and processed for immunodetection of total VCAM-1. Shown are average densitometric values of TNFα-induced VCAM-1. *P<0.01 respect to control and P<0.05 respect to the matching time-point in wild-type HEK293 cells. **P<0.05 respect to control and P<0.06 (not quite statistically different) respect to matching time-point in wild-type HEK293 cells; ***P<0.05 respect to control and to matching time-point in the absence of Gd<sup>3+</sup>; ns: not significantly different from control. B) HCAECs were transfected with human TRPC3-HA (“T3-HA-Tx”, see text for details) or vector alone (pcDNA3.1, “Mock-Tx”). 48 hours later cells were treated with TNFα (10 ng/ml, 3 h) and processed for immunodetection of total VCAM-1. *P<0.01 respect to control; ** P<0.01 respect to control and P<0.05 respect to TNFα-treated mock-transfected cells; ns: not significantly different from control. Shown are results from three independent experiments.

Figure 5.

HCAECs were transfected with non-specific oligonucleotides (NSsiRNA) or siRNA specific for TRPC3 (TRPC3siRNA) and 48 hours later treated with TNFα (10 ng/ml, 5 min) and processed for immunodetection of phosphorylated IκBα (pIκBα, panel A) and total IκBα (panel B). Membranes were reprobed for GAPDH to control for protein loading. Blots are representative from three independent experiments. Position of the 38 Kda and 52 Kda molecular weight markers is indicated.
Figure 6.
HCAECs transiently transfected with human TRPC3-HA (“T3-HA-Tx”) or vector alone (pcDNA3.1, “Mock-Tx”) were treated with TNFα (10 ng/ml, 5 min) and processed for immunodetection of phosphorylated (pIκBα) and total IκBα (IκBα). Blots are representative from three independent experiments. Membranes were reprobed for GAPDH to control for protein loading. Position of the 38 Kda and 52 Kda molecular weight markers is indicated.

Figure 7.
HCAECs were treated with TNFα (10 ng/ml, 5 min) in the absence or presence (15 min pre-incubation) of W-7 (50 μM), KN-62 (10 μM), SKF96365 (“SKF”, 30 μM) or apocynin (0.5 mM); alternatively, cells were transfected with non-specific oligonucleotides (NSsiRNA) or TRPC3 siRNA (TRPC3siRNA) and 48 hours later treated with TNFα (10 ng/ml, 5 min). Cells were then processed for immunodetection of A) phosphorylated IKKβ (pIKKβ), or B) phosphorylated IκBα (pIκBα). Membranes were reprobed for GAPDH to control for protein loading. Representative blots are shown (n=3). Relevant molecular weight markers are indicated.
Novelty and Significance

What is known?

- Recruitment of monocytes to the subendothelial milieu is a key event in atherogenesis and is mostly mediated by vascular cell adhesion molecule-1 (VCAM-1).
- Transient Receptor Potential Canonical (TRPC) channels are among the most important Ca\(^{2+}\) permeable cation channels in endothelium where they participate in both physiological and pathophysiological processes.
- In recent work we identified TRPC3, a member of the TRPC family, as an obligatory component in the mechanism underlying regulated expression of VCAM-1 in coronary endothelium, pointing for a potential role of this channel in atherogenesis.

What new information does this article contribute?

- The present work shows that in coronary endothelium pro-atherogenic stimuli induce expression of TRPC3 and the increase in plasma membrane constitutively active TRPC3 channels mediates the Ca\(^{2+}\) influx that supports regulated expression of VCAM-1, ICAM-1 and monocyte adhesion.
Evidence is provided indicating that TRPC3-mediated constitutive Ca\textsuperscript{2+} influx is coupled to activation of Nuclear Factor kappa B (NFkB) through Ca\textsuperscript{2+}-dependent activation of the calmodulin/calmodulin-dependent kinase axis.

Expression of TRPC3 in coronary endothelium is upregulated by pro-atherogenic factors. Because TRPC3 exhibits high constitutive, non-regulated activity, it was important to determine if TRPC3’s contribution to VCAM-1 expression and monocyte adhesion was through regulated or non-regulated function and how such activity couples to intracellular signaling. We show that not only VCAM-1 but also ICAM-1 expression has an obligatory requirement for TRPC3 constitutive function. This is a highly novel concept as the participation of TRPCs in vascular inflammation has been associated to receptor dependent channel activation, rather than constitutive activity, underscoring potential pathophysiological relevance of increased endothelial expression of constitutively active TRPC channels. Mechanistic insight is provided indicating that TRPC3-mediated constitutive Ca\textsuperscript{2+} influx couples to activation of the canonical NFkB pathway through Ca\textsuperscript{2+}-dependent activation of calmodulin and calmodulin-dependent kinases. Identifying novel players in the signaling controlling expression of cell adhesion molecules is imperative to expand the spectrum of existing potential molecular targets that can be exploited for development of effective anti-inflammatory therapies for atherosclerosis. The recognition of TRPC3 as a novel component in such processes represents a major contribution into that direction.
Figure 1

A

VCAM-1

GAPDH

+Ca\(^{2+}\)

- Ca\(^{2+}\)

Control

TNF

Control

TNF

Control

TNF


B

Cell surface VCAM-1

(fold induction over control)

+Ca\(^{2+}\)

- Ca\(^{2+}\)


C

Monocyte adhesion

(fold over control)

Control

TNF

TNF, no Ca

TNF+VCAM1-Ab

TNF+VEGFR1-Ab

- Ca\(^{2+}\)
Figure 2
Figure 3

A

B

C

D

Rate of Barium Influx
([F340/F380]/min)

0.8
0.85
0.9
0.95
1
1.05
1.1
1.15
1.2

0 100 200 300 400 500 600 700

Time (seconds)

Ratio (F340/F380)

Nominally Ca\(^{2+}\)-free
10 mM Ba\(^{2+}\)
2 mM Ca\(^{2+}\)

Control
+TNF
+TNF+TRPC3siRNA

Control
TNF
TNF+TRPC3 siRNA

Current density (pA/pF)

-60 mV
+60 mV

0.5
1
1.5
2

Control
TNF
TNF+TRPC3 siRNA

TRPC3 protein/beta actin (fold increase over control)

0
0.5
1
1.5
2

Control
TNF

β-actin

0
0.5
1
1.5
2

Control
TNF

Rate of Barium Influx
([F340/F380]/min)

* ns
Figure 4

A

- **Wild-type HEK293**
- **T3-HEK293**

B

- **Mock-Tx**
- **T3-HA-Tx**
Figure 5

A

NSsiRNA  TRPC3siRNA

pIkBα

GAPDH

Control  TNF  Control  TNF

52 Kda

38 Kda

B

NSsiRNA  TRPC3siRNA

IkBα

GAPDH

Control  TNF  Control  TNF

52 Kda

38 Kda
Figure 6

The figure shows the results of a Western blot analysis. The blot is probed with antibodies specific for pIkBα (phosphorylated IkBα) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). The figure compares the expression levels of these proteins under different conditions: Control, TNF, Mock-Tx, and T3-HA-Tx.

- **pIkBα Blot**: The blot is divided into two panels. The upper panel shows pIkBα with a molecular weight of 52 Kda and 38 Kda. The lower panel shows GAPDH with a molecular weight of 52 Kda and 38 Kda.

- **GAPDH Blot**: The blot is also divided into two panels. The upper panel shows pIkBα with a molecular weight of 52 Kda and 38 Kda. The lower panel shows GAPDH with a molecular weight of 52 Kda and 38 Kda.

The blot is normalized by GAPDH expression in each lane, and the results are presented for control and TNF-treated samples with and without the T3-HA-Tx treatment.
Figure 7

A

B

pIkBα
GAPDH
52 Kda
38 Kda
Control
W7 KN62 SKF
+TNF
TNF
pIkBα
GAPDH
52 Kda
38 Kda
Control
W7 KN62 SKF
+TNF
TNF
pIKKβ
GAPDH
102 Kda
76 Kda
Control
W7 KN62 TNF SKF
NSsiRNA TRPC3siRNA
Control
Control
+TNF
+Apocynin

102 Kda
76 Kda
102 Kda
76 Kda
52 Kda
38 Kda
52 Kda
38 Kda
Supplementary information

Methods

Cells and transfections: HCAECs (Lonza, CA) and U937 human monocytic cells (ATCC, VA) were grown as described in [1]. Human embryonic kidney (HEK293) cells stably expressing human TRPC3 (kindly provided by Dr. J.W. Putney Jr., NIEHS, NC) were grown as in [2]. siRNA oligonucleotides for TRPC3, 6 or 7 (siGENOME SMART pool duplexes, Dharmacon), TRPC3-HA (into pcDNA3.1, kindly provided by Dr. Lutz Birnbaumer, NIEHS, NC) or pcDNA3.1 were delivered to cells with Lipofectamine2000 (Invitrogen, CA). For either TRPC3, 6 or 7 siRNA duplexes were used as a pool of four duplexes (target sequences below) at a total final concentration of 100 nM.

siRNA target sequences:

Scrambled (non-specific oligonucleotide) 5’-UAGCGACUAAACACAUCA

TRPC3-1 5’-PGUAUUCCCUAGGUCCUUCCUU

TRPC3-2 5’-PCAUUCCAAGAACCAGACCUU

TRPC3-3 5’-PAUUGCAUGGAGAGCUUCCGUU

TRPC3-4 5’-PUGAUAGCUAUGGUCUGCUCUU

TRPC6-1 5’-GCAUACAUUUUAGUGUAUC

TRPC6-2 5’-UGAACGCGCCUCAUGAUAAU
TRPC6-3      5'-CAUCAUCAUUGCGAGAUU
TRPC6-4      5'-UAAAGGUUAUGUUCGGAUU
TRPC7-1      5'-AAACAAAUCUUCAGAGUA
TRPC7-2      5'-GAAAUGGGCAUGCUGAAUU
TRPC7-3      5'-GGAUCAAACUCGCCAUUAA
TRPC7-4      5'-CGGCUAUCUGAAGUAAC

Transfected cells were used for experiments 48 hours after transfection. We have previously shown that the siRNA protocol used here efficiently and selectively knocks down TRPC3 in HCAECs without altering expression of other TRPCs\(^1\). Knockdown of TRPC3, 6 and 7 at the protein level is documented in Figures IV and V. For rescue experiments, HCAECs were co-transfected with TRPC3 siRNA (100 nM) plus 1 μg of TRPC3-HA cDNA (into pcDNA3.1) and 48 hours later processed for immunodetection of VCAM-1, surface VCAM-1 or monocyte adhesion. The siGENOME SMART pool duplexes used here, target 3’-UTR (untranslated regions) sequences in TRPC3 mRNA; the cDNA encoding for TRPC3-HA contains only the ORF sequence for TRPC3-HA and thus it is not targeted by the siRNA oligos; therefore, concomitant expression of TRPC3-HA results in the observed “rescued” phenotype. Human recombinant tumor necrosis factor α (TNFα, Calbiochem, CA) was from *E. coli*, and contamination with bacterial LPS was <<0.1 ng/µg of TNFα. At the concentration of TNFα used in the present studies (10 ng/ml) LPS contamination is estimated to be <<1 pg/ml; even at concentrations of LPS as high as 1 μg/ml we did not observe significant effects of LPS on VCAM-1 expression in HCAECs (Figure VIII). Therefore, any potential effect from LPS
contamination during TNFα treatments can be neglected. LPS (from *E. coli*) and interleukin-1β were from Calbiochem.

**Immunoblotting:** Cells were processed for SDS-PAGE (10% acrylamide) and immunoblotting as in¹. Primary antibodies were: anti-VCAM-1 (clone E-10, Santa Cruz, CA); anti-ICAM-1 (clone 0.N.146, Santa Cruz, CA); anti-E selectin (clone 1.BB.613, Santa Cruz, CA); anti-TNFRI (Calbiochem); anti-TRPC1, anti-TRPC3 and anti-TRPC4 (Alomone Labs, Israel); anti-TRPC7 (kindly provided by Dr. W. Schilling, Case Western University School of Medicine, OH); anti-IκBα, anti-phosphoIkBα, anti-phospho-IKKβ (Ser177/181) (Cell Signaling, MA); anti-GFP (clone B-2, Santa Cruz, CA); anti-HA (clone 3F10, Roche, Germany); anti-beta actin (Millipore, MA); anti-GAPDH (clone 0411, Santa Cruz, CA). After incubation with the appropriate HRP-labelled secondary antibody, immunoreactive bands were visualized by ECL (Amersham, PA) and quantified by densitometry within the linear range of the film. All blots for a particular experimental condition were analyzed by densitometry and the values for the band of interest were normalized by the densitometric values obtained for beta-actin or GAPDH, as indicated; means ± SEM were obtained and corresponding fold change values respect to controls calculated.

**Cell ELISA:** The protocol for cell surface VCAM-1, ICAM-1 or E-selectin (cell ELISA) was essentially as previously described¹. HCAECs were grown to confluence in 96-well plates, and after the indicated treatments cells were fixed in 0.5% glutaraldehyde, non-specific sites blocked with 0.5% bovine serum albumin, and then sequentially incubated (1 h, 37°C) with either VCAM-1 (R&D Systems, MN), ICAM-1 or E-selectin monoclonal antibodies and peroxidase-conjugated anti-mouse antibody (Amersham).
Peroxidase reaction was performed with 3,3′,5,5′-Tetramethylbenzidine (Sigma) and stopped with 2 N HCl within the linear range of color development (10-15 min). Cell surface VCAM-1, ICAM-1 or E-selectin was estimated as optical density at 450 nm after background subtraction (O.D. in the absence of primary antibody).

**Monocyte adhesion:** This was essentially as in ¹. HCAECs were grown to confluence in 24-well plates. After indicated treatments calcein-loaded U937 cells were added (50,000/well) and incubation proceeded for 45 min at 37°C. Following washes with PBS, bound monocytes were counted (3 fields/well, triplicates/condition). In siRNA experiments, transfected HCAECs were plated onto 24-well plates for 48 hours and then processed as above.

**Real-time Fluorescence:** Coverslip-plated cells loaded with Fura-2 were used to monitor changes of intracellular Ca²⁺ or Ba²⁺ by real-time fluorescence with a CCD camera-based imaging system (Intracellular Imaging Inc., Cincinnati OH) as previously described¹, ². Measurements were at room temperature and treatments were in HEPES-buffered saline solution (HBSS, in mM: 140 NaCl, 4.7 KCl, 1 MgCl₂, 10 glucose, 10 HEPES pH 7.4, 2 CaCl₂). When indicated, Fura-2 measurements were performed in the presence of N-methyl-D-glucamine (NMDG, 140 mM) as a substitute for external Na⁺ to prevent or minimize Na⁺-influx dependent membrane depolarization. We did not observe significant differences in the effect of TNFα or ATP¹ when Ca²⁺/Ba²⁺ measurements were performed at 37°C compared to room temperature; because cellular loading of Fura-2/AM was performed at room temperature -to minimize dye compartmentalization, see for instance ³- we proceeded with the recordings at room temperature to avoid additional and unnecessary temperature changes immediately before the measurements. “Nominally
Ca\(^{2+}\)-free medium” means HBSS with no Ca\(^{2+}\) added (free Ca\(^{2+}\) << 5 µM). In transfection experiments GFP was used as a marker and measurements were on GFP\(^+\) cells. Basal cytosolic Ca\(^{2+}\) levels were 96 ± 7 nM throughout several independent calibrations; however, because tabulated K\(_d\) values for Fura-2/Ca\(^{2+}\) were used –we have not determined the actual K\(_d\) in HCAEC’s cytosolic environment - Ca\(^{2+}\) values are only estimative; therefore, and for simplicity, the ratio of Fura-2 fluorescence (510 nm emission) when excited at 340 and 380 nm (F340/F380) was used to calculate peaks or rates of changes in fluorescence, as in \(^2\).

**Electrophysiology:** Ionic currents were recorded using the patch-clamp technique in its whole-cell configuration using an Axopatch-200B amplifier (Axon Instruments, USA) essentially as in \(^4\). The resistance of the pipettes (borosilicate glass capillaries, World Precision Instruments, FL) varied between 3-5 MΩ when filled with intracellular solution (composition below). The extracellular solution (osmolarity 310 mosmol/l) contained (in mM): NaCl, 145; KCl, 5; CsCl 10, HEPES, 10; MgCl\(_2\), 1; CaCl\(_2\), 2; pH, 7.3 (adjusted with NaOH). The intracellular (pipette) solution contained (in mM): 145 Cs\(^+\) methanesulfonate; 10 BAPTA; 10 HEPES; 1 MgCl\(_2\); 2 MgATP; 3.2 CaCl\(_2\), pH 7.2; the free Ca\(^{2+}\) concentration of this solution was 100 nM, calculated with MaxChelator program Winmaxc v2.4 \(^5\). All measurements were performed at room temperature (22 °C). After establishing the whole cell configuration, membrane potential was clamped to 0 mV, and 250-ms voltage ramps (-80 to +80 mV) were applied every 2 s. When needed, acute addition of TNF\(\alpha\) to the external solution was carried out using a multibarrel puffing micropipette with common outflow positioned in close proximity to the cell under examination, and the cell was constantly superfused with bath solution to reduce
potential artifacts related to switching from static to moving solution. Signals were low-pass filtered (1 kHz), digitized (20 kHz) and analyzed with Clampfit 9.2 software (Axon Instr., USA). In transfection experiments GFP was used as a marker and measurements were on GFP\(^+\) cells. Upon establishment of the whole-cell configuration, capacitive currents were compensated and measurements started within 30 seconds. Cell capacitances ranged between 40-75 pF.

**Mice, aortic root sectioning and immunohistochemistry:** All animal studies meet NIH guidelines and corresponding protocols have been approved by University of Toledo IACUC. We maintain our own colonies of C57BL/6 and ApoE\(^{-/-}\) mice (originally obtained from Jackson Labs, ME) at the Division of Laboratory and Animal Research of the University of Toledo Health Science Campus. Starting at 6 week-old, male and female ApoE-deficient mice (ApoE\(^{-/-}\), on C57BL/6 background) were kept on Western type diet (D12079B, Research Diets) during 21 weeks. Under these conditions, as expected, ApoE\(^{-/-}\) mice developed significant lesions along the aortic root segment (Figure X); lesion development in ApoE\(^{-/-}\) mice has been thoroughly characterized\(^6\)-\(^8\). C57BL/6 mice were kept on regular chow diet for 21-weeks and used as controls. At the end of the 21 weeks, we evaluated lesion size and TRPC3 expression in aortic root sections. Gender differences in lesion size are not consistent throughout studies and some published evidence suggests that female mice tend to develop larger lesions than males (discussed in\(^9\)). Under our own experimental conditions we found no significant differences in lesion area between male and females from ApoE\(^{-/-}\) on Western type diet (lesion area: 742,300 ± 89,076 and 668,600 ± 73,500 µm\(^2\), for male and female ApoE\(^{-/-}\), respectively, \(P<0.538; n=6/\text{group}\)).
At euthanasia mice were perfused through the left ventricle with heparinized-PBS followed by 4% paraformaldehyde. The heart was removed and cut in the plane between the lower tips of the right and left atria, allowing cross-sections of all three aortic valves to be in the same geometric plane; the aorta was cut at the point where it emerges from the ventricle. The upper portion of the heart was put into a tissue mold, embedded in O.C.T. and frozen in the Peltier stage of the cryostat (Thermo Scientific R. Allan HM550 Cryostat). Blocks were processed immediately or stored at -20°C until sectioning. 10 µm thick cross-sections were collected starting at the point where aorta emerges from the ventricle (beginning of ascending aorta, sections round in appearance: stage I) and moving towards the aortic sinus (insinuation of aortic valves: stage II; appearance of clearly identifiable aortic valves: stage III; aortic sinus: stage IV), covering a distance of ~650-700 µm. Sections were collected onto Fisher Superfrost Plus-coated slides following a scheme similar to that described by Daugherty and Whitman\textsuperscript{10}. Each slide (at least 5 slides per animal) contained 12 sections at 40 µm interval throughout the entire aortic root. Additional sections were collected at the end to be used as controls in subsequent immunostaining procedures. Sections were air-dried and stored (-80°C) until processed. Slides were processed for Oil Red O (ORO) staining or immunohistochemistry as described below.

**Oil Red O (ORO) staining:** Sections were fixed with 10% formalin, stained with ORO and counterstained with hematoxylin. Images of each section on a slide were captured using a digital camera (Micropublisher 3.3 Megapixel Cooled CCD Color Digital Camera coupled to Zeiss Axiovert 40CL inverted microscope) and lesion area was measured with image analysis software (NIS Elements D) by manually outlining each lesion as defined
by the lumen boundary and the internal elastic lamina (ApoE<sup>−/−</sup> mice) using ORO staining as a visual aid only. Lesion size for each mouse was obtained as the total lesion area (in square micrometers) in 12 sections from the same mouse (at 40 µm interval throughout the aortic root).

**Immunohistochemistry:** Sections were fixed in acetone (-20°C, 10 min) and processed for immunostaining for TRPC3 using rabbit anti-TRPC3 (Alomone) followed by incubation with polyclonal swine anti-rabbit biotinylated immunoglobulins (F(ab’)<sub>2</sub> (Dako, Denmark); immunostaining for MOMA-2 was performed using clone MCA519G (Serotec) followed by incubation with biotinylated rabbit anti-rat antibody (Dako). In both instances, treatment with secondary antibodies was followed by incubation with alkaline phosphatase-conjugated streptavidin (Dako). The blocking step included 1% BSA plus normal swine serum (for TRPC3 staining) or 1% BSA alone (for MOMA-2 staining). Counterstaining was performed with hematoxylin and mounting with Ultramount (Dako). Negative controls were performed by omitting primary antibody.

**Statistical analysis:** Mean rates of Ba<sup>2+</sup> entry and means for normalized densitometric values were compared using a two-tailed t test for two means, using Graph Pad InStat version 3.00 for Windows 95 (Graph Pad Software, San Diego CA). Averaged results are from 3-5 independent experiments. P<0.05 was considered significant.

**Figure Legends**

**Supplemental Figure I.**

HCAECs were treated with TNFα (10 ng/ml) for the indicated times and processed for immunodetection of VCAM-1 in whole cell lysates as described in Methods. The doublet
(~90-105 kDa) in the immunoblot represents different extents of glycosylation of VCAM-1 (see for instance\textsuperscript{11, 12}). For reference, the position of the 76 Kda and 102 Kda molecular weight markers is indicated.

**Supplemental Figure II.**

**A)** Time course of spontaneous, constitutive whole-cell currents in HCAECs, before and after acute addition of TNF\(\alpha\) (10 ng/ml) to the external bath. Shown are average inward (-60 mV) and outward (+60 mV) currents from 4 individual cells. X-axis tick mark labels are shown in low position respect to the axis to avoid overlap with the inward current trace. **B)** Current-voltage (I/V) relationship for spontaneous, constitutive whole-cell currents before (black trace) and after (light and dark gray traces) 3 hour pre-treatment with TNF\(\alpha\) (10 ng/ml), as indicated. It should be noted that, contrary to experiments in “A”, light and dark gray I/V curves correspond to cells that were continuously exposed to TNF\(\alpha\) for 3 hours before measurement of whole-cell currents (\textit{i.e.}, no acute stimulation). The “Control” I/V curve is indistinguishable from the I/V relationships in experiments like those in “A” (\textit{i.e.}, spontaneous whole-cell currents with or without acute TNF\(\alpha\) addition). Whole-cell currents were measured as described in Methods and normalized by cell capacitance (pA/pF). Data are representative of independent measurements performed on at least eight single cells.

**Supplemental Figure III.**

**A)** In HCAECs activation of P2Y\(_2\) receptors by ATP promotes a biphasic Ca\(^{2+}\) response, that consists of a transient PLC-dependent IP\(_3\)-mediated release of Ca\(^{2+}\) from internal
Ca$^{2+}$ stores followed by Ca$^{2+}$ influx across the plasma membrane$^1$. The Fura-2 measurements shown in panel A illustrate how when cells are challenged with ATP in nominally Ca$^{2+}$-free medium (filled squares trace) a rapid and transient increase in cytosolic Ca$^{2+}$ takes place (IP$_3$-induced Ca$^{2+}$ release). In the experiments shown here, once this Ca$^{2+}$ transient was over, Ba$^{2+}$ (10 mM) was added to the bath to evidence operation of a cation (Ca$^{2+}$ and Ba$^{2+}$ permeable$^1$) influx route. Alternatively, cells were maintained in nominally Ca$^{2+}$-free medium (without ATP stimulation) and when indicated Ba$^{2+}$ (10 mM) was added to the bath to evidence the existence of constitutive, non-regulated cation influx (empty squares trace). Traces are averages of 15-22 cells and are representative of at least four independent experiments. Values for rates of Ba$^{2+}$ influx for ATP-induced (regulated) and constitutive (non-regulated) cation entry are shown as means ± SEM of fluorescence ratio per minute. 

