THE ROLE OF PROTEIN S-GLUTATHIONYLATION ON Ca\textsuperscript{2+} SIGNALING IN CULTURED AORTIC ENDOTHELIAL CELLS

By

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The Role of Protein S-Glutathionylation on Ca$^{2+}$ Signaling in Cultured Aortic Endothelial Cells

Abstract

By

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In non-excitable cells, oxidative stress impacts both basal and agonist-mediated changes in cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}]_i$). An increase in protein S-glutathionylation is a common response to oxidative stress; however, the role of glutathionylation in Ca$^{2+}$ homeostasis and Ca$^{2+}$ signaling processes is not well understood. Diamide, a membrane-permeable oxidizing reagent, rapidly and reversibly converts glutathione (GSH) to its disulfide (GSSG), and promotes glutathionylation. In the present Dissertation, the acute effect of diamide on the [Ca$^{2+}]_i$ of cultured aortic endothelial cells (ECs) was examined at the single-cell level using the fluorescent Ca$^{2+}$ indicator, fura-2 and time-lapse video microscopy. Low concentrations (50, 100 µM) of diamide reversibly increased spontaneous Ca$^{2+}$ oscillations, whereas high concentrations (250, 500 µM) of diamide caused an immediate, synchronized Ca$^{2+}$ oscillation in essentially all cells examined followed by a time-dependent rise in basal [Ca$^{2+}]_i$. Most strikingly, diamide evoked a dose-dependent increase in single-cell Ca$^{2+}$ oscillations in both the presence and absence of extracellular Ca$^{2+}$. Oscillations in [Ca$^{2+}]_i$ were due to inositol 1,4,5-trisphosphate (IP$_3$) receptor (IP$_3$R) activation, since they were attenuated by pharmacological inhibition of either phospholipase C or IP$_3$Rs themselves. However,
diamide, even at high concentrations, did not increase PIP$_2$ hydrolysis, suggesting diamide increases the sensitivity of IP$_3$Rs to activation. In agreement with this, diamide enhanced both IP$_3$-induced Ca$^{2+}$-release (IICR) and Ca$^{2+}$-induced Ca$^{2+}$-release (CICR) via IP$_3$Rs. Since IP$_3$ is not changing during challenge with diamide, our results indicate diamide primarily increases the sensitivity of IP$_3$Rs to cytosolic Ca$^{2+}$, i.e. enhanced CICR. Consistent with GSH-dependent thiol-modification, this oscillatory response was dependent upon both the cellular GSH concentration and the ability to recycle GSSG back to GSH. Moreover, exogenous exposure of cells to hydrogen peroxide (H$_2$O$_2$) produced similar changes in IP$_3$R activity and in biochemical assays we found that the IP$_3$R could be reversibly glutathionylated in response to either diamide or H$_2$O$_2$. When taken together, our data demonstrate oxidative stress increases CICR via the IP$_3$R, and reveals an important role for glutathionylation in the coordination of cellular Ca$^{2+}$ dynamics during both physiological redox signaling processes and during the pathological response to oxidative stress.
Chapter 1

INTRODUCTION
1.1: Ca\textsuperscript{2+} HOMEOSTASIS & SIGNALING

The calcium ion (Ca\textsuperscript{2+}) is a universal signaling molecule involved in virtually every aspect of cellular behavior. Changes in cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) control extremely rapid events, from synaptic transmission in neurons and excitation-contraction coupling in muscle, to processes occurring over longer durations, including fertilization, cellular proliferation, differentiation, gene transcription, protein synthesis and metabolism. Aberrant changes in [Ca\textsuperscript{2+}]\textsubscript{i} can lead to the deregulation of Ca\textsuperscript{2+}-dependent signaling processes and if unabated ultimately cellular death through apoptotic or necrotic pathways. Ca\textsuperscript{2+} is thus “a double edged sword,” essential to both the life and death of the cell.

Cellular Ca\textsuperscript{2+} homeostasis and Ca\textsuperscript{2+} signaling mechanisms are coordinated by an exquisite array of Ca\textsuperscript{2+}-permeable ion channels, transporters, and exchangers located in the plasmalemmal and intracellular membranes, as well as a plethora of regulatory/accessory proteins and molecules (for reviews see (Bootman & Berridge, 1995; Berridge et al., 2000; Berridge et al., 2003)). Individual cell types express a unique complement of these proteins and factors which customize downstream Ca\textsuperscript{2+}-dependent signal transduction to the specific stimuli responsible for initiating the Ca\textsuperscript{2+} signaling cascade, and to the specific needs of the cell. This diversity tailors the speed, amplitude, duration, and spatial-temporal pattern of intracellular Ca\textsuperscript{2+} dynamics to each cell’s unique physiological functions.

In mammalian cells, [Ca\textsuperscript{2+}]\textsubscript{i} is maintained at ~100 nM during resting conditions. In contrast, the [Ca\textsuperscript{2+}] of the extracellular milieu is typically 1-2 mM. In the
endoplasmic/sarcoplasmic reticulum (ER/SR), the primary Ca\textsuperscript{2+} storage site of the cell, free [Ca\textsuperscript{2+}] is \(\sim 0.5 - 1.0\) mM; achieved by Ca\textsuperscript{2+} binding proteins located in the ER/SR lumen. The 1,000 – 10,000 fold difference in [Ca\textsuperscript{2+}] across the ER/SR and plasma membranes provides a strong driving force for Ca\textsuperscript{2+} influx into the cytosol. Ca\textsuperscript{2+} efflux mechanisms are therefore critical in preserving these chemical gradients (Brini & Carafoli, 2009). Ca\textsuperscript{2+} is primarily removed from the cytosol by the concerted actions of two ubiquitous membrane transport proteins: the plasmalemmal Ca\textsuperscript{2+}-ATPase (PMCA) pump, responsible for extrusion of Ca\textsuperscript{2+} from the cell (Di Leva \textit{et al.}, 2008); and the sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) pump, responsible for the sequestration of Ca\textsuperscript{2+} into ER/SR stores (MacLennan \textit{et al.}, 1997). In some cell types (e.g. cardiac myocytes), the Na\textsuperscript{+},Ca\textsuperscript{2+}-exchanger (NCX) present in the surface membrane plays an important role in Ca\textsuperscript{2+} removal from the cytoplasm (Philipson & Nicoll, 2000).

Mitochondria can also sequester Ca\textsuperscript{2+}, but their Ca\textsuperscript{2+} uptake mechanisms are generally of low affinity and under normal conditions are not thought to play a prominent role in maintenance of basal [Ca\textsuperscript{2+}], (Duchen, 2000; Brini, 2003). Finally, acidic compartments (i.e. the Golgi apparatus, endosomes, and lysosomes) contain Ca\textsuperscript{2+} pumps and Ca\textsuperscript{2+} exchangers capable of removing Ca\textsuperscript{2+} from the cytosol, but their contribution to the preservation of basal [Ca\textsuperscript{2+}], in mammalian cells remains poorly understood (Morgan \textit{et al.}, 2011).

During a Ca\textsuperscript{2+} signaling event, [Ca\textsuperscript{2+}], can transiently rise into the micro-molar range by the activation of Ca\textsuperscript{2+}-permeable ion channels present in the ER/SR and/or the plasma membrane. In non-excitable cells, Ca\textsuperscript{2+} signal transduction is typically initiated by stimulation of cell surface receptors linked to the activation of phospholipase C
isoforms β or γ (PLC-β/γ; (Berridge, 1993)). PLC-β is activated following stimulation of G-protein coupled receptors linked to the hetero-trimeric Gq family proteins, whereas PLC-γ is turned on by phosphorylation in response to tyrosine kinase receptor stimulation. PLC isoforms can also be activated independent of receptor stimulation by Ras (PLC-ε), or by a rise in [Ca^{2+}]_{i} itself (PLC-δ). Activation of PLC leads to the generation of inositol 1,4,5-trisphosphate (IP_{3}) and diacylglycerol (DAG) following hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_{2}). Membrane delimited DAG activates protein kinase C (PKC), which can then phosphorylate downstream target proteins. IP_{3} liberated from the inner leaflet of the plasmalemmal diffuses into the cytoplasm and binds to IP_{3} receptors (IP_{3}R) located in the ER membrane (see below). IP_{3}R activation stimulates the release of intracellular Ca^{2+} leading to a rise in [Ca^{2+}]_{i} and a concomitant reduction in ER Ca^{2+}. The depletion of ER Ca^{2+} stores initiates store operated Ca^{2+} entry (SOCE; (Parekh & Putney, 2005)), presumably coordinated by STIM and ORAI proteins located in the ER and plasma membranes, respectively. Elevated [Ca^{2+}]_{i} is then restored to basal levels by the re-uptake of Ca^{2+} back into the ER by the SERCA pumps, and by the extrusion of Ca^{2+} out of the cell by PMCA pumps. Re-uptake of Ca^{2+} back into the ER is critical in maintaining the cyclical influx and efflux of Ca^{2+} into and out of the cytosol necessary for the propagation of regenerative oscillations in [Ca^{2+}]_{i}. For the most part, the information encoded by Ca^{2+} oscillations have yet to be unraveled; however, the involvement of IP_{3}Rs and alterations in IP_{3}R behavior represents a potential mechanism for the transmission of this Ca^{2+} “code” into the appropriate cellular response.
1.2: IP$_3$ Receptors (IP$_3$R)

IP$_3$Rs are a ubiquitously expressed family of Ca$^{2+}$-permeable ion channels found in the ER membrane of nearly all known cells (for reviews see (Taylor, 1998; Foskett et al., 2007; Mikoshiba, 2007)). The first evidence for an intracellular Ca$^{2+}$ release channel activated by IP$_3$ was demonstrated by Michael Berridge in experiments that began at Case Western Reserve University. Berridge showed the activation of cell surface receptors lead to the formation of a diffusible second messenger (i.e. IP$_3$) capable of eliciting a robust release of Ca$^{2+}$ from intracellular stores (Streb et al., 1983). Shortly thereafter, what is now known as the IP$_3$ receptor type 1 (IP$_3$R$_1$) isoform was purified (Maeda et al., 1988) and cloned (Furuichi et al., 1989) from mouse cerebellum, and functionally expressed in neuroblastoma/glioma (Furuichi et al., 1989) and fibroblast (Miyawaki et al., 1990) cell lines by Mikoshiba’s groups. Both purified and recombinant IP$_3$Rs exhibited a high affinity (~100 nM) for IP$_3$ binding to the receptor/channel. Ca$^{2+}$ release from isolated microsomes in response to IP$_3$ was enhanced by IP$_3$R overexpression (Miyawaki et al., 1990), and the incorporation of purified IP$_3$R into lipid bilayers produced Ca$^{2+}$-permeable ion channel activity in the presence of IP$_3$ (Maeda et al., 1991). The culmination of these studies provides some of the initial evidence that the IP$_3$R is an intracellular Ca$^{2+}$ channel activated by IP$_3$.

Functional IP$_3$Rs are tetramers, with each subunit consisting of a large cytosolic N-terminus, 6 trans-membrane (TM) segments containing the channel pore, and a short cytosolic C-terminus (Foskett et al., 2007). The N-terminus contains a number of regulatory domains essential for receptor/channel function, including the IP$_3$ binding region and several putative Ca$^{2+}$ binding motifs. Each monomer is composed of ~ 2,700
amino acids with a molecular weight between 250-300 kDa; leading to a channel mass
greater than 1 MDa. In human, three separate gene products (IP₃R types 1-3) and a
number of splice variants have been identified to date. At the protein level, IP₃R
isoforms are 60-80% homologous, and exhibit distinct, overlapping expression patterns
with most cell types expressing more than one isoform. The contribution of individual
IP₃R isoforms to specific cellular functions is an area of active investigation.

IP₃Rs are primarily regulated by IP₃ and Ca²⁺ in a complex manner that is still not
completely understood (Marchant & Taylor, 1997; Mak et al., 1998; Shinohara et al.,
2011). Both IP₃ and Ca²⁺ are required for channel activation (Iino, 1990; Bezprozvanny
et al., 1991). In general, IP₃Rs are activated by IP₃ in a graded fashion with a greater
number of channels responding to increasing concentrations of IP₃, i.e. IP₃-induced Ca²⁺
release (IICR). Cytosolic Ca²⁺, on the other hand, exhibits biphasic effects on IP₃R
activity. Low concentrations of Ca²⁺ are required for IP₃ binding and can stimulate IP₃R
activation through a process of Ca²⁺-induced Ca²⁺ release (CICR), whereas high Ca²⁺
concentrations eventually inhibit channel activity (Bezprozvanny et al., 1991). Although
somewhat variable between studies, IP₃R isoforms exhibit different sensitivities to
activation by IP₃ and Ca²⁺. The relative affinity of IP₃R isoforms for IP₃ follows the
sequence type-2 > type-1 > type-3 (Tu et al., 2005b), whereas the relative affinity to Ca²⁺
is reported as type-2 > type-3 > type-1 (Tu et al., 2005a). Regulation of IP₃R activity by
IP₃ and Ca²⁺ is further complicated at the cellular and tissue level by the disparate
distribution of IP₃R isoforms and their splice variants, the possibility of
erhomultimerization of individual IP₃R isoforms (Foskett et al., 2007), and an apparent
heterogeneous sensitivity of individual IP$_3$R isoforms to activation by IP$_3$ and Ca$^{2+}$ (Vais et al., 2010).

The fundamental basis for how activation of IP$_3$Rs by IP$_3$ and Ca$^{2+}$ leads to the generation and propagation of global changes in [Ca$^{2+}$]$_i$ has been organized into three broad levels of stimulation (Fig 1.1; (Parker et al., 1996)). At low levels of IP$_3$, the activation of single IP$_3$Rs produce a localized rise in [Ca$^{2+}$]$_i$ in the immediate vicinity of the activated channel; commonly referred to as a Ca$^{2+}$ “blip”. Blips are rapidly dissipated due to feedback inhibition of the channel by Ca$^{2+}$ and by the Ca$^{2+}$ buffering capacity of the cytosol limiting its diffusion. At intermediate levels of IP$_3$, Ca$^{2+}$ “puffs” originate from the coordinated opening of several IP$_3$Rs in close proximity to one another, referred to as a cluster. The coordinated release of Ca$^{2+}$ from clustered IP$_3$Rs is facilitated by CICR. Both blips and puffs occur over very short time-scales (µs-ms) and alone do not cause global changes in [Ca$^{2+}$]$_i$. At high levels of IP$_3$, a large number of IP$_3$Rs are activated in separate clusters, with the Ca$^{2+}$ released from a one cluster triggering the release of Ca$^{2+}$ from adjacent clusters by CICR, leading to regenerative, global changes in [Ca$^{2+}$]$_i$ referred to as Ca$^{2+}$ “waves”. It is these Ca$^{2+}$ waves, or global Ca$^{2+}$ oscillations, that are responsible for activating most downstream Ca$^{2+}$-dependent signaling pathways.