B) Summarized data of the effect of the channel blockers Gd$^{3+}$ (Gd3+, 10 µM) and SKF96365 (SKF, 30 µM) on either ATP-induced (Receptor-activated influx) or constitutive influx, expressed as percent of the rate of Ba$^{2+}$ influx in the absence of blockers (“Control”). Results are from three independent experiments, each performed on at least 15 cells; *P<0.001. 

C) HCAECs were transfected with siRNA specific for TRPC3, 6 or 7 or non-specific oligonucleotides (“Control”) and 48 hours later loaded with Fura-2 to evaluate constitutive Ba$^{2+}$ influx as in “A”. Results are from three independent experiments, each performed on at least 8-10 transfected cells; *P<0.001; ns: not significantly different from control.
Supplemental Figure IV.

HCAECs were transfected with TRPC3 siRNA or non-specific oligonucleotides (NSsiRNA) and 48 hours later treated with TNFα (10 ng/ml, 3 h). Lysates were obtained and cellular proteins were immunoblotted for detection of TRPC3 as described in Methods. The lower portion of the membranes was cut and processed for immunodetection of beta-actin (β-actin) to control for protein loading. Shown is a representative blot from five independent experiments. In this particular case, normalized densitometric values (TRPC3/β-actin) showed a 1.7-fold increase in TRPC3 protein respect to control, which falls within the range of 1.95 ± 0.25 fold-increase respect to control in cells treated with TNFα (Figure 3D). The immunoblots were performed on lysates from the same batch of cells that were used for “NSsiRNA” and “TRPC3siRNA” conditions in the experiments in Figures 2B, 2C and 3A. For reference, the position of the 76 Kda and 102 Kda molecular weight markers is indicated.

Supplemental Figure V.

HCAECs were transfected with siRNA specific for TRPC6 or 7, or non-specific oligonucleotides (NSsiRNA). 48 hours later lysates were obtained and cellular proteins were immunoblotted for detection of TRPC6 and 7 as described in Methods. The lower portion of the membranes was cut and processed for immunodetection of GAPDH to control for protein loading. Shown are representative blots from three independent experiments. The immunoblots were performed on lysates from the same batch of cells that were used for “TRPC6siRNA” and “TRPC7siRNA” conditions in the experiments in
Figures 2B, 2C and IIC. For reference, the position of the 76 Kda and 102 Kda molecular weight markers is indicated.

**Supplemental Figure VI.**
HCAECs were transfected with TRPC3 siRNA, non-specific oligonucleotides (NSsiRNA) or co-transfected with TRPC3 siRNA plus TRPC3-HA cDNA (“Rescue”). Forty eight hours later cells were treated with TNFα (10 ng/ml, 3 h), lysed, and cellular proteins were immunoblotted for detection of VCAM-1 as described in Methods. The lower portion of the membranes was cut and processed for immunodetection of GAPDH to control for protein loading. TNFα-induced VCAM-1 in the “Rescue” condition seems more robust than in NSsiRNA-transfected cells, probably reflecting the enhancing effect of TRPC3 overexpression on TNFα action (see for instance Figure 4B). Shown are blots representative from three independent experiments. For reference, the position of the 76 Kda and 102 Kda molecular weight markers is indicated.

**Supplemental Figure VII.**
HCAECs were transfected with TRPC3 siRNA or non-specific oligonucleotides (NSsiRNA) and 48 hours later treated with TNFα (10 ng/ml, 3 h). Lysates were obtained and cellular proteins were immunoblotted for detection of ICAM-1 as described in Methods. The lower portion of the membranes was cut and processed for immunodetection of GAPDH to control for protein loading. Shown are blots representative from two independent experiments. For reference, the position of the 76 Kda and 102 Kda molecular weight markers is indicated.
Supplemental Figure VIII.

HCAECs were treated with TNFα (10 ng/ml), LPS (from *E. coli*, 1 µg/ml) or IL-1β (human recombinant, 10 ng/ml) during 3 hours and then processed for immunodetection of VCAM-1 in whole cell lysates as described in Methods. The lower portion of the membrane was cut and processed for immunodetection of GAPDH to control for protein loading. Shown is a representative blot from two independent experiments. For reference, the position of the 76 Kda and 102 Kda molecular weight markers is indicated.

Supplemental Figure IX.

HCAECs were treated with TNFα (10 ng/ml, 3 h) and processed for immunodetection of all TRPC family members. Shown are representative blots for those TRPCs that exhibited significant change after treatment (see text for details). The lower portion of the membranes was cut and processed for immunodetection of beta-actin (β-actin) to control for protein loading. For reference, the position of the 76 Kda and 102 Kda molecular weight markers is indicated.

Supplemental Figure X.

Photomicrographs of aortic root sections from ApoE−/− mice showing intima thickening, presence of lipid deposits and macrophage infiltration. A) Oil Red O staining of neutral lipids; original magnification X5. B, C) Macrophages were stained with MOMA-2 antibody; macrophage accumulation can be seen under the endothelium, with some immunoreactive areas separated by regions of non-stained cells and matrix. Original magnification X20. D-F) Immunostaining for TRPC3 in aortic root sections from wild-
type (D) and ApoE−/− mice (E, F). In wild-type animals TRPC3 staining, when present, was manifested as few positive cells (arrowheads) or was barely visible at low power. TRPC3 staining was more notorious in lesions of ApoE−/− mice, with a patchy (E) or diffuse (F) staining. No gender differences in TRPC3 immunostaining were found for either wild-type or ApoE−/− animals. Original magnification X20.

Supplementary Figure XI.

Wild-type HEK293 cells (WtHEK293, panel A) or T3-HEK293 cells (B) were treated with TNFα (10 ng/ml) for the indicated times and processed for immunodetection of TRPC3. T3-HEK293 cells stably express a GFP-tagged version of TRPC3 (T3-GFP), and therefore anti-GFP was used for detection of overexpressed T3-GFP. Endogenous TRPC3 in wild-type HEK293 cells was detected with anti-TRPC3 antibody (Alomone Labs, Israel). The lower portion of the membranes was cut and processed for immunodetection of GAPDH to control for protein loading. C) HCAECs transiently expressing human TRPC3-HA (“T3-HA-Tx”, see text for details) or vector alone (pcDNA3.1, “Mock-Tx”) were lysed, TRPC3-HA was immunoprecipitated with anti-HA monoclonal antibody (Roche, Germany) and the membranes immunoblotted with the same antibody. **Note:** there is clearly a TNFα-dependent upregulation of T3-GFP in T3-HEK293 cells, likely due to the known responsiveness of the CMV promoter—which drives expression of the T3-GFP construct- to this cytokine\textsuperscript{13}. There seemed to be a trend for TNFα to induce a slight increase (~15% above basal when normalized densitometric values are compared; n=2) in endogenous TRPC3 in wt-HEK293 cells after 16 hours of
treatment, which coincides with the time at which VCAM-1 expression is significantly upregulated following cytokine treatment (see Figure 4A).

**Supplemental Figure XII.**
HCAECs were treated with TNFα (10 ng/ml) for the indicated times and then processed for immunodetection of phosphorylated IκBα (pIκBα, panel A) and total IκBα (panel B). Representative blot from three independent experiments. Membranes were reprobed for immunodetection of GAPDH to control for protein loading. For reference, the position of the 38 Kda and 52 Kda molecular weight markers is indicated.

**Supplemental Figure XIII.**
A) HCAECs were treated during 5 min with TNFα (10 ng/ml), PMA (0.1 mM) or TNFα+PMA. B) HCAECs were treated during 5 min with TNFα (10 ng/ml) in the presence or absence of Gö6976 (“Go+TNF”, 1 μM, 15 min pre-treatment). In all instances cells were processed for immunodetection of phosphorylated IκBα (pIκBα) and membranes reprobed for immunodetection of GAPDH to control for protein loading. Shown are representative blots from two independent experiments. The position of the 38 Kda and 52 Kda molecular weight markers is indicated. **Note:** in “B” the blot corresponding to “Go+TNF” was boxed to denote the fact that it was cut from a different region of the same membrane and pasted next to “Control” and “TNF” for comparison purposes; however, all experimental conditions shown in “B” were blotted on the same PVDF membrane and ECL-developed simultaneously on the same film.
References


Supplementary Figure I

VCAM-1

β-actin

Control, TNF, 3 h, TNF, 6 h, TNF, 16 h

102 Kda
76 Kda
Supplementary Figure II

A

B

-10
-8
-6
-4
-2
0
2
4
6
8
10
-80 -60 -40 -20 0 20 40 60 80
mV
pA/pF
Control
+TNF
+TNF+TRPC3siRNA

Time (seconds)
Current density (pA/pF)

0 50 100 150 200 250 300 350

0 50 100 150 200 250 300 350

0 50 100 150 200 250 300 350

0 50 100 150 200 250 300 350
Supplementary Figure III

A

Nominally Ca\(^{2+}\)-free

10 mM Ba\(^{2+}\)

100 µM ATP

+ATP = 0.06 ± 0.004

Constitutive = 0.016 ± 0.002

B

Receptor-activated influx

Constitutive influx

Rate of Ba\(^{2+}\) influx (% of Control)

Control

TRPC3siRNA

TRPC6siRNA

TRPC7siRNA

ns

ns

C

Rate of Ba\(^{2+}\) influx (% of Control)

Control

Gd3+

SKF

* * *
Supplementary Figure IV

NSsiRNA

Control

TNF

TRPC3

β-actin

TRPC3siRNA

Control

TNF

102 Kda
76 Kda
Supplementary Figure V

[Image of Western blot analysis showing TRPC6, TRPC7, and GAPDH proteins with molecular weights of 102 Kda and 76 Kda.]

NSsiRNA, TRPC6siRNA, TRPC7siRNA
Supplementary Figure VI

![Image of a Western blot showing the expression of VCAM-1 and GAPDH in different conditions: Control, TNF, NSsiRNA, TRPC3siRNA, and "Rescue". The blot shows bands at 102 Kda and 76 Kda.]
Supplementary Figure VII

NSsiRNA

ICAM-1

GAPDH

TRPC3siRNA

102 Kda

76 Kda

Control

TNF

Control

TNF
Supplementary Figure VIII

VCAM-1

GAPDH

Control
TNF
LPS
IL-1β

102 Kda
76 Kda
Supplementary Figure IX
Supplementary Figure X
Supplementary Figure X continued
Supplementary Figure XI

A  WtHEK293 cells
   +TNFα
   TRPC3
   GAPDH
   Control  3 h  6 h  16 h

B  T3-HEK293 cells
   +TNFα
   T3-GFP
   GAPDH
   Control  3 h  6 h  16 h

C  HCAECs
   IP: anti-HA
   IB: anti-HA
   T3-HA
   Mock-Tx cells
   T3-HA-Tx cells
Supplementary Figure XII

A

B

GAPDH

IkBα

Control 5 min 10 min 30 min 45 min 60 min

52 Kda

38 Kda

+TNFα
Supplementary Figure XIII

A

pIkBα

GAPDH

52 Kda

38 Kda

Control

TNF

PMA

TNF+PMA

B

pIkBα

GAPDH

52 Kda

38 Kda

Control

TNF

Go+TNF

Go+TNF
Chapter 5.

EVIDENCE FOR OPERATION OF NICOTINIC AND MUSCARINIC ACETYLCOLINE RECEPTOR-DEPENDENT SURVIVAL PATHWAYS IN HUMAN CORONARY ARTERY ENDOTHELIAL CELLS


Kathryn Smedlund¹, Jean-Yves Tano¹, Joseph Margiotta² and Guillermo Vazquez¹,*

¹Department of Physiology and Pharmacology, ²Department of Neurosciences, University of Toledo College of Medicine, Health Science Campus, 3000 Arlington Av, Toledo, Ohio 43614 USA

*To whom correspondence should be addressed at: Dept. of Physiology and Pharmacology, UTHSC Mail stop 1008, Toledo OH 43614 USA. E-mail: Guillermo.Vazquez@utoledo.edu

Tel: 419-383 5301

Fax: 419-383 2871

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**ABSTRACT**
Nicotinic acetylcholine receptors (nAChRs) have recently emerged as critical players in modulation of endothelial function. In particular, studies on endothelial cells from different vascular beds have shown anti-apoptotic actions of nicotinic stimulation, but whether there is actually activation of survival signaling downstream nAChR function has not been explored. In the present work we used human coronary artery endothelial cells (HCAECs) and a pharmacological approach to examine the impact of cholinergic stimulation on survival signaling pathways. Our findings show that cholinergic receptors promote activation of three typical survival routes: the phosphatidylinositol-3-kinase (PI3K)/AKT axis, activated downstream muscarinic and nicotinic acetylcholine receptors; the JAK2/STAT3 axis, activated downstream nAChR; and ERK1/2 MAP kinases, activated by both mAChR and nAChR. Based on their sensitivity to α-bungarotoxin, nicotinic regulation of JAK2/STAT3 and ERK1/2 occurs downstream α7-nAChRs. The present findings suggest that in HCAECs the two cholinergic receptors may act concertedly to induce an efficient survival response of coronary cells when exposed to pro-apoptotic stimuli.
INTRODUCTION

Over the last decade we have witnessed a significant progress in our knowledge of physiological and pathophysiological processes that are modulated by nicotinic acetylcholine receptors (nAChR) in non-neuronal tissues and organ systems. Indeed, many of the nAChR subtypes identified in neurons are also expressed in non-neuronal cells [Wessler and Kirkpatrick, 2008]. Among those is vascular endothelium, which exhibits a variety of functions that are, directly or indirectly, influenced by nicotinic cholinergic signaling, such as proliferation, migration, permeability changes in response to pro-inflammatory cytokines and angiogenesis, among others [Cooke and Ghebremariam, 2008; de Jonge and Ulloa, 2007]. Importantly, nAChR function is emerging as a critical modulator of cellular and molecular mechanisms linked to the pathogenesis of inflammatory vascular disease, such as atherosclerosis. Recent studies indeed indicate that nAChR function may play a role in atherogenesis by modulating plaque neovascularization [Zhang et al.], oxidative and phagocytic activity of macrophages [Wilund et al., 2009] and the pro-inflammatory and pro-apoptotic actions of cytokines on endothelial cells [Hakki et al., 2002]. The latter is of particular relevance to atherogenesis, as endothelial cell apoptosis is determinant in progression and fate of the lesion, particularly in advanced stages where the integrity of the endothelium covering or surrounding the plaque can be a defining factor to plaque stability and the precipitation of an acute coronary syndrome [Virmani et al., 2006]. It is thus of central importance to improve our understanding of the mechanisms underlying endothelial survival in order to identify components that might eventually be targeted to manipulate the endothelial cell’s life at the lesion site. Here we report that in human coronary artery endothelial cells
HCAECs) cholinergic stimulation results in activation of three major signaling limbs associated to survival: a phosphatidyl-inositol-3-kinase (PI3K)/AKT axis downstream stimulation of muscarinic (mAChR) and nicotinic (nAChR) acetylcholine receptors, nAChR-dependent activation of JAK2/STAT3 axis, and nicotinic and muscarinic-dependent stimulation of ERK1/2 MAP kinases. These findings suggest that in HCAECs nicotinic and muscarinic cholinergic signaling may act in a concerted manner to promote an efficient survival response of the coronary endothelium when cells are exposed to pro-apoptotic stimuli.

MATERIALS AND METHODS

Cell culture, immunoblotting and TUNEL assay were essentially as described by us [Smedlund et al., 2010; Smedlund and Vazquez, 2008; Tano and Vazquez, 2011]. Protocol details and antibody sources are provided in the online supplementary material.

RESULTS

In several cell types, including endothelial cells, activation of the phosphatidylinositol-3-kinase (PI3K)/AKT axis plays a key role in cell survival [Amaravadi and Thompson, 2005]. Based on existing evidence indicating an anti-inflammatory role of nicotinic acetylcholine receptors (nAChRs) and considering that those receptors are expressed in endothelial cells from diverse vascular beds [Cooke and Ghebremariam, 2008] we examined whether stimulation of nAChRs in HCAECs exerts a modulatory role on survival signaling. We first used the cholinomimetic agonist carbachol (CCh), which stimulates both muscarinic (mAChR) and nicotinic acetylcholine receptors and thus
better resembles the physiological scenario, in which the two receptor pathways would be activated by their natural ligand acetylcholine. As shown in Figure 1A, CCh (100 µM) induced a time-dependent activation of AKT, as indicated by the extent of phosphorylation of AKT on Ser473. AKT phosphorylation occurred as early as 5 min, peaked at 10-15 min (4-5 fold over control) and declined to pre-stimulation levels after 30-60 min (see Suppl. Fig. IA for densitometric analysis). When cells were pre-treated with the mAChR antagonist atropine (1 µM) the effects of CCh were drastically reduced, particularly at the 10-15 min time points (Fig. 1B and Suppl. Fig. IB), while AKT phosphorylation was still noticeable at later times (30-60 min). This indicated a priori that both mAChR and nAChR contributed, although to different extents and with a different temporal profile, to AKT activation in HCAECs (see Discussion). To specifically examine the involvement of nAChR, we next exposed HCAECs to nicotine (10 µM). Interestingly, this resulted in biphasic AKT phosphorylation, with a first peak at 5 min (~2-3 fold over control) and a delayed effect at ~60 min (Fig. 1C and Suppl. Fig. IC), reflecting a pattern similar to that observed with CCh plus atropine. These actions of nicotine were almost completely reduced by the nAChR antagonist d-tubocurarine (dTC, 100 µM; Fig. 1D and Suppl. Fig. ID), confirming that the atropine-insensitive activation of AKT that followed CCh treatment occurred downstream nAChR. Pre-treatment of HCAECs with the PI3K inhibitor LY294002 (10 µM) abrogated cholinergic-induced AKT phosphorylation (not shown) indicating that activation of AKT took place downstream PI3K. A major mechanism of AKT-dependent cell survival operates through AKT-mediated phosphorylation of its downstream target BAD, a pro-apoptotic Bcl-2 family member, on Ser136 [Amaravadi and Thompson, 2005]. Supplemental figure II
shows that CCh induced atropine-sensitive increase in phosphorylation of BAD (Ser136) which correlated well with the CCh-dependent activation of AKT. Nicotine treatment did not affect BAD phosphorylation (not shown).

In cell types other than endothelial, v.g., neurons and macrophages ([Marrero et al., 2011] and references therein) activation of STAT3 and the MAP family kinases ERK1/2 has been shown to occur downstream of AChR stimulation and represents an alternative pro-survival pathway to the PI3K/AKT axis. Notably, CCh treatment of HCAECs resulted in time-dependent activation of STAT3, as indicated by an increase in the extent of STAT3 phosphorylation on Tyr705 (Fig. 2A), which peaked around 5-10 min (~4-5-fold increase over control, see Suppl. Fig. IIIA) and rapidly declined to pre-stimulation levels. CCh-induced STAT3 phosphorylation was insensitive to atropine (Fig. 2B and Suppl. Fig. IIIB) suggesting stimulation downstream of AChRs. In line with this notion, levels of phospho (Tyr705)-STAT3 were significantly elevated following treatment with nicotine, and this effect was fully prevented by dTC (Fig. 2C and Suppl. Fig. IIIC). Nicotine-induced activation of STAT3 was markedly diminished by pre-treating cells with the JAK2 selective inhibitor AG490 (10 µM, Fig. 2C) suggesting operation of a nAChR/JAK2/STAT3 axis. In line with this, HCAECs treated with nicotine exhibited a time-dependent increase in JAK2 (Tyr221) phosphorylation that was abrogated by AG490 (Suppl. Fig. IVA-C). Notably, nicotine-dependent phosphorylation of JAK2 and STAT3 was suppressed by the α7-nAChR selective blocker α-bungarotoxin (100 nM; Fig. 2C and Suppl. Fig. IVD).

CCh treatment of HCAECs also resulted in time-dependent activation of ERK1/2 MAP kinases, manifested as an increase in ERK1/2 phosphorylation (Thr202/Tyr204 of ERK1,
Thr185/Tyr187 of ERK2) that peaked at 5 min following cholinergic stimulation (Fig. 3A). This effect was partially decreased by atropine (≈50% reduction; Suppl. Fig. VA) indicating nicotinic and muscarinic cholinergic contribution to ERK1/2 stimulation. In line with this, nicotine treatment also augmented the levels of phospho-ERK1/2 in a dTC-sensitive manner (Fig. 3B and Suppl. Fig. VB). Remarkably, nicotinic activation of ERK1/2 was completely prevented by α-bungarotoxin (100 nM; Fig. 3C) suggesting involvement of α7-nAChRs. In agreement with this interpretation, challenging HCAECs with the α7-nAChR selective agonist GTS-21 (30 µM; [Kem, 1997; Nai et al., 2003]) promoted ERK1/2 phosphorylation – although somewhat delayed compared to nicotine-in an α-bungarotoxin-sensitive manner (Suppl. Fig. VI). We recently showed that in HCAECs pro-inflammatory actions of the atherorelevant cytokine TNFα causes rapid (within 5 min) and sustained (up to 1 hour) activation of NFκB, based on the extent of IκBα phosphorylation/degradation ([Smedlund et al., 2010], and Suppl. Fig. VII).

Moreover, it has been shown that cholinergic stimulation can prevent endothelial cell activation and leukocyte recruitment through inhibition of NFκB [Saeed et al., 2005]. When HCAECs were challenged with carbachol (100 µM) or nicotine (10 µM) no significant changes were observed in IκBα phosphorylation or degradation, nor in the ability of TNFα (10 ng/ml) to activate NFκB (Suppl. Fig. VII). Also, cholinergic stimulation of HCAECs failed to induce VCAM-1 expression (not shown) in line with our previous finding that in these cells VCAM-1 is under complete control of NFκB [Smedlund et al., 2010].

Finally, we wanted to explore if stimulation of cholinergic receptors had an impact on the rate of apoptosis of HCAECs. To answer this important question we examined TNFα-
induced apoptosis in HCAECs in the absence or presence of cholinergic stimulation, by using a terminal deoxynucleotidyl-transferase-dUTP-nick end labeling (TUNEL) assay. HCAECs were incubated for 48 hours in endothelial basal medium with or without (control) TNFα (10 ng/ml), and with or without concomitant incubation with muscarinic or nicotinic acetylcholine receptor agonists and/or antagonists. As expected, TNFα treatment resulted in appearance of a significant number of apoptotic cells (~4-fold increase in TUNEL-positive cells) compared to control (Fig. 4). Cholinergic agonists or antagonists did not induce apoptosis when added alone to the medium. However, simultaneous incubation of cells with TNFα in the presence of nicotine or CCh, resulted in a statistically significant reduction of the number of TUNEL-positive cells compared to cells treated with the cytokine alone. Notably, blocking mAChR or nAChR completely prevented the protective actions of the cholinergic agonists.

DISCUSSION

The present work provides for the first time evidence showing that stimulation of nicotinic and muscarinic acetylcholine receptors triggers pro-survival signaling in endothelial cells derived from human coronary arteries (HCAECs). When the cholinomimetic carbachol was used to activate nAChR and mAChR, three survival signaling branches were activated: PI3K/AKT, JAK2/STAT3 and the MAPK family members ERK1/2 (see model, Suppl. Fig. VIII). An immediate speculation can be made, and is that under physiological conditions of receptor stimulation with acetylcholine as the endogenous ligand, concomitant cholinergic-dependent activation of those signaling
pathways likely accounts for an efficient survival response of coronary endothelial cells when exposed to an inflammatory setting.

Our results show that whereas both mAChR and nAChR activated PI3K/AKT in HCAECs, nicotinic stimulation of AKT exhibited a biphasic temporal course compared to a monophasic effect of muscarinic signaling. One interpretation is that muscarinic stimulation also triggers compensatory mechanisms that turn off AKT – not unusual in G protein coupled receptor modulation of AKT [Liu et al., 2004]-; as stimulation persists, deactivation takes over and masks the delayed nicotinic signaling when both mAChR and nAChR are simultaneously activated. Despite these differences, the characteristics of mAChR and nAChR stimulation of AKT were highly comparable to that of other well known activators of AKT in the coronary circulation [Erdogdu et al., 2010; Mahadev et al., 2008; Teng et al., 2011]. In several cell types activation of AKT by muscarinic receptors is subsequent to direct actions of either G \( g_{\alpha i} \) or G \( g_{\alpha q} \) –depending on the type of mAChR- and G \( g_{\beta\gamma} \) on both PI3K and AKT [Resende and Adhikari, 2009]. As for the nAChR, in rat pheochromocytoma PC12 cells nicotine-dependent stimulation of PI3K/AKT occurs downstream JAK2, and involves direct interaction between \( \alpha 7 \)-nAChR and JAK2; this is followed by tyrosine phosphorylation of JAK2, activation of PI3K and recruitment and activation of AKT [Shaw et al., 2002]. In non-neuronal cell types, such as macrophages or microglia, nAChR activation of PI3K/AKT seems to be \( \text{Ca}^{2+} \)-dependent, but is not clear if this is subsequent to ion channel \( \text{Ca}^{2+} \) fluxes or related to nAChR-dependent activation of PLC and \( \text{Ca}^{2+} \) release from stores ([de Jonge and Ulloa, 2007] and references therein). Molecular and pharmacological studies will be required to
determine how in HCAECs activation of mAChR or nAChR couples to activation of PI3K/AKT.

In macrophages, cholinergic activation of the JAK2/STAT3 pathway takes place downstream α7-nAChR [Marrero et al., 2011]. Our observation that nicotine-induced phosphorylation of JAK2 and STAT3 are inhibited by α-bungarotoxin, represent the first evidence to date indicating activation of the JAK2/STAT3 axis downstream α7-nAChRs in human coronary endothelial cells. Unlike our findings in HCAECs, recent work showed that nicotine treatment of human umbilical vein and dermal microvascular endothelial cells inhibits JAK2/STAT3 [Chatterjee et al., 2009]. Rather than contradictory, these findings likely reflect heterogeneity of endothelial cells derived from different vascular beds in regards to their responsiveness to cholinergic stimulation.

Similar to the PI3K/AKT axis, cholinergic activation of ERK1/2 in HCAECs is contributed by both mAChR and nAChR. Notably, nicotinic stimulation of ERK1/2 was also completely abolished by α-bungarotoxin, again indicating involvement of α7-nAChRs. Interestingly, growth factor and activity-dependent survival of chick ciliary ganglion neurons is promoted by α7-nAChRs, at least in part, through ERK1/2-mediated signaling [Pugh and Margiotta, 2000; Pugh and Margiotta, 2006; Pugh et al., 2006]. It is thus tempting to speculate that α7-nAChR-dependent modulation of JAK2/STAT3 and ERK1/2 pathways represents a general mechanism of nicotinic cholinergic regulation of survival in different cell types. In the case of HCAECs, the question arises as to how persistent activation of nAChRs, as expected to occur in smokers which have sustained circulating levels of nicotine, affects the balance of mAChR vs. nAChR survival signaling in the context of coronary pathology.
Importantly, the present studies also show a *bona fide* anti-apoptotic effect of sustained cholinergic stimulation when HCAECs were exposed to inflammatory conditions. In HCAECs nicotine has been shown to reduce apoptosis induced by combined treatment with TNFα and dexamethasone [Hakki et al., 2002]. However, in these cells dexamethasone interferes with inflammatory signaling [Zouki et al., 2000] making it difficult to conclude if those results were subsequent to a genuine anti-apoptotic action of nicotine rather than a combined action of nicotinic and glucocorticoid signaling on expression/function of pro-apoptotic molecules. Our findings showing lack of cholinergic activation of the NFκB pathway or VCAM-1 induction in HCAECs are in line with the notion of a pro-survival, protective rather than pro-inflammatory role of cholinergic signaling in these cells. In the particular case of nAChR signaling in endothelial cells from coronary circulation, studies aimed at evaluating nicotine effects have yielded rather controversial results. For example, the expression of VCAM-1 and ICAM-1, key mediators of monocyte recruitment, was shown to be augmented by nicotine treatment of HCAECs in one study [Cirillo et al., 2007] while work from other laboratory showed no effect [Hakki et al., 2002]. Whereas these controversial findings may be partly due to differences in experimental conditions, it is also possible that the high membrane permeability of nicotine may induce effects beyond those strictly related to its actions on nAChRs, especially during prolonged incubation times with the alkaloid. Within this context, by showing equivalent outcomes when stimulating nAChR with either nicotine or carbachol - but blocking mAChR signaling with atropine- our findings strongly indicate operation of a genuine nicotinic cholinergic effect on survival signaling rather than non-specific effects of nicotine.
We have not been able to detect cholinergic-induced Ca\(^{2+}\) influx in HCAECs by means of conventional Fura-2-based Ca\(^{2+}\) imaging techniques (not shown). However, these cells have a very efficient Ca\(^{2+}\) buffering system [Smedlund and Vazquez, 2008] and thus the possibility exists that Ca\(^{2+}\) entering through nAChRs is rapidly coped by the buffering apparatus and goes undetectable. Using rat coronary microvascular endothelial cells Adams and colleagues [Moccia et al., 2004] showed the existence of functional nAChRs which mediate inward currents carried primarily by Na\(^+\). Additional studies are needed to define if in HCAECs nAChRs exhibit similar permeability properties. This will also define whether the role of nAChR in survival signaling in HCAECs is related to channel function or if it merely reflects a signaling role independent of channeling properties, as shown in cell types other than endothelial [de Jonge and Ulloa, 2007]. Given the fundamental role of endothelial cell survival/apoptosis in atherogenesis, the present findings, although strictly pharmacological, represent a timely and novel contribution to our understanding of molecular events that might be of relevance for cholinergic-dependent modulation of coronary endothelial cell survival in the inflammatory setting of atherosclerosis.

**ACKNOWLEDGEMENTS**
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LITERATURE CITED


FIGURE LEGENDS

Figure 1.
HCAECs were treated with carbachol (CCh, 100 µM, “A” and “B”) or nicotine (10 µM, “C” and “D”) for the indicated times in the absence (“A” and “C”) or presence of atropine (“Atr”, 1 µM, “B”) or d-tubocurarine (“dTC”, 100 µM, “D”). In all instances, cells were processed for immunodetection of phospho-AKT (Ser473; 60 kDa) in whole cell lysates. When indicated, atropine and dTC were added 15 min before stimulation and kept throughout the entire duration of the experiment. Membranes were reprobed for GAPDH (37 kDa) to control for protein loading. Blots are representative from at least three independent experiments. In “A”, positions of the 52 Kda and 76 Kda molecular weight markers are indicated on right side of the blot for reference.

Figure 2.
HCAECs were treated with carbachol (CCh, 100 µM) for the indicated times in the absence (“A”) or presence (“B”) of atropine (“Atr”, 1 µM) and then processed for immunodetection of phospho-STAT3 (Tyr705; ~80 kDa) in whole cell lysates. Alternatively, cells were exposed to nicotine (10 µM, “C”) for the indicated times in the absence or presence of d-tubocurarine (“dTC”, 100 µM), AG490 (10 µM) or α-bungarotoxin (“αBgt”, 100 nM, 15 min pre-incubation) before immunodetection of phospho-STAT3 (Tyr705). When indicated, dTC and AG490 were added 15 min before stimulation. Membranes were reprobed for total STAT3 to control for protein loading. Blots are representative from at least three independent experiments. In “A”, positions of
the 76 Kda and 102 Kda molecular weight markers are indicated on right side of the blot for reference.

**Figure 3.**

**A)** HCAECs were treated with carbachol (CCh, 100 µM) for the indicated times in the absence or presence of atropine (“Atr”, 1 µM, 15 min pre-treatment) and then processed for immunodetection of phospho-ERK1/2 (Thr202/Tyr204 of ERK1, Thr185/Tyr187 of ERK2; 42/44 kDa) in whole cell lysates. Alternatively, cells were exposed to nicotine (10 µM, “B”) for the indicated times in the absence or presence of d-tubocurarine (“dTC”, 100 µM, 15 min pre-treatment) or **C)** α-bungarotoxin (“αBgt”, 100 nM, 15 min pre-incubation), as indicated, before immunodetection of phospho-ERK1/2. Membranes were reprobed for total ERK1/2 to control for protein loading. Blots are representative from at least three independent experiments. In “A”, positions of the 38 Kda and 52 Kda molecular weight markers are indicated on right side of the blot for reference.

**Figure 4.**

HCAECs were grown on 24-well plates and exposed for 48 hours (37°C in humidified air/5% CO₂ atmosphere) to endothelial basal medium in the absence (“Control”) or presence of TNFα (“TNF”, 10 ng/ml), nicotine (“N”, 10 µM), carbachol (“CCh”, 100 µM), atropine (“Atr”, 1 µM) or d-tubocurarine (“dTC”, 100 µM), or the indicated combinations. Following treatments HCAECs were processed for evaluation of apoptosis by TUNEL assay, as described in Methods. aP<0.01 respect to “Control”; bP<0.05 respect to “TNF” alone; cP<0.05 respect to “N + TNF” or “CCh + TNF”. For each experimental
condition the change in number of apoptotic (TUNEL-positive) cells was normalized to
the respective control and results expressed as fold respect to control.
**Figure 1**

(A) +CCh  
(B) +CCh + Atr  
(C) +Nicotine  
(D) +Nicotine + dTC  

(A) pAKT  
(B) pAKT  
(C) pAKT  
(D) pAKT  

GAPDH  
GAPDH  
GAPDH  
GAPDH  

Control  5 min  10 min  15 min  30 min  60 min  
Control  5 min  10 min  15 min  30 min  60 min  
Control  5 min  10 min  15 min  30 min  60 min  
Control  5 min  10 min  15 min  30 min  60 min  

---

145
Figure 2
Figure 3

A

+CCh  +CCh + Atr

pERK1/2

ERK1/2

Control  5 min  10 min  Control  5 min  10 min

B

+Nicotine  + dTC

pERK1/2

ERK1/2

Control  5 min  10 min  Control  5 min  10 min

C

+Nicotine + αBgt

pERK1/2

ERK1/2

Control  5 min  10 min  15 min  30 min  60 min
Figure 4

TUNEL positive HCAECs (fold change over control)
Supplementary Figure I

A +CCh

B +CCh + Atr

C +Nicotine

D +Nicotine + dTC

(pAKT/GAPDH (fold change over control))

- Control
- 5
- 10
- 50
- 100

- Control
- 5
- 10
- 50
- 100

- Control
- 5
- 10
- 50
- 100

- Control
- 5
- 10
- 50
- 100
Supplementary Figure II

A

<table>
<thead>
<tr>
<th>+CCh</th>
<th>pBAD</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 min</td>
<td>10 min</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>+CCh + Atr</th>
<th>pBAD</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 min</td>
<td>10 min</td>
</tr>
</tbody>
</table>
Supplementary Figure III

A

B

C

** +CCh

+Nicotine
Supplementary Figure IV

A

+Nicotine

pJAK2

GAPDH

Control 5 min 10 min 15 min 30 min 60 min

B

pJAK2/GAPDH (fold change over control)

0 1 2 3 4 5 6

C

+Nicotine + AG490

pJAK2

GAPDH

Control 5 min 10 min 15 min 30 min 60 min

D

+Nicotine + aBgt

pJAK2

GAPDH

Control 5 min 10 min 15 min 30 min 60 min
Supplementary Figure V

A

B

**+Nicotine**

**+CCh**

**+CCh + Atr**

*pERK1,2/ERK1,2 (fold change over control)*

0

0.5

1

1.5

2

2.5

3

3.5

4

5

6

**+CCh**

**+CCh + Atr**

*pERK1,2/ERK1,2 (fold change over control)*

0

0.5

1

1.5

2

2.5

3

3.5

4

5

6

**+Nicotine**

*pERK1,2/ERK1,2 (fold change over control)*

0

0.5

1

1.5

2

2.5

3

3.5

4

5

6
Supplementary Figure VI

A

+GTS-21

pERK1/2

ERK1/2

Control

5 min

10 min

15 min

30 min

60 min

B

+GTS-21 + αBgt

pERK1/2

ERK1/2

Control

5 min

10 min

15 min

30 min

60 min

C

[Graph showing fold change over control for pERK1,2/ERK1,2]

0

0.5

1

1.5

2

2.5

3

3.5

4

Fold change over control
Supplementary Figure VII

A
B
C
D
E

pIkBa
GAPDH
Control  TNF
Nicotine

IkBa
GAPDH
Control  5 min 10 min 15 min 30 min 60 min

pIkBa
GAPDH
Control 5 min 10 min 15 min 30 min 60 min

IkBa
GAPDH
Control  5 min 10 min 15 min 30 min 60 min

IkBa
GAPDH
Control  5 min 10 min 15 min 30 min 60 min

IkBa
GAPDH
Control  5 min 10 min 15 min 30 min 60 min

+CCh

+CCh

+CCh
Supplementary Figure VIII

mACHR

PI3K

AKT

BAD

ERK 1/2

ERK 1/2

JAK2

STAT3

Apoptosis

Survival genes
Chapter 6.

Molecular and Cellular Aspects of Atherosclerosis: Emerging Roles of TRPC Channels

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Guillermo Vazquez, Kathryn Smedlund, Jean-Yves K. Tano and Robert Lee
Department of Physiology and Pharmacology, University of Toledo Health Science Campus, 3000
Arlington Av, Toledo OH 43614

Author Contributions:

Section 3.1. TRPC3 and regulated expression of VCAM-1.

1. Overview of molecular and cellular events in atherogenesis

1.1. Endothelial inflammatory signaling and monocyte recruitment

Recruitment of circulating monocytes to activated areas of the endothelium and their migration to the subintimal inflammatory foci represents one of the earliest events in atherogenesis (Linton and Fazio 2003; Hansson 2005). Importantly, monocyte recruitment can be recognized throughout all lesional stages including advanced lesions, where plaque infiltration and neovascularization occur. Indeed, available experimental evidence supports the notion that in advanced stages monocyte infiltration contributes to
plaque instability and rupture (Virmani, Burke et al. 2006). Monocyte recruitment to the subendothelial milieu implies a sequence of events that begin with monocyte rolling along and tethering to the endothelial surface, firm adhesion and activation, and ultimately migration to the subintima. At the molecular level, the entire sequence entails interaction of integrins on the monocyte surface with cell adhesion molecules (CAMs) expressed on the endothelial cell. Monocyte rolling and tethering is mainly mediated by CAMs from the selectin group (v.g., E-selectin) while firm adhesion and migration are mostly mediated by CAMs from the immunoglobulin (Ig) superfamily, such as intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Compelling evidence accumulated over the last decade has clearly established that VCAM-1 (CD106) has a prominent role in mediating attachment and migration of monocytes (the Cluster of Differentiation nomenclature (Zola, Swart et al. 2005) is given for reference, but “VCAM-1” will be used throughout the text).

1.1.1. VCAM-1 expression and atherosclerosis

Although other adhesion molecules, such as ICAM-1 (CD54) or E-selectin, also contribute to adhesion of monocytes to endothelial cells, VCAM-1 is unique in that its expression level and pattern are highly sensitive to the action of several pro-inflammatory/pro-atherogenic stimuli. While other CAMs are constitutively expressed in non-activated endothelium, VCAM-1 is virtually absent, but its markedly upregulated when endothelium is exposed to atherorelevant stimuli (Galkina and Ley 2007). For example, in hypercholesterolemic animals both ICAM-1 and VCAM-1 are induced in
early lesions. However, VCAM-1 expression is largely restricted to lesions, or to sites prone to lesion formation, and can also be detected even before the onset of visible fatty streaks, while ICAM-1 also extends into uninvolved aorta and lesion-protected regions (Iiyama, Hajra et al. 1999). This dissimilar pattern immediately suggested different functions for ICAM-1 and VCAM-1, at least in lesion initiation. A more direct comparison of VCAM-1 and ICAM-1 in atherosclerosis was possible through generation of mice homozygous for a VCAM-1 molecule lacking the Ig-like extracellular domain 4 (Vcam1^{D4D/D4D}) which partially circumvents the embryonic lethality of Vcam-1^{-/-} mice. Using such mouse model it was possible to show that whereas both ICAM-1 and VCAM-1 are upregulated in lesions, VCAM-1 has a dominant role in early lesion formation (Cybulsky, Iiyama et al. 2001; Dansky, Barlow et al. 2001). Atherorelevant stimuli such as tumor necrosis factor-α (TNFα), locally released nucleotides, vascular endothelial growth factor (VEGF) or oxidized low density lipoprotein (oxLDL), are potent inducers of VCAM-1 expression (Galkina and Ley 2007). Depending on the stimulus and/or the regional location of the endothelial cell along the vascular bed, VCAM-1 expression is regulated through Nuclear Factor kappaB (NFκB), Nuclear Factor of Activated T cells (NFAT) or both (Armesilla, Lorenzo et al. 1999; Kim, Moon et al. 2001; Yao and Duh 2004).

1.1.2. VCAM-1 structure and function

Human VCAM-1 is a single transmembrane protein with seven Ig-like extracellular domains (1-7) but can also be expressed as a form with only domains 1-3 and 5-7 (Chuluyan, Osborn et al. 1995). The extracellular domains 1 and 4 mediate specific
binding to the integrin $\alpha_4\beta_1$ (Very Late Antigen 4, VLA-4) on the monocyte facilitating firm adhesion to the endothelium. The cytoplasmic domain is only 19 amino acids long, and has a PDZ-binding motif, but specific interactions or functional relevance remain unknown. Interaction of VCAM-1 with $\alpha_4\beta_1$ conveys a conformational message through the transmembrane domain towards the cytosolic region, triggering intracellular signaling events. Importantly, VCAM-1-dependent signaling is a prerequisite for successful migration of bound monocytes (Matheny, Deem et al. 2000; Deem, Abdala-Valencia et al. 2007). Some salient features within this signaling are the recruitment and activation of the Rho-family GTPase Rac-1, Rac-1 dependent stimulation of NADPH oxidase and production of hydrogen peroxide ($H_2O_2$) (van Wetering, van den Berk et al. 2003). VCAM-1-dependent release of $H_2O_2$ to the extracellular milieu is thought to contribute to activation of matrix metalloproteases and the increased endothelial permeability observed during atherogenesis. This may be particularly important in advanced lesions by creating additional endothelial damage and plaque instability (Virmani, Burke et al. 2006). Calcium ($Ca^{2+}$) release and influx subsequent to VCAM-1-$\alpha_4\beta_1$ interaction or antibody-induced VCAM-1 crosslinking have also been related to the signaling required for monocyte migration (Isabelle Ricard 1997; Cook-Mills 2002; Cook-Mills, Johnson et al. 2004). However, neither the nature of the channels involved nor the underlying mechanism/s are yet known. In coronary artery endothelial cells of human origin (HCAECs) activation of VCAM-1 by antibody-induced crosslinking results in approximately three-fold increase in the rate of cation influx compared to non-treated cells (Smedlund and Vazquez, unpublished observations). Such increase in cation influx occurs over the existing constitutive influx, and thus is possible that both constitutive and
regulated activity of cation channels play a role. Because TRPC3 accounts for most of the constitutive cation entry in HCAECs and also significantly contributes to regulated influx (Vazquez and Putney 2006; Smedlund and Vazquez 2008) it is likely that TRPC3 represents a component of the signaling underlying VCAM-1-dependent monocyte transmigration.

1.2. Role of Macrophage survival and apoptosis in lesion development

As mentioned above, monocyte recruitment to the subintima is a fundamental event in atherogenesis (Linton and Fazio 2003). Transmigration of the monocyte to the subendothelial milieu is followed by its differentiation into macrophage, which is now recognized as a key cell component in determining lesion progression and fate. Lesional macrophages engulf modified lipoproteins, mostly oxLDL, becoming lipid-laden macrophages; this results in a lipid overload of the macrophage which imposes a significant stress to the endoplasmic reticulum (ER), mostly due to accumulation of free cholesterol. Consequently, an irreversible ER-stress response is triggered leading to macrophage apoptosis. Indeed, the majority of apoptotic lesional cells are macrophages (Linton and Fazio 2003; Tabas 2010). Clearance of apoptotic cells is conducted by resident phagocytes, which phagocytose the apoptotic macrophage and exit the lesion site through lymphatic circulation or by migrating back to the blood stream. This clearing process, known as efferocytosis, is crucial in preventing the apoptotic cells from dying in situ which would otherwise lead to post-apoptotic necrosis and the subsequent exacerbation of the inflammatory response. Notably, the balance between production of apoptotic macrophages and their clearance by efferocytosis constitutes a defining factor.
in lesion formation, remodeling and progression (Tabas 2010) and references therein. For instance, macrophage apoptosis in early lesions is beneficial in that reduces lesion cellularity and size and plaque progression (Arai, Shelton et al. 2005; Liu, Thewke et al. 2005; Babaev, Chew et al. 2008; Wang, Liu et al. 2008) while increased apoptosis in advanced plaques enlarges the necrotic core and promotes plaque instability (Linton, Babaev et al. 1999; Seimon, Wang et al. 2009; Yancey, Blakemore et al. 2010). Thus, altered expression and/or deregulation of signaling proteins directly or indirectly involved in macrophage survival/apoptosis can have a significant impact on lesion progression.

2. TRPC channels

2.1. Structure, function and role in cardiovascular disease

Calcium influx has long been recognized as an essential component of physiological and pathophysiological events. Changes in intracellular Ca\(^{2+}\) concentration that follow Ca\(^{2+}\) influx through plasma membrane Ca\(^{2+}\) channels not only modulate a myriad of Ca\(^{2+}\)-dependent signaling pathways but also affect the driving force for other ions by modifying the membrane potential. Ca\(^{2+}\) influx is of particular importance in vascular function and cardiovascular disease, where the effects of Ca\(^{2+}\) influx can be seen throughout the entire cardiovascular system, in smooth muscle and endothelial cells, cardiomyocytes, lymphocytes, monocytes, macrophages, among other cell types. Of the many Ca\(^{2+}\) channels identified in the last half century, Transient Receptor Potential Canonical (TRPC) channels are recognized as major contributors to Ca\(^{2+}\) influx and play a role in various physiological and pathological states. The TRPC family belongs to the TRP superfamily of ion channel forming proteins, and are the most closely related to the
founding member *Drosophila* TRP protein (Vazquez, Wedel et al. 2004). TRPC proteins can be grouped into four subgroups, TRPC1, TRPC2 (a pseudogene in humans), TRPC3/6/7 and TRPC4/5 (Trebak, Vazquez et al. 2003; Vazquez, Wedel et al. 2004). Despite some structural variation across the subgroups of the TRPC family, there are several structural motifs which are conserved throughout members. The cytoplasmic N- and C-termini are separated by six transmembrane domains (TM1-TM6), with a re-entry loop between TM5 and TM6 which is thought to line the wall of the channel pore ((Vazquez, Wedel et al. 2004) and references therein). Other shared structural motifs of TRPCs include ankyrin repeats and a putative caveolin binding site on the N-terminus, and on the C-terminus the so called TRP signature motif (EWKFAR), a proline-rich motif and a calmodulin/IP₃ receptor binding (CIRB) site; predicted coiled-coil regions are present on both N- and C-termini, and in TRPC4 and 5, an extended C-terminus includes a PDZ binding motif (Vazquez, Wedel et al. 2004). An examination of the function of the TRPC cytoplasmic motifs hints at mechanisms of channel activation and signaling pathways. For instance, ankyrin repeats form specialized structures with the repeated units stacking against one another to form a protein-binding interface; this allows for interaction with other proteins and seems to play a role in channel trafficking to the plasma membrane. Coiled-coil regions are commonly associated with oligomerization and may contribute to formation of specific homo- and heterotetramers of TRPCs or association with other proteins containing coiled-coil motifs (Vazquez, Wedel et al. 2004). The proline-rich motif and CIRB region have also been associated to interactions with different signaling molecules, with variations existing throughout individual TRPC members. The mechanisms underlying activation and regulation of TRPC channels has
been matter of extensive research and debate, with efforts mostly centered at elucidating whether they form store-operated (activated by mere depletion of internal $\text{Ca}^{2+}$ stores) or non-store-operated channels (discussed in (Trebak, Vazquez et al. 2003; Vazquez, Wedel et al. 2004; Smyth, DeHaven et al. 2006). Whereas some properties of TRPC channels observed in heterologous expression systems correlate well with those of TRPCs expressed under native conditions, many others do not. It is imperative to elucidate the mechanism/s underlying regulation of TRPC channels in their native environment, as this would greatly contribute to assign definitive roles to individual TRPC members.

Nevertheless, equally important to comprehend their role in cardiovascular physiology and disease is to identify cellular and molecular events which, directly or indirectly, may rely upon appropriate TRPC function.