In addition to IP$_3$ and Ca$^{2+}$, IP$_3$R activity can be modulated by a large and growing list of regulatory/accessory proteins and molecules which further contribute to the complexity of IP$_3$R function in vivo (Foskett et al., 2007). For example, IP$_3$R channel activity is potentiated by ATP, phosphorylation by cAMP dependent protein kinase (PKA), PKC, and tyrosine kinases. IP$_3$Rs are attenuated by interaction with calmodulin (CaM), IP$_3$R binding protein released by IP$_3$ (IRBIT), ERp44, and phosphorylation by
CaM-dependent kinase (CaMK). There is also substantial evidence demonstrating IP₃R activity is sensitive to changes in the redox status of the intracellular environment. In this regard, both thiol-oxidizing and reducing agents have been shown to alter IP₃R function. The molecular basis governing redox regulation of IP₃Rs is not known, but it appears to be isoform specific since IP₃R₁ and IP₃R₂ exhibit redox-dependent changes in activity, whereas IP₃R₃ is much less sensitive to oxidative stress (Bultynck et al., 2004).

1.3: OXIDATIVE STRESS

Oxidative stress is defined as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage” (Dalle-Donne et al., 2008). This relatively new definition for what constitutes oxidative stress reflects the growing appreciation that the redox status of the cell is a dynamic balance between the intrinsic production of, and extrinsic exposure to, reactive species and the cellular capacity to neutralize these threats. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are constantly being produced within cells, and the generation of superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) is an inescapable consequence of cellular respiration. As such, mitochondria are the major site of ROS generated in the cell. ROS are also formed in the ER during protein folding and maturation by protein disulfide isomerases and oxidoreductases (Frand et al., 2000; Hebert & Molinari, 2007). Moreover, the ER is the major site of drug detoxification by the cytochrome P450 (CYP) superfamily which catalyzes the oxidation of substrates by O₂ (Anzenbacher & Anzenbacherova, 2001). Cells also express specialized enzymes
whose sole purpose is the production of ROS or RNS. For example, NADPH oxidase (NOX) and NO synthase (NOS) enzymes are responsible for producing \( \text{O}_2^- \) and NO, respectively (Knowles & Moncada, 1994; Bedard & Krause, 2007). Given the constant generation of endogenous ROS/RNS, as well as exposure to exogenous reactive species, cells have developed sophisticated mechanisms to defend themselves from the damaging effects of these molecules using a wide array of enzymes and antioxidants.

1.4: GLUTATHIONE

Glutathione (GSH) is the most abundant antioxidant in mammalian cells. GSH, a tri-peptide composed of the amino acids cysteine, glutamate and glycine, and its oxidized disulfide (GSSG) are ubiquitously expressed (Fig 1.2). GSH has a molecular weight of 307 Da and is negatively charged at neutral pH. A \( \gamma \)-peptide bond linking the side-chain carboxylate of glutamate and the amino group of cysteinyl residue protects GSH against protease degradation. The thiol group of the cysteinyl residue functions as an electron donor and \( \text{O}_2 \) accepter during redox reactions; when oxidized, GSH dimerizes to form GSSG.

In most cells, the total [GSH] is typically in the milli-molar range, whereas the total [GSH] of plasma varies from 1 – 100 µM. Consistent with a highly reducing nature of the cytosol, cytosolic GSH ranges from 1-10 mM, and at a 100:1 ratio relative to GSSG (Kosower & Kosower, 1978; Meister & Anderson, 1983). In addition to the cytosol, GSH and GSSG are also found in intracellular organelles and the nucleus. Nuclear GSH content is predicted to be similar to the cytosol since the small size and water solubility of GSH would facilitate its passive diffusion into the nucleus through
nucleoporins. The [GSH] in mitochondria is reportedly higher than the cytosol (ranging from 5-10 mM), but the GSH/GSSG ratio is lower (~10:1 GSH to GSSG; (Dalle-Donne et al., 2008)). In the ER, the total [GSH] is not known; however, by best estimates the GSH/GSSG ratio of the ER is 3:1 to 5:1 (Hwang et al., 1992; Dixon et al., 2008). These values are consistent with the more oxidizing environment in the ER, however substantial \textit{ex vivo} oxidation of GSH during sample preparations makes quantification of total [GSH] and the GSH/GSSG ratio in the ER and other intracellular organelles difficult. Given this, few studies have attempted to quantify the total [GSH], or the relative GSH/GSSG ratio of these compartments. Finally, a large pool of intracellular GSH is associated with proteins in the form of protein-glutathione (P-SSG) mixed disulfides (Hansen et al., 2009), and one study suggests that the majority of GSH present in the ER may in fact be P-SSG (Bass et al., 2004).

Cellular GSH homeostasis is controlled by a number of linked enzyme systems regulating its biosynthesis, recycling, and degradation (Meister & Anderson, 1983). The synthesis of GSH occurs only in the cytosol, and is controlled by the \( \gamma \)-glutamyl cycle. Individual cysteine and glutamate amino acids are combined by \( \gamma \)-glutamylcysteine synthetase, and glycine is attached by glutathione synthetase; the rate limiting enzymes controlling GSH production. GSH synthesis is regulated by feedback inhibition by GSH itself. When oxidized, GSH forms the disulfide GSG, and GSSG can be recycled back to GSH by glutathione reductase at the expense of NADPH. GSH and GSSG are transported out of the cell for degradation by \( \gamma \)-glutamyl transpeptidase located on the outer leaflet of the plasma membrane. Degradation products are then transported back into the cell where they are subsequently used to synthesize new GSH. Although the
importance of the GHS/GSSG ratio in maintaining cellular redox status is well-established, the mechanisms regulating transport of GSH and GSSG into and out of the cytoplasm and the lumen of intracellular organelles remain elusive, but may occur through the multi-drug resistant family of transport proteins (Cole & Deeley, 2006).

GSH, along with its associated enzyme networks, serves as the primary cellular antioxidant defense system. GSH serves a critical function as a scavenger of ROS and RNS, as a substrate for glutathione peroxidase-dependent reduction of $\text{H}_2\text{O}_2$, and as a cofactor in the detoxification of xenobiotics by glutathione S-transferases and CYP family members. Thus, both free radical and non-radical oxidants increase the formation of GSSG, which must be recycled back to GSH or transported out of the cell in order to maintain GSH/GSSG balance. Disruption of the GSH/GSSG ratio can lead to formation of P-SSG mixed disulfides, i.e. protein S-glutathionylation.

1.5: PROTEIN S-GLUTATHIONYLATION

Protein S-glutathionylation is a reversible, redox-regulated post-translational modification of sensitive cysteine (Cys) thiol (-SH) groups by GSH (Dalle-Donne et al., 2007; Dalle-Donne et al., 2008; Mieyal et al., 2008). Protein modification by GSH was first described 60 years ago as a prosthetic group attached to glyceraldehyde-3-phosphate dehydrogenase (Krimsky & Racker, 1952). Shortly thereafter, P-SSG mixed disulfides were reported to be constitutively present in a wide variety of tissues (Harrap et al., 1973), and were increased following exposure of cells to ionizing radiation (Modig, 1968) and ROS/RNS (Brigelius et al., 1983). These early studies suggested that the
modification of protein-SH (P-SH) groups by GSH served a protective role in preventing the loss of protein function that occurred in response to oxidative stress. More recent studies indicate protein S-glutathionylation also plays an important role in redox signaling processes. In this regard, glutathionylation can serve as an activator or an inhibitor of protein function. It is now appreciated that a significant number of intracellular proteins form mixed disulfides with GSH, including; components of the cytoskeleton (Wang et al., 2001; Landino et al., 2004), enzymes (Manevich et al., 2004; Chen et al., 2010), kinases (Humphries et al., 2002; Kambe et al., 2010; Zmijewski et al., 2010), phosphatases (Barrett et al., 1999), ion channels (Aracena-Parks et al., 2006; Yang et al., 2010), transporters (Adachi et al., 2004; Wang et al., 2005; Figtree et al., 2009), and transcription factors (Pineda-Molina et al., 2001; Velu et al., 2007). The modification of P-SH groups by GSH exhibits a high degree of specificity. The susceptibility of proteins to glutathionylation, the sites of modification, and the molecular basis leading to the formation of P-SSG mixed disulfides are areas of active investigation. Although the precise mechanism/s regulating the formation of P-SSG mixed disulfides in vivo is controversial, protein S-glutathionylation is predicted to occur through a number of biochemical pathways including; direct thiol-disulfide exchange, sulfinic acid intermediates, thiyl radicals, and S-nitrosylated intermediates. A basic scheme for how ROS and RNS can lead to the formation of P-SSG mixed disulfides is illustrated in Fig 1.3. \( \text{O}_2^{1-} \), primarily generated within the cell by mitochondria and oxidase enzymes, is rapidly converted to \( \text{H}_2\text{O}_2 \) by superoxide dismutase (SOD). GSH-dependent peroxidase (GPx) enzymes subsequently metabolize \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) in a two-step reaction that leads to the formation of one molecule of GSSG. GSSG must be
recycled back to GSH by glutathione reductase (GR), or transported out of the cell in order to maintain the GSH/GSSG balance. GSSG, under some conditions, may react with P-SH groups to produce P-SSG, but direct thiol-disulfide exchange is not thought to play a prominent role in P-SSG formation in vivo (Fig 1.3; pathway 1). If H$_2$O$_2$ exceeds the buffering capacity of GPx, H$_2$O$_2$ can oxidize GSH or P-SH groups to the sulfenic acid intermediates GSOH or P-SOH, respectively (Fig 1.3; pathway 2). Sulfenic acid intermediates are highly reactive and further oxidation to sulfinic (P-SO$_2$H) or sulfonic (P-SO$_3$H) acid moieties is irreversible. Thus, both GSH and PSH will readily react with either P-SOH or GSOH, respectively, to produce P-SSG. The hydroxyl radical (OH$^\cdot$), formed intracellularly from H$_2$O$_2$ by Fenton reactions and by UV radiation, and O$_2^\cdot$ can be directly scavenged by GSH or can react with P-SH groups leading to the formation of the thyl radicals GS$^\cdot$ and P-S$^\cdot$ (Fig 1.3; pathway 3). These radicals are extremely unstable and can subsequently react with either GSH or P-SH groups to form P-SSG mixed disulfides. P-SSG mixed disulfide formation can also occur through the reaction of NO with either P-SH or GSH leading to the S-nitrosylated intermediates P-SNO and GSNO, respectively (Fig 1.3; pathway 4). GSH can subsequently react with P-SNO groups, and GSNO can react with P-SH groups, to form P-SSG. Alternatively NO can also react with O$_2^\cdot$ to form the highly reactive species peroxynitrite (ONOO$^\cdot$). ONOO$^-$ can lead to the formation of both sulfenic acid intermediates (P-SOH, GSOH) and S-nitrosylated intermediates (P-SNO, GSNO) which can subsequently form P-SSG mixed disulfides, as described above.

In contrast to the many possible ways ROS/RNS can lead to the formation of P-SSG mixed disulfides, de-glutathionylation (i.e. the reversal of P-SSG back to P-SH) is
controlled primarily by glutaredoxin (Grx) enzymes. Grx use GSH as a cofactor to catalyze the reduction of P-SSG to P-SH. This reaction forms one molecule of GSSG which also must be reduced to GSH or transported out of the cell in order to maintain cellular glutathione homeostasis. The length of time a protein is glutathionylated in vivo is almost entirely dependent upon enzymatic activity of Grx and the availability of intracellular GSH.

1.6: DIAMIDE

Diamide (diazenedicarboxylic acid bis (N,N-dimethylamide)) is a widely used, cell-permeable, thiol-oxidizing agent first described by Kosower et al. in 1969 (Kosower et al., 1969). The development of diamide was propelled by the desire for a reagent that would preferentially oxidize intracellular GSH without the spurious production of reactive species that could cause cellular damage independent of perturbations in the GSH/GSSG ratio. In their initial characterization of diamide in red blood cells (RBCs), Kosower and colleagues found diamide oxidized GSH to GSSG in a stoichiometric two-step reaction (Fig 1.4). In this reaction, diamide initially reacts with one GSH molecule forming a diamide-GS radical intermediate, which then reacts with a second GSH; thereby producing one molecule of GSSG and an unreactive hydrazine metabolite of diamide. Although, theoretically 0.5 moles of diamide is necessary to completely oxidize 1 mole of GSH, experimentally ~0.6 moles of diamide were found to be required. The effect of diamide was reversible, and RBCs depleted of all intracellular GSH regenerated > 90% of their original GSH content within 30 min following removal of the reagent. Moreover, diamide did not cause any gross cellular or molecular damage to RBCs. In
vitro, diamide preferentially oxidizes GSH when compared to other physiological reducing equivalents such as NADH, NADPH, and coenzyme A (Kosower et al., 1972). In accordance with the initial observations of Kosower and colleagues, studies on cultured cells have shown that diamide treatment causes a decrease in GSH, an increase in GSSG, and an increase in the formation of P-SSG mixed disulfides (Hansen et al., 2009). Consistent with diamide’s specificity for GSH, brief (5 min) exposure of Hela or HEK cell lines to massive concentrations of diamide (5 mM) was not lethal, and following the removal of diamide, cells continued propagating in culture (Hansen et al., 2009). Given these results, it is unlikely that diamide causes irreversible damage to proteins, lipids, nucleic acids, or small molecules. The ability of diamide to selectively perturb GSH homeostasis and promote the formation of P-SSG has been exploited to identify protein targets of glutathionylation and to examine the effect of glutathionylation on protein and/or cellular function (for recent examples see (Fratelli et al., 2002; Humphries et al., 2002; Lind et al., 2002; Caplan et al., 2004; Wang et al., 2005; Gilge et al., 2008; Hill et al., 2010).

1.7: THE PURPOSE OF THIS STUDY

Exogenous application of ROS/RNS to tissues and cells in culture has been used for decades as an experimental tool to tip the redox balance towards oxidative stress. In addition, specific inhibitors of the glutathione redox pathway have also been used to alter the antioxidant defenses of the cell. Collectively, these studies have provided a wealth of information related to both how cells normally handle oxidants and how a shift in the
redox balance can lead to pathological changes. However, it is often difficult to identify the molecular events attributable solely to protein S-glutathionylation during the exogenous application or even endogenous generation of ROS/RNS because these reactive species not only modify proteins, but also affect membrane lipids and nucleic acids. Moreover, ROS/RNS can cause thiol modification other than glutathionylation. For example ROS can oxidize both Cys and methionine (Met) residues to sulfenic, sulfinic or sulfonic acids, whereas RNS can nitrosylate not only Cys but also tyrosine (Tyr) residues. Additionally, it is becoming increasingly evident that small changes in redox balance can lead to modification of specific signaling pathways which in turn, elicit a physiological response, i.e., redox signaling (Hess et al., 2005; Hidalgo & Donoso, 2008; Jones, 2008). These reversible molecular switches appear to be flipped by thiol modification of cellular proteins. Currently, a major challenge is to understand how glutathionylation regulates protein function and the molecular events associated with conversion from physiological redox signaling processes to pathological changes related to oxidative injury and cellular damage.