TRPC proteins are ubiquitously expressed throughout the cardiovascular system and hematopoietic cells and all members have been implicated not only in physiological cardiovascular functions but most importantly, in the pathogenesis of cardiovascular disease. Indeed, TRPCs have been implicated in a variety of processes known to be critical in cardiovascular pathology such as endothelial dysfunction, vascular relaxation, oxidative stress, and angiogenesis among others. This has recently been reviewed by us (Tano, Smedlund et al. 2010) and others (Abramowitz and Birnbaumer 2009) and the reader is referred to those for further details. The following sections focus on recent findings that specifically point to a potential role of TRPC3 in atherorelevant processes.

3. Participation of TRPC3 in atherorelevant molecular/cellular processes

3.1. TRPC3 and regulated expression of VCAM-1
Calcium signaling is an important component of the mechanism by which several inflammatory factors induce VCAM-1 expression. For instance, changes in intracellular Ca\(^{2+}\) associated to Ca\(^{2+}\) release from internal stores have been linked to the ability of Substance P to induce VCAM-1 subsequent to Ca\(^{2+}\)-dependent activation of NFAT and NFκB in microvascular endothelium (Quinlan, Naik et al. 1999), and of β\(_2\)-microglobulin to induce VCAM-1 expression in synovial fibroblasts (Chen, O’Neill et al. 2002). In the human coronary endothelial cells HCAEC, Ca\(^{2+}\) mobilization contributes to lipoprotein A- and ATP-dependent VCAM-1 expression (Allen, Khan et al. 1998; Seye, Yu et al. 2003). Nevertheless, the specific role of Ca\(^{2+}\) influx in VCAM-1 expression was never directly examined before. In recent work we specifically explored the impact of Ca\(^{2+}\) influx in regulated expression of VCAM-1 in HCAECs (Vazquez and Putney 2006; Smedlund and Vazquez 2008). Our studies demonstrated that, whereas HCAECs express all members of the TRPC family, only TRPC3 forms, or is part of, endogenous Ca\(^{2+}\)-permeable channels that contribute to ATP stimulated Ca\(^{2+}\) influx. Such mechanism occurs downstream ATP-dependent activation of purinergic P2Y\(_2\) receptors and results in upregulation of VCAM-1 total and plasma membrane associated levels with subsequent increase in monocyte adhesion (Smedlund and Vazquez 2008). This represented the first direct indication that Ca\(^{2+}\) influx plays a role in the signaling driving VCAM-1 expression and that TRPC3 forms native Ca\(^{2+}\)-permeable channels whose activity is fundamental within the signaling underlying VCAM-1 expression and monocyte recruitment. Because TRPC3 is the only TRPC member whose high constitutive, non-regulated activity has been shown to operate under either heterologous or native expression conditions (Trebak, Vazquez et al. 2003) those findings raised the question
whether TRPC3 contributes to expression of VCAM-1 through regulated activity, constitutive activity, or both. In a follow up study using TNFα to induce VCAM-1 expression and a combination of real-time fluorescence and silencing RNA approaches, we conclusively showed that it is the constitutive function of TRPC3 which mediates most of the Ca\(^{2+}\) influx required for regulated expression of VCAM-1 in HCAECs (Smedlund and Vazquez 2008).

In most endothelial cells VCAM-1 expression is regulated, at the transcriptional level, by nuclear factor kappa B (NFkB)(Zhang 2008) and we have shown that this is also the case in HCAECs. NFkB activation involves its release from the inhibitory protein IkBα and then the subsequent translocation of NFkB to the nucleus where it modulates transcriptional activity of target genes. Release of NFkB from IkBα is preceded by phosphorylation of IkBα by IkBα kinases (IKKs) followed by its ubiquitination and proteosomal degradation (Tergaonkar 2006). Because in most cells types examined so far NFkB activation depends, directly or indirectly, on Ca\(^{2+}\) influx, and TNFα-induced VCAM-1 requires constitutive Ca\(^{2+}\) influx (Smedlund, Tano et al. 2010), we examined whether TRPC3, through its constitutive function, contributes to the mechanism by which NFkB modulates VCAM-1 expression in HCAECs. Interestingly, our studies showed that knockdown of TRPC3 in HCAECs drastically reduced the ability of TNFα to induce phosphorylation of IkBα and its upstream regulator IKKβ (Smedlund, Tano et al. 2010), and this correlated with an inhibition of IkBα degradation. These findings indicated for the first time that TRPC3 constitutive function is an obligatory component in the signaling driving TNFα-dependent activation of NFkB. In addition, we showed that TRPC3-mediated Ca\(^{2+}\) entry is fundamental to activate the calmodulin
(CAM)/calmodulin kinase II (CAMKII) axis in a NADPH oxidase-dependent manner, and this signaling axis in turn activates NFkB (Smedlund, Tano et al. 2010). Importantly, our studies brought about a conceptually novel perspective on the role of TRPCs in cardiovascular disease, as they underscored for the first time, the potential pathological impact of upregulated expression of a TRPC channel endowed with high constitutive activity and how this may relate with pathological Ca\(^{2+}\)-dependent signaling, independently of the canonical pathway driven by receptor stimulation. This is of particular interest to the field, as in those instances where TRPCs participate in mechanisms associated to inflammatory vascular disease (reviewed by us in (Tano, Smedlund et al. 2010)) their contribution relates to regulated, or receptor-dependent channel function, rather than constitutive activity. In vivo studies are underway using mouse models of atherosclerosis with genetically manipulated levels of endothelial TRPC3 in order to determine the potential impact of TRPC3 expression and constitutive function in the context of the molecular and cellular events that lead to atherosclerotic lesion development in the intact vessel.

3.2. TRPC3 and macrophage survival
As stated earlier (section 2.2) the balance between apoptotic macrophages and their clearance by resident phagocytes at the lesion site is determinant in the progression of the atherosclerotic lesion. Within this context, signaling events that modulate the survival rate of the macrophage have a tremendous impact on such balance, provided efferocytic properties of resident phagocytes remain unaffected. Recent studies in our laboratory have implicated non-regulated, constitutive Ca\(^{2+}\) influx in the signaling associated with
macrophage survival. Two major pathways are essential for the survival of macrophages in the atherosclerotic lesions: the phosphatidylinositol-3-kinase (PI3K)/AKT axis and the NFκB route. In the PI3K/AKT pathway, macrophage survival signals (e.g., insulin-like growth factor, prostaglandin E2) acting through either receptor tyrosine kinases or G-protein coupled receptors induce activation of PI3K in the plasma membrane and the subsequent generation of 3’-phosphorylated phosphoinositides such as phosphatidylinositol 3, 4 bisphosphate/3,4,5 trisphosphate. These phosphoinositides allow for recruitment and activation of PDK-1 which then leads to full activation of AKT kinase. One of the major mechanisms of AKT-dependent survival takes place through AKT-mediated phosphorylation of the pro-apoptotic protein BAD, a member of the Bcl-2 family. Upon AKT-mediated phosphorylation, BAD releases the anti-apoptotic proteins Bcl-2 and Bcl-x, preventing mitochondrial release of cytochrome c and thus progression of the mitochondrial apoptotic pathway (Datta, Brunet et al. 1999). As for macrophage survival through the transcription factor NFκB, it is known that activation of NFκB exquisitely regulates the transcriptional status of several survival genes. Both of the survival pathways described above are highly active in THP-1 derived macrophages (TDMs) upon exposure to the atherorelevant cytokine TNFα (Tano and Vazquez 2011). Notably, maneuvers that prevent constitutive Ca^{2+} entry through Ca^{2+} permeable channels drastically reduce the phosphorylation of IkBα, AKT and its downstream target BAD, with the subsequent increase in macrophage apoptotic rate (Tano and Vazquez 2011). In addition, when TDMs are pre-treated with pharmacological inhibitors of CAM and CAMKII, activation of survival signaling is prevented as efficiently as blockade of constitutive Ca^{2+} influx does. These findings indicated for the first time that activation of
macrophage survival pathways depends, to a significant extent, on constitutive Ca\(^{2+}\) influx presumably through a mechanism involving the CAM/CAMKII axis (Tano and Vazquez 2011). A particularly interesting observation derived from those studies was that inhibition of PI3K function completely abrogated TNFα-dependent NFkB activation suggesting that the PI3K/AKT axis exerts a regulatory action on the NFkB pathway. Operation of such crosstalk has been demonstrated in cell types other than macrophages, where AKT-dependent transactivation of NFkB acts as an alternative AKT-dependent anti-apoptotic route independently of the AKT/BAD axis (Romashkova and Makarov 1999; Madrid, Wang et al. 2000; Bai, Ueno et al. 2009). In summary, our studies suggest that in human macrophages a CAM/CAMKII axis links constitutive Ca\(^{2+}\) influx to activation of AKT, which then serves as a signaling node to promote survival through NFkB and/or phosphorylation of BAD.

Macrophages from both human and mouse origin express TRPC3, and TRPC3 constitutive function has been shown to be operational in different cell types from these two mammalian species. These attributes make TRPC3 a great candidate to mediate the constitutive Ca\(^{2+}\) influx that supports the macrophage survival mechanisms described above. We indeed examined this possibility in recent studies that made use of bone-marrow derived macrophages obtained from mice globally deficient in TRPC3 (Tano, Smedlund et al. 2011). Macrophages derived from TRPC3 deficient bone marrow (TRPC3\(^{-/-}\)) exhibited a significant reduction in constitutive cation influx compared to TRPC3\(^{+/+}\) cells (Tano, Smedlund et al. 2011). Most importantly, the number of apoptotic macrophages in response to TNFα was significantly higher in TRPC3\(^{-/-}\) cultures than in those of TRPC3\(^{+/+}\) macrophages, indicating a diminished survival in macrophages lacking
TRPC3. Importantly, these observations correlated very well with the activation status of survival signaling: the phosphorylation of IkBα, AKT and BAD was severely reduced in TRPC3−/− macrophages (Tano, Smedlund et al. 2011). Altogether, these findings indicated that TRPC3 has an obligatory role in macrophage survival and that TRPC3 is likely to mediate the constitutive Ca^{2+} influx required for proper operation of survival signaling.

As described above, clearance of apoptotic macrophages by resident phagocytes at the lesion site is fundamental for appropriate inflammation resolution, and is a major factor in determining lesion cellularity. By means of an in vitro efferocytosis assay in which TRPC3^{+/+} and TRPC3^{−/−} were used as either phagocytes or apoptotic cells, we observed that the phagocytic function of TRPC3^{−/−} macrophages is drastically impaired when compared to that of TRPC3^{+/+} phagocytes; interestingly, apoptotic TRPC3^{−/−} cells seem to be poor substrates for phagocytosis regardless of the phagocyte’s TRPC3 expression status (Tano, Smedlund et al. 2011). Although additional studies are required to clarify TRPC3’s role in efferocytosis, these findings suggest a critical requirement for TRPC3 within the signaling associated to phagocytic activity and/or cell-cell recognition processes that underlie efferocytosis.

4. TRPC3 as a prospective target in atherosclerosis: roadmap of an exciting TRiP

The rapid advance in elucidating signaling mechanisms associated to atherogenesis was enthusiastically perceived as an opportunity to develop anti-inflammatory strategies to manage the disease. However, the multifactorial nature of atherosclerosis makes such therapeutic strategies, often aimed at interfering on single targets, of limited efficacy and
it is likely that multiple targeting is necessary to achieve clinically significant outcomes (Yonekawa and Harlan 2005; Preiss and Sattar 2007; Recio-Mayoral, Kaski et al. 2007). This is not surprising if we take in consideration the multifactorial nature of atherosclerosis and the diverse repertoire of signaling molecules and cell types that contribute to its pathogenesis. In that context, identifying new components of signaling events linked to monocyte recruitment and/or modulation of macrophage survival/apoptosis at the lesion site is of fundamental importance to move forward in the search for additional potential targets that could make those alternative therapeutic strategies a reality. Furthering our knowledge on the potential new roles of TRPC3, as well as other TRPC members, in atherogenesis can make a significant contribution to the search for new targets for the disease. Although ubiquitously expressed throughout tissues, TRPC3 on endothelial or macrophage surface could be used as a molecular target of relatively easy access for therapeutic and/or diagnostic purposes or may be exploited as a marker in non-invasive imaging, as it has been applied to other cell surface proteins (Kaufmann, Carr et al.; Saraste, Nekolla et al. 2009). The potential advantages of TRPC3 vs. traditional channel blockers as a prospective target for atherosclerosis has recently been discussed by us (Vazquez 2011). The exploration of TRPC3 as an atherorelevant signaling molecule is at its infancy, and several additional studies will be required to determine the impact of TRPC3 expression/function on atherorelevant events. The generation and characterization of new mouse models of atherosclerosis with genetically manipulated levels of TRPC3 in the atherorelevant cell or tissue of interest (i.e., conditional knockouts or transgenics) will be a unique contribution to that goal.
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Chapter 7.

Endothelial TRPC3/6/7 Proteins
at the Edge of Cardiovascular Disease

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J.Y. Tano, K. Smedlund, *G. Vazquez

Department of Physiology and Pharmacology, and the Center for Diabetes and Endocrine Research, College of Medicine, University of Toledo Health Science Campus, 3000 Arlington Av, Toledo, Ohio 43614-5804, USA

Correspondence to:

Guillermo Vazquez, Department of Physiology and Pharmacology, UTHSC Mailstop 1800, Toledo OH 43614 USA. FAX: 419 383 2871; e-mail address: Guillermo.Vazquez@utoledo.edu

Author Contribution:

Sections titled “BASIC STRUCTURAL FEATURES OF TRPC3/6/7 PROTEINS” and “TRPC3/6/7 AND ENDOTHELIAL DYSFUNCTION”.
ABSTRACT

An impressive amount of experimental evidence accumulated over the last two decades has clearly demonstrated that Ca$^{2+}$ influx through plasma membrane Ca$^{2+}$-permeable channels plays a critical role in endothelial cell physiology and pathophysiology. Research efforts aimed at understanding the role of Ca$^{2+}$ influx within the signaling events underlying endothelial dysfunction have grown at a fast pace over more recent years. Transient Receptor Potential Canonical (TRPC) proteins, which belong to the larger TRP superfamily of channel forming proteins, form Ca$^{2+}$-permeable cation channels in vascular endothelium and there is currently no question about their involvement in Ca$^{2+}$ influx associated with endothelial cell’s physiology. It is also becoming evident that TRPCs are important players in the pathogenesis of cardiovascular disease. Therefore, it is imperative to elucidate the mechanism/s underlying regulation of endothelial TRPC channels as well as to identify signaling events downstream TRPC activation in order to better comprehend their role in cardiovascular physiology and disease. This review focuses on members of the TRPC3/6/7 group of TRPC proteins, revises current knowledge on their expression and regulation in endothelium, and discusses their role in cardiovascular disease as it relates to endothelial dysfunction.

**Key words:** TRPC channels; endothelial dysfunction; cardiovascular disease; cation channels; Ca$^{2+}$ influx
INTRODUCTION

The myriad of roles played by Ca\textsuperscript{2+}-dependent signaling in endothelial cell physiology and pathophysiology is now largely recognized. In particular, the importance of the Ca\textsuperscript{2+} influx component of the endothelial Ca\textsuperscript{2+} response has received a great deal of attention over the last decade (reviewed in [1-4]). Regardless of the regional location of endothelial cells along the vascular tree, changes in intracellular Ca\textsuperscript{2+} levels associated to receptor-regulated Ca\textsuperscript{2+} influx exert a tremendous impact on many endothelial functions [1,5-7]. For instance, immediate consequences of this Ca\textsuperscript{2+} influx include short and long term cellular responses, such as activation of kinases, synthesis and/or release of vasoactive molecules, activation of pro-inflammatory signaling and gene transcription. Thrombin, nucleotides, bradykinin, prostaglandins and vascular endothelial growth factor (VEGF), among many others, act on their specific receptors on the endothelial cell membrane to promote Ca\textsuperscript{2+} influx, in most instances as part of the so called “biphasic” Ca\textsuperscript{2+} response, i.e., Ca\textsuperscript{2+} release from intracellular stores and Ca\textsuperscript{2+} influx from the outside. In intact endothelium Ca\textsuperscript{2+} influx can take place immediately or after a short delay following receptor stimulation and it can be required early, late or continuously throughout the signaling process for a proper Ca\textsuperscript{2+}-dependent response to be accomplished. Because of the wide repertoire of signaling events that can directly or indirectly be affected by Ca\textsuperscript{2+} influx, it is not surprising that modulation of Ca\textsuperscript{2+}-permeable channels is followed by changes in critical endothelial functions such as regulation of vascular tone, permeability and angiogenesis, to name a few. Transient Receptor Potential Canonical (TRPC) channels are among the most important Ca\textsuperscript{2+}-permeable cation channels in vascular endothelium. Regardless of the chronic controversy on the activating mechanism of
individual TRPC members (TRPC1-7, *vide infra*) there is no question about their participation in Ca\(^{2+}\) influx associated with endothelial cell physiology [8,9]. Most importantly, increasing evidence indicates that TRPCs are important protagonists in cardiovascular disease (CVD; [10-13]). Without doubt, elucidation of the mechanism/s underlying receptor-dependent regulation of TRPC channels would greatly contribute to assign definitive functions to individual TRPC members in endothelial Ca\(^{2+}\) signaling. However, equally important to comprehend their role in cardiovascular physiology and disease is to identify and characterize those Ca\(^{2+}\)-dependent events activated downstream to TRPC-mediated Ca\(^{2+}\) entry. Here we focus on members of the TRPC3/6/7 subfamily, discuss their role in CVD in relation to endothelial dysfunction and propose avenues to be followed that may improve our chances to exploit these proteins as molecular targets with potential therapeutic and/or diagnostic use.

**BASIC STRUCTURAL FEATURES OF TRPC3/6/7 PROTEINS**

TRPC3/6/7 proteins constitute a subcategory of the TRPC family of cation channel forming proteins which in turn is part of the larger TRP superfamily ([14-16] and references therein). The TRPC family contains those TRP members most closely related to the original *Drosophila melanogaster* TRP protein and the subdivision in the following groups: TRPC1; TRPC2; TRPC3/6/7; and TRPC4/5, is mainly based on structural and/or pharmacological similarities. TRPC2 is a pseudogene in humans, old world monkeys and apes, but can form functional channels in rat, mouse and other mammals. Like the other members of the TRPC group, TRPC3/6/7 proteins are thought to share a common membrane topology (see Figure 1) which includes six predicted transmembrane domains
(TM1-TM6) separating the cytoplasmic N- and C-termini, and a putative pore region between TM5 and TM6 [17,18]. On the N-terminus, three to four ankyrin repeats precede a predicted coiled-coil region and a putative caveolin binding region; the C-terminus shows a conserved EWKFAR sequence (TRP signature motif), a highly conserved proline rich motif, a calmodulin/IP_3 receptor binding domain and a predicted coiled-coil region. In native and heterologous expression systems functional TRPC3/6/7 channels are thought to oligomerize into homo- or hetero-tetrameric arrangements of four TRPC proteins [16,17,19] by means of interactions between specific domains in the N-terminus, the pore region and C-terminal domain [20,21]. Although certain specificity of assembly for different TRPC members has been shown –e.g., TRPC1 associates with 4 and 5, whereas TRPC3, 6 and 7 interact among them ([16] and references therein)-, other combinations can certainly take place. For instance, in porcine aortic endothelial cells (PAECs) native TRPC3 and 4 associate to form functional cation channels [22], while in bovine aortic endothelial cells (BAECs) TRPC5 and 6 associate and translocate to the plasma membrane in a lysophosphatidylcholine (lysoPC)-dependent manner ([23] and vide infra). Thus, it seems that the combinatorial alternatives under native expression conditions might not necessarily follow -not surprisingly- those predicted by observations made in overexpression systems. The possibility that yet to be known ancillary molecules are required in the assembling process makes it likely that distinct TRPC combinations occur on different cell types depending on expression level and/or repertoire of both TRPC and ancillary proteins.
EXPRESSION OF TRPC3/6/7 PROTEINS IN ENDOTHELIUM

Most endothelial cells examined so far appear to express all members of the TRPC family, \textit{i.e.}, TRPC1 through 7 [9,24]. As mentioned above, TRPC2 is a pseudogene in humans and thus not expressed in endothelial cells of that origin. Nevertheless, most studies evaluated the presence of RNA message (mRNA) and a systematic examination at the protein level is not available. Yip \textit{et al.} [25] provided the first expression analysis of TRPC homologs in different sized human intact vessels. By means of \textit{in situ} hybridization they showed that TRPC1, 3-6 are abundantly expressed in endothelium and \textit{tunica media} from cerebral and coronary arteries, whereas TRPC7 was found in endothelium but not in smooth muscle cells. A similar expression pattern was observed in small sized coronary arterioles and coronary artery \textit{vasa vasorum} [25] and importantly, the mRNA expression pattern for TRPC1, 3-6 was confirmed at the protein level by immunohistochemistry of arterial cross sections. In rats, expression of TRPC7 in small muscular or partially muscularized extra-alveolar vessels and conduit pulmonary artery was also found, almost exclusively, in endothelium [26]. This awaits confirmation as the antibody used in the latter studies cross reacts with TRPC3 and 6. If TRPC7 expression is truly confined to the endothelial layer, future \textit{in vitro} and \textit{in vivo} studies should explore whether such restriction is associated with Ca\textsuperscript{2+} influx-dependent endothelial functions that are specifically mediated by TRPC7. Recent work from our lab showed, for the first time, the expression of TRPC7 protein in primary cultures of human coronary artery endothelial cells (HCAECs, [27]) in line with the findings of Yip \textit{et al.} [25] in intact coronary vessels. A thorough evaluation of the expression of twenty two TRP genes has recently been conducted in mouse by means of RT-PCR and \textit{in situ} hybridization [28].
While a systematic analysis of vessels from different vascular beds was not performed, that study shows that TRPC3 is particularly abundant in aortic *tunica muscularis* and endothelium of C57BL/10, Balb/c and NOD mice.

Although suggestive, information on mRNA levels cannot be simply extrapolated to the protein scenario, and in many instances they might even be poorly indicative of the channel protein repertoire present at the plasma membrane. Differences in the turn-over rates of message *versus* protein and/or regulation of channel trafficking and membrane insertion (*vide infra*) may significantly affect TRPC abundance in the plasma membrane at a given time. Information at the protein level has also been somewhat limited and/or questionable by the variable success attained with commercially available antibodies and, in some instances (*e.g.*, TRPC7) by the lack of a readily available antibody. At any rate, whenever information at the protein level exists, TRPC expression pattern seems to vary significantly depending upon the cell line and/or the vascular bed where the endothelial cells are derived from. For example, while TRPC1 and 4 are frequently found in lung and umbilical vein endothelium (see [9,29] and references therein), coronary artery endothelial cells of human origin (HCAECs), despite the presence of message for all TRPCs [25,27] show quite significant levels of TRPC3 protein and to a lesser extent, TRPC1, 4 and 7, while TRPC5 is barely detectable [27]. To add to this complexity, most studies on primary endothelial cell cultures do not state the passage number of the cells used for experiments. This is important, as the possibility that primary cells become adoptive to the culture conditions and therefore, that changes in TRPC protein expression pattern may occur, should be considered. For instance, in HCAECs, TRPC6 expression seems to vary dramatically with passage number and culture conditions, while TRPC1 is
downregulated to almost undetectable levels when cells are exposed to pro-inflammatory conditions (Smedlund and Vazquez, unpublished observations). This is of particular significance in studies using cultured endothelial cells isolated from diseased arteries, where culture-dependent changes in TRPC expression may mask potential differences between normal cells and those derived from pathological specimens. Immunohistochemical analysis of TRPC expression in tissue sections derived from normal versus diseased subjects rather than immunoblotting of lysates from cultured cells, would provide a more realistic perception of in vivo expression levels as well as a framework to validate in vitro studies on cell cultures. For instance, specimens from atherectomy of peripheral vessels during percutaneous procedures are attractive sources of samples to examine TRPC expression in intact endothelium in patients with symptomatic atherosclerotic plaque; however, symptomatic lesions often have denuded endothelium and also, observations are limited by lack of proper controls -all samples from cases-.