Despite wide use of diamide as a thiol oxidant, the effect of diamide on Ca\(^{2+}\) homeostasis and Ca\(^{2+}\) signaling mechanisms has remained virtually unexplored. Prior to the inception of this study, only one previous report examining the effect of diamide on the [Ca\(^{2+}\)]\(_i\) of intact cells had been published (Fowler & Tiger, 1998). As discussed above, diamide selectively perturbs intracellular GSH homeostasis and increases P-SSG formation without generating ROS/RNS. These previous studies provide the basis for using diamide to examine the regulation of Ca\(^{2+}\) homeostasis and Ca\(^{2+}\) signaling mechanisms by protein S-glutathionylation. In the present Dissertation, we explore the
effect of diamide on the $[\text{Ca}^{2+}]_i$ in cultured vascular endothelial cells (ECs) by single-cell imaging of the fluorescent $\text{Ca}^{2+}$ indicator fura-2 using time-lapse video microscopy. In Chapter 2, we investigate the acute effect of diamide on the $[\text{Ca}^{2+}]_i$ of cultured vascular ECs over a range of concentrations. The most striking effect of diamide was an increase in spontaneous $\text{Ca}^{2+}$ oscillations. In Chapter 3, we dissect the mechanism responsible for this oscillatory response. To our knowledge, these are the first studies examining the effect of diamide on the $[\text{Ca}^{2+}]_i$ of intact cells at the single-cell level.
Figure 1.1: Fundamental basis for activation of IP$_3$Rs by IP$_3$ and Ca$^{2+}$.

See text for explanation.
Figure 1.2: Structure of GSH and its disulfide GSG.
Figure 1.3: Potential pathways leading to the formation of protein S-glutathionylation in vivo.

1) Direct thiol-disulfide exchange; 2) Sulfenic acid intermediates; 3) Thiyl radicals; 4) and S-nitrosylated intermediates. Abbreviations: SOD, superoxide dismutase; Gpx, glutathione-dependent peroxidase; GR, glutathione reductase; Grx, glutaredoxin. See text for explanation.
1.8.4: Figure 1.4

**Diamide**

\((\text{CH}_3)_2\text{NCON=NCON(CH}_3)_2\)

**Oxidation Reaction**

\[(\text{CH}_3)_2\text{NCON=NCON(CH}_3)_2 + \text{GSH} \rightarrow (\text{CH}_3)_2\text{NCON(GS)HNCON(CH}_3)_2\]

\[(\text{CH}_3)_2\text{NCON(GS)HNCON(CH}_3)_2 + \text{GSH} \rightarrow \text{GSSG} + (\text{CH}_3)_2\text{NCONHNHCON(CH}_3)_2\]  
(Toxazine)

**Figure 1.4:** Diamide composition and reaction with GSH.

Oxidation of GSH by diamide occurs by a two-step addition and displacement reaction; theoretically 0.5 moles of diamide oxidizes 1 mole of GSH.
CHAPTER 2

Effect of Protein S-Glutathionylation on Ca$^{2+}$ Homeostasis in Cultured Aortic Endothelial Cells

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2.1: INTRODUCTION

The vascular endothelium is a continuous, single-cell thick sheet, comprised predominantly of endothelial cells (ECs), which lines the interior of the entire mammalian circulatory system and forms the capillaries beds where gas and solute exchange occur. As the interface between the circulation and surrounding tissues, vascular ECs are vulnerable to oxidative insults arising from the byproducts of drug metabolism and infiltrating neutrophils and macrophages. Additionally, ROS and RNS normally generated during the reduction of molecular oxygen, and the formation of NO contribute to the oxidant burden of the endothelium. Although the underlying basis remains unknown, oxidative stress is implicated in a wide range of disease processes; from acute events such as ischemia-reperfusion injury and infection, to chronic conditions such as diabetes, atherosclerosis, and hypertension.

One of the earliest events in ECs associated with an oxidative insult is a disruption of Ca\(^{2+}\) homeostasis (Schilling & Elliott, 1992). Ca\(^{2+}\) signal transduction plays an essential role in vascular EC function. Receptor-mediated changes in \([\text{Ca}^{2+}]_i\) are important for controlling the production and release of paracrine factors critical to the regulation of vascular permeability and tone, platelet aggregation and thrombosis, fibrinolysis, angiogenesis, mechanoreception, and inflammation. Physiologically occurring oxidants known to decrease GSH such as \(\text{O}_2^\cdot\), \(\text{H}_2\text{O}_2\), and \(\text{ONO}^\cdot\), can significantly affect both basal and agonist-mediated changes in EC \([\text{Ca}^{2+}]_i\) (Elliott et al., 1989; Elliott, 1996; Graier et al., 1998; Hu et al., 1998; Hu et al., 2000). Previous studies have shown that oxidant-induced changes in EC \([\text{Ca}^{2+}]_i\) are related to the oxidation of intracellular GSH to GSSG (Elliott & Schilling, 1990; Elliott et al., 1995; Henschke &
Additionally, oxidative stress is known to increase the formation of P-SSG mixed disulfides in ECs (Schuppe et al., 1992), but the role of protein S-glutathionylation in Ca\(^{2+}\) homeostasis and signaling of vascular ECs has not been explored.

As discussed in Chapter 1 section 1.6, diamide is a widely used, cell-permeable, thiol-oxidizing agent that rapidly converts intracellular GSH to GSSG and promotes the formation of P-SSG mixed disulfides without generating ROS/RNS (Kosower et al., 1969; Kosower & Kosower, 1995). Since the cytosolic concentration of GSH is normally in the milli-molar range, low concentrations of diamide can be used to alter the redox balance in a graded fashion and thus, to interrogate the consequences of P-SSG formation over both the physiological and pathological range of oxidative challenge, without the added complexities associated with direct application or generation of ROS/RNS. To determine if protein S-glutathionylation alters the function of ion channels and pumps associated with Ca\(^{2+}\) signaling, the acute effect of diamide on \([Ca^{2+}]_i\) of cultured bovine aortic endothelial cells (BAEC) was examined in the present study at the single cell level using the fluorescent Ca\(^{2+}\) indicator, fura-2 and time-lapse video microscopy. A preliminary report of this study has been published in abstract form (Lock & Schilling, 2010).
2.2: METHODS

2.2.1: Cell Culture. The isolation, culture, and characterization of the bovine aortic endothelial cell (BAEC) line used in this study were extensively described in previous reports (Colden-Stanfield et al., 1987; Schilling et al., 1988; Schilling et al., 1989). Briefly, BAECs were cultured as monolayers on 100 mm plastic cell culture dishes in low glucose DMEM containing L-glutamine (Invitrogen), and supplemented with 10% heat inactivated fetal bovine serum (FBS), 15 mM HEPES, 28.6 mM sodium bicarbonate, and 1% penicillin-streptomycin-neomycin solution (PSN; Gibco). BAEC monolayers, which exhibited a cobblestone appearance typical of contact-inhibited endothelial cell cultures, were used for experimentation between passages 13-20. LLC-PK1 cells stably-expressing the GFP-tagged pleckstrin homology domain of PLCγ were a generous gift of Dr. Jeffrey Schelling (MetroHealth Medical Center, Cleveland OH). LLC-PK1 cells were cultured in DMEM-F12 supplemented with 10% FBS, 1% PSN, and 400 µg/ml G418.

2.2.2: Solutions and Reagents. Normal Ca\(^{2+}\) buffer (Ca\(^{2+}\)-ECS) contains in mM: 10 HEPES, 140 NaCl, 5.4 KCl, 1 MgCl\(_2\), 1.8 CaCl\(_2\), and 10 glucose, pH 7.4 at 37\(^\circ\)C. Zero Ca\(^{2+}\) buffer (zero Ca\(^{2+}\)-ECS) contains in mM: 10 HEPES, 140 NaCl, 5.4 KCl, 1 MgCl\(_2\), 0.3 EGTA, and 10 glucose, pH 7.4 at 37\(^\circ\)C. Phosphate buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na\(_2\)HPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\), pH adjusted to 7.4 or 8.0 with NaOH as indicated. Diamide (diazenedicarboxylic acid bis (NN,-dimethylamide)), 2-APB (2-aminoethyl diphenyl borate), BCNU (N,N’-bis(2-chloroethyl)-N-nitroso urea), BSO (l-buthionine–sulfoximine), and DMSO (dimethyl sulfoxide) were purchased from Sigma-Aldrich. U73122 and U73343 were obtained
from Calbiochem. Fura-2 acetoxyethyl ester (fura-2/AM), and pluronic F-127 were obtained from Invitrogen. Biotinylated-GSH ethyl ester (BioGEE) was synthesized as previously described (Figtree et al., 2009). The 5F10 mouse monoclonal anti-PMCA antibody was from Affinity Bioreagents (catalogue no. MA3-914). The anti-IP$_3$ receptor type I antibody was from Millipore (catalogue no. 07-514). Stock solutions of 2-APB (50 mM), U73122 (10 mM) and U73343 (10 mM) were prepared in DMSO. BCNU stock (37.5 mM) was prepared in an aqueous solution with 10% ethanol and was stored at -20°C (up to one month). BSO was prepared as an aqueous 100 mM stock solution and was stored at 4°C (up to one month). Fura-2/AM was reconstituted using DMSO and 10% pluronic F-127 at a 1:1 ratio to yield a 1 mM stock solution. Diamide stock solutions were prepared in either Ca$^{2+}$-ECS or zero Ca$^{2+}$-ECS at a final concentration of 10 mM; both fura-2/AM and diamide were prepared fresh each day of experimentation.

**2.2.3: Ca$^{2+}$Imaging.** Time-dependent changes in [Ca$^{2+}$], were measured in BAEC monolayers as previously described (Goel & Schilling, 2010). Briefly, BAEC monolayers grown on glass coverslips were loaded with fura-2, mounted in a temperature-controlled perfusion chamber, and placed on the stage of a Leica DMIRE2 inverted microscope. At 6 sec intervals, excitation wavelength alternated between 340 and 380 nm and emission was recorded at 510 nm using filters appropriate for fura-2. Epifluorescence was recorded using a SPOT-RT camera (Diagnostic Instruments, Sterling Heights, MI, USA) and images were acquired and analyzed using SimplePCI imaging software (Compix Inc., Cranberry Township, PA, USA). Solutions were perfused into the recording chamber via an inline heater; all fura-2 imaging experiments were performed at 37°C.
2.2.4: Data analysis. Over the course of these experiments, slight variation in the sensitivity of the BAECs to diamide was noted. For this reason, controls were always performed in parallel for each experimental protocol. For example, the effect of 250 µM diamide on Ca^{2+} oscillations is reported in Figs 2.4, 2.11D, and 2.12D; these represent independent data sets. The figures show [Ca^{2+}]i responses from individual cells (40-80 cells per field of view) as different gray-scale lines. Oscillation frequency was determined for each individual cell and subsequently binned into three frequency categories based on the number of oscillations observed during the test period; no oscillations, 1-2 oscillations, or 3+ oscillations (expressed as a percent of the total cells counted per monolayer). Histograms of oscillation frequencies show the average values from multiple monolayers under each condition reported as mean ± SE with n equal to the number of monolayers examined under each condition. The total number of cells and the total number of monolayers counted for each condition is given in the figure legend. Statistical analysis was performed using the paired Student’s t-test with Bonferonni’s correction for multiple comparisons; p value < 0.05 was considered to be statistically significant.

2.2.5: Isolation of membranes from BAECs. Membranes were isolated as previous described (Sinkins et al., 2009). Briefly, confluent BAEC monolayers were harvested from the culture dishes by scrapping, subjected to centrifugation at 500 × g for 5 min, and resuspended in lysis buffer containing 20 mM Tris-Cl, 5 mM EDTA, 1 mM EGTA, and protease inhibitor mixture. The cell suspension was sonicated on ice using a sonic dismembranator (Fisher) on a power setting of 2.5. The cell suspension was sonicated three times for 10 s with a 10-s rest between pulses. The cell lysate was subjected to
centrifugation at 6000 × g for 10 min at 4 °C. The resulting pellet was discarded, and the 
supernatants were centrifuged at 50,000 × g for 30 min. The microsomal pellets were 
resuspended in lysis buffer at a protein concentration of 5-10 mg/ml.

2.3.6: Synthesis of biotinylated GSH. Reduced glutathione (GSH) was labeled with 
sulfo-link-NHS-LC-biotin (Pierce). The reaction was performed by combining 10 mM 
GSH with 10 mM of biotin reagent in phosphate buffered saline (PBS, pH 8.0). After 1 
hour at room temperature, 50 mM TRIS was added to remove any excess sulfo-link 
reagent.

2.2.7: Glutathionylation of the IP₃R and PMCA in BAEC microsomal 
membranes. Membrane proteins were glutathionylated by incubating microsomes with 
125 µM biotin-GSH and 100 µM diamide for 10 min at room temperature in PBS (pH 8.0 or 7.4). Some samples were treated with dithiothreitol (DTT) for an additional 15 min at 
room temperature. Following incubation, membranes were pelleted by centrifugation and 
washed 3 times with PBS to remove excess reagents. Washed membrane pellets were 
solubilized in PBS containing 1% Triton X-100 for 30 minutes on ice. Lysates were 
cleared by centrifugation at 50,000 x g for 60 minutes at 4°C, and glutathionylated 
proteins were extracted by overnight incubation with streptavidin-agarose beads (Pierce).

2.2.8: Glutathionylation of IP₃R and PMCA in vivo. BAECs, harvested by 
scraping, were washed and re-suspended in 1.5 ml Ca²⁺-ECS without BSA (pH 7.4). The 
cells were incubated with 250 or 500 µM BioGEE for 60 or 180 min at 37°C. BioGEE-
loaded cells were washed with Ca²⁺-ECS and incubated for 15 minutes at room 
temperature in the absence or presence of 500 µM diamide. Following incubation, the
cells were washed with diamide-free Ca$^{2+}$-ECS and membrane preparations were generated as described above. Membrane aliquots were solubilized in PBS containing 1% Triton X-100 for 30 min on ice. Lysates were cleared by centrifugation, and biotinylated proteins were extracted with streptavidin-agarose beads. Where indicated, dithiorthreitol (DTT; 20 mM) was added prior to pull-down.

2.2.9: Immunoblots. Following in vitro or in vivo glutathionylation reactions, proteins captured on streptavidin beads were fractionated by SDS-PAGE and electrotransferred to PVDF membrane (100V for 1hr) in CAPS/methanol buffer. Blots were probed with anti-PMCA or anti-IP$_3$ receptor antibody and detected, following incubation with HRP-conjugated IgG, by SuperSignal® West Pico chemiluminescent substrate (Pierce). For comparison and statistical analysis, band intensities from avidin pull-down samples were quantified by densitometry, and normalized to input controls.
2.3: RESULTS

2.3.1: Diamide increases Ca\textsuperscript{2+} oscillations and elevates basal [Ca\textsuperscript{2+}]\textsubscript{i}. The acute effect of diamide on [Ca\textsuperscript{2+}]\textsubscript{i} of BAECs was examined by single-cell Ca\textsuperscript{2+} imaging. Low concentrations of diamide (50 μM, 100 μM) progressively increased the number of cells exhibiting spontaneous asynchronous Ca\textsuperscript{2+} oscillations and increased the oscillation frequency of individual cells. As seen in Fig 2.1A-C and quantified in Fig 2.1D, 95% of cells failed to exhibit even a single Ca\textsuperscript{2+} oscillation during the recording period in the absence of diamide. However, addition of 50 μM diamide to the extracellular solution caused ~30% of the cells to exhibit one or two Ca\textsuperscript{2+} spikes, and 12% of cells to exhibit three or more Ca\textsuperscript{2+} oscillations during the treatment period. Raising the concentration of diamide to 100 μM caused ~80% of the cells to oscillate with ~40% of the cells showing three or more oscillations. Upon washout of diamide, there was an immediate cessation of Ca\textsuperscript{2+} oscillations and a return of [Ca\textsuperscript{2+}]\textsubscript{i} to basal levels, demonstrating that the effects of diamide are rapidly reversible under control conditions. To determine the source of Ca\textsuperscript{2+} responsible for the oscillations observed, the experiments were repeated in the absence of extracellular Ca\textsuperscript{2+} (zero Ca\textsuperscript{2+}-ECS). As seen in Fig 2.2A-C and quantified in Fig 2.2D, ~20% of cells treated with 50 μM diamide responded with one or two Ca\textsuperscript{2+} oscillations, whereas exposure to 100 μM diamide produced a more robust response with over 90% of cells exhibiting at least one Ca\textsuperscript{2+} spike during the treatment period. Once again this response was reversed upon washout of diamide. These results demonstrate that the diamide-induced Ca\textsuperscript{2+} oscillations primarily reflect the release of Ca\textsuperscript{2+} from internal stores. The only notable difference in the diamide response observed in the absence versus the presence of extracellular Ca\textsuperscript{2+} was the number of cells exhibiting
higher frequency oscillations; e.g. ~10% versus ~40%, respectively at 100 µM diamide. Thus, in the absence of extracellular Ca\(^{2+}\), the internal stores will eventually deplete and oscillations will cease. For comparison, diamide-induced Ca\(^{2+}\) oscillations in both the presence and absence of extracellular Ca\(^{2+}\) were similar in magnitude to the Ca\(^{2+}\) transients observed in response to bradykinin, a potent endothelial-dependent vasodilator (Fig 2.3).

Higher concentrations of diamide (250 µM, 500 µM) effectively synchronized the observed Ca\(^{2+}\) oscillations (Fig 2.4). Upon exposure to the oxidant, nearly all cells monitored exhibited a single Ca\(^{2+}\) oscillation followed by a steady rise in basal [Ca\(^{2+}\)]\(_i\) throughout the remainder of the treatment period. This response was observed in both the presence (Ca\(^{2+}\)-ECS) and absence (zero Ca\(^{2+}\)-ECS) of extracellular Ca\(^{2+}\). In the presence of extracellular Ca\(^{2+}\), the [Ca\(^{2+}\)]\(_i\) of some cells remained elevated or continued to oscillate after the initial spike, but the oscillations if present occurred in an asynchronized fashion. At the end of the 10 min treatment period, basal [Ca\(^{2+}\)]\(_i\) of diamide-treated BAECs was significantly elevated when compared to matched untreated-controls. In the presence of extracellular Ca\(^{2+}\) (Fig 2.4C), cells challenged with either 250 µM or 500 µM diamide exhibited a 25% and 30% increase in their baseline fluorescence ratio at the end of the treatment period, respectively. Likewise, in the absence of extracellular Ca\(^{2+}\) (Fig 2.4F), cells treated with 250 µM or 500 µM diamide produced a 22% and 35% increase in baseline fluorescence ratio, respectively. The rise in basal [Ca\(^{2+}\)]\(_i\) was completely reversed following washout of the reagent from cells treated with 250 µM diamide. However, following removal of 500 µM diamide, 170 out 281 cells (in Ca\(^{2+}\)-ECS) and
140 out of 322 cells (in zero Ca\(^{2+}\)-ECS) failed to return to baseline levels during the 5 minute washout period.

The products of the reaction of diamide with GSH are GSSG and a hydrazine byproduct of diamide (Kosower et al., 1969; Kosower & Kosower, 1995). A number of control experiments were therefore performed to determine the specificity of the diamide response. First, cell-free experiments confirmed that the changes in [Ca\(^{2+}\)]\(_i\) were not due to a direct effect of diamide or the hydrazine byproduct on fura-2 fluorescence per se (data not shown). Second, the diamide-induced changes in [Ca\(^{2+}\)]\(_i\) of BAECs were prevented by premixing diamide with GSH (at 1:4 molar ratio) to inactivate diamide, demonstrating that the thiol-oxidizing effect of diamide, and not the hydrazine byproduct, is responsible for the observed changes in Ca\(^{2+}\) dynamics (Fig 2.5). Third, both the release of Ca\(^{2+}\) from stores and the rise in basal [Ca\(^{2+}\)]\(_i\) in response to diamide were prevented by loading BAECs with the Ca\(^{2+}\) chelator, BAPTA (Fig 2.6). Since BAECs express purinergic receptors which when stimulated initiate a robust Ca\(^{2+}\) response, we considered the possibility that diamide may cause the release of ATP. However, addition of 5 units/ml of apyrase to the bath solution during treatment with 100 µM diamide did not affect the observed Ca\(^{2+}\) oscillation frequency when compared to matched controls, indicating that a diamide-induced ATP release is not responsible for the changes seen in [Ca\(^{2+}\)]\(_i\) (Fig 2.7). Collectively, these results show that diamide at low concentrations dynamically alters the release of Ca\(^{2+}\) from internal stores, presumably via activation of IP\(_3\) receptors (IP\(_3\)R), and at higher concentrations, inhibits Ca\(^{2+}\) efflux from the cytosol. Since BAECs lack a Na\(^+\),Ca\(^{2+}\)-exchanger (Schilling & Elliott, 1992), the rise in basal
[Ca^{2+}]_{i} in the absence of extracellular Ca^{2+} presumably reflects inhibition of the plasmalemmal Ca^{2+}-ATPase (PMCA) pump.

2.3.2: Diamide-induced Ca^{2+} oscillations are attenuated by the IP_{3}R inhibitor 2-APB. To determine the role of the IP_{3}R in the diamide-induced Ca^{2+} oscillations, the effect of 2-APB, a receptor antagonist (Maruyama et al., 1997) was examined in the absence of extracellular Ca^{2+}. As shown in Fig 2.8A-B and quantified in 2.8C, pretreatment of BAECs for 10 min with 50 μM 2-APB, immediately prior to challenge with 100 μM diamide, significantly attenuated Ca^{2+} oscillations when compared to matched vehicle-pretreated controls. Pretreatment of the cells with 2-APB also blocked the synchronized oscillation seen upon exposure to 500 µM diamide (Fig 2.8D-E). These results confirm that diamide-induced Ca^{2+} oscillations are due to Ca^{2+} release from internal stores via the IP_{3}R. Interestingly, 2-APB did not block the effect of diamide on the reversible rise in basal [Ca^{2+}]_{i} (Fig 2.8F), suggesting that this phase is independent of the IP_{3}R.

2.3.3: Diamide-induced Ca^{2+} oscillations are prevented by inhibition of PLC. Previous studies have shown that the thiol-reagent thimerosal, stimulates Ca^{2+} oscillations by sensitizing the IP_{3}R to basal levels of IP_{3} i.e., without increasing the formation of IP_{3} (Bootman et al., 1992). To evaluate the role of IP_{3} in the diamide-induced changes in [Ca^{2+}]_{i}, we monitored Ca^{2+} oscillations in BAECs pretreated with U73122 (10 μM), an inhibitor of PLC (Bleasdale et al., 1990), or its inactive analogue U73343 (10 μM) prior to challenge with 100 μM diamide. As seen in Fig 2.9, diamide-induced Ca^{2+} oscillations measured in zero Ca^{2+}-ECS were essentially eliminated by U73122, but not by U73343, when compared to vehicle-pretreated controls. To
determine if diamide activates PLC and increases phosphoinositide hydrolysis at the single cell level, we examined fluorescence distribution of the GFP-tagged pleckstrin homology domain of PLC-γ (GFP-PH). This reporter protein binds to PIP₂ and associates with the plasmalemma under basal conditions, but re-distributes to the cytosol following activation of PLC and hydrolysis of PIP₂ (Varnai & Balla, 2008). These experiments were performed on an LLC-PK1 cell line stably expressing GFP-PH. As seen in Fig 2.10 (upper panels), stimulation of the LLC-PK1 cells with ATP (100 µM), which activates PLC via P2Y purergic receptors and increases [Ca²⁺]i (Weinberg et al., 1989; Anderson et al., 1991; Himpens et al., 1993), caused the rapid and reversible redistribution of GFP-PH from the plasma membrane to the cytosol, but the subsequent application of diamide (500 µM; Fig 2.10, lower panels), had no effect on the distribution of GFP-PH, demonstrating that diamide does not stimulate the hydrolysis of PIP₂. In parallel experiments, a second application of ATP again produced a redistribution of the probe (not shown). Control experiments also showed that diamide increased Ca²⁺ oscillations in the LLC-PK1 cells similar to that observed in BAECs (not shown). Together these results demonstrate that diamide increases Ca²⁺ release from IP₃-sensitive internal Ca²⁺ stores without increasing PIP₂ hydrolysis.

2.3.4: Inhibition of glutathione reductase by BCNU potentiates diamide-induced changes in [Ca²⁺]ᵢ. Diamide rapidly converts GSH into GSSG. However, when diamide is added to cells at low concentrations, the GSSG produced is rapidly converted back to GSH by glutathione reductase at the expense of NADPH. Thus, diamide-induced changes in the [Ca²⁺]ᵢ of BAECs may reflect a change in the GSH/GSSG ratio or a decrease in cellular NADPH. Inhibition of glutathione reductase would be expected to
augment the effects of diamide if they are related to a change in the GSH/GSSG ratio rather than the loss of NADPH. BCNU, a well-established inhibitor of glutathione reductase, has previously been shown to increase the sensitivity of a variety of cells, including endothelial cells (Elliott & Schilling, 1990), to oxidative stress. As shown in Fig 2.11, the effects of both low and high concentrations of diamide on the [Ca^{2+}]_i of BAECs are enhanced following pretreatment of cells with 75 μM BCNU when compared to matched vehicle-pretreated controls. Cells challenged with 25 μM diamide pretreated with vehicle only, had no effect on oscillation frequency, i.e., ~95% of the cells failed to oscillate, a value similar to controls shown in Fig 2.2. Pretreatment of the cells with BCNU also had no effect on oscillation frequency, however the subsequent addition of 25 μM diamide to BAECs pretreated with 75 μM BCNU caused ~55% of the cells to oscillate (Fig 2.11A-B and quantified in 2.11C). Interestingly, in some experiments the BCNU-treated cells continued to exhibit Ca^{2+} oscillations even during the washout period suggesting that reversal of the diamide effect requires reduced GSH.

BAECs challenged with 250 μM diamide pretreated with vehicle only, exhibited a rise in basal [Ca^{2+}]_i similar to that shown in Fig 2.4, which upon washout of the reagent returned back to basal levels. However, although BCNU-pretreated cells displayed a rise in basal [Ca^{2+}]_i similar to that observed in non-BCNU treated cells when challenged with 250 μM diamide, the elevated [Ca^{2+}]_i failed to reverse upon washout of the oxidant (Fig 2.11D-E and quantified in 2.11F). These results suggest that the diamide-induced changes in [Ca^{2+}]_i is not related to changes in NADPH, but rather is linked to the GSH/GSSG ratio. Furthermore, the ability of the cells to recover from the diamide-induced insult (both Ca^{2+} release from intracellular stores and the elevation of basal
[Ca^{2+}]_i) requires reduced GSH, which presumably is needed to reverse protein S-glutathionylation.

2.3.5: Inhibition of γ-glutamylcysteine synthetase by BSO also enhances diamide-induced changes in [Ca^{2+}]_i. The generation of γ-glutamylcysteine is the first and rate-limiting step in the de novo synthesis of GSH. The enzyme controlling this reaction, γ-glutamylcysteine synthetase, can be inhibited by BSO (Griffith & Meister, 1979). Previous studies in cultured cells have shown BSO reduces total cellular GSH (Bootman et al., 1992; Henschke & Elliott, 1995) without affecting GSSG (Khamaisi et al., 2000; Gilge et al., 2008). As shown in Fig 2.12, the effects of both low and high concentrations of diamide on the [Ca^{2+}]_i of BAECs are enhanced following pretreatment of cells with 500 μM BSO when compared to matched untreated-controls. Untreated cells challenged with 50 μM diamide exhibited oscillation frequencies similar to those shown in Fig 2.2, i.e., ~30% of the cells exhibit one or two oscillation during the treatment period in zero Ca^{2+}-ECS. However, the addition of 50 μM diamide to BAECs pretreated with 500 μM BSO caused ~80% of cells to oscillate (Fig 2.12A-B and quantified in 2.12C). Although BSO pretreated cells displayed a rise in basal [Ca^{2+}]_i in response to 250 μM diamide similar to that observed in non-BSO treated cells, [Ca^{2+}]_i did not return to baseline following washout of the oxidant (Fig 2.12D-E and quantified in 2.12F). These results provide additional evidence that a decrease in GSH renders BAECs more susceptible to diamide-induced changes in [Ca^{2+}]_i, and emphasize the importance of the reduced GSH in reversal of the diamide response. To demonstrate that the changes observed indeed reflect a thiol modification, we examined the effect of dithiothreitol (DTT) added during the washout of diamide from BCNU-and BSO-pretreated cells. As seen in Fig 2.13A-B...
and 2.13D-E and as quantified in Fig 2.13C and 2.13F, the rise in basal [Ca\(^{2+}\)]\(_i\) seen at the higher diamide concentration was rapidly reversed when the wash buffer contained 2 mM DTT, supporting the hypothesis that the effects of diamide reflect a reversible thiol modification.