REGULATION OF ENDOTHELIAL TRPC3/6/7 CHANNELS

Whether belonging to the G-protein coupled (GPCR) or receptor tyrosine kinase (RTK) group, Ca$^{2+}$ mobilizing receptors activate a phosphoinositide-specific phospholipase C (PI-PLC, e.g., PLC$\beta$, PLC$\gamma$) that then mediates generation of inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ induces release of Ca$^{2+}$ from endoplasmic reticulum Ca$^{2+}$ stores resulting in a transient increase in cytosolic Ca$^{2+}$ concentration; this is accompanied, or followed by Ca$^{2+}$ entry through plasma membrane Ca$^{2+}$ channels, which provides a more sustained elevation of cytosolic Ca$^{2+}$. As in most non-excitable cells,
Ca\(^{2+}\) entry in endothelium occurs predominantly through two major routes: store-operated Ca\(^{2+}\) (SOC) entry (SOCE, initiated by depletion of Ca\(^{2+}\) stores) and/or through non-store-operated Ca\(^{2+}\) (non-SOC) entry (non-SOCE; [1,7]). All TRPCs are activated downstream PI-PLC and thus represent excellent candidates for SOCE and non-SOCE under physiological conditions of receptor stimulation [14]. Indeed, a variety of Ca\(^{2+}\) mobilizing receptors are able to activate TRPC channels in endothelial cells from different vascular beds (a thorough list of TRPC-activating agonists and receptors is provided in [13]). As a result, it is not surprising that TRPCs take part in a diversity of receptor-modulated vascular functions, including regulation of vascular tone and permeability, secretion, endothelial cell proliferation and apoptosis [7-9]. Acting themselves as Ca\(^{2+}\) entry gates is not the only way TRPCs can contribute to changes in intracellular Ca\(^{2+}\). Because their permeability is non-selective for cations, TRPCs can alter membrane potential by mediating Na\(^{+}\) influx, which in turn modulates the driving force for Ca\(^{2+}\) entry and/or affects the activity of voltage-gated Ca\(^{2+}\) channels. For instance, TRPC6-mediated Na\(^{+}\) influx accounts for membrane depolarization and activation of L-type Ca\(^{2+}\) channels in smooth muscle cells [30], while TRPC3-mediated Na\(^{+}\) influx activates the reverse mode —i.e., Ca\(^{2+}\) “in”- of the Na\(^{+}/Ca^{2+}\) exchanger (NCX) in both HEK293 cells and rat cardiac myocytes, thus indirectly altering intracellular Ca\(^{2+}\) levels [31,32]. Interestingly, in the axial component of the transverse tubular system in rat ventricular myocytes, TRPC3 is part of a signaling complex that includes NCX and Na\(^{+}/K^{+}\) ATPase [33]. Operation of similar mechanisms in endothelial cells has not yet been reported. Unless proven otherwise, all signaling pathways triggered by GPCR or RTK stimulation can, on a theoretical basis, activate and/or modulate TRPC function.
The discussion here is focused on those mechanisms that, directly or indirectly, have been shown to, or eventually may, modulate the function of TRPC3/6/7 channels in endothelium. Information on regulation of TRPCs in other cell types, tissues or heterologous expression systems can be found in several recent reviews [15,16,34,35].

**A Shakespearean Dilemma: Store-Operated Or Non-Store-Operated?**

Whether expressed in heterologous expression systems or, as far as it has been explored, in their native environment, all TRPC proteins form non-voltage gated, non-selective cation channels that are activated downstream stimulation of PI-PLC (PLCβ, PLCγ; the role of PLD in regulation of TRPCs has been scarcely explored, but see [36]). As mentioned above, this is a feature that makes them potentially suitable to operate as either SOC or non-SOC channels. This is mainly the reason why we keep saying, now for more than a decade, that TRPC proteins - and other members of the TRP superfamily- can be envisioned as distinctive molecular candidates for SOC channels in physiological situations [14,16,37]. However, despite the fact that some TRPCs, at least under certain circumstances, do form store-operated Ca\(^{2+}\)-permeable channels, their role as genuine SOCs, particularly under physiological conditions, is still debatable –TRPC3 being the inveterate example of this; discussed in [16,38]-. It is actually conceivable that under physiological stimulation some TRPC channels are modulated by combinations of the two aforementioned mechanisms. The realization that channels with biophysical properties indistinguishable from the archetypical store-operated Ca\(^{2+}\)-release activated Ca\(^{2+}\) (CRAC) channels can be reconstituted by the integrated function of stromal interaction molecule 1 (STIM1), a Ca\(^{2+}\)-binding protein which functions as the sensor of Ca\(^{2+}\) store content, and Orai1 functioning as the channel itself ([39,40] and references
therein), somewhat dimmed the idea of TRPC proteins as components of CRAC channels. Nevertheless, SOC channels are heterogeneous in respect to cation selectivity and pharmacological profile in different cell types [41], and within that context CRAC simply constitutes a particular case within the SOC channel category. It is then reasonable that TRPC proteins may indeed be part of SOC channels other than CRAC. Importantly, recent studies suggest that both STIM1 and Orai proteins interact with TRPCs in instances where the latter are found to be legitimately store-operated ([42-45] and vide infra). In vitro, the distinction between SOC and non-SOC mechanisms is rather simple to establish by combining pharmacological maneuvers with fluorescence measurements of cytosolic Ca\(^{2+}\) and/or by monitoring membrane currents under controlled cytosolic Ca\(^{2+}\) buffering [46]. However, the scenario under physiological conditions is more complex and likely implies a combination of the two influx routes and/or a spatiotemporal overlap of the Ca\(^{2+}\) gradients derived from them.

Great deal of our knowledge on the role of TRPCs in endothelial SOCE comes from in vivo and in vitro studies on TRPC1 and 4 ([5,8,47-49], but see [50]). The regulation of endothelial TRPC3 however, is less clear, despite the fact that its function is unequivocally important for sustained Ca\(^{2+}\) entry in endothelium. In human umbilical vein endothelial cells (HUVECs) for example, SOC currents induced either actively (IP\(_3\)-induced Ca\(^{2+}\) store depletion) or passively (thapsigargin-dependent Ca\(^{2+}\) store depletion) are abolished by expression of a dominant-negative N-terminal fragment of TRPC3, suggesting that native TRPC3 proteins might be part of SOC channels [51]. However, in the HUVEC-derived Ea.hy926 cells, endogenous cation currents reminiscent of those rendered by heterologously expressed TRPC3, are clearly activated by receptor
stimulation but not by store-depletion [52]. In the latter, adaptation to culture conditions during establishment of the HUVEC-derived cell line may in part account for differences in channel assembly and/or regulation. Comparing SOCE and non-SOCE in uterine artery endothelial cells (UAECs) from pregnant versus non-pregnant ewes, Bird et al. [53] showed that store-operated Ca\(^{2+}\) influx is increased in UAECs derived from pregnant animals compared to non-pregnant controls through a mechanism involving augmented interaction between TRPC3 and type 2 IP\(_3\) receptor without changes in TRPC3 expression [53,54]. It remains to be studied if similar changes in regulated Ca\(^{2+}\) signaling and/or TRPC3 coupling mechanism occur in human UAECs.

As for TRPC6, existing evidence so far indicates that heterologous expression of human or murine TRPC6 protein renders a non-selective cation channel whose activation is linked to PI-PLC-derived products but is independent of Ca\(^{2+}\) store depletion (reviewed in [16,38]). This seems to be the case also for native endothelial TRPC6. In studies aimed at characterizing the Ca\(^{2+}\) influx pathway accompanying VEGF-dependent increase in permeability in frog mesenteric microvessels, Pocock et al. [55] identified TRPC6 as a likely candidate to mediate the required Ca\(^{2+}\) influx in a store-independent but DAG-operated manner. More recently, a VEGF-activated non-selective cation current with pharmacological and biophysical properties reminiscent of those from heterologously expressed TRPC3/6 channels was characterized in HMVECs, a human neonatal endothelial cell line derived from foreskin microvessels [56]. Activation of this current was independent of the filling status of intracellular Ca\(^{2+}\) stores and its properties were almost indistinguishable from those of a current activated by the DAG analog 1-oleoyl-2-acetyl-sn-glycerol (OAG). Subsequent demonstration that overexpression of a dominant
negative TRPC6 construct abrogated VEGF-dependent increase in Ca\textsuperscript{2+} influx in HMVECs [57] strongly supported the notion that TRPC6 is part of the non-store operated DAG-sensitive channels in these cells. By combining pharmacological and siRNA approaches Leung et al. [58] showed that TRPC6 forms, or is part of, channels activated by bradykinin in primary cultures of rat aortic endothelial cells and in H5V cells – a mouse heart microvessel endothelial cell line-. Once again, the signaling mechanism linking bradykinin receptor stimulation and TRPC6 activation was independent of store-depletion but dependent upon PI-PLC activity and DAG formation, pointing to endogenous DAG as the likely physiological activator. Singh et al. [59] showed that TRPC6 mediates the Ca\textsuperscript{2+} influx underlying thrombin-induced endothelial cell contraction in human pulmonary artery endothelial cells (HPAECs) through a mechanism that is G\textsubscript{q}-dependent but store-independent. To what extent the expression level of TRPC6 affects its regulation –i.e., whether it operates in store-operated versus non-store operated mode- as reported for TRPC3 and 7 (vide infra) has not yet been addressed. Recent work from Birnbaumer’s lab ([45,60], and vide infra) shows that in HEK293 cells stably expressing TRPC6 the cell’s availability of Orai1 determines whether TRPC6 operates in a store-dependent manner, providing the first example of store-operated TRPC6. As of this writing, there is no information available on regulation or functions of endothelial TRPC7.

**Interaction Of TRPC3/6/7 Proteins With STIM1 And Orai1**

As mentioned above several recent studies indicate that both STIM1 and Orai proteins (Orai1-3) interact with TRPC channels, whether TRPCs are operating as SOCs or non-SOCs [43,45,60-68]. Whereas those findings relate to the behavior of overexpressed
TRPCs in cell types other than endothelial, the recent identification of STIM1 and Orai1 as the fundamental components of SOC/CRAC channels in HUVECs [50] invites to explore not only the role of these proteins in specific endothelial functions but also the possibility that interactions between TRPCs, STIM1 and/or Orai1 may also take part in endothelium. The study by Abdullaev et al. [50] shows that in HUVECs passive depletion of intracellular Ca\textsuperscript{2+} stores with thapsigargin or activation of thrombin or VEGF receptors activates SOCE with typical characteristics of CRAC channels. Furthermore, siRNA experiments show that STIM1 and Orai1 are part of HUVEC’s SOC/CRAC currents with no contribution of TRPC1 or 4 [50]. The latter is in contrast with previous observations indicating a role for TRPC1 and 4 in SOCE (\textit{vide supra}). It should be kept in mind however, that the findings by Abdullaev \textit{et al.} do not exclude the possibility that interactions may exist between STIM1, Orai1 and other members of the TRPC family also expressed in HUVECs (\textit{e.g.}, TRPC3, 5 or 6; [51,69,70]). Within this context, it is important to consider the work by Liao \textit{et al.} [45,60] indicating that not only TRPCs functionally interact with Orai1-3 and STIM1 to form SOC channels, but also that the resultant channels recapitulate characteristics of CRACs in a manner that strictly depends upon the expression level of Orai1. Based on their observations Liao \textit{et al.} [45,60] proposed that Orai1 is the rate limiting step in determining the store-operated behavior of TRPC1, 3, 6 or 7, which carries the implicit idea that when TRPCs are expressed in excess of the cell’s available Orai1, they function in non-store operated mode [45]. This in a way illustrates the concept introduced by Vazquez \textit{et al.} [71] that the expression level of a TRPC protein can determine the channel’s ability to operate as SOC or non-SOC. However, in recent work using HEK293 cells stably overexpressing TRPCs, DeHaven \textit{et al.}
al. [72] found that TRPC1, 3, 5-7 channels are activated by mechanisms dependent on PI-PLC without involvement of STIM1 or Orai1. Clearly, studies aimed at addressing potential interactions between STIM1, Orai1 and TRPCs under native expression conditions are needed before the in vivo relevance of findings derived from overexpression systems can be understood.

**Regulation By Diacylglycerol and Kinases**

The role of receptor-dependent, PLC-mediated generation of DAG in the activation of members of the TRPC3/6/7 subfamily –and likely others, see for instance [73] - is perhaps the most widely accepted –and to some extent, assumed- elemental signaling path underlying receptor-dependent activation of TRPCs. Other mechanisms have been proposed, such as store-operation, IP$_3$, Ca$^{2+}$ itself, phosphatidylinositol 4,5-bisphosphate, or receptor-induced membrane insertion of pre-existing intracellular channels, but their operation has not been as consistently established as that of DAG [38,44,74,75]. Nevertheless, most mechanisms examined so far seem to be severely affected by expression conditions (i.e., native vs. overexpression), experimental assay (e.g., real-time fluorescence vs. patch-clamp) and cell type (i.e., primary vs. immortalized cell lines) among others. The existence of multiple potential phosphorylation sites in the primary sequence of TRPC3/6/7 proteins, which includes consensus motifs for protein kinases A, C, G, myosin light chain kinase and tyrosine kinases, has also pointed to phosphorylation/dephosphorylation as a regulatory mechanism (reviewed in [16]). As mentioned above, there is compelling evidence favoring the notion that PLC-derived DAG is the activating signal for TRPC3/6/7 channels [38,76]. Despite the fact that the fatty acid composition of OAG (1-oleoyl-2-acetyl-sn-glycerol) poorly resembles that
from endogenous PLC-derived DAG species (predominantly, 1-stearoyl-2-arachidonyl-sn-glycerol), this membrane permeable DAG analogue became a traditional pharmacological tool to activate ectopically expressed TRPC3/6/7 channels. Notably, in most cell types known to express TRPC3/6/7 proteins, native channels are generally unresponsive to exogenous application of OAG [16,36,38,77]. For instance, whereas HCAECs express TRPC3, 6 and 7, there is no detectable cation influx by simply challenging the cells with OAG [78]. The simplest interpretation is that native channels are insensitive to DAG. However, TRPC3/6/7 channels are potently inhibited by PKC [73,79,80] which is massively activated by OAG, and this could mask the ability of OAG to activate native channels. In line with this, avian B lymphocytes exhibit OAG-activated TRPC7-mediated cation influx and currents only under conditions of PKC inhibition [81]. Similarly, when HCAECs are pre-treated with a cocktail of PKC inhibitors, OAG promotes a robust cation influx that is not observed without PKC inhibition [78]. These findings also suggest that if DAG derived from receptor-stimulated PI-PLC is responsible for activation of native TRPC3/6/7 channels, in order to prevent concomitant inhibition by PKC either considerable compartmentalization of the signaling route exists under physiological conditions, or the lipid composition of native DAG species allows for a more fine spatiotemporal control of channel and kinase activation.

**Translocation And Membrane Insertion**

Regulated translocation to and insertion into the plasma membrane of TRPC channels is another established regulatory mechanism (reviewed in [82]) and its operation has also been demonstrated in endothelial cells. Mehta et al. [83] showed that in HPAECs
activation of protease-activated-receptor type 1 by thrombin promotes rapid association of RhoA with TRPC1 and IP$_3$ receptors to form a supramolecular complex that translocates to the plasma membrane. Singh et al. [59] later observed, again in HPAECs, that the signaling underlying thrombin-dependent activation of RhoA and myosin light chain kinase also requires functional TRPC6, as knockdown of native TRPC6 abolished the pro-inflammatory actions of thrombin. In BAECs, lysoPC, a phospholipid that exerts an inhibitory action on endothelial cell migration in a Ca$^{2+}$-influx dependent fashion [84], promotes the rapid and sequential translocation of TRPC6 and 5 to the plasma membrane [23]. TRPC6 has an obligatory role in such mechanism, as lysoPC effects are dramatically reduced in aortic endothelial cells from TRPC6 knockout mice [23] and TRPC5 translocation is abrogated when TRPC6 expression is knocked down. Translocation of TRPC6 to the plasma membrane also occurs as part of the mechanism underlying endothelial cell hyperpolarization. Fleming et al. [85] showed that in both native and cultured endothelial cells cytochrome P450-derived epoxyeicosatrienoic acids induce TRPC6 translocation to caveolin-1 rich regions in the plasma membrane, and that TRPC6-mediated Ca$^{2+}$ influx in turn accounts for prolonged activation of Ca$^{2+}$-dependent K$^+$ channels.

### Expression Level And Constitutive Activity

From studies aimed at understanding the discrepancy regarding the ability of TRPC3 to operate as either SOC or non-SOC channel, it became evident that changes in the level of TRPC3 protein within the cell may switch the coupling mechanism underlying channel activation ([71,86], discussed in [87]). Ectopic expression of TRPC3 in avian B lymphocytes renders channels that can operate as either SOCs or non-SOCs depending on
the amount of channel protein. While store-operation was observed only at low TRPC3 expression [71,86], high TRPC3 levels favored the receptor-regulated mode of TRPC3 activation [71,88]. Similar observations were made regarding regulation of TRPC7 [89]. These findings added a novel concept: a TRPC protein functioning in two distinct ways depending on expression level. Whereas it remains to be explored what factors are implicated in regulation of these changes, recent evidence suggests that they may have pathophysiological significance (vide infra). We propose that this may be particularly relevant when one considers channels endowed with significant constitutive, or non-regulated activity (e.g., TRPC3, 7), as increased channel protein levels would impose a gain in constitutive channel function that may result in pathological Ca^{2+}-dependent signaling, independently of receptor stimulation. Indeed, despite high structural and functional similarity, TRPC3, 6 and 7 differ substantially in their constitutive activities. TRPC6 is a tightly regulated receptor-activated cation channel, while TRPC3 and 7 display important constitutive function [16,38,76]. N-glycosylation of the channel protein seems to be a major determinant for the differences in constitutive activity between TRPC3 and 6 [90]. Introduction of an additional glycosylated site in the second extracellular loop of TRPC3, which mimics the glycosylation status of TRPC6, reduces dramatically TRPC3 constitutive activity, while channel responsiveness to agonists or OAG is preserved [90]. Conversely, removal of a glycosylation site from TRPC6 is sufficient to confer this channel with constitutive activity indistinguishable from that of TRPC3. Whether N-glycosylation modulates constitutive activity of TRPC7 remains to be addressed. Are there in vivo examples of biological consequences of TRPC3 or 7 constitutive activities? The best available case derives from studies on TRPC6 knockout
mice, in which compensatory upregulated expression of TRPC3 results in elevated contractility of aorta and cerebral arteries [91]. In mouse skeletal myocytes TRPC3 expression is upregulated by neuromuscular activity in a calcineurin-dependent manner, and enhanced expression of TRPC3 facilitates NFAT activity [92] in the absence of receptor stimulation, suggesting that the gain in constitutively active channels drives Ca$^{2+}$-dependent activation of NFAT. Finally, in GABA projection neurons of the substantia nigra pars reticulata from C57BL/6 mice [93] Na$^+$ permeation through constitutively active TRPC3 mediates the membrane depolarization that triggers the firing of action potentials. Are there similar examples in endothelial cells? In HCAECs activation of TNFα or P2Y$_2$ receptors [27] induces expression of TRPC3, and the subsequent gain in TRPC3-dependent constitutive cation influx is obligatory in the signaling underlying regulated expression of vascular cell adhesion molecule-1 (VCAM-1; *vide infra*).

**OVERVIEW OF TRPC3/6/7 CHANNELS IN CARDIOVASCULAR DISEASE**

By now it is evident that members of the TRPC3/6/7 group play critical roles in signaling events associated with the pathogenesis of CVD. For instance, the contribution of TRPC6 to the proliferation of vascular smooth muscle cells implicates TRPC6 in the development of both idiopathic and hypoxia-induced –acute and chronic- pulmonary hypertension [94-97] as well as in the pathogenesis of intima hyperplasia [98,99]. Ca$^{2+}$ entry through TRPC3, 6 and 7 underlies the Ca$^{2+}$ signaling associated to cardiac hypertrophy [100-103]. TRPC3 and 7 may participate in immune responses mediated by B and T lymphocytes [81,86,104] what might be potentially relevant to the pathogenesis
of inflammatory vascular disease. Expression of TRPC3, by yet to be known mechanisms, is upregulated in monocytes from spontaneously hypertensive rats [105,106] and this correlates with increased regulated Ca$^{2+}$ influx. Similarly, increased store-operated and DAG-regulated Ca$^{2+}$ influx parallel augmented expression of TRPC3 - and 5- in circulating monocytes from patients with essential hypertension [107,108]. Constitutively active TRPC3 is upregulated in TRPC6 knockout mice and is responsible for enhanced contractility of aorta and cerebral arteries and elevated blood pressure [91].

The section below revises existing evidence linking TRPC3/6/7 proteins to CVD as it relates to endothelial dysfunction. Literature on the role of TRPs in general and TRPCs other than TRPC3/6/7 in CVD has been reviewed in [9,12,109,110].

TRPC3/6/7 AND ENDOTHELIAL DYSFUNCTION

At the luminal edge of the vascular wall the endothelium is an extremely versatile and multifunctional tissue that is committed to an enormous variety of functions. Endothelial cells participate in regulation of vascular tone, synthesis and/or release of antithrombotic and prothrombotic molecules, synthesis and degradation of extracellular matrix components, regulation of inflammation and immunity and neovascularization, to name a few [111]. Furthermore, it has considerable phenotypic variability depending upon anatomic site and dynamic adaptation to local environmental factors. Endothelial dysfunction can be defined as an imbalance of one or more of the endothelial duties required for maintenance of vascular homeostasis, and occurs in most, if not all, states of CVD, whether cardiovascular is the primary problem or a complication of a pre-existing
pathology. Most endothelial functions are highly interconnected and interdependent and thus the dysfunctional state is usually manifested as a complex altered phenotype that revolves around a combination of oxidative stress, inflammation, thrombosis and impaired nitric oxide (NO) bioavailability. Because TRPC3/6/7 proteins are expressed along the entire vascular tree, it is thus not surprising that their deregulation and/or altered expression are associated to endothelial dysfunction and vascular disease (see Figure 2). For example, there is evidence favoring the notion that TRPC3 is likely to participate not only in normal redox-dependent endothelial functions but also in oxidative stress, known to constitute a link between vascular inflammation and redox imbalance. Using a dominant-negative N-terminal fragment of TRPC3, Balzer et al. [112] showed for the first time, that native TRPC3 proteins in PAECs form, or are part of, the channels mediating oxidative-stress. Whereas slightly different in terms of electrophysiological properties, the studies by Balzer et al. [112] also suggest that TRPC3 is a likely candidate for the molecular make up of a redox-sensitive channel identified in calf pulmonary artery endothelial cells, where a tert-butylhydroperoxide-activated non-selective cation conductance mediates Na\(^+\) influx and the subsequent breakdown of membrane potential [113,114]. Subsequent work from the same group suggests that in PAECs native TRPC3 and 4 associate to form the redox-sensitive channel [22]. The mechanism by which oxidative stress activates TRPC3-based channels remains largely unknown, but redox-dependent modifications of lipids in the channel’s microdomains or oxidation of critical sulfhydryls on the channel protein, are some possibilities [112,115]. For a discussion on the role of TRP channels other than TRPCs in oxidative stress see [116] and references therein.
Regulated expression of endothelial cell adhesion molecules (CAMs) and recruitment of circulating leukocytes to the arterial intima constitutes one of the earliest molecular/cellular events in the pathogenesis of inflammatory vascular disease such as atherosclerosis [117]. In HCAECs pro-atherogenic actions of ATP include induced expression of VCAM-1, a critical CAM mediating monocyte adhesion and transendothelial migration [118], through a mechanism that requires P2Y$_2$ receptor activation and changes in intracellular Ca$^{2+}$ levels [119,120]. In a recent study, we showed that in HCAECs native TRPC3 forms, or is part of, native Ca$^{2+}$-permeable channels that contribute to ATP-stimulated Ca$^{2+}$ influx [78], and that TRPC3 expression and constitutive function are obligatory for ATP-induced VCAM-1 and monocyte adhesion, underscoring a potential novel function of TRPC3 in atherogenesis. LysoPC inhibits the migration of BAECs through a mechanism that is Ca$^{2+}$-influx dependent and has an obligatory requirement for functional TRPC6 [23,84]. Because LysoPC is a major component of oxidized low-density lipoproteins, endothelial TRPC6 might be part of the inflammatory signaling underlying pro-atherogenic actions of LysoPC. In HMVECs the angiogenic actions of VEGF require Ca$^{2+}$ influx, and TRPC6 is a constituent of VEGF-activated non-selective cation channels in these cells [57]. Indeed, TRPC6 activity is obligatory in the mechanism underlying VEGF-dependent migration, sprouting and proliferation of these cells [57]. A role for TRPC6 in VEGF-dependent angiogenesis was also shown in HUVECs [70]. Thus, besides being part of physiological actions of VEGF it is plausible that TRPC6 might contribute to pathological angiogenesis, as it occurs in advanced atherosclerotic lesions. In fact, unstable atherosclerotic plaques that are prone to rupture are characterized by a large necrotic core with significant neovascularization,
apoptotic macrophages, increased VEGF levels and frequently intraplaque haemorrhage [121]. A negative correlation between TRPC3 expression and VEGF-dependent proliferation of HUVECs and the HUVEC-derived cell line EAhy926 was recently described [122]. There is an important amount of evidence on the role of TRPC3/6/7 proteins in smooth muscle cells of the systemic and pulmonary circulation in relation to systemic blood pressure, hypoxia-induced –acute and chronic- pulmonary hypertension and idiopathic chronic pulmonary hypertension (reviewed in [12,123]). Conversely, information on the role of endothelial TRPC3/6/7 channels in endothelial processes related to hypertension is scarce. A positive correlation has been found in the expression of TRPC3 with increased store-operated and DAG-regulated Ca\(^{2+}\) influx in monocytes isolated from patients with essential hypertension [107,108]. This prompted the authors to examine if TRPC3 levels were altered in malignant hypertension, for which essential hypertension is a contributing factor. Thilo et al. [124] evaluated expression of endothelial TRPC3 and 6 proteins by immunostaining cross sections from pre-glomerular arterioles obtained from six patients with malignant hypertension. They found that TRPC3, but not TRPC6, was significantly higher in malignant hypertension specimens compared to samples from patients with diarrhea-associated hemolytic-uremic syndrome, a disease that also presents with endothelial injury/dysfunction but of different pathogenesis [124]. More recently, the same group examined expression of TRPC3 in vascular endothelium of normal renal tissue obtained from patients with renal cell carcinoma and found that TRPC3 is augmented in patients with a history of systolic blood pressure (SBP) >140 mm Hg but not in those with SBP ≤140 mm Hg [122]. Although these findings suggest an association between TRPC3 expression and
hypertension, the question remains whether increased TRPC3 is a contributing factor to the pathogenesis of the disease rather than the consequence of an altered endothelial phenotype. Evidence on the modulation of NO bioavailability as a mechanism linking TRPC3/6/7 function with vasorelaxation and blood pressure is not yet available.