2.3.6: Diamide increases glutathionylation of the IP\(_3\)R and the PMCA both in vivo and in vitro. To determine if diamide causes glutathionylation of the PMCA and/or the IP\(_3\)R, BAECs were loaded intracellularly with BioGEE, a membrane permeable ethyl-ester form of biotin-GSH that is trapped within the cell by the action of cellular esterases (Sullivan et al., 2000). Following treatment with diamide, membranes isolated from BioGEE-loaded cells were solubilized in lysis buffer and glutathionylated proteins were extracted using avidin-conjugated agarose beads as described in Experimental Procedures. A small amount of each protein was present in pull-downs from untreated control cells indicative of some basal level of glutathionylation. However, following treatment of the cells for 10 minutes with 500 µM diamide, the amount of recovered IP\(_3\)R (Fig 2.14A) and PMCA (Fig 2.14B) significantly increased 2.83 ± 0.54-fold (p < 0.03) and 7.99 ± 1.1-fold (p < 0.001), respectively, consistent with increased glutathionylation. To determine if the IP\(_3\)R and the PMCA could also be glutathionylated in vitro, membrane preparations isolated from BAECs were treated with biotin-GSH alone or biotin-GSH plus diamide. As seen in Fig 2.14C and 2.14D, no apparent glutathionylation was observed in the absence of diamide, whereas a substantial amount of both the PMCA and the IP\(_3\)R were recovered in the avidin pull-downs following treatment of isolated membranes with biotin-GSH plus diamide. Consistent with thiol-
modification, both the *in vivo* and *in vitro* glutathionylation was reversed by addition of excess DTT prior to pull-down.
2.4: DISCUSSION

Diamide produced two major effects on Ca$^{2+}$ homeostasis in BAECs. First, diamide caused a dramatic, concentration-dependent increase in Ca$^{2+}$ oscillation frequency, and second, diamide increased basal [Ca$^{2+}$]. The later effect became particularly obvious at higher diamide concentrations. Diamide is a membrane permeable thiol-oxidizing agent that has been used for over 40 years to alter the redox balance in a variety of cell types. Diamide rapidly and reversibly oxidizes intracellular GSH to GSSG and causes formation of P-SSG. It is thought that most if not all of the effects of diamide at the cellular level reflect thiol modifications, but the actual mechanism by which diamide increases formation of P-SSG is not entirely clear. The oxidation of GSH by diamide occurs by a well-studied two-step reaction (Kosower & Kosower, 1995). The first step involves the formation of a diamide-GS intermediate which then rapidly reacts with a second molecule of GSH yielding GSSG plus the hydrazine metabolite of diamide (Fig 2.15). As the concentration of GSSG increases in response to diamide, protein S-glutathionylation might occur via a disulfide exchange reaction (shaded dashed line in Fig 2.15). However, as discussed by Gallogly and Mieyal (Gallogly & Mieyal, 2007; Gallogly et al., 2009), for most protein thiols, the cellular GSH/GSSG ratio must decrease from the normal value of ~100 to less than 1 for significant disulfide exchange to occur. In this regard, previous studies have shown that the GSH/GSSG ratio was only reduced to ~35 following treatment of human umbilical vein endothelial cells with 200 µM diamide for 15 minutes (Schuppe et al., 1992). Since diamide preferentially reacts with cellular GSH and exhibits limited reactivity towards protein thiol groups (Kosower & Kosower, 1995), it seems likely that the diamide-induced glutathionylation occurs by
an exchange reaction between the diamide-GS intermediate and accessible Cys residues in cellular proteins. The lifetime of the P-SSG formed by this reaction is dependent on the activity of cellular glutaredoxins which de-glutathionylate P-SSG at the expense of GSH (Gallogly & Mieyal, 2007). The GSSG product of this reaction, and the GSSG directly produced by diamide, is then rapidly reduced back to GSH by the action of glutathione reductase at the expense of NADPH. Thus, by adding low concentrations of diamide to the cell, the equilibrium is shifted towards P-SSG. It is easy to see how inhibition of glutathione reductase or inhibition of GSH synthesis would enhance the effect of diamide; both maneuvers decrease the availability of cellular GSH and thus limit the ability of the cell to de-glutathionylate the modified protein, i.e. the equilibrium is shifted even further towards P-SSG. Indeed, in the present study, the effects of diamide on Ca$^{2+}$ oscillations and basal [Ca$^{2+}$], were greatly enhanced by inhibition of glutathione reductase by BCNU or by inhibition of GSH synthesis by BSO. Furthermore, the effects of diamide were irreversible when the cellular capacity to re-generate reduced GSH was compromised, but importantly, could be reversed under these conditions by DTT. Taken together, these results provide strong support for the hypothesis that the effect of diamide on Ca$^{2+}$ homeostasis reflects thiol modification. The reversal by DTT also shows that diamide does not cause irreversible damage to the cellular mechanism responsible for returning [Ca$^{2+}$], to the normal resting level.

What is the molecular mechanism by which diamide-induced glutathionylation increases Ca$^{2+}$ oscillation frequency? The diamide-induced change in Ca$^{2+}$ oscillation frequency was 1) unaffected by removal of extracellular Ca$^{2+}$, 2) inhibited by the IP$_3$ receptor antagonist 2-APB, and 3) blocked by inhibition of PLC with U73122. Clearly,
diamide stimulates the release of Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive internal Ca\textsuperscript{2+} stores, but since diamide had no effect on PIP\textsubscript{2} hydrolysis, it seems unlikely that diamide stimulates PLC in a fashion analogous to receptor stimulation. However, it is well-established that the IP\textsubscript{3}R is sensitive to Ca\textsuperscript{2+} concentration within the lumen of the ER, and that spontaneous release can occur when the ER becomes overloaded with Ca\textsuperscript{2+} (Missiaen \textit{et al.}, 1991, 1992). Previous studies have shown that ONOO\textsuperscript{-} activates the SERCA pump via glutathionylation of Cys-674 (Adachi \textit{et al.}, 2004; Cohen & Adachi, 2006; Lancel \textit{et al.}, 2009). Thus, it is possible that a diamide-induced glutathionylation of SERCA in the BAECs might elevate luminal Ca\textsuperscript{2+}, and thus increase spontaneous Ca\textsuperscript{2+} release. In preliminary BAEC experiments we found that bradykinin- and thapsigargin-induced Ca\textsuperscript{2+} release from internal stores is increased after 15 minutes in 500 µM diamide (in the presence of extracellular Ca\textsuperscript{2+}) consistent with an increased Ca\textsuperscript{2+} load of the ER (Lock and Schilling, unpublished observations). However, it seems unlikely that the effect of diamide on the [Ca\textsuperscript{2+}]\textsubscript{i} dynamics in the present study is due to activation of SECRA pumps, since the majority of experiments were performed in the absence of extracellular Ca\textsuperscript{2+} which would limit Ca\textsuperscript{2+} uptake into the ER.

Another possibility is that diamide may sensitize the IP\textsubscript{3}R to basal levels of IP\textsubscript{3}. Consistent with this hypothesis, diamide did not itself activate PLC and the effects of diamide were completely blocked by inhibition of PLC by U73122, showing that some basal level of IP\textsubscript{3} generation is required for the diamide effects. These results are reminiscent of the effects of thimerosal which has been shown to initiate Ca\textsuperscript{2+} oscillations without stimulating phosphoinositide hydrolysis (Bootman \textit{et al.}, 1992). Thimerosal causes an increase in affinity of the IP\textsubscript{3}R such that channel activation occurs at the basal
resting levels of IP$_3$. The effects of thimerosal are also observed in partially purified IP$_3$R preparations reconstituted into either lipid vesicles or planar lipid bilayers, consistent with direct modification of critical sulfhydryl groups of the IP$_3$R (Kaplin et al., 1994; Thrower et al., 1996). Ca$^{2+}$ release via the IP$_3$R has also been observed in permeabilized cells challenged with high (milli-molar) concentrations of GSSG (Missiaen et al., 1991, 1992). The effect of GSSG may also be related to a change in IP$_3$R affinity for IP$_3$ (Lopez-Colome & Lee, 1996), but GSSG appears to be much less effective compared to thimerosal and the effects may be indirect (Renard-Rooney et al., 1995; Missiaen et al., 1996). To examine the possibility that diamide promotes the direct glutathionylation of the IP$_3$R, we probed for the presence of the receptor in avidin-pulldown assays from BAECs loaded intracellularly with biotin-GSH and from lysate of BAEC microsomal membranes reacted with diamide in the presence of biotin-GSH. Indeed, the IP$_3$R was present in the pull-downs both in vivo and in vitro, and in both assays, the glutathionylation was reversed by excess DTT. These results suggest that diamide stimulates the reversible glutathionylation of the IP$_3$R or some tightly associated regulatory protein. Whether or not glutathionylation changes affinity of the receptor for IP$_3$ remains to be determined, but given the well-known effects of thimerosal, this seems to be a plausible mechanism.

Higher concentrations of diamide caused a significant time-dependent increase in basal resting [Ca$^{2+}$]. The increase in [Ca$^{2+}$]$_i$ was seen in both the presence and importantly, in the complete absence of extracellular Ca$^{2+}$. As mentioned above, the BAECs used in the present study do not express the Na$^+$.Ca$^{2+}$-exchanger. Thus, the rise in basal [Ca$^{2+}$]$_i$ may be explained by a diamide-induced inhibition of the PMCA. A
similar rise in basal [Ca\textsuperscript{2+}] was previously reported in aortic and pulmonary artery EC treated with tert-butyl-hydroperoxide (t-BOOH; (Elliott et al., 1989; Elliott & Schilling, 1990)), and more recently in pancreatic acinar cells challenged with H\textsubscript{2}O\textsubscript{2} (Baggaley et al., 2008). It is well-established that the PMCA is sensitive to oxidant stress. Specifically, it has been shown that ROS inhibit Ca\textsuperscript{2+} pump function leading to inhibition of Ca\textsuperscript{2+} extrusion across the plasma membrane (for reviews see (Squier & Bigelow, 2000; Waring, 2005)). Consistent with this, the ATPase activity of PMCA pumps was reduced in response to a wide variety of oxidizing agents including H\textsubscript{2}O\textsubscript{2}, t-BOOH, O\textsubscript{2}\textsuperscript{•−}, and ONOO\textsuperscript{−} (Bellomo et al., 1983; Zaidi & Michaelis, 1999; Zaidi et al., 2009). However, at least in part, the effects of ROS on PMCA may be attributable to changes in calmodulin (CaM). The PMCA is a Ca\textsuperscript{2+}-CaM regulated protein (Carafoli, 1991). In the absence of CaM, the affinity of the PMCA for Ca\textsuperscript{2+} is 10-20 μM, whereas in the presence of CaM the affinity of the pump for Ca\textsuperscript{2+} is greatly increased to 0.4-0.5 μM. Oxidative stress will inactivate CaM through modification of highly conserved methionine residues (Bigelow & Squier, 2005). The oxidized form of CaM essentially acts as a dominant-negative, preventing the binding of native CaM to the PMCA, blocking activation of the pump. Thus, oxidative stress appears to have both direct effects on the PMCA and indirect effects via CaM, both of which will attenuate Ca\textsuperscript{2+} pump function contributing to the rise in basal [Ca\textsuperscript{2+}]. The ability of glutathionylation to alter PMCA pump function however, has never been described. To determine if the PMCA is glutathionylated in response to diamide, we probed the avidin-pull-downs from biotin-GSH-loaded BAECs for the PMCA. Diamide increased the PMCA captured in these pull-downs, consistent with increased direct glutathionylation of the PMCA or a tightly associated regulatory protein.
The rise in basal $[\text{Ca}^{2+}]_i$ and the glutathionylation of the PMCA were reversed by excess DTT, again consistent with thiol modification.

Although inhibition of the PMCA by thiol modification has been proposed as a potential mechanism contributing to a rise in $[\text{Ca}^{2+}]_i$, previous studies have shown that loss of PMCA ATPase activity in response to t-BOOH was prevented by GSH or DTT (Bellomo et al., 1983). Moreover, by themselves GSH, DTT, and GSSG have been shown to stimulate the ATPase activity of PMCA pumps (Bellomo et al., 1983; Nicotera et al., 1985a; Nicotera et al., 1985b). In preliminary studies in BAECs, we found that the rise in basal $[\text{Ca}^{2+}]_i$ in response to high concentrations of diamide occurs within a subcellular compartment that is shielded from global changes in $[\text{Ca}^{2+}]_i$ during $\text{Ca}^{2+}$ signaling events, and is not cleared by $\text{Ca}^{2+}$ sequestration into ER, mitochondrial or lysosomal $\text{Ca}^{2+}$ stores (data not shown). Moreover, depletion of internal $\text{Ca}^{2+}$ stores failed to abrogate the diamide-induced rise in basal $[\text{Ca}^{2+}]_i$. However, the role of the PMCA in this response remains elusive, and the effect of diamide may be indirect. High concentrations of diamide will rapidly deplete intracellular GSH, and eventually overwhelm the capacity of the cell to regenerate GSH from GSSG. With little to no GSH left to react with, diamide could begin to directly modify proteins; for example, by promoting intra- and inter- molecular disulfide bond formation. Thus diamide, at high concentrations, may cause changes to $[\text{Ca}^{2+}]_i$, unrelated to GSH oxidation and P-SSG formation. Further studies are necessary to determine the underlying mechanisms responsible for the time dependent rise in basal $[\text{Ca}^{2+}]_i$ in ECs during treatment with diamide, and the functional consequence of glutathionylation on PMCA function.
In summary, diamide enhances $\text{Ca}^{2+}$ oscillations and increases basal $[\text{Ca}^{2+}]_i$ in the absence of receptor stimulation or phosphoinositide hydrolysis. To our knowledge, these are the first experiments showing that glutathionylation alters PMCA and IP$_3$R function possibly via direct protein modification. At least over the time frame examined, the effects of diamide were reversible suggesting that the change in oscillation frequency observed at the lower concentrations and the rise in basal $[\text{Ca}^{2+}]_i$ seen at the higher concentrations of diamide reflect physiological redox signaling mechanisms rather than pathological oxidative stress. Distinct information is encoded by frequency versus amplitude modulation of $\text{Ca}^{2+}$ signals (Parekh, 2011). Thus, redox signaling via glutathionylation may differentially activate multiple downstream pathways responsive to even small changes in cytosolic $\text{Ca}^{2+}$ dynamics.
2.5: FIGURES

2.5.1: Figure 2.1

A. No treatment

B. 50 μM diamide

C. 100 μM diamide

D. Cells counted (% of total)
**Figure 2.1:** Low concentrations of diamide cause asynchronous Ca$^{2+}$ oscillations when measured in the presence of extracellular Ca$^{2+}$ (Ca$^{2+}$-ECS).

A-C) Fura-2 fluorescence ratio (340nm/380nm) was recorded from single BAECs in the absence (A) and presence of 50 μM (B) or 100 μM (C) diamide as indicated by the horizontal bar at the top of each panel. Individual cells are shown as different grey-scale traces. D) Single cell [Ca$^{2+}$]$_i$ oscillation frequency was quantified as the number of cells exhibiting 0, 1-2, and 3+ Ca$^{2+}$ spikes during the 10 min treatment period. Values represent the mean ± SE of 3-4 experiments; 259 (untreated), 204 (50 μM), and 197 (100 μM) cells were counted per treatment with 42-63 cells counted per experiment. Asterisks (*) indicate p < 0.0005 when compared to matched untreated-controls.
2.5.2: Figure 2.2

A. No treatment

B. 50 μM diamide

C. 100 μM diamide

D. Cells counted (% of total)
Figure 2.2: Low concentrations of diamide cause asynchronous $Ca^{2+}$ oscillations when measured in the absence of extracellular $Ca^{2+}$ (zero $Ca^{2+}$-ECS).

The protocol was the same as Fig 2.1 with the exception that 0.3 mM EGTA was added and CaCl$_2$ was omitted from the extracellular buffer (zero $Ca^{2+}$-ECS). Values represent the mean ± SE of 3-5 experiments; 336 (untreated), 205 (50 μM), and 177 (100 μM) cells were counted per treatment with 54-80 cells counted per experiment. Asterisks (*) indicate $p < 0.0001$ compared to matched untreated-controls.
2.5.3: Figure 2.3

A. Ca\textsuperscript{2+}-ECS

B. Zero Ca\textsuperscript{2+}-ECS

C. Mean peak BK response
Figure 2.3: Effect of bradykinin on $[Ca^{2+}]_i$ in BAECs.

Bradykinin produced the typical Ca$^{2+}$ response; a transient component indicative of Ca$^{2+}$ release from internal Ca$^{2+}$ stores and a more sustained component indicative of Ca$^{2+}$ influx from the extracellular space. The two components are separated by using the Ca$^{2+}$ add-back protocol shown in B. A) Fura-2 fluorescence ratio (340nm/380nm) was recorded from single BAECs bathed in normal Ca$^{2+}$-containing ECS. Bradykinin (BK; final concentration 50 nM) was added during the time indicated by the horizontal bar at the top of the panel. B) Same protocol as in panel A with the exception that the bath solution was zero Ca$^{2+}$-ECS. At the time indicated, the bath was changed to normal Ca$^{2+}$-ECS. Each figure shows results from a representative experiment with individual cells shown as different color traces. C) The peak BK response was quantified for each cell. Values represent the mean ± SE of 3 experiments; a total of 172 (normal Ca$^{2+}$-ECS) and 213 (zero Ca$^{2+}$-ECS) cells were analyzed per treatment with 56-74 cells analyzed per experiment. Note that the peak BK response is similar in magnitude to the diamide-induced oscillatory increases in $[Ca^{2+}]_i$, shown in Figs 2.1 and 2.2.
2.5.4: Figure 2.4

A. Ca$^{2+}$-ECS

B. 500 µM diamide

C. % change in baseline ratio

D. zero Ca$^{2+}$-ECS

E. 500 µM diamide

F. % change in baseline ratio
Figure 2.4: High concentrations of diamide elevate basal $[Ca^{2+}]_i$ when measured in both the presence and absence of extracellular $Ca^{2+}$.

Fura-2 fluorescence ratio (340nm/380nm) was recorded from single BAECs in normal $Ca^{2+}$ containing buffer ($Ca^{2+}$-ECS) or in the absence of extracellular $Ca^{2+}$ (zero $Ca^{2+}$-ECS), in the presence of 250 μM (A, D) or 500 μM diamide (B, E). Basal $[Ca^{2+}]_i$ (C, F) was quantified as the percent change in the baseline fluorescence ratio at the end of the ten minute treatment period for each experimental condition. C) Values represent the mean ± SE of 5-6 experiments; 261 (untreated), 354 (250 μM) and 281 (500 μM) cells were analyzed per treatment with 43-64 cells analyzed per experiment. F) Values represent the mean ± SE of 4-5 experiments; 263 (untreated), 258 (250 μM), and 332 (500 μM) cells were analyzed per treatment, with 54-74 cell analyzed per experiment. Asterisks (*) indicate p < 0.0001 when compared to matched untreated-controls.
2.5.5: Figure 2.5

A. 100 μM diamide

B. Inactivated diamide

C. Cells counted (% of total) vs. Ca²⁺ spikes/10 min

- 100 μM diamide
- Inactivated diamide

* Denotes significant difference.
Figure 2.5: Comparison of active versus inactivated diamide on Ca\textsuperscript{2+} oscillations in BAECs.

The general protocol was the same as that described in Fig 2.1; extracellular buffer was zero Ca\textsuperscript{2+}-ECS. A) Diamide (100 µM) was added during the time indicated by the horizontal bar above the graph. B) Same protocol as in panel A with the exception that diamide (100 µM final concentration) was first mixed with an excess of reduced glutathione (GSH) in a 1:4 molar ratio immediately prior to addition to the bath. Diamide reacts with GSH within seconds to produce GSSG plus a hydrazine byproduct. C) Single cell [Ca\textsuperscript{2+}], oscillation frequency was quantified as the number of cells exhibiting 0, 1-2, and 3+ Ca\textsuperscript{2+} spikes during the 10 min treatment period. Values represent the mean ± SE of 3 experiments; 189 (diamide) and 174 (inactivated diamide) cells were counted per treatment with 53-76 cells counted per experiment. Asterisks (*) indicate p < 0.001 when compared to diamide treated cells.
2.5.6: Figure 2.6

![Graph showing R/R₀ over time](image)
**Figure 2.6:** *BAPTA loading prevents diamide-induced change in \([Ca^{2+}]_i\).*

Fura-2 fluorescence ratio was recorded from single BAECs pretreated with either vehicle (*closed circles*) or BAPTA-AM (*open circles*) for 30 min prior to challenge with 500 µM diamide in zero Ca\(^{2+}\)-ECS. Values represent the mean ± se normalized fluorescence ratio (R/R₀) from 4 independent experiments for each condition; a total of 286 (Vehicle) and 279 (BAPTA) individual cells were recorded with 54-86 cells analyzed per experiment. Where error bars are absent the se is smaller than the symbol.
2.5.7: Figure 2.7

A. 

100 μM diamide

340/380

0.9

0.8

0.7

0.6

0.5

0.4

0.3

0 2 4 6 8 10 12 14 16

Time (min)

B. 

100 μM diamide + 5 U/ml Apyrase

340/380

0.9

0.8

0.7

0.6

0.5

0.4

0.3

0 2 4 6 8 10 12 14 16

Time (min)

C. 

100 μM diamide

untreated

5 U/ml Apyrase

Cells counted (% of total)

0 20 40 60 80 100

0 1-2 3+

Ca²⁺ spikes/10 min

0 2 4 6 8 10 12 14 16

Time (min)
Figure 2.7: Effect of apyrase on diamide-induced Ca^{2+} oscillations in BAECs

The general protocol was the same as that described in Fig 2.1; extracellular buffer was zero Ca^{2+}-ECS. **A**) Diamide (100 µM) was added during the time indicated by the horizontal bar above the graph. **B**) Same protocol as in panel A with the exception that 5 Units/ml apyrase was added along with diamide. **C**) Single cell [Ca^{2+}]_i oscillation frequency was quantified as the number of cells exhibiting 0, 1-2, and 3+ Ca^{2+} spikes during the 10 min treatment period. Values represent the average of 2 experiments; 144 (diamide) and 131 (diamide plus apyrase) cells were counted per treatment with 58-79 cells counted per experiment.
2.5.8: Figure 2.8

A. Vehicle

B. 50 μM 2-APB

C. 100 μM diamide

D. Vehicle

E. 50 μM 2-APB

F. 500 μM diamide

C. Cells counted (% of total)

F. % change in baseline ratio

Ca²⁺ spikes/10 min
Figure 2.8: Inhibition of the IP₃R by 2-APB attenuates diamide-induced Ca²⁺ oscillations when measured in the absence of extracellular Ca²⁺.

Protocol was the same as in Fig 2.2 with the exception that BAECs were pretreated for 10 min with vehicle (A), or 50 μM 2-APB (B) immediately prior to challenge with 100 μM diamide in zero Ca²⁺-ECS. C) Diamide-induced single cell [Ca²⁺]ᵢ oscillation was quantified as described in the legend to Fig 2.1 for each experimental condition. Values represent the mean ± se of 3-5 experiments; 314 (vehicle) and 222 (50 μM 2-APB) cells were counted per treatment with 58-82 cells counted per experiment. Asterisks (*) indicate p < 0.00001 compared to matched vehicle-controls. D, E) Same protocol as in A and B with 500 μM diamide added during the time indicated. F) Basal [Ca²⁺]ᵢ from experiments shown in panels D and E were quantified as the percent change in the baseline fluorescence ratio at the end of the ten minute treatment period. Values represent the mean ± se of 3-4 experiments; 201 (vehicle), 261 (50 μM 2-APB) cells were counted per treatment with 53-75 cells per experiment; NS, not significant.
2.5.9: Figure 2.9

A. **Vehicle**

B. **10 μM U73122**

C. **10 μM U73343**

D. **100 μM diamide**

- **Vehicle**
- **10 μM U73122**
- **10 μM U73343**

**Cells counted (% of total)**

- **Ca^{2+} spikes/10 min**

0 1-2 3+
Figure 2.9: Inhibition of PLC by U73122 prevents diamide-induced Ca\textsuperscript{2+} oscillations when measured in the absence of extracellular Ca\textsuperscript{2+}.

Protocol was the same as in Fig 2.2 with the exception that BAECs were pretreated for 4 min with vehicle (A), 10 μM U73122 (B), or 10 μM U73343 (C) immediately prior to challenge with 100 μM diamide in zero Ca\textsuperscript{2+}-ECS. D) Diamide-induced single cell [Ca\textsuperscript{2+}]\textsubscript{i} oscillations were quantified as described in Fig 2.1 for each experimental condition. Values represent the mean ± se of 4-5 experiments; 331 (vehicle), 279 (U73122), 252 (U73343) cells were counted per treatment with 56-77 cells counted per experiment. Asterisks (*) indicate p < 0.001 compared to matched vehicle-controls.
2.5.10: Figure 2.10
Figure 2.10: Diamide does not stimulate PIP$_2$ hydrolysis.

Fluorescence images were acquired from LLC-PK1 cells stably expressing the GFP-tagged pleckstrin homology domain of PLC$_\gamma$ (GFP-PH). In the representative experiment shown, the cells, perfused with normal Ca$^{2+}$-ECS, were challenged with ATP (100 µM) for ten minutes followed by a washout period. The cells were then challenged with diamide (500 µM) for 10 min followed by a final wash period. Approximately 10 min elapsed between the end of the ATP wash and the beginning of the challenge with diamide. Selected images are shown before, 5 min after each agent, and 5 min after initiation of the wash as indicated at the top. Diamide had no effect on the distribution of GFP-PH when tested on naive cells (n=3) or when applied after ATP (n=3).
2.5.11: Figure 2.11

A. Vehicle

B. 75 μM BCNU

C. 25 μM diamide
   - Vehicle
   - 75 μM BCNU

D. Vehicle

E. 75 μM BCNU

F. 250 μM diamide
   - Vehicle
   - 75 μM BCNU

- Cells counted (% of total)
  - Ca²⁺ spikes/10 min

- % change in baseline ratio
  - Δ[Ca²⁺]
  - Washout
Figure 2.11: Inhibition of glutathione reductase by BCNU potentiates diamide-induced changes when measure in the absence of extracellular Ca²⁺.

BAECs in normal Ca²⁺-ECS were either pretreated for 30 min with vehicle or 75 μM BCNU prior to challenging with diamide. Fura-2 fluorescence ratio was recorded from single BAECs in zero Ca²⁺-ECS in the presence of 25 μM (A, B), or 250 μM diamide (D, E) as indicated by the horizontal bar at the top of each panel. C) Single cell [Ca²⁺]ᵢ oscillation frequency was quantified as described in the legend to Fig 2.1. Values represent the mean ± se of 4 experiments; 260 (vehicle), and 261 (75 μM BCNU) cells were counted per experimental condition, with 54-76 cells counted per experiment. Asterisks (*) indicate p value < 0.001 compared to matched-controls. F) The change in basal [Ca²⁺]ᵢ was quantified as the percent change in the baseline fluorescence ratio at the end of the ten minute treatment period (Δ[Ca²⁺]ᵢ) and at the end of a 5 minute washout period (Washout); values represent the mean ± se of 3-4 experiments; 169 (vehicle), and 236 (75 μM BCNU) cells were analyzed per experimental condition with 51-64 cells analyzed per experiment. Asterisks (*) indicate p < 0.001 compared to matched controls.
2.5.12: Figure 2.12

A. No pretreatment

D. No pretreatment

B. 500 μM BSO

E. 500 μM BSO

C. 50 μM diamide

F. 250 μM diamide

- No pretreatment
- 500 μM BSO

Percentage of cells counted (% of total)

% change in baseline ratio

Ca²⁺ spikes/10 min

Δ[Ca²⁺]ₗ, Washout
**Figure 2.12:** *Inhibition of γ-glutamylcysteine synthetase by BSO enhances diamide-induced changes in [Ca^{2+}]_{i} when measured in the absence of extracellular Ca^{2+}.*

BAECs were pretreated for 24 hrs with 500 μM BSO prior to challenge with diamide. Fura-2 fluorescence ratio was recorded from single BAECs in zero Ca^{2+}-ECS in the presence of 50 μM (A, B), or 250 μM diamide (D, E) as indicated by the horizontal bar at the top of each panel. C) Single cell [Ca^{2+}]_{i} oscillation frequency was quantified as described in the legend to Fig 2.1. Values represent the mean ± se of 4-5 experiments; 246 (no treatment), and 296 (500 μM BSO) cells were counted per experimental condition, with 59-65 cells counted per experiment. Asterisks (*) indicate p value < 0.001 compared to matched-controls. F) The change in basal [Ca^{2+}]_{i} was quantified as described in Fig 2.10. Values represent the mean ± se of 3-4 experiments; 244 (no treatment), and 196 (500 μM BSO) cells were analyzed per experimental condition with 58-66 cells analyzed per experiment. Asterisks (*) indicate p < 0.001 compared to matched controls.
2.5.13: Figure 2.13

A. 75 μM BCNU

D. 500 μM BSO

B. 75 μM BCNU

E. 500 μM BSO

C. 75 μM BCNU

F. 500 μM BSO
Figure 2.13: Effects of diamide on \([Ca^{2+}]_i\) are reversed by DTT.

A, B) BAECs in normal Ca\(^{2+}\)-ECS were pretreated for 30 min with 75 μM BCNU prior to challenge with diamide as described in the legend to Fig 2.10. DTT (2 mM) was added to the wash buffer in B. C) The change in basal \([Ca^{2+}]_i\) was quantified as described in Fig 2.10; values represent the mean ± SE of 3 experiments; 169 (No DTT), and 236 (2 mM DTT) cells were analyzed per treatment with 51-64 cells analyzed per experiment. Asterisks (*) indicate p < 0.001 compared to no DTT. D, E) BAECs were pretreated with 500 μM BSO prior to challenge with diamide as described in the legend to Fig 2.11. DTT (2mM) was added to the wash buffer in E. F) The change in basal \([Ca^{2+}]_i\) was quantified as described in Fig 2.10; values represent the mean ± SE of 3 experiments; 190 (No DTT), and 207 (2 mM DTT) cells were analyzed per treatment with 55-85 cells analyzed per experiment. Asterisks (*) indicate p < 0.0002 compared to no DTT.
2.5.14: Figure 2.14

A. BioGEE-loaded BAEC cells

B. BioGEE-loaded BAEC cells

C. BAEC membranes

D. BAEC membranes
Figure 2.14: Diamide increases glutathionylation of IP$_3$R and PMCA both in vivo and in vitro.

A, B) BAECs, loaded with the cell-permeable form of biotinylated-GSH (BioGEE), were suspended in normal Ca$^{2+}$-ECS and divided into 3 aliquots as indicated above each lane; 1 aliquot was left untreated (BioGEE alone) and the remaining 2 aliquots were incubated with diamide (500 µM) for 10 min. The membrane lysate from one aliquot of diamide-treated cells was incubated with DTT prior to pull-down. Biotinylated proteins were extracted from the cleared lysates with avidin-agarose beads and probed for either the IP$_3$R or PMCA as described in Experimental Procedures. C, D) Membranes isolated from BAECs were treated with glutathionylation reagents as indicated above each lane (125 µM biotin-GSH alone, 100 µM diamide plus 125 µM biotin-GSH, and diamide plus biotin-GSH followed by 20 mM DTT prior to pull-down). Biotinylated proteins were extracted from the cleared membrane lysates with avidin-agarose beads and probed for either the IP$_3$R or PMCA. Samples of membrane lysates were reserved prior to pull-down (input). Results shown are representative of 3-6 independent experiments.
Figure 2.15

Diamide

\[ \gamma \text{-glutamyl cycle} \]

\[ \gamma \text{-GS} \rightarrow \text{BSO} \]

\[ \text{ADP} \]

GSH

Diamide-GS intermediate

GSH

\[ \text{GSSG} \]

\[ \text{GR} \]

\[ \text{BCNU} \]

\[ \text{NADPH} \]

PSH

\[ \text{Grx} \]

Protein S-glutathionylation (P-SSG)

\[ \text{IP}_3R \]

increased \( \text{Ca}^{2+} \) oscillations

\[ \text{PMCA} \]

elevated basal \( [\text{Ca}^{2+}]_i \)
**Figure 2.15:** *Metabolism of diamide leads to increased protein S-glutathionylation (P-SSG).*

The schematic diagram shows the enzymatic pathways responsible for generation and maintenance of cellular GSH and two pathways by which diamide increases P-SSG formation. The GSSG disulfide exchange reaction (shaded arrows) is unlikely to occur at low concentrations of diamide (see text for details). The \( \gamma - \text{glutamylcysteine synthetase} \) (\( \gamma - \text{GS} \)) catalyzes the rate-limiting step in GSH synthesis and is inhibited by BSO. Glutathione reductase (GR) catalyzes the conversion of GSSG to GSH at the expense of NADPH and is inhibited by BCNU. Glutaredoxin (Grx) catalyzes protein de-glutathionylation at the expense of GSH.
CHAPTER 3

Protein S-Glutathionylation Enhances Ca\(^{2+}\)-Induced Ca\(^{2+}\) Release via the IP\(_3\)R in Cultured Aortic Endothelial Cells

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3.1: INTRODUCTION

In most non-excitable cells, including vascular ECs, the generation and propagation of oscillations in \([\text{Ca}^{2+}]_i\) are predominantly driven by the release of \(\text{Ca}^{2+}\) from the ER via the IP_3R, as discussed in Chapter 1 section 1.2 (for reviews see (Bootman et al., 1995; Berridge et al., 2000; Foskett et al., 2007)). At the cellular level, signal transduction commonly involves not just the generation of \(\text{Ca}^{2+}\) oscillations, but rather changes in both the frequency and amplitude of the oscillatory response (Parekh, 2011), both of which are controlled to a great extent by the integrated regulation of the IP_3R by IP_3 and \(\text{Ca}^{2+}\). The interplay between IP_3 and \(\text{Ca}^{2+}\) is critical to the “all-or-nothing” generation of a \(\text{Ca}^{2+}\) oscillation. IP_3 increases IP_3R channel open probability which initiates IIICR, whereas \(\text{Ca}^{2+}\) activates the IP_3R at low concentration by CICR, but inhibits channel activity at high concentrations. Thus, feed-forward activation of the IP_3R by CICR plays a critical role in the rising phase of a global \(\text{Ca}^{2+}\) oscillation, whereas feedback inhibition by \(\text{Ca}^{2+}\) helps terminate IP_3R activity and contributes to the falling phase of a \(\text{Ca}^{2+}\) oscillation.