Altered endothelial permeability also contributes to the pathogenesis of CVD. Both transcellular and paracellular pathways are involved in regulation of endothelial barrier function, and TRPCs have been linked to those processes [2]. Indeed, compelling evidence supports a critical role for TRPC1 and 4 in receptor-regulated endothelial permeability (reviewed in [29]); however, studies on members of the TRPC3/6/7 group are limited. Using frog mesenteric microvessels as a model to examine the role of Ca\(^{2+}\) influx in VEGF-dependent vascular permeability, Pocock et al. [55] identified TRPC6 as the likely candidate mediating VEGF-dependent, DAG-sensitive Ca\(^{2+}\) influx. In HMVECs, the actions of VEGF on endothelial permeability are accompanied by activation of a non-selective cation current reminiscent of heterologously expressed TRPC3/6 channels [56]. That TRPC6 is indeed part of those channels was subsequently confirmed by a dominant negative approach [57]. More recently, Singh et al. [59] showed that TRPC6-mediated Ca\(^{2+}\) influx contributes to the signaling underlying thrombin-induced cell contraction in HPAECs, an event also leading to increased permeability.

**CONCLUDING REMARKS**

The last decade has witnessed a significant growth of our understanding on how TRPC3/6/7 proteins contribute to vascular physiology and to the pathogenesis of several forms of CVD, particularly in relation to altered expression and/or regulation of these proteins in smooth muscle from cardiopulmonary vasculature. Nevertheless, our current
knowledge on their roles in vascular endothelium is still in its infancy. Do endothelial TRPC3/6/7 proteins have a future as molecular targets in CVD? The activity of TRPC3/6/7 channels is susceptible to the action of several organic and inorganic compounds, but none of them has proven to be specific enough to be considered for therapeutic purposes. Moreover, the search for new compounds that would specifically interact with TRPC3/6/7 proteins is at a primitive stage. Future efforts should be oriented into two – not mutually exclusive - main directions. First, in vitro and in vivo studies should aggressively address not only the mechanism/s underlying regulation of native, rather than ectopically expressed TRPC3/6/7 proteins within the context of endothelial signaling, but also identify molecular/cellular events downstream channel activation. Second, the development of endothelial-specific, rather than global transgenic and knockout animals, would provide a unique opportunity to assess the contribution of individual TRPC3/6/7 proteins to the pathogenesis of endothelial-related CVD within the context of the multiple mechanisms that take place in vivo in intact vessels. The latter is of particular importance for: a) determining if correlations exist between expression/function of a particular channel protein and the severity of the disease and/or with the regional location of damaged vascular areas, so that the channel in question might be exploited as a marker with diagnostic and/or prognostic use; b) outlining a molecular framework within the vascular tree to rationally decide as to which of the TRPC3/6/7 proteins has real potential as a target with therapeutic utility.

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Figure Legends:

Figure 1:
Membrane topology and salient structural features of TRPC3/6/7 proteins. TRPC3/6/7 proteins share a common membrane topology with six predicted transmembrane domains (TM1-TM6), cytoplasmic N- and C-termini and a putative pore region (“pore”, delineated by the re-entry loop between TM5-TM6). The ankyrin repeats (“A” in boxes) on the N-terminus precede the coiled-coil (“CC”) and caveolin binding (circle) regions. Salient features on the C-terminus include the TRP signature motif (EWKFAR sequence), a highly conserved proline rich motif (circle), a CIRB (calmodulin/IP₃ receptor binding) domain and another coiled-coil region. The glycosylation site (Gly) in the first extracellular loop is present in TRPC3/6/7 whereas that in the second extracellular loop is present in TRPC6/7. Functional TRPC3/6/7 channels are formed by homo- or hetero-tetrameric arrangements of four TRPC proteins (see text for details).

Figure 2:
Schematic cartoon summarizing some of the salient molecular, cellular and signaling events that directly or indirectly can be affected by the activity of endothelial TRPC3/6/7 channels. NCX: Na⁺/Ca²⁺ exchanger; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; CAMs: cell adhesion molecules; PKCs: protein kinase C; CAM: calmodulin; CAMK: calmodulin-dependent kinase; PI3K: phosphatidylinositol-3-kinase; NFkB: nuclear factor kappa B; NFAT: nuclear factor of activated T cells; ROS: reactive oxygen species.
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Figure 1
Figure 2

TRPC3/6/7

Membrane potential (L-type channels, NCX)

Regulated expression of CAMs (VCAM-1), inflammatory cell recruitment

Endothelial cell retraction/permeability

Pro-inflammatory signaling (NFkB, calcineurin/NFAT)

Oxidative stress (ROS sensing, NADPH oxidase)

Ca²⁺-dependent signaling (PKCs, CAM/CAMK, PI3K/Akt, etc.)

Na⁺/Ca²⁺
Chapter 8.

On The Potential Role of Source and Species of Diacylglycerol in Phospholipase-Dependent Regulation of TRPC3 Channels


by

Guillermo Vazquez\textsuperscript{1,2,\dagger}, Jean-Yves Tano\textsuperscript{1} and Kathryn Smedlund\textsuperscript{1}

\textsuperscript{1}Department of Physiology and Pharmacology and \textsuperscript{2}Center for Diabetes and Endocrine Research at the University of Toledo College of Medicine, Health Science Campus, 3000 Arlington Av, Toledo, Ohio 43614 USA

\dagger To whom correspondence should be addressed at: Dept. of Physiology and Pharmacology, UTHSC Mail stop 1008, Toledo OH 43614 USA.

E-mail: Guillermo.Vazquez@utoledo.edu

Tel: 419-383 5301

Fax: 419-383 2871

Author contribution:

Figures 1 and 2.
Key words: TRPC3; phospholipases; diacylglycerol; Ca$^{2+}$ influx; cation channels; calcium signaling; channel modulation
Abstract

Members of the Transient Receptor Potential Canonical (TRPC) family of channel forming proteins are among the most important $\text{Ca}^{2+}$-permeable cation channels in non-excitable cells. Physiologically, TRPC channels are activated downstream receptor-dependent stimulation of phospholipases, either by store-operated or non-store operated mechanisms. TRPC3, a member of the TRPC3/6/7 subfamily, has been largely studied mostly due to its ability to function in one or the other modes, depending on cell type and expression conditions. The role of TRPC3 as a non-store operated channel has been attributed to its ability to respond to diacylglycerol (DAG) either exogenously applied or endogenously produced following activation of receptor-stimulated phospholipases. Despite the vast amount of information accumulated on this topic, some critical aspects related to phospholipase-dependent DAG-mediated regulation of TRPC3 remain unclear and/or unexplored. Among these, the source and species of native DAG, modulation by different DAG-generating phospholipases and protein kinase C-dependent inhibition of TRPC3 in its native environment are just few examples. The present essay is intended to compile existing knowledge on the nature of phospholipase-derived DAGs, their biophysical properties and current evidence on phospholipase-dependent regulation of TRPC3, to speculate on potential scenarios that may eventually provide answers to some of the above questions.

Running Title: TRPC3 and diacylglycerol species
1. Introduction

In non-excitable cells calcium (Ca\(^{2+}\)) mobilizing agonists acting on G-protein coupled (GPCR) or receptor tyrosine kinase (RTK) membrane receptors typically activate a phosphoinositide-specific phospholipase C (PI-PLC, e.g., PLC\(\beta\), PLC\(\gamma\)) that catalyzes the hydrolysis of phosphatidylinositol 4,5-bis-phosphate (PIP\(_2\)) to generate inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG). IP\(_3\) triggers the release of Ca\(^{2+}\) from endoplasmic reticulum Ca\(^{2+}\) stores which results in a transient increase in cytosolic Ca\(^{2+}\) concentration. In the so called biphasic Ca\(^{2+}\) response, such Ca\(^{2+}\) transient is simultaneously accompanied, or followed by Ca\(^{2+}\) influx through plasma membrane Ca\(^{2+}\)-permeable channels which provides a more sustained elevation of cytosolic Ca\(^{2+}\) \(^1\). When present, the Ca\(^{2+}\) influx component can take place either immediately or after a short delay following receptor activation; therefore, Ca\(^{2+}\) influx can participate in Ca\(^{2+}\)-dependent events that occur very early, late or in a sustained manner throughout the signaling process. This Ca\(^{2+}\) entry can occur through a store-operated Ca\(^{2+}\) (SOC) entry route (SOCE, triggered by depletion of Ca\(^{2+}\) stores) and/or through a non-store-operated Ca\(^{2+}\) (non-SOC) entry path (non-SOCE) \(^2\) \(^3\) and the channels involved may exhibit more or less selectivity towards Ca\(^{2+}\) depending on their molecular structure and/or the cell type being examined.

Transient Receptor Potential Canonical (TRPC) channels are now recognized amongst the most important Ca\(^{2+}\)-permeable cation channels in nearly all non-excitable cells \(^4\) \(^6\). Regardless of expression conditions \(-i.e.,\ heterologous vs. native-\) TRPC proteins form non-voltage gated, Ca\(^{2+}\)-permeable non-selective cation channels that are activated downstream stimulation of PI-PLCs and therefore, now for more than a decade,
they have been considered good candidates for mediating SOCE and/or non-SOCE under physiological conditions of receptor stimulation \(^4\). In this context TRPC3, a member of the TRPC3/6/7 subfamily, has maintained a leading role, but its ability to be activated by mere depletion of Ca\(^{2+}\) stores – *i.e.*, to operate as a SOC channel- has been, and still is, a controversial issue (reviewed in \(^5, 7, 8\)). The demonstration that the activity of TRPC3 – as well as that of its close relatives TRPC6 and 7 - can be enhanced by direct application of synthetic DAG analogues or by increasing endogenous DAG levels with the combined use of DAG lipase and DAG kinase inhibitors \(^9-11\), provided a reasonable explanation as to how those channels could be activated physiologically in a PI-PLC-dependent but store-independent manner. Nevertheless, whereas the role of TRPC3 as a non-SOC channel has been somewhat less questioned than its role as a genuine SOC (discussed in \(^5, 7\)), TRPC3 activation by DAG is not exempt of mysteries. Indeed, some critical aspects related to phospholipase-dependent, DAG-mediated regulation of TRPC3, have received little attention and several questions remain to be answered. Do the source and species of endogenous DAG have an impact on TRPC3 function? Can TRPC3, in its physiological environment, be subject to simultaneous modulation by different DAG-generating phospholipases? How does protein kinase C (PKC)-dependent inhibition of TRPC3 fit within a physiological model whereby DAG is the endogenous activator and/or modulator of the channel? In the following sections we take in consideration existing knowledge on the nature of phospholipase-derived DAGs, their biophysical behavior in biological membranes and current evidence on phospholipase-dependent regulation of TRPC3 to speculate on potential scenarios that may eventually provide answers –or at least provoke new thoughts and/or experimental efforts- to some of the above questions.
The focus will be on TRPC3 because the vast majority of experimental data on DAG-sensitive TRPCs comes from studies on this member of the TRPC family. However, many of the concepts or speculations made here can eventually apply to its close DAG-sensitive relatives TRPC6 and 7.

2. DAG sources, DAG species and regulation of TRPC3

Whereas there is compelling evidence supporting a role for DAG, particularly that derived from PI-PLC activity, as an endogenous activating signal for TRPC3 channels (see 7, 8 and references therein), it is still debatable whether the action of DAG results from direct interaction with the channel or if ancillary channel proteins endowed with DAG binding properties are responsible for mediating DAG’s activating effect. The latter scenario is supported by the following: a) DAG-dependent regulation of TRPC7, a close TRPC3 relative, is lost in excised patches 11-13 – this however is not the case for TRPC6; an explanation to this apparent discrepancy was recently discussed in 12 - or by preventing resynthesis of polyphosphoinositides 12; b) TRPC3 activity induced by 1-oleyl-2-acetyl-sn-glycerol (OAG) exhibits an obligatory requirement for the tyrosine kinase Src 14; c) TRPC3 channels newly inserted into the plasma membrane show constitutive activity but are DAG-insensitive 15; d) so far, no canonical DAG binding sites have been identified on the TRPC3 protein –candidate DAG-binding auxiliary proteins have not been found either.-
2.1. Synthetic vs. native DAG species in regulation of TRPC3

Experimental evidence showing DAG-induced activation of TRPC3 comes mostly from studies involving exogenous application of synthetic membrane permeable analogues of DAG, such as OAG or 1,2-dieocanoyl-sn-glycerol (DOG). Importantly, both the fatty acid composition of this class of DAG analogues and probably their mode of accumulation and/or distribution in the plasma membrane poorly resemble that of endogenous PI-PLC-derived DAG species (vide infra). Moreover, in those instances where activation by endogenous DAG has been presumed – e.g., using inhibitors of DAG metabolism- the ultimate source of DAG responsible for channel activation is either uncertain, or it is assumed that DAG derives from PI-PLC-mediated hydrolysis of PIP$_2$. However, activation of either GPCRs or RTKs results in most instances in a biphasic production of DAG, characterized by a rapid and transient phase, mostly due to PI-PLC activity, and a second sustained phase that is associated to activation of PLD- and/or PLC-mediated hydrolysis of phosphatidylcholine (PC)\textsuperscript{16, 17}. Activation of PC-PLC represents an alternative direct source of receptor-regulated production of DAG from PC, as PC-PLC catalyzes the hydrolysis of PC - and other phosphatides, e.g., sphingomyelin, phosphatidylethanolamine- to generate phosphocholine and DAG\textsuperscript{18}. PLD-dependent generation of DAG from PC however, is a two step process, with PLD-mediated hydrolysis of PC producing phosphatidic acid (PA) which then is converted to DAG by the action of a phosphatidate phosphohydrolase (PAP)\textsuperscript{19}. Unlike PI-PLC, the role of DAGs derived from the activity of PC-PLD in regulation of TRPC3 has been scarcely explored. It was not until recently that some basic evidence became available suggesting a link between PC-PLD and TRPC3 function in both native and expression systems (vide
infra). As for PC-PLC, whereas its activity and regulation by GPCRs and RTKs is well described in mammalian cells \(^2\), there is no evidence yet on a potential role of this phospholipase in TRPC3 function.

OAG, a synthetic membrane permeable analogue of DAG, is by far the most popular pharmacological tool to induce TRPC3 activity, particularly when TRPC3 is ectopically expressed in heterologous cell systems. This is so despite the poor resemblance of OAG’s fatty acid composition to that of endogenous DAG species generated upon receptor-dependent stimulation of phospholipases. As mentioned above, endogenous DAG species can derive from different sources – e.g., PI-PLC, PC-PLD, and PC-PLC- and they differ in their fatty acid profiles (Figure 1A). DAGs accumulated upon receptor stimulation generally contain: a) a saturated fatty acid at position \textit{sn}-1 of glycerol - e.g., stearoyl- and an unsaturated fatty acid in position \textit{sn}-2 – e.g., arachidonoyl or oleyl-; or b) a saturated acyl chain in position \textit{sn}-1 and a mono- or unsaturated fatty acid in position \textit{sn}-2. The relative abundance of one or the other fatty acids is dictated by the original phospholipase substrate, \textit{i.e.}, PIP\(_2\) or PC, whether PI-PLC or PC-PLD/PC-PLC are involved, respectively. It has been demonstrated that DAGs derived from PI-PLC activation are, for the most part, polyunsaturated, while DAGs generated by PC-PLD are saturated or monounsaturated \(^2\). Of relevance within the context of TRPC3 regulation, this differential lipid profile of DAGs is known to strongly affect their ability to activate PKC (\textit{vide infra}). A native-like fatty acid profile resembling PI-PLC-derived DAGs is well preserved in the DAG analogue SAG (1-stearoyl-2-arachidonoyl-snglycerol). Nevertheless, the poor membrane permeability of SAG compared to OAG has limited its use in intact cells, where bath application of SAG has shown, if anything at all,
much less potency than OAG to activate cation influx and/or currents (see for instance 22). Of note, the original study by Hoffmann et al. 11 on the activating effect of DAG on TRPC3 and 6 clearly showed a more efficient activation of TRPC6 with SAG than with OAG or 1-stearoyl-2-linoleoyl-sn-glycerol (SLG) –this was not tested on TRPC3- already suggesting the importance of preserving native fatty acid composition. However, the vast majority of studies that came thereafter opted for OAG as the DAG analogue of preference (see 7, 8 and references therein), even if the possibility existed for the investigator to add membrane impermeant DAGs by either accessing the cell’s interior or the cytosolic leaflet of the membrane in the whole-cell or excised patch configurations, respectively, of the patch-clamp technique.

Based on the above considerations, it becomes intuitive to think on the potential effect of DAG’s fatty acid composition on TRPC3 function and its modulatory consequences, particularly under physiological situations. How and at which stage of channel activation/regulation do DAG species play a role? Regardless of the action of DAGs on TRPC3 being direct or indirect (vide supra) it is plausible that the fatty acid composition of a particular DAG species may affect: a) its affinity for the channel protein, should DAG/TRPC3 interactions occur; b) its affinity for the ancillary DAG-binding protein, should the action of DAG be indirect; c) the orientation of DAG within the inner membrane leaflet and consequently, its interaction with surrounding lipids, TRPC3 or TRPC3-auxiliary proteins. In fact, the nature of the fatty acids attached to the glycerol backbone of DAG – i.e., saturated vs. unsaturated- is determinant for DAG’s actual membrane miscibility and will determine the resultant spatial structure and accommodation of DAG within the membrane (23 and references therein). Another aspect
to be considered is that exogenously applied DAG analogues are unlikely to accumulate in and/or distribute within the plasma membrane as endogenous DAGs do. Of importance, studies on membrane localization of PI-PLC-derived DAG indicate that this lipid accumulates in the membrane in the form of discrete droplets that localize within the microdomains of the site of its generation, a situation that dramatically contrasts with the massive and extensive membrane insertion that may follow exogenous addition of OAG.

2.2. Biophysical properties of DAGs in biological membranes and regulation of TRPC3

An interesting property of DAGs, at least in synthetic lipid bilayers is that as their concentration increases, they induce lateral phase separation and, subsequently, an alteration in membrane curvature. This is believed to be due to the immiscibility of DAG with phospholipids, which favors the occurrence of hydrophobic interactions between DAG and intrinsic membrane proteins. This property of DAGs explains the fact that when they are incorporated in artificial phospholipid bilayers their presence promotes transitions from lamellar to non-lamellar phases, such as inverted hexagonal and inverted cubic phases. In any case, the practical consequence of an increase in DAG concentration in a biological membrane is that the cytosolic membrane leaflet will “tend” to transition from a lamellar to a non-lamellar state; whereas such change does not actually take place in a cell’s membrane, the direct consequence of the “transitional propensity” is a change in what is referred to as “membrane curvature packing stress”. DAGs derived from either PI-PLC, PC-PLC or PC-PLD activity have indeed been shown to affect curvature packing stress of native membranes, altering the activity of
glycosyltransferases, vesicle fusion at synapses, and the binding of ArfGAP1 to membranes in HeLa cells. In the case of PC-PLD, the immediate products of PC hydrolysis are PA and phosphocholine. PA exhibits a conical shape that fits well in membranes exhibiting negative curvature, such as hexagonal H-II phase. Further conversion of PA to DAG by the action of PAP increases both the hydrophobicity and stability of the membrane surface particularly in regions where membrane curvature is already negative. Instead, DAG derived from PIP2 fits better in planar phospholipid bilayers, due to higher degree of unsaturation in the fatty acids. On a theoretical basis, TRPC3 protein, or the ancillary channel proteins that bind DAG, may exhibit more or less preference for one or the other DAG species, or the efficiency of the DAG recognizing site to interact with DAG –whether on TRPC3 or on the DAG binding protein- may vary depending upon the extent of hydrophobicity imposed to the membrane by the newly formed DAG.

A model intended to explain how changes in membrane curvature may affect ion channel function through membrane stretching has been recently discussed (and references therein). Briefly, this model postulates that if the thickness of the transmembrane portion of the channel were to increase when the channel opens, then the phospholipid bilayer would have to stretch to prevent exposure of hydrophobic areas of the channel protein to water or to the polar head groups of the membrane phospholipids. Because such a stretching requires curving of the membrane immediately surrounding the channel, lipids endowed with a conical shape, such as PA or DAGs, would favor membrane curvature. In a study aimed at determining the effects of mechanical stretching on the function of TRPC6 channels, Spassova et al. proposed a model in which PI-
PLC-derived DAG, generated in the inner leaflet of the membrane, would increase membrane curvature as a major determinant of the physical stretch leading to channel activation. The conclusion was also made that OAG, because of its membrane permeability, would reach the inner bilayer leaflet and affect membrane curvature in a DAG-like manner. Whereas is possible that endogenously generated DAG impacts membrane curvature in the channel’s vicinity, it is highly unlikely that OAG, which massively enters the cell and partitions throughout all cellular membranes (vide supra), will end up selectively localized in a delimited area of the inner membrane leaflet. Notably, the idea that DAGs may exert a physiological role through their ability to modify membrane curvature was proposed by Michell more than thirty years ago. Whereas the findings by Spassova et al. are in line with operation, at least for TRPC6, of a membrane stretching model, further studies are required to determine if this is a general phenomenon or a feature of certain TRPCs. Nevertheless, operation of a membrane stretching mechanism could be of particular relevance for channels endowed with a lipid annulus. The lipid annulus –annular or boundary lipids- is present in many ion channels and transporters such as the nicotinic acetylcholine receptor, the potassium channel KcsA from Streptomyces lividans, the Ca\(^{2+}/Mg\(^{2+}\)-ATPase or the mechanosensitive channel of large conductance (MscL) from Mycobacterium tuberculosis, among others, and is critical for proper function. It can be defined as a lipid shell surrounding, to different extents, the transmembrane segment of the channel protein, and is immobilized relative to the bulk membrane phospholipids. The lipid annulus is present in a liquid-ordered (L\(_{o}\)) phase with a higher degree of order than the bulk lipid in the membrane, and plays a critical role in modulating channel gating as well.
as the interaction of the channel protein with bulk lipids, ligands, and pharmacological agents (see \(^{30, 37, 38}\) and references therein). It remains to be explored if a *lipid annulus* exists for TRPC3 channels when embedded in biological membranes, and if so, how DAGs, either from outside or inside the annular lipid, may influence channel function.