Although the IP_3R is typically activated by a rise in IP_3 following receptor-initiated hydrolysis of PIP_2 by PLC (Berridge, 1993), \(\text{Ca}^{2+}\) release from IP_3-sensitive stores is reported to occur in the absence of an increase in IP_3 formation during periods of oxidative stress (Rooney et al., 1991; Bird et al., 1993). In non-excitable cells, including ECs, ROS and RNS, as well as a variety of pharmacological oxidants, cause an increase in \(\text{Ca}^{2+}\) oscillations under basal conditions and enhance agonist-stimulated changes in \([\text{Ca}^{2+}]_i\). Oxidant-induced changes in IP_3R activity can be reversed by reducing agents such as DTT, suggesting that alterations in thiol chemistry are responsible for this
enhanced Ca$^{2+}$ response (Bootman et al., 1992; Bird et al., 1993). Furthermore, studies examining the effects of oxidizing reagents on purified IP$_3$Rs indicate that this enhanced activation by IP$_3$ reflects direct modification of the channel protein (Kaplin et al., 1994; Thrower et al., 1996; Poirier et al., 2001). In this regard, a number of residues have been identified on the IP$_3$R as potential sites of redox modification (Joseph et al., 2006; Kang et al., 2008). However, despite the general consensus that oxidative stress sensitizes the IP$_3$R to activation by IP$_3$, the molecular basis for this response remains unknown.

Previously, we showed that low concentrations of diamide cause a dramatic increase in asynchronous single-cell Ca$^{2+}$ oscillations in cultured aortic endothelial cells (Chapter 2; (Lock et al., 2011)). Diamide, even at high concentrations, did not increase PIP$_2$ hydrolysis indicating that diamide does not activate PLC. However, the diamide response was observed even in the absence of extracellular Ca$^{2+}$, and was attenuated by inhibition of the IP$_3$R with 2-APB or by inhibition of PLC by U73122 suggesting that diamide sensitizes the IP$_3$R to basal levels of IP$_3$. Consistent with an important role for protein S-glutathionylation, the effect of diamide on Ca$^{2+}$ dynamics was reversed by DTT, and was dependent upon intracellular GSH and the capacity of the cell to regenerate GSH from GSSG. Finally, biochemical studies showed that glutathionylation of the IP$_3$R was significantly increased following exposure of the cells to diamide. Together these results demonstrate that diamide mobilizes Ca$^{2+}$ from IP$_3$-sensitive internal Ca$^{2+}$ stores and suggest that oxidant-induced glutathionylation sensitizes the IP$_3$R. To test this hypothesis, the effect of diamide and H$_2$O$_2$ on IICR and CICR via the IP$_3$R was examined in the present study in two cultured aortic EC lines using single-cell
Ca\textsuperscript{2+} imaging. Our results show that both diamide and H\textsubscript{2}O\textsubscript{2} primarily increase sensitivity of the IP\textsubscript{3}R to Ca\textsuperscript{2+}, i.e. enhanced CICR.
3.2: METHODS

3.2.1: Cell Culture. BAECs were cultured as described in section 2.2.1 and used for experimentation between passages 12-17. HAECs were purchased from Lonza, and cultured on 100 mm plastic dishes in endothelial cell basal medium-2 (EMB-2; Lonza, cat. no. CC-3156) supplemented with growth factors, cytokines, and chemicals (2% FBS, hydrocortisone, human recombinant fibroblast growth factor-B, human recombinant vascular endothelial growth factor, recombinant Long R insulin like growth factor-1, ascorbic acid, human recombinant epidermal growth factor, gentamicin sulfate/amphotericin-B, and heparin) from Lonza (SingleQuots; cat. no. CC-4176). HAECs were cultured as sub-confluent monolayers according to the company’s instructions, and used for experimentation between passages 2-5.

3.2.2: Solutions and Reagent. Ca$^{2+}$-ECS and zero Ca$^{2+}$ buffer were described in 2.2.2. Diamide (diazenedicarboxylic acid bis (NN,-dimethylamide)), 30% hydrogen peroxide (H$_2$O$_2$), histamine (HIST), bradykinin (BK), Poly-D-Lysine (PDL), thapsigargin (TG), ionomycin, DTT, and DMSO were purchased from Sigma-Aldrich. Ryanodine (Ryn) and bafilomycin were from Calbiochem, and xestospongin C (XeC) was from Cayman Chemicals. Fura-2 acetoxymethyl ester (fura-2/AM), and pluronic F-127 were obtained from Invitrogen. Fura-2/AM was reconstituted using DMSO and 10% pluronic F-127 at a 1:1 ratio to yield a 1 mM stock solution. Diamide, H$_2$O$_2$, and ryanodine stock solutions were prepared in aqueous solution, whereas TG, ionomycin, bafilomycin, and XeC were reconstituted in 100% ethanol. All stock solutions were prepared at a final concentration of 10-100 mM.
**3.2.3: Ca^{2+} Imaging.** Time-dependent changes in [Ca^{2+}]_{i}, were measured in both HAEC and BAEC monolayers, as previously above in section 2.2.3. For HAECs, coverslips were charged with 1 mg/ml PDL to improve cell attachment. Oxidant and agonist responses did not differ in HAECs cultured on PDL-coated coverslips in comparison to cells grown on untreated coverslips. BAECs and HAECs were loaded for 30 min at 37°C in normal Ca^{2+} containing solution (Ca^{2+}-ECS) with 6 µM and 3 µM fura-2/AM, respectively. Cells were then washed for 30 min at room temperature in Ca^{2+}-ECS before recording. All recordings were made in the absence of extracellular Ca^{2+} (zero Ca^{2+}-ECS) to specifically monitor changes in [Ca^{2+}]_{i}, related to the release of Ca^{2+} from internal stores.

**3.2.4: Data analysis.** Over the course of these experiments, slight variation in the sensitivity of HAECs and BAECs to chemical oxidants and receptor agonists were noted. For this reason, controls were always performed in parallel for each experimental protocol. For example, the dose-dependent effect of histamine on Ca^{2+} oscillations in untreated HAEC is reported in Fig 3.2 and Fig 3.12; these represent independent data sets. Traces show [Ca^{2+}]_{i} responses from individual cells as gray-scale and black lines. A range of 24-53 HAECs and 46-93 BAECs were monitored per experiment. The difference in the range of cells monitored per experiment in HAECs versus BAECs reflects the different density in which the cell lines are maintained in culture, i.e. ~70% confluent versus fully confluent, respectively. The number of cells oscillating in response to oxidant or agonist challenge was quantified for each individual experiment as the percent of the total cells monitored exhibiting at least one [Ca^{2+}]_{i} oscillation during the indicated treatment period. Histograms and individual data points generating agonist-
dose response curves represent the average values from multiple monolayers under each condition reported as mean ± SE with n equal to the number of monolayers examined under each condition. The total number of cells and the total number of monolayers tested for each condition is given in the figure legend. Statistical comparison of means was performed using the paired Student’s t-test or ANOVA with post-hoc Tukey’s test for pair-wise comparisons; p value < 0.05 was considered to be statistically significant. The phenotypic shift in the TG response depicted in Figs 3.5, 3.6, 3.8, 3.9, and 3.13 was quantified by cumulative frequency analysis of the peak change in fura-2 fluorescence ratio (Peak Ratio), and the length of time from TG exposure to the peak change (Latency to Peak). Differences were identified using the Kruskal-Wallis test and post-hoc pair-wise comparisons were made using the Mann-Whitney U-test with Bonferonni’s correction for multiple comparisons; p < 0.01 was considered significant.

3.2.5: Synthesis of BioGEE. GSH ethyl ester was labeled with sulfo-link-NHS-LC-biotin (Pierce) as previously described (Figtree et al., 2009). Essentially, the reaction was performed by combining 10 mM GSH ethyl ester with 10 mM of biotin reagent in phosphate buffered saline (PBS, pH 8.0). After 1 hour at room temperature, 50 mM TRIS was added to remove any excess sulfo-link reagent.

3.2.6: Isolation of membranes from BAECs. Membranes were isolated as described above in section 2.2.5.

3.2.7: Glutathionylation of IP₃R₁ in vivo: BAECs, harvested by scraping, were washed and re-suspended in 1.5 ml Ca²⁺-ECS and incubated with 500 µM BioGEE for 180 minutes at 37°C. BioGEE-loaded cells were washed with Ca²⁺-ECS and either left
untreated or subjected to oxidative challenge with either 0.1 mM H$_2$O$_2$, or 1 mM H$_2$O$_2$ for 10 min at room temperature. Following treatment, the cells were washed with Ca$^{2+}$-ECS and membrane preparations were generated as in section 2.2.5. For each sample, membrane preparations were divided into two aliquots; one aliquot from each sample was incubated with 20 mM DTT. Membrane aliquots were then solubilized in PBS containing 1% Triton X-100 for 30 min on ice. Following solubilization membrane lysates were cleared by centrifugation, and biotinylated proteins were extracted with streptavidin-agarose beads by overnight incubation at 4$^\circ$C.

3.2.8: Immunoblots. Following in vivo glutathionylation reactions, proteins captured on streptavidin-agarose were processed, probed, and detected as described in section 2.2.9 using an anti-IP$_3$ receptor type 1 antibody (Millipore; cat. no. 07-514).
3.3: RESULTS

3.3.1: Effect of diamide on [Ca\textsuperscript{2+}]\textsubscript{i} in cultured aortic endothelial cells. The acute effect of diamide on [Ca\textsuperscript{2+}]\textsubscript{i} of two independent cultured EC lines – bovine (BAECs) and human (HAECs) aortic endothelial cells – was investigated by single-cell imaging of fura-2-loaded monolayers. Previously, we reported that diamide produced a concentration-dependent increase in asynchronous Ca\textsuperscript{2+} oscillations in BAECs by stimulating the release of Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive stores (Lock et al., 2011). HAECs, however, were unexpectedly resistant to diamide-induced oscillations in [Ca\textsuperscript{2+}]\textsubscript{i}. As shown in Fig 3.1A and quantified in Fig 3.1C, HAECs challenged with 100 µM diamide exhibited Ca\textsuperscript{2+} oscillations in only 1.5% ± 0.9% (mean ± SE) of the total cells monitored, whereas oscillations were not observed in paired controls. Challenge with increasing concentrations of diamide (up to 1.0 mM) had no effect on the number of HAECs responding (data not shown). In comparison, BAECs challenged with 100 µM diamide displayed a significant increase in the number of cells exhibiting asynchronous Ca\textsuperscript{2+} oscillations when compared to paired untreated controls (Fig 3.1B and quantified in Fig 3.1C). In addition to BAECs, diamide mobilized Ca\textsuperscript{2+} from the internal stores of a mouse inner medullary collecting duct cells (IMCD-3), pig proximal tubule cells (LLC-PK1), and Chick DT-40 B-cells (data not shown).

3.3.2: Diamide increases the sensitivity of cultured aortic endothelial cells to receptor-stimulated Ca\textsuperscript{2+} oscillations. Our previous studies showed that the diamide-induced change in [Ca\textsuperscript{2+}]\textsubscript{i} in BAECs is blocked by inhibition of PLC by U73122 or by inhibition of the IP\textsubscript{3}R by 2-APB suggesting that diamide sensitizes the IP\textsubscript{3}R to basal levels of IP\textsubscript{3}. Thus, the lack of sensitivity of HAECs to diamide may reflect the lack of
sufficient IP$_3$ to initiate an oscillation under resting conditions. If this is true, we reasoned that low concentrations of diamide that have little or no effect on Ca$^{2+}$ oscillations, should enhance the response to receptor agonists that generate IP$_3$. To test this hypothesis, HAECs bathed in Ca$^{2+}$ free media, were left untreated or treated with diamide for 5 minutes immediately prior to stimulation with HIST in the continued absence or presence of diamide (Fig 3.2). Under control conditions, 30 nM HIST had no effect on [Ca$^{2+}$]$_i$ (Fig 3.2A). However, pretreatment with 100 µM diamide dramatically increased in the number cells oscillating in response to 30 nM HIST (Fig 3.2B). Diamide pretreatment significantly increased the number of HAECs responding to every sub-maximal concentration of HIST tested, whereas neither untreated cells, nor cells treated with diamide alone exhibited Ca$^{2+}$ oscillations in the absence of receptor stimulation. This increased sensitivity to HIST-stimulated Ca$^{2+}$ oscillations is reflected in a 3- to 5-fold leftward shift in the HIST dose-response curve in HAECs pretreated with diamide relative to paired-untreated controls (Fig 3.2C). In a parallel set of experiments (Fig 3.3), we confirmed that this effect of diamide is related to a change in IP$_3$R function since the response was unaffected by Ryn (Fig 3.3C and 3.3D) and significantly attenuated by XeC (Fig 3.3B and 3.3D); XeC is a cell-permeable IP$_3$R inhibitor (Gafni et al., 1997).