Interestingly, OAG-induced lateral phase separation of phospholipids is facilitated by the presence of \(\text{Ca}^{2+}\) in the surrounding medium \(^{39}\). This could explain some disparate effects of OAG on TRPC3 in the presence or absence of extracellular \(\text{Ca}^{2+}\). Lintschinger *et al.* observed that when \(\text{Ca}^{2+}\) was present in the bath at physiological concentrations (e.g., 2 mM), OAG-induced currents in HEK293 cells stably overexpressing human TRPC3 were smaller than when nominally \(\text{Ca}^{2+}\)-free medium was used, and this behavior was different whether TRPC3 was alone or forming heteromultimers with TRPC1 \(^{40}\). It is possible that when \(\text{Ca}^{2+}\) is present OAG-dependent disturbance of phospholipids surrounding the channel occurs, which in turn interferes with channel gating. The interpretation provided by Lintschinger *et al.* still applies, in that TRPC3 homomultimers vs. TRPC3/TRPC1 heteromultimers likely exhibit differential sensitivity towards OAG-induced disturbances of the phospholipid microenvironment. Functional TRPC3 channels are thought to be formed by either homo- or hetero-tetrameric arrangements of four TRPC proteins by means of interactions between specific domains in the N-terminus, the pore region and C-terminal domain \(^5\). If we consider an idealized combinatorial arrangement of all possible tetramers made of DAG sensitive TRPCs — *i.e.*, TRPC3, 6 and 7— twenty one different oligomers are theoretically possible — assuming that all combinations are feasible and that all three members co-exist within the same cell at a particular time and in stoichiometrically sufficient proportions -. Based on this, it is
reasonable to consider that different tetrameric arrangements may alter the DAG affinity of individual TRPC subunits, or alternatively, their ability to interact more or less efficiently with ancillary DAG-binding proteins.

2.3. Role of DAG in the negative regulation of TRPC3 by PKC

As mentioned above, endogenously generated DAGs are believed to accumulate within short distances of the site of production, within the boundaries of the inner leaflet of the plasma membrane. This is a situation that contrasts dramatically with the massive and extensive membrane insertion that likely follows exogenous addition of membrane permeable DAG analogues. And it could in part explain a puzzling observation related to DAG-dependent regulation of TRPC3, which is the fact that in most cells known to endogenously express TRPC3 – the same applies for most cells expressing TRPC6 and/or 7 proteins-, native channels are unresponsive to DAG analogues in that no cation influx and/or currents are detected upon application of OAG\(^5,7,41-43\) – some exceptions exist, see for instance \(^22,44,45\). This has been logically interpreted as a manifestation of the well known sensitivity of TRPC3/6/7 proteins to PKC-dependent inhibition \(^9,46,47\). Whereas TRPC3 activation by DAG analogues is independent of PKC (see \(^7,8\) and references therein), massive PKC activation by OAG –or other DAG analogues- will mask the ability of OAG to activate native channels, as channel inhibition will prevail over stimulation. That native TRPC3 –and TRPC7- channels are however able to respond to exogenously applied OAG has been shown by measuring OAG-activated cation influx and currents under conditions of PKC inhibition \(^43,48\). However, if phospholipase-derived DAG is responsible for activation of native channels, those findings and the above
interpretation do not explain how concomitant channel inhibition by PKC is prevented or defeated under physiological conditions. One possibility is that the fatty acid composition of native DAG species may allow for a fine spatiotemporal control of channel vs. kinase activation (see model in Figure 1B). It is reasonable to consider that different DAG species might exhibit differential affinity towards the TRPC3 protein, the hypothetical ancillary DAG binding protein or the DAG-sensitive PKCs that mediate channel inhibition. It is also possible that a significant degree of compartmentalization of the signaling route exists, so that DAGs act on the immediate channel’s vicinity but remain inaccessible to PKC. For example, if a lipid annulus exists for TRPC3 and DAG is generated within the boundaries of the annulus, the intra-annular DAG would be unlikely to be available for PKC activation (Figure 2). Moreover, if TRPC3 and the phospholipase responsible for DAG generation exist in the membrane as a signaling complex – TRPC3 has indeed been shown to function within Ca\(^{2+}\) signaling complexes assembled in plasma membrane microdomains; see \(^{49}\) and references therein- then DAG would be expected to accumulate in the lipid microenvironment of the channel until an activating threshold is reached; beyond that point, further DAG accumulation results in successful PKC recruitment and/or activation and the subsequent negative feedback on the channel’s activity. This model is somewhat supported by studies on DAG-dependent regulation of constitutively active TRPC3-like cationic currents (I\(_{\text{cat}}\)) in rabbit myocytes isolated from portal vein and ear artery \(^{42, 50, 51}\). In these cells bath application of OAG evoked biphasic effects on I\(_{\text{cat}}\) via PKC-independent and PKC-dependent mechanisms. Interestingly, concentrations of OAG below ten micromolar increased I\(_{\text{cat}}\) currents in cell-attached patches while those above ten micromolar resulted in a decrease in steady-state
constitutive channel activity\textsuperscript{42}. The channel’s response to OAG exhibited a bell-shaped dose-response with EC\textsubscript{50} ~2 μM and IC\textsubscript{50} ~32 μM for this DAG analogue\textsuperscript{42}. Although this biphasic regulation corresponds to an analogue with poor resemblance to native DAGs, it clearly illustrates what may well be a more general situation that applies to endogenous DAGs. Finally, in line with the notions above of signaling compartmentalization and TRPC3 forming part of signaling microdomains, it is possible that acute channel activation occurs subsequently to DAGs generated by phospholipases in close proximity to the channel microenvironment—with DAG metabolizing systems accounting for rapid DAG conversion and thus minimal PKC activation—while distal DAG-generating phospholipases account for the more sustained phase of channel function and eventually, PKC activation.

2.4. DAG species, PKC function and negative regulation of TRPC3

Notably, whereas \textit{in vitro} data shows that essentially all DAG species are able to activate DAG-sensitive PKCs, it is not clear if that is the case \textit{in vivo}. In fact, it seems that in intact cells PI-PLC-generated DAG species are more efficient in terms of PKC activation than PC-PLD-derived DAGs\textsuperscript{21}, a phenomenon likely related to the nature of the acyl chains in the glycerol backbone. For instance, DAGs containing polyunsaturated fatty acids in \textit{sn}-2 position, like those derived from PI-PLC-mediated hydrolysis of PIP\textsubscript{2}, are more efficient in activating PKCs than DAGs containing saturated or monounsaturated fatty acids—derived from PC hydrolysis. This is well exemplified by the observation that 1-stearoyl-2-arachidonoyl-\textit{sn}-glycerol (SAG) and 1-stearoyl-2-docosahexaenoyl-\textit{sn}-glycerol (SDG) both exhibit similar potencies towards PKC
activation, while the effects of 1-stearoyl-2-eicosapentaenoyl-sn-glycerol (SEG) are significantly lower \(^{52}\). Therefore, whenever PC-PLD-derived DAGs were to activate TRPC3, it is plausible to consider that the inefficiency of those DAG species to support PKC activity would allow for a more sustained activation of the channel, as PKC-mediated inhibition will be minimal or inexistent. However, whenever robust and/or sustained PC-PLD activation occurs, this may give rise to concentrations of DAG in the channel’s microdomain high enough to activate PKC, as the higher DAG level would compensate for the lower affinity of PKC for that particular DAG species (see model in Figure 1B).

There is no question on the inhibitory action of PKC on TRPC3 activity, and a PKC phosphorylation site responsible for such effect has been unequivocally identified \(^{46}\). However, the identity of the PKC isoform/s involved remains unknown. So far, available functional evidence linking PKC with TRPC3 activity is for the most part pharmacological. Despite the limited isoform selectivity of the PKC inhibitors traditionally used in those studies, their spectrum of action suggests that the isoform/s involved are likely to belong to the Ca\(^{2+}\)- and DAG-dependent category. If this is so, it is then possible that rapid DAG-dependent activation of the channel, a membrane delimited step, will drive the Ca\(^{2+}\) influx that, in concert with rising DAG levels, will provide the appropriate environment for PKC recruitment and activation. This scenario would be compatible with a temporal sequence of events where DAG-dependent activation precedes DAG-mediated stimulation of PKC and subsequent negative feedback regulation of the channel.
3. PI-PLC and PC-PLD in DAG-dependent regulation of TRPC3: mutually exclusive, complementary or alternating?

Activation of PI-PLC by membrane receptors is subject to rapid desensitization, and DAG generated through PIP$_2$ hydrolysis accumulates in a rapid but transient manner, declining to pre-stimulation levels in a matter of seconds to few minutes (see for instance $^{21,53}$). As mentioned above, receptor-dependent generation of DAG is biphasic, with the second, more delayed phase of DAG production occurring right about when the concentration of PI-PLC-dependent DAG begins to subside $^{17}$. This second phase, which accounts for sustained DAG levels in the cell, is for the most part associated to activation of PLD-mediated hydrolysis of PC $^{16}$. The scenario for phospholipase-dependent regulation of TRPC3 gets puzzling when we consider receptors exhibiting dual coupling in terms of those two DAG-generating phospholipases –i.e., PI-PLC and PC-PLD coupled receptors-. Considering the above mentioned biphasic nature of phospholipase-mediated generation of DAG in biological membranes, one would intuitively think that PIP$_2$-derived DAG would be responsible for early channel activation and as stimulation persists, PC-derived DAG may take over to compensate for the desensitization of the PI-PLC pathway (Figure 2, panel B). Based on the above considerations on fatty acid composition of PI-PLC vs. PC-PLD derived DAGs, such a model implies two main assumptions: a) the channel –or the ancillary DAG binding protein- should be able to interact equally well with DAGs regardless of their origin; or b) channel activation and regulation are differentially managed by different DAG species – interestingly, TRPC3-mediated Ca$^{2+}$ influx and whole-cell currents in general exhibit a somewhat biphasic time
course; it remains to be determined whether this is related to intrinsic channel gating properties or the resultant of bimodal regulation by different DAG species.

Some available evidence shows TRPC3’s ability to concomitantly couple to signaling from different phospholipases—at least from within the same class-. For instance, in HEK293 cells stably expressing TRPC3, stimulation of the epidermal growth factor receptor (EGFR) results in robust TRPC3-mediated Ca\(^{2+}\) influx\(^ {10, 11}\), clearly indicating that DAG derived from PLC\(\gamma\)–the PI-PLC recruited and activated upon EGFR activation\(^ {54}\)–can activate TRPC3. In the same expression system, TRPC3 is also efficiently activated by stimulation of GPCRs such as the muscarinic receptor type-5, which couples to PI-PLC\(\beta\)\(^ {10, 55}\). Similarly, TRPC3 ectopically expressed in avian B lymphocytes is robustly activated by antibody-induced crosslinking of surface IgM, which triggers a series of tyrosine phosphorylation events that drive recruitment and activation of PLC\(\gamma\)\(^ {56, 57}\). This is not surprising, as PLC\(\beta\) and PLC\(\gamma\) both use PIP\(_2\) as a substrate and thus, DAG species derived from their activities are expected to be similar, if not identical, in regards to fatty acid composition and biological properties. Interestingly, IgM-dependent activation of TRPC3 is lost in a PLC\(\gamma\)-deficient variant of DT40 cells whereas stimulation of the M5 muscarinic receptor, which in those cells also couples to PI-PLC\(\beta\), is preserved and results in efficient channel activation\(^ {58}\). In HEK293 cells EGFR also couples to PC-PLD\(^ {59}\) and therefore it is plausible that DAG generated from EGF stimulation is contributed by both PI-PLC\(\gamma\) and PC-PLD–this may actually be the case for several of the receptors known to activate TRPC3-. In that situation, it remains to be determined if EGFR-dependent activation of TRPC3 is the result of the action of PI-
PLCγ or PC-PLD derived DAG, a concerted effect of both DAGs, or if, as discussed above, they act sequentially during early and sustained activation of the channel.

The majority of studies examining regulation of TRPC3 by store-dependent or store-independent mechanisms give little consideration to the possibility that those may actually be alternative and/or concomitant routes of channel regulation. In fact, TRPC3 can function either as SOC or as non-SOC channel, with existing variations and discrepancies mostly due to the characteristics of the expression system and/or channel expression level, among others (discussed in 5, 60-63). But is likely that the scenario under physiological conditions implies a combination of both influx routes, with a particular receptor triggering the store-operated, store-independent or both mechanisms of channel regulation depending on the coupling phospholipase and/or the sequential stimulation of different phospholipases, whenever dual coupling exists –which in turn, may be cell type dependent or expression level dependent, among other variables-. In the case of receptors coupling to PC-PLD rather than PI-PLC, activation of the phospholipase will result in an increase in the local DAG concentration but no IP₃ will be produced; consequently, the system is not expected to promote store depletion and DAG will be the dominating signal. However, in mammalian cells many receptors that promote PC-PLD activity also couple to PI-PLC, giving rise to two potential scenarios: either both PI-PLC and PC-PLD participate in regulation of TRPC3, or only one of them contributes to the process. Recent experimental findings suggest that both scenarios are possible.
3.1. Regulation of TRPC3 by PI-PLC and PC-PLD

This situation is illustrated by the recent findings by Kwan et al. Working with HEK293 cells transiently overexpressing TRPC3, these authors showed that histamine promoted Ca\textsuperscript{2+} influx through a non-capacitative pathway subsequent to activation of H\textsubscript{2} type histamine receptors. H\textsubscript{2} histamine receptors couple to both G\textsubscript{s} and G\textsubscript{q} proteins, and therefore downstream signaling events can be associated to the adenylyl cyclase/cyclicAMP/PKA route, the PI-PLC/DAG/IP\textsubscript{3}/Ca\textsuperscript{2+} path, or a combination of both. The results showed that histamine-dependent activation of TRPC3 did not involve the cyclic AMP/PKA pathway, but rather was subsequent to activation of both PI-PLC and PC-PLD. The PI-PLC inhibitor U73122 or the PC-PLD inhibitor 1-butanol, when used separately, caused a partial reduction of the non-capacitative TRPC3-mediated Ca\textsuperscript{2+} influx, whereas simultaneous inhibition of both phospholipases resulted in complete suppression of channel function. Although the conclusions from this work were based solely on pharmacological inhibition of phospholipase activity, the findings were indicative of a potential involvement of PC-PLD in receptor-activated TRPC3 in HEK293 cells. All experiments in the study by Kwan et al. were conducted in cells in which Ca\textsuperscript{2+} stores were depleted by thapsigargin-mediated inhibition of sarcoplasmic endoplasmic reticulum Ca\textsuperscript{2+} pumps (SERCAs), a maneuver that may mask potential effects of SERCA inhibition and/or store depletion on the activity and regulation of membrane phospholipases and/or the channel itself –see for instance. Therefore, it remains to be determined the extent of contribution of PI-PLC and PC-PLD to receptor- and OAG-stimulated TRPC3 function under normal intracellular buffering conditions. Of note, TRPC6 was not affected by inhibition of PC-PLD, suggesting that the role of PC-
derived DAG might be specific for TRPC3. If the latter is true, it implies either that highly selective structural recognition site/s exist on the TRPC3 protein –should DAG’s effect on TRPC3 be direct- or the hypothetical DAG-binding ancillary proteins are not only specific for PC-derived DAG but also selectively interact with TRPC3 and not TRPC6.

As mentioned above, DAG-dependent regulation of TRPC7 is lost in excised patches\textsuperscript{11-13} or in cells in which resynthesis of polyphosphoinositides is blocked\textsuperscript{12}. Interestingly, all members of the TRPC3/6/7 group have been shown to be sensitive, to different extents, to activation by PIP\textsubscript{2} in excised patches. It is well established that PIP\textsubscript{2} is a critical cofactor for PLD, to the extent that it can profoundly affect the activity, trafficking and receptor-dependent activation of the enzyme (see\textsuperscript{66} and references therein). Therefore, a decrease in cellular PIP\textsubscript{2} levels\textemdash via PI-PLC dependent hydrolysis of PIP\textsubscript{2}, PIP\textsubscript{2} scavenging or phosphatase mediated degradation of PIP\textsubscript{2} as it may occur in excised patches\textemdash can inhibit PLD activity. It remains to be explored whether PIP\textsubscript{2}-dependent activation of TRPC3/6/7 channels is the result of direct channel activation by the lipid, or if it reflects restoration and/or activation of PLD activity that may potentially be required for channel function. The latter is somewhat supported by the observation that addition of PIP\textsubscript{2} to patch pipettes in the whole-cell configuration did not affect pre-existing TRPC7 currents\textsuperscript{12}. This was indeed interpreted by Lemonnier \textit{et al.}\textsuperscript{12} as indicative of PIP\textsubscript{2} acting as a co-factor, as sufficient PIP\textsubscript{2} will be present in intact cells to preclude an additional effect from exogenously added lipid to be detected.
3.2. Regulation of TRPC3 by PC-PLD

In a recent work aimed at examining the effects of phospholipase inhibitors on excitatory postsynaptic currents (EPSC) induced by metabotropic glutamate receptor type 1 (mGluR1) in rat Purkinje cells, Glitsch \(^{67}\) showed that glutamate-dependent activation of these currents involved PC-PLD-derived DAG without participation of PI-PLC. When cells were pre-treated with the PI-PLC inhibitor U73122, but not the less active analogue U73343, EPSC currents were not affected. In contrast, incubation with the PC-PLD inhibitor 1-butanol, but not the inactive isomer 2-butanol, completely abrogated EPSC currents \(^{67}\). Because in HEK293 cells stably overexpressing human TRPC3 1-butanol did not affect OAG-stimulated Ca\(^{2+}\) influx, the conclusion was made that the effects of the alcohol were not the result of direct channel inhibition. But this was not directly tested in Purkinje cells \(–i.e.,\) the native channel environment- and thus the possibility remains that channel expression level and/or the stoichiometric arrangement of the native channel may exhibit a somewhat different sensitivity to pharmacological manipulations as compared to overexpression conditions (see for instance \(^{62}\)). Despite this, perhaps the most intriguing observation was that simultaneous inhibition of PI-PLC and PC-PLD did not result in any further reduction in mGluR1-dependent EPSC compared to PC-PLD inhibition alone, suggesting that in Purkinje cells mGluR1-induced activation of TRPC3 \(–i.e.,\) regulated channel function- was entirely dependent upon PC-PLD activity, despite the fact that mGluR1 also couples to PI-PLC in those cells. These findings in Purkinje cells somehow exemplify the notion discussed above that TRPC3 either exhibits a high degree of selectivity towards PC-PLD-derived DAG species compared to those from PI-PLC, or a tight association and/or compartmentalization exists between the channel and PC-PLD,
so that only locally produced PC-derived DAG within the immediate channel’s vicinity is able to reach the activating threshold for DAG.

4. TRPC3 constitutive, non-regulated function and phospholipase activity

The first, and as of this writing the only attempt to address the potential role of phospholipase-associated signaling on TRPC3 constitutive function derives from studies by Albert and Large. These authors described the existence of constitutive TRPC3-like non-selective cation currents ($I_{\text{cat}}$) in rabbit ear artery myocytes and showed that constitutive activity of these currents were likely to be supported by PC-PLD-derived DAG, but not DAG from PI-PLC. In the initial description of $I_{\text{cat}}$ a dual effect of OAG on these currents was noticed, i.e., stimulation followed by inhibition. OAG-dependent stimulation of $I_{\text{cat}}$ was PKC-independent, whereas OAG-induced inhibition of the current required PKC activity. Under native conditions PKC activation was mediated by PI-PLC-derived DAG. Importantly, PKC-mediated inhibition was found to be tonic, i.e., already operating under constitutive conditions. The fact that $I_{\text{cat}}$ was endowed with high constitutive activity that was spontaneously detectable -without the need of PKC inhibition, cf. 48, 68- implied the existence of an additional activating signal to overcome the tonic inhibition by PKC. Moreover, because PKC activity was dependent upon PI-PLC-derived DAG, the channel activating signal should be generated through a pathway other than $G_q$/PI-PLC. Indeed, pharmacological inhibition of PI-PLC enhanced the already constitutively active $I_{\text{cat}}$, in line with the notion of a non-PI-PLC derived signal responsible for supporting $I_{\text{cat}}$ constitutive activity. Albert and Large tested the hypothesis that DAG derived from an alternative source was responsible for $I_{\text{cat}}$
constitutive activity by a combination of pharmacological and biochemical approaches. First, the PC-PLD inhibitors 1-butanol -but not 2-butanol- and C2-ceramide significantly reduced I_{cat}, whereas D-609 and AACOF3 - PC-PLC and PLA2 inhibitors, respectively- were devoid of effect on the current 42. At the single channel level the activity of I_{cat} was strongly augmented by addition of purified PC-PLD to excised membrane patches. Finally, that constitutive currents were supported by PC-PLD-derived DAG and not PA, was demonstrated by the inhibition of I_{cat} by DL-propranolol, an inhibitor of PAP, confirming that I_{cat} activity required conversion of PA to DAG. It remains to be explored if tonic production of DAG through PC-PLD activity also accounts for TRPC3 constitutive activity in other cell types and expression systems.

5. Concluding remarks

Receptor dependent regulation of TRPC3 occurs downstream stimulation of phosphoinositide- or phosphatidylcholine-specific phospholipases, with DAG being recognized as the likely activating signal, at least when the channel operates in non-store dependent mode. Although useful to pharmacologically assess TRPC3 function, the widespread use of DAG analogues has somehow distracted us from the instinctive notion that native DAG species derived from different and/or alternative sources, the way they partition within the membrane and their ability to more or less efficiently activate DAG-dependent PKCs may have a significant impact on the behavior of native TRPC3 channels under conditions of physiological stimulation.

As the field of TRPC research grows at a fast pace towards understanding their role in physiology and disease, particularly in relation of Ca^{2+}/Na^{+}-influx dependent
events located downstream channel activation, our knowledge on the paths leading to activation/regulation in native systems remains poor and still subject to conservative experimental perspectives. Biophysical approaches combined to existing molecular/structural information on the TRPC3 protein are likely to provide a more refined information on the influence of DAG species –as well as other potential modulatory lipids- on TRPC3 activation and regulation in its native environment. Such information is critical not only to improve our understanding of TRPC3 regulation in vivo but also to better focus existing efforts aimed at designing specific pharmacological modulators of TRPC3 function with potential therapeutical use in human disease.

6. Acknowledgments

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**Figure legends**

**Figure 1**

A) DAG can be generated by receptor dependent stimulation of PI-PLCs (e.g., PLCα, PLCγ), PC-PLC or PC-PLD. PIP2-derived DAG (DAG\(_1\)) mostly contains a saturated fatty acid at position \(sn\)-1 of glycerol and an unsaturated fatty acid in position \(sn\)-2. Instead, PC-derived DAG (DAG\(_2\)) in general presents with a saturated fatty acid at \(sn\)-1 and a mono- or unsaturated fatty acid at \(sn\)-2. Designation as DAG\(_1\) or DAG\(_2\) is arbitrary to emphasize the fact that they represent different DAG species. For comparison, the acyl chain composition of most commonly used DAG analogues (SAG, DOG, OAG; see text for details) is shown. PC: phosphatidylcholine; PI: phosphoinositide; PIP\(_2\): phosphatidylinositol 4,5-bis-phosphate; PA: phosphatidic acid; PAP: phosphatidate phosphohydrolase; p-choline: phosphocholine.

B) Receptors (R) coupling to both PI-PLC and PC-PLD (or PC-PLC) can generate two different DAG species in terms of their fatty acid profiles (DAG\(_1\) and DAG\(_2\)). DAGs can act directly on TRPC3 (T3) or indirectly through a DAG binding ancillary protein (DBAP). The cartoon at the bottom provides an idealized model of the potential temporal interplay between DAG species, PKC activity and TRPC3 function (see text for details): TRPC3 basal function (stage 1 in model) is increased by receptor-generated DAGs (stages 2 and 3 in model) with DAG\(_1\) and DAG\(_2\) accounting, respectively, for rapid and sustained channel activation. DAG\(_1\) species are good activators of PKC, but their cellular levels decrease rapidly due to the action of DAG metabolizing systems and desensitization of the PI-PLC pathway; while DAG\(_2\) species are not potent activators of PKC, as DAG\(_2\) levels increase this may compensate for their lower affinity towards PKC.
and thus support kinase activity, which down regulates channel function (stage 4 in model). PKC activity and the decrease of DAG levels eventually lead to the return of TRPC3 function to basal levels (stage 5 in model).

Figure 2

A) Signaling compartmentalization (black bordered box, expanded in B and C) and/or the existence of a lipid annulus (symbolized by darker phospholipids) may explain the paradoxical regulation of TRPC3 by DAGs. DAGs can be generated by receptor dependent stimulation of PI-PLC or PC-PLD (see text and Figure 1). PIP$_2$-derived DAGs (DAG$_1$), of a more transient nature than PC-derived DAGs (DAG$_2$), may account for initial channel activation, while DAG$_2$ can support more sustained channel activity (B). When generated within the immediate channel’s vicinity DAGs remain inaccessible to PKC. As DAGs accumulate in the channel’s lipid microenvironment, an activating threshold is reached and further DAG accumulation may result in PKC activation (C; see text for details). DAGs can act directly on TRPC3 or indirectly through a DAG binding ancillary protein (DBAP).
Figure 1A.

A

\[
\begin{align*}
\text{PIP}_2 & \xrightarrow{\text{PI-PLC}} \text{DAG}_1 + \text{IP}_3 \\
\text{PC} & \xrightarrow{\text{PC-PLC}} \text{DAG}_2 + \text{Choline} \\
\text{PC} & \xrightarrow{\text{PC-PLD}} \text{PA + p-Choline} \\
& \quad \downarrow \text{PAP} \\
& \quad \text{DAG}_2 \\
\end{align*}
\]

<table>
<thead>
<tr>
<th>DAG</th>
<th>sn-1</th>
<th>sn-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAG$_1$</td>
<td>18:0</td>
<td>20:4 (n-6) or 18:1 (n-9)</td>
</tr>
<tr>
<td>DAG$_2$</td>
<td>16:0 or 18:0</td>
<td>18:1 (n-9)</td>
</tr>
<tr>
<td>SAG</td>
<td>18:0</td>
<td>20:4 (n-6)</td>
</tr>
<tr>
<td>DOG</td>
<td>8:0</td>
<td>8:0</td>
</tr>
<tr>
<td>OAG</td>
<td>18:1 (n-9)</td>
<td>2:0</td>
</tr>
</tbody>
</table>
Figure 1B.

B

R

\[ \text{PI-PLC} \rightarrow \text{DAG}_1 \]

\[ \text{PC-PLD} \rightarrow \text{DAG}_2 \]

\[ \text{DBAP} \rightarrow \text{T3} \rightarrow \text{PKC} \]

(1) (3) (4) (5)

T3 function
Figure 2.
Chapter 9.

Discussion and Conclusion

9.1. The requirement of TRPC3 constitutive activity in VCAM-1 expression and function in human coronary artery endothelial cells (HCAECs).

Over the last decade, it has been revealed that VCAM-1 has a prominent role in mediating attachment and migration of monocytes to endothelium, particularly in areas where endothelial cell activation occurs. Attachment of circulating monocytes to the endothelium requires interaction of the integrin $\alpha_4\beta_1$ on the monocyte with VCAM-1 on the endothelial surface. VCAM-1 is unique in regards to the regulation of its expression when compared to other adhesion molecules that also contribute to monocyte recruitment, such as ICAM-1 and E-selectin. The latter are constitutively expressed in non-activated endothelium, whereas VCAM-1 is virtually absent; when the endothelium is exposed to pro-inflammatory stimuli or other mechanical or chemical insults, VCAM-1 expression increases dramatically (Galkina, 2007). Therefore, this variation of expression implies different functions for VCAM-1 versus the other adhesion molecules. In the vascular endothelium, nucleotides (i.e. ATP, UTP) that are released to the extracellular milieu in response to different stressors, such as ischemia, hypoxia,
chemical, or mechanical stress, are all known to exert a strong pro-inflammatory effect and induce expression of VCAM-1.

Over the last decade VCAM-1 has been envisaged as a potential candidate for molecular targeting in anti-inflammatory therapies for atherosclerosis. However, preliminary basic and translational studies suggest that targeting VCAM-1 alone might not be sufficient to obtain results of clinical significance. This is probably due to the multifactorial nature of atherogenesis, and is likely that dual- or multi-targeting might be necessary to achieve efficient anti-inflammatory therapy. Therefore, it is important to identify new components in the mechanism underlying VCAM-1 expression and monocyte recruitment that can then be exploited as alternative and/or concomitant targets in the design of anti-inflammatory strategies for atherosclerosis [discussed in (Vazquez, 2012)].

Endothelial cell dysfunction is now recognized as a major component in the pathogenesis of various cardiovascular diseases, including hypertension, atherosclerosis, coronary syndrome and heart failure, among others (Kwan, 2007). Transient Receptor Potential Canonical (TRPC) proteins are amongst the most important channel forming proteins in the vascular endothelium. Furthermore, these channels participate in the Ca\(^{2+}\) influx that is required for the modulation of diverse vascular functions (Tano, 2010). Many studies have made it evident that TRPC-mediated Ca\(^{2+}\) influx also plays a major role in cardiovascular disease. Because endothelial cells express all members of the TRPC family—the ultimate combination and/or repertoire depending, among other factors, upon the vascular bed from where the endothelial cell is derived—is not surprising
that malfunction, dysregulation and/or changes in expression level of these channels has been associated with endothelial dysfunction (Vazquez, 2010).

Over the last few years, several studies have made evident the importance of Ca\(^{2+}\) signaling in regulated expression of VCAM-1. For instance, in microvascular endothelium (Quinlan et al., 1999) and synovial fibroblasts (Chen et al., 2002) changes in intracellular Ca\(^{2+}\) associated to Ca\(^{2+}\) release from internal stores have been linked to the ability of Substance P or β2-microglobulin to induce VCAM-1. Furthermore, in human coronary artery endothelial cells (HCAECs) Ca\(^{2+}\) mobilization has been related to the mechanism by which ATP induces VCAM-1 expression (Seye CL, 2004). Recently, our laboratory has shown that constitutive function of a native TRPC channel, in particular TRPC3, plays an obligatory role in the mechanism regulating expression of VCAM-1 in HCAECs (Smedlund et al., 2010; Smedlund, 2008). HCAECs express all members of the TRPC family (Yip et al., 2004; Smedlund, 2008). Using a silencing RNA approach combined with real-time fluorescence monitoring of Ca\(^{2+}\) influx, it was shown that among all TRPC members expressed in HCAECs only TRPC3 forms, or is part of, endogenous Ca\(^{2+}\)-permeable channels that contribute to ATP stimulated Ca\(^{2+}\) influx (Vazquez and Putney, 2006; Smedlund, 2008), a process occurring downstream activation of purinergic P2Y\(_2\) receptors. Notably, those studies also showed that constitutive Ca\(^{2+}\) influx in HCAECs is contributed, for the most part, by endogenous TRPC3 channels. In addition, we have shown that TRPC3 expression is essential in the signaling underlying ATP induced VCAM-1 and monocyte adhesion (Smedlund, 2008). Moreover, treatment of HCAECs with ATP also significantly enhances TRPC3 protein expression which correlates with a marked increase in constitutive cation entry. This
strongly suggests a link between native TRPC3 channels expressed in the coronary endothelium and regulated VCAM-1 expression and function, a critical event in the initiation and development of atherosclerotic lesions.

The observation that TRPC3 plays a critical role in ATP-induced VCAM-1 expression/function and that this is paralleled with increased TRPC3 expression and constitutive cation influx, raised the important question whether TRPC3 contribution to regulated expression of VCAM-1 takes place through regulated activity, constitutive activity, or both. Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine that induces VCAM-1 expression in endothelial cells from several vascular beds (Ley and Huo, 2001) including HCAECs (Smedlund, 2008; Smedlund et al., 2010). However, TNFα signaling is not associated to regulation of Ca²⁺ influx (Bradley, 2008). While studying the role of Ca²⁺ influx in ATP-dependent VCAM-1 expression in HCAECs, we observed that maneuvers that prevent Ca²⁺ entry into the cell (i.e., omission of extracellular Ca²⁺, use of channel blockers) significantly impairs the ability of TNFα to induce VCAM-1 in these cells (Smedlund et al., 2010; Smedlund, 2008). Notably, in HCAECs TNFα-induced VCAM-1 is also accompanied by an increase in TRPC3 expression and the magnitude of constitutive cation influx. This increase in constitutive cation influx is not manifested when TRPC3 is knocked down. Altogether, these findings suggest that the gain in constitutive cation influx is a result of an increase in the number of constitutively active TRPC3 channels in the membrane (Smedlund et al., 2010). To directly examine if constitutive, non-regulated function of TRPC3 is required for VCAM-1 expression and function, we evaluated the role of extracellular Ca²⁺ on TNFα-induced VCAM-1. TNFα induces VCAM-1 as early as three hours; however, in the absence of
extracellular Ca\(^{2+}\) or in the presence of various channel blockers (i.e. Gd\(^{3+}\), SKF96365) VCAM-1 expression, both in whole cell lysates or plasma membrane resident VCAM-1, is significantly reduced. Furthermore, when TRPC3 is knocked down by using silencing RNA specific for TRPC3, VCAM-1 expression and monocyte adhesion are also markedly reduced. Importantly, when HCAECs were cotransfected with TRPC3 siRNA and a TRPC3 encoding plasmid resistant to the silencing oligonucleotides, the siRNA effect was reverted (“rescue” experiment) indicating that the results observed with TRPC3 siRNA were indeed specifically due to TRPC3 knockdown. Altogether, these findings strongly support the notion that TRPC3, presumably through its constitutive function, contributes the Ca\(^{2+}\) influx that is required to support VCAM-1 expression and monocyte adhesion (Smedlund et al., 2010).

Other groups have demonstrated TNFα-dependent modulation of Ca\(^{2+}\) influx in relation to TRPC function. For example, in airway smooth muscle cells, TNFα increased constitutive Ca\(^{2+}\) entry and this was completely abolished by knockdown of TRPC3 (White et al., 2006). In the same study, it was also demonstrated that TNFα increased TRPC3 expression, whereas that of TRPC1, 4, 5, and 6 remained unchanged (White et al., 2006). However, in endothelial cells derived from dermal microvessels, umbilical vein (HUVECs) and pulmonary artery (HPAECs), TNFα clearly induced expression of TRPC1 (Paria et al., 2003; Paria et al., 2004). Additionally, in HUVECs and HPAECs increased TRPC1 expression was also accompanied by augmented receptor regulated Ca\(^{2+}\) influx. In HCAECs when examining TNFα actions on individual TRPC members, we found that expression of TRPC3, 4, and 7 was increased, whereas that of TRPC1 was drastically reduced. These apparently contradictory results could be due to not only
experimental variability but to differential responsiveness of individual TRPCs to TNFα actions in different vascular beds throughout the arterial tree.

In most endothelial cells, VCAM-1 expression is regulated by Nuclear Factor kappa B (NFkB) (Zhang, 2008). NFkB activation involves the release of the inhibitory protein IkBα and the subsequent translocation of NFkB to the nucleus where it modulates the transcription of target genes. Release of NFkB from IkBα, requires phosphorylation of the IkBα by IkBα kinase (IKK)β followed by ubiquitination and proteasomal degradation of the IkBα protein (Tergaonkar, 2006). We observed that treatment of HCAECs with TNFα resulted in a rapid and sustained activation of NFkB. This was manifested as an increase in IkBα phosphorylation and its degradation (Smedlund et al., 2010), two of the earliest events in the cascade leading to NFkB activation. Moreover, when HCAECs were pretreated with hypoestoxide, a selective inhibitor of IkBα phosphorylation (Ojo-Amaize, 2001), TNFα-induced VCAM-1 was completely abrogated, indicating that in HCAECs VCAM-1 expression is mostly under control of the NFkB pathway.

Considering that: a) NFkB activation depends, directly or indirectly, on Ca^{2+} influx (Han and Logsdon, 2000); b) that TNFα-induced VCAM-1 requires constitutive Ca^{2+} influx, presumably through TRPC3; and c) that VCAM-1 activation in HCAECs is driven by NFkB, we next examined the requirement of TRPC3 for NFkB signaling in HCAECs. Using the silencing approach described above, we observed that when TRPC3 was knocked down, TNFα-induced phosphorylation of IkBα and its upstream regulator IKKβ, was completely prevented. Furthermore, IkBα degradation was also significantly reduced. Altogether, these findings suggest that TRPC3 expression/function is indeed
required for proper TNFα-dependent activation of NFkB pathway (Smedlund et al., 2010).

It is known that in several cell types, including endothelium (Bair et al., JBC 2009) Ca^{2+} influx dependent activation of NFkB is often associated to calmodulin (CAM) and calmodulin-dependent kinases (CAMKs). Therefore, using a pharmacological approach, we examined the role of CAM using the CAM inhibitor W7, and CAMKs using the CAMK inhibitor KN62, in NFkB activation in HCAECs. When HCAECs were pretreated with W7 or KN62 a dramatic reduction in TNFα-dependent phosphorylation of IkBα and its upstream regulator IKKβ was observed. Although pharmacological in nature, this represents the first evidence to date for a role of the Ca^{2+}/CAM/CAMK axis in TNFα-dependent activation of NFkB in coronary endothelial cells. Additionally, when HACECs were treated with the channel blocker SKF96365, similarly to W7, TNFα-induced phosphorylation of IkBα and IKKβ were completely prevented, indicating a mandatory requirement for constitutive Ca^{2+} entry in activation of NFkB. Moreover, inhibition of NADPH oxidase with apocynin resulted in a partial decrease of IkBα phosphorylation, indicating that NADPH oxidase-mediated production of ROS is also necessary for full NFkB activation.

The requirement of constitutive Ca^{2+} influx presumably through TRPC3, for VCAM-1 expression, monocyte adhesion and NFkB activation in HCAECs, prompted us to evaluate if increased expression of TRPC3 and thus increased constitutive Ca^{2+} influx is sufficient to promote VCAM-1 expression and NFkB activation. Using two different TRPC3 expression systems, we evaluated VCAM-1 expression following TNFα stimulation. First, VCAM-1 expression and NFkB activation were examined in HEK293
cells (non-endothelial) stably overexpressing TRPC3 (T3-HEK293). T3-HEK293 cells exhibit a robust constitutive Ca\(^{2+}\) entry when compared to wild-type HEK293 cells because of their high expression level of TRPC3 channels (Trebak, 2003). In the wild-type cells VCAM-1 expression was significantly augmented after 16 hours of treatment with TNF\(\alpha\). Notably, the time course for TNF\(\alpha\)-induced VCAM-1 in T3-HEK293 was clearly left shifted, with a trend to be increased as early as 3 hours following exposure to the cytokine and a statistically significant upregulation at 16 hours of TNF\(\alpha\) treatment when compared to wild-type cells. Notably, basal VCAM-1 expression in T3-HEK293 cells was not significantly different from that in wild-type cells. In addition, in both wild-type and T3-HEK293 cells, TNF\(\alpha\)-induced VCAM-1 was markedly decreased in the presence of the channel blocker Gd\(^{3+}\), confirming the requirement for constitutive Ca\(^{2+}\) influx in regulated expression of VCAM-1. Importantly, a similar effect was observed for NFkB activation, where T3-HEK293 cells exhibited a significantly enhanced IkB\(\alpha\) phosphorylation and degradation compared to wild-type cells, while basal levels remained unchanged.

Second, basal and TNF\(\alpha\)-induced VCAM-1 and NFkB activation were examined in HCAECs transiently overexpressing T3-HA, a hemagglutinin epitope (HA)-tagged version of human TRPC3, shown to be fully functional and with properties indistinguishable from the non-tagged channel protein (Vazquez et al., 2004; Wedel et al., 2003). As expected, T3-HA-transfected cells exhibited a robust increase in constitutive cation entry compared to mock-transfected cells (Smedlund et al., 2010; Vazquez et al., 2004). While no differences were observed in basal VCAM-1 expression or basal NFkB activation, TNF\(\alpha\)-induced VCAM-1 and IkB\(\alpha\)
phosphorylation/degradation were significantly increased in T3-HA transfected HCAECs compared to mock transfected cells, once again suggesting that increased TRPC3 expression, and thus increased constitutive Ca\(^{2+}\) influx, enhances TNF\(\alpha\)-induced VCAM-1 expression and NFkB activation. Because TRPC3 overexpression, regardless of the cell system used, did not alter basal levels of VCAM-1 nor basal NFkB activation, we conclude that TRPC3 expression and its constitutive function are necessary but not sufficient to drive inflammatory signaling, and that a priming signal, such as TNF\(\alpha\), may be required.

TRPC channels participate in numerous Ca\(^{2+}\) dependent events that are part of endothelial cell physiology and, over recent years, have emerged as critical players in signaling events associated to cardiovascular disease (reviewed by us in (Tano, 2010). Our findings, described and discussed here (and see Chapters 3, 4, 6, and 7 for details), underscore novel aspects within the molecular mechanism underlying regulated expression of VCAM-1 and monocyte adhesion, two of the most critical events in initiation and progression of atherosclerotic lesions in vivo. Our studies on the role of Ca\(^{2+}\) influx in purinergic-dependent expression of VCAM-1 in HCAECs, have demonstrated that native TRPC3 proteins form, or are part of, Ca\(^{2+}\)-permeable channels that contribute to the Ca\(^{2+}\) influx that follows P2Y\(_2\) receptor activation, and that TRPC3 expression has a mandatory role in ATP-induced VCAM-1 expression and monocyte adhesion. Furthermore, using TNF\(\alpha\), a cytokine involved in the pathogenesis of atherosclerosis but not associated with regulated Ca\(^{2+}\) influx, we demonstrated that it is the constitutive function of TRPC3 channels that contributes to VCAM-1 expression and monocyte adhesion. Altogether, our findings constitute novel evidence illustrating how a
gain in the expression level of TRPC3, a TRPC channel endowed with high constitutive function, may result in exacerbated inflammatory signaling with potential pathological impact even in the absence of receptor stimulation. These observations underscore the potential significance of endothelial TRPC3 channels within the context of molecular and cellular events that are critical in the pathogenesis of coronary artery disease (Smedlund et al., 2010; Smedlund, 2008).

9.2. Cholinergic dependent survival signaling in HCAECs.

The vascular endothelium possesses a number of important properties to maintain endothelial cell homeostasis and delay the development of atherosclerosis. However, different chemical, biological, and/or mechanical insults can result in endothelial dysfunction, which, in the context of other existing risk factors, may favor the development of atherosclerotic lesions. Recently, our knowledge of the role of nAChRs in physiological and pathophysiological processes in non-neuronal tissues and organ systems has significantly increased. For instance, the vascular endothelium exhibits a variety of functions that are influenced by cholinergic signaling, including proliferation, survival, migration and angiogenesis (Cooke, 2007). Moreover, it has been demonstrated that in human microvascular endothelial cells, nicotinic or cholinergic - via vagus nerve stimulation- inhibits leukocyte recruitment during local inflammation via the so called cholinergic anti-inflammatory pathway (Saeed et al., 2005).

Our lab has recently provided evidence for the first time that stimulation of nicotinic- and muscarinic-acetylcholine receptors (respectively, nAChR and mAChR) triggers pro-survival signaling in human coronary endothelial cells (HCAECs). When
stimulating HCAECs with the cholinomimetic agent carbachol to simultaneously activate nAChRs and mAChRs, three survival signaling pathways are activated, namely: PI3K/AKT, JAK2/STAT3, and the MAP Kinase family members ERK1/2; this results in concomitant reduction of the apoptosis rate of these cells when exposed to the proatherogenic cytokine TNFα. These observations suggest that cholinergic-dependent activation of these survival signaling pathways likely accounts for an efficient survival response of coronary endothelial cells when exposed to an inflammatory environment (Smedlund et al., 2011).

Our findings, described in detail in chapter five, show that both nAChRs and mAChRs activate the PI3K/AKT axis in HCAECs; however, nicotinic stimulation of AKT demonstrated a biphasic activation of AKT compared to the monophasic effect of muscarinic signaling. One explanation for this observation is that muscarinic stimulation can also trigger compensatory mechanisms that turn off AKT, a phenomenon that is not unusual in G protein coupled receptor modulation of AKT (Liu et al., 2004). As the stimulation persists, deactivation of AKT occurs which can then mask the delayed nicotinic signaling when both receptors are simultaneously activated. Despite these differences, the activation profile of AKT by mACHR and nAChR stimulation was reminiscent of that reported for other well known activators of AKT in the coronary circulation (Mahadev et al., 2008; Teng et al., 2011).

Upon examining the JAK2/STAT3 survival signaling in HCAECs we demonstrated for the first time, that nicotine-induced phosphorylation of JAK2 and STAT3 is inhibited by α-bungarotoxin, indicating activation of JAK2/STAT3 axis downstream α7-nAChRs. Interestingly, it has been observed in macrophages that
cholinergic activation of the JAK2/STAT3 pathway occurs downstream α7-nAChR
(Jonge et al., 2005). However, in other vascular endothelial beds, such as human
umbilical vein and dermal microvascular endothelial cells, nicotine treatment was shown
to inhibit JAK2/STAT3 phosphorylation (Chatterjee et al., 2009). These observations
may be due to the heterogeneity of endothelial cells derived from different vascular beds
in regards of their responsiveness to cholinergic stimulation.

Furthermore, cholinergic stimulation of the MAP kinase ERK1/2 in HCAECs
exhibited similar activation features as that observed for the PI3K/AKT axis, where both
mAChR and nAChR contribute to its activation. In addition, ERK1/2 phosphorylation
was completely abolished in the presence of α-bungaratoxin, suggesting involvement of
α7-nAChR in the activation process.

In the endothelium the balance between apoptosis and survival is critical in
maintaining endothelial cell homeostasis. Our studies show three efficient survival
signaling pathways triggered in HCAECs in response to cholinergic stimulation.
Moreover, our findings show lack of cholinergic activation of the NFκB pathway or
induction of VCAM-1 in HCAECs, which goes in line with the notion of a pro-survival
and protective role, rather than a pro-inflammatory action, of cholinergic signaling in
these cells. In the case of nAChR, other studies have demonstrated rather controversial
results when examining nicotine effects on HCAECs. For example, the expression of
VCAM-1 and ICAM-1 was shown to be enhanced by nicotine treatment of HCAECs
(Cirillo et al., 2007), whereas, Hakki et al. reported that nicotine had no effect at all
(Hakki et al., 2002). These controversial findings could be due partially to differences in
experimental conditions, or it is also possible that the high membrane permeability of
nicotine could induce effects beyond those strictly related to its actions on nAChRs, especially with prolonged incubation times with this alkaloid.

In addition, when examining apoptosis in HCAECs we observed that cholinergic stimulation does not induce apoptosis; rather, a significant reduction in apoptosis was found when the cells were exposed to cholinergic agonists in the presence of the pro-inflammatory cytokine TNFα. Furthermore, when stimulating nAChR with either nicotine or carbachol -but in the presence of the muscarinic inhibitor atropine- we found equivalent outcomes, strongly supporting the notion of a genuine nicotinic cholinergic effect on survival signaling rather than a non-specific action of nicotine.

Using a Fura-2-based Ca$^{2+}$ imaging technique we were not able to detect cholinergic induced Ca$^{2+}$ influx in HCAECs. However, this could be due to the fact that these cells have a very efficient Ca$^{2+}$ buffering system (Smedlund, 2008). Therefore, the possibility exists that Ca$^{2+}$ entering through the nAChR is rapidly buffered out and goes undetectable under our experimental conditions. Future studies need to be conducted to define whether the role of nAChR survival signaling is related to channel permeation properties or if the signaling role is independent of channel gating and cation permeation, as shown in cell types other than endothelial cells (Jonge et al., 2005). In summary, our findings represent a novel contribution to our understanding of the events that may be of relevance for cholinergic dependent modulation of coronary endothelial cell survival. Future experimental designs using mouse models of atherosclerosis will address the question whether this cholinergic dependent survival signaling is of relevance in endothelial integrity during the course of atherosclerosis.
9.3. Concluding Remarks

Our studies provide, for the first time, experimental evidence underscoring the potential impact of upregulated expression of constitutively active TRPC3 channels within the context of the inflammatory molecular/cellular events that are critical in the pathogenesis of coronary artery disease. Interestingly, immunohistochemical analysis of aortic root sections from ApoE<sup>−/−</sup> mice, a recognized model of atherosclerosis, showed upregulated expression of TRPC3, compared to that in sections from wild-type animals. The latter findings, although suggestive, do not establish a causal link between increased TRPC3 expression and atherogenesis due to the multitude of inflammatory mediators that underlie lesion development in vivo. Therefore, generation of mouse models of atherosclerosis in which TRPC3 expression has been genetically manipulated (i.e. conditional transgenic or knockout animals for TRPC3; see section 2.2.) is required to properly examine the contribution of TRPC3 to lesion development in vivo. The in vitro studies here described using primary endothelial cells from an atherorelevant vascular bed, namely coronary endothelium; provide a molecular framework for a logical and rational design of in vivo studies using the novel mouse models described in sections 2.2.1. and 2.2.2. above. Furthermore, identifying new players within the signaling mechanisms of VCAM-1 expression and monocyte recruitment is imperative to develop alternative therapeutic strategies for effective treatment and/or diagnosis of atherosclerosis, particularly at early stages of the disease. By examining the novel role of TRPC3 in the pathogenesis of the atherosclerotic lesion by using both in vitro and in vivo approaches, the findings derived from our studies can certainly make a significant contribution into that direction.
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