A similar effect of diamide on BK-mediated changes in [Ca$^{2+}$]$_i$ was observed in BAECs (Fig 3.4). Cells, which did not respond to 100 pM BK under control conditions (Fig 3.4A), exhibited a robust Ca$^{2+}$ response to 100 pM BK when briefly pretreated with 20 µM diamide, a threshold concentration that has only modest effect on Ca$^{2+}$ oscillations (Fig 3.4B). Once again, diamide pretreatment significantly increased the number of cells oscillating in response to every sub-maximal agonist concentration tested, and altogether
produced a 2- to 3-fold leftward shift in the BK dose-response relationship when compared to paired-untreated controls (Fig 3.4C). Since BAECs do not express Ryn receptors (Schilling & Elliott, 1992), collectively these results demonstrate that diamide increases the sensitivity of cultured aortic endothelial cells to receptor-stimulated Ca\textsuperscript{2+} oscillations, and suggests that P-SSG formation enhances the sensitivity of the IP\textsubscript{3}R to IICR.

In order to determine if diamide affected other parameters of the Ca\textsuperscript{2+} oscillatory response, we examined the time from agonist exposure to the peak change in [Ca\textsuperscript{2+}]\textsubscript{i} (Latency) and the peak amplitude of the Ca\textsuperscript{2+} oscillation (Amplitude) for the first oscillation of every cell responding to receptor stimulation in both untreated and diamide treated cells (Table 3.1). In both cell lines, a graded Ca\textsuperscript{2+} response was observed in which oscillations increased in amplitude and decreased in latency time in response to stimulation with increasing concentrations of receptor agonist. Pretreatment of HAECs with diamide slightly reduced the Latency and increased the Amplitude of cells responding to submaximal [HIST] in comparison to paired untreated controls stimulated with HIST, whereas no difference in the Latency or Amplitude was observed at a maximal [HIST]. A similar effect of diamide on the reduction in Latency to submaximal [BK] was observed in BAECs. However, there was no consistent trend in the Amplitude of the Ca\textsuperscript{2+} oscillations in untreated versus diamide-treated BAECs. These results are consistent with the observation that diamide increased the sensitivity of the IP\textsubscript{3}R to IICR, but since but since the generation of a Ca\textsuperscript{2+} oscillation reflects both IICR and CICR; diamide may also be increasing the sensitivity of the receptor to Ca\textsuperscript{2+}.
3.3.3: Diamide enhances thapsigargin-mediated changes in the $[\text{Ca}^{2+}]_i$ of cultured aortic endothelial cells. Inhibition of the sarcoplasmic/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) pump by thapsigargin (TG) is a commonly used method for increasing $[\text{Ca}^{2+}]_i$ in the absence of receptor stimulation and in the absence of phosphoinositide hydrolysis (Lytton et al., 1991). It is thought that TG-induced rise in $[\text{Ca}^{2+}]_i$ reflects the passive leak of Ca$^{2+}$ from the ER following SERCA inhibition. However, the results of our next experiments revealed a surprising finding. As previously reported in most non-excitable cell types, in the absence of extracellular Ca$^{2+}$, TG elicited a near homogenous Ca$^{2+}$ response in HAECs under control conditions (Fig 3.5A, black traces). The response in each cell was characterized by a relatively slow rate of rise and small peak change in $[\text{Ca}^{2+}]_i$. In contrast, the TG response of HAECs briefly pretreated with a low concentration of diamide could be segregated into two phenotypic profiles. The first profile was that seen in controls, i.e., slow rate of rise and low amplitude, whereas the second profile was characterized by a rapid rate to rise and large peak change in $[\text{Ca}^{2+}]_i$ (Fig 3.5B; gray-scale traces), suggesting that diamide may sensitize the IP$_3$R to CICR. If this is true we reasoned that a low concentration of HIST, which would increase IP$_3$ but not produce oscillations, would also sensitize HAECs to TG. As seen in Fig 3.5C, the TG-induced Ca$^{2+}$ response in HIST pre-treated cells were phenotypically similar both in magnitude and time course to that observed in the diamide-treated group, consistent with enhanced CICR. In HAECs, TG evoked a CICR response in 87.1% ± 3.5% of the total cells pretreated with 100 µM diamide and 58.8% ± 9.3% of cells pretreated with 30 nM HIST, whereas a CICR profile in response to TG was observed in only 1 out of 121 cells analyzed under control conditions. The
phenotypic change in the TG response (i.e. enhanced CICR) was quantified in an unbiased manner using cumulative frequency analysis of the Peak Ratio and the Latency to Peak. As seen in Fig 3.5D and 3.5E, both diamide and HIST produced a significant increase in the Peak Ratio and a significant decrease in the Latency to Peak following TG exposure in HAECs.

Similar to the Ca$^{2+}$ response to TG observed in HAECs, BAECs briefly pretreated with a either a sub-threshold concentration of diamide (Fig 3.6B) or BK (Fig 3.6C) also exhibited an enhanced Ca$^{2+}$ response to TG. However, unlike HAECs, BAECs exhibited a heterogeneous response, consisting of both slow (black traces) and fast (gray traces) Ca$^{2+}$ profiles, even under control conditions (Fig 3.6A). In some cells, a rapid release of Ca$^{2+}$ was observed after a slow rise in [Ca$^{2+}$], providing further evidence that the fast response reflects all-or-nothing CICR from stores (inset Fig 3.6A). Altogether, a CICR profile in response to TG was observed in 74.4% ± 4.9% of diamide-treated and 52.4% ± 9.3% of BK-treated cells, whereas only 32.3% ± 4.5% of untreated BAECs exhibited a CICR response to TG. As seen in Fig 3.6D and 3.6E, both diamide and BK significantly increased the Peak Ratio and significantly decreased the Latency to Peak in response to TG in BAECs.

Diamide is expected to increase glutathionylation of a number of cellular proteins, some of which may impact Ca$^{2+}$ homeostasis. For example, diamide may affect the Ca$^{2+}$ buffer capacity of the cytoplasm and/or alter the Ca$^{2+}$ load of the internal stores by stimulation of SERCA, both of which may contribute to the enhanced response to TG. However, at the concentrations used, diamide had no significant effect on basal [Ca$^{2+}$], or on the peak response to maximum concentrations of HIST, BK, or ionomycin (Fig 3.7).
Furthermore, diamide had no significant effect on the kinetics of these responses. Thus, alterations in stored Ca\(^{2+}\) or in the buffer capacity of the cytoplasm seem unlikely. Additionally, the diamide-induced change in TG response was unaffected by blockade of the Ryn receptor, or by inhibition of lysosomes by bafilomycin, but could be partially (but significantly) attenuated by XeC (Fig 3.8 and Fig 3.9). To further demonstrate the impact of diamide on CICR via the IP\(_3\)R, we examined a near threshold concentration of TG. As seen in Fig 3.10, 3 nM TG produced a slow gradual rise in [Ca\(^{2+}\)]\(_i\) in HAECs when measured in the absence of extracellular Ca\(^{2+}\) under control conditions. However, in the presence of diamide, CICR was seen in 57.0 ± 2.1% of cells examined (Fig 3.10B and 3.10D) and 14.1 ± 1.3% exhibited repetitive Ca\(^{2+}\) oscillations over the recording period (Fig 3.10C). Collectively these results demonstrate IP\(_3\)R activation can shape the change in [Ca\(^{2+}\)]\(_i\) following depletion of the ER Ca\(^{2+}\) store by a process of CICR, and suggest that diamide increases the sensitivity of the IP\(_3\)R to cytosolic Ca\(^{2+}\). Moreover, these results indicate that the stimulatory effect of diamide on IP\(_3\)R activity is downstream of receptor activation.

3.3.4: Effect of H\(_2\)O\(_2\) on [Ca\(^{2+}\)]\(_i\) of cultured aortic endothelial cells. To determine if a physiologically relevant oxidant produces similar changes in IP\(_3\)R activity, the effect of hydrogen peroxide (H\(_2\)O\(_2\)) on the [Ca\(^{2+}\)]\(_i\) of HAECs and BAECs was investigated under identical conditions to those described above for diamide. In the absence of extracellular Ca\(^{2+}\), HAECs challenged with 100 µM H\(_2\)O\(_2\) did not exhibit oscillations in [Ca\(^{2+}\)]\(_i\), nor were Ca\(^{2+}\) oscillations observed in paired-untreated controls (0% ± 0% vs. 0% ± 0%, respectively; Fig 3.11A and quantified in 3.11C). BAECs, on the other hand, exhibited a significant increase in the number of cells oscillating in
response to 100 µM H₂O₂ when compared to paired-untreated controls (10.3% ± 0.8% vs. 1.2% ± 0.7%, respectively; Fig 3.11B and quantified in 3.11C).

3.3.5: H₂O₂ increases the sensitivity of HAECs to HIST- and TG- stimulated changes in [Ca²⁺]ᵢ. To determine if H₂O₂-induced Ca²⁺ oscillations reflect an increased sensitivity of the IP₃R, the effect of H₂O₂ on HIST- and TG-mediated changes in [Ca²⁺]ᵢ was examined in HAECs. Similar to the effects seen with diamide, brief pretreatment of HAECs with 100 µM H₂O₂ significantly increased the number of cells oscillating in response to a sub-maximal concentration of HIST, and altogether resulted in a 2- to 3-fold shift in the HIST dose-response relationship when compared to paired-untreated controls (Fig 3.12). Again, analogous to the effect of diamide, HAECs pretreated with H₂O₂ exhibited an enhanced response to TG, indicative of IP₃R activation by CICR, when compared to paired-untreated controls (Fig 3.13). Collectively, these results demonstrate that H₂O₂, like diamide, can increase the sensitivity of the IP₃R to activation by IICR and CICR.

3.3.6: Oxidative stress promotes glutathionylation of the IP₃R₁ in vivo. Despite substantial evidence that thiol-oxidizing agents directly modify the IP₃R, the molecular nature of the modification/s responsible for changes in IP₃R activity are still not well understood. The type 1 IP₃R (IP₃R₁) is expressed in vascular endothelial cells (Grayson et al., 2004) and we previously reported that glutathionylation of native type 1 IP₃R (IP₃R₁) is increased in BAECs treated with diamide (Lock et al., 2011). To determine if glutathionylation occurs in response to a physiological oxidant, the effect of H₂O₂ on glutathionylation of native IP₃R₁ was investigated in BAECs loaded with biotin-GSH ethyl ester (BioGEE). BioGEE is a membrane-permeable form of biotin-labeled GSH
that is trapped within the cell by the action of cellular esterases. Following 10 min treatment with H$_2$O$_2$, glutathionylated proteins from BioGEE-loaded cells were captured using streptavidin-agarose beads and probed for IP$_3$R$_1$ by Western Blot as described in Material and Methods. As seen in Fig 3.14, IP$_3$R$_1$ glutathionylation was increased in response to H$_2$O$_2$. Consistent with thiol modification, oxidant-induced glutathionylation of the IP$_3$R was reversed by addition of excess DTT prior to avidin-pull-down. These results suggest that glutathionylation of IP$_3$R$_1$ maybe a common response to oxidative stress.
3.4: DISCUSSION

In the present study, we utilized the thiol-oxidant diamide to investigate the consequences of protein S-glutathionylation on IP$_3$R function in intact cultured aortic ECs. Diamide reacts with GSH and promotes P-SSG formation in a well-established two-step reaction (Kosower et al., 1969; Kosower & Kosower, 1995). Intracellularly, diamide rapidly and preferentially reacts with GSH producing a diamide-GS intermediate which can either react with another molecule of GSH (producing GSSG) or react with a protein thiol (P-SH) giving rise to P-SSG. Protein de-glutathionylation is achieved primarily through the enzymatic action of glutaredoxin which uses GSH to produce P-SH and GSSG (Shelton et al., 2005). Reduced GSH is regenerated from GSSG by glutathione reductase at the expense of NADPH. Since the concentration of GSH in the cytosol is generally 1-10 mM (Meister & Anderson, 1983), low concentrations of diamide (e.g. 20-100 µM) are thought to shift the steady-state equilibrium in favor of P-SSG. Previously we found that diamide produced asynchronous Ca$^{2+}$ oscillations in BAECs (Lock et al., 2011). This effect of diamide was prevented by inhibition of PLC or by blockade of the IP$_3$R, but diamide did not increase hydrolysis of PIP$_2$. Together these results suggested that diamide sensitizes the IP$_3$R to basal levels of IP$_3$. However, the extent to which this reflects enhanced sensitivity to IP$_3$ or to cytosolic Ca$^{2+}$ is difficult to distinguish at the cellular level. Since IP$_3$ is not changing during challenge with diamide, an increase in Ca$^{2+}$ oscillations likely reflects an increased sensitivity of the IP$_3$R to Ca$^{2+}$, i.e. enhanced CICR. The results of the present study demonstrate that CICR is indeed enhanced following diamide treatment. Furthermore, we also found that exogenous application of H$_2$O$_2$ produced similar changes in IP$_3$R activity and, like
diamide, H$_2$O$_2$ increased glutathionylation of native IP$_3$R$_1$ in BAECs. Although an increased sensitivity of the IP$_3$R to IP$_3$ in response to oxidative stress has been reported previously (Rooney et al., 1991; Bird et al., 1993), to our knowledge this is the first demonstration that thiol-oxidizing agents enhance the sensitivity of the IP$_3$R to CICR. This may reflect a direct modification of the IP$_3$R by glutathionylation.

The mobilization of intracellular Ca$^{2+}$ stores in response to thiol-oxidizing agents has been reported in a wide range of non-excitable cell types including hepatocytes (Rooney et al., 1991; Bird et al., 1993), Hela cells (Bootman et al., 1992), hamster eggs (Miyazaki et al., 1992), pancreatic acinar cells (Klonowski-Stumpe et al., 1997), platelets (vanGorp et al., 1997), and aortic ECs (Hu et al., 1998). Although H$_2$O$_2$ was shown to stimulate single-cell Ca$^{2+}$ oscillations in cultured HAECs in a prior study, in our hands neither H$_2$O$_2$, nor diamide evoked an oscillatory response in HAECs under basal conditions. In contrast, both diamide and H$_2$O$_2$ were effective in eliciting Ca$^{2+}$ oscillations in BAECs. These results are reminiscent of a study by Bird et al, in which they observed Ca$^{2+}$ oscillations in response to t-BOOH in primary rat hepatocytes, but not in hepatocytes isolated from guinea pig (Bird et al., 1993). In their study, if the intracellular IP$_3$ was elevated by a low concentration of receptor agonist or by microinjection of IP$_3$, t-BOOH-induced Ca$^{2+}$ oscillations were then observed in guinea pig hepatocytes. Similarly, we found that brief (5 min) pretreatment of either HAECs or BAECs with diamide, at a concentration which did not stimulate Ca$^{2+}$ oscillations, significantly increased the number of cells oscillating in response to either HIST or BK, respectively. Moreover, H$_2$O$_2$ pretreatment increased the sensitivity of HAECs to HIST-induced oscillations. Since both HIST and BK initiate Ca$^{2+}$ oscillations by activating
PLC and elevating IP₃, both diamide and H₂O₂ may decrease the threshold for activation of the IP₃R by IP₃. However, since a local rise in [Ca²⁺]ᵢ in response to IP₃ binding can stimulate Ca²⁺ release through a feed-forward process involving CICR, the increased IP₃R activity during an oxidative challenge could also reflect changes in the Ca²⁺ sensitivity of the receptor.

It is well established that IP₃R activity is regulated by cytosolic Ca²⁺ (Taylor, 1998). In both single channel studies (Bezprozvanny et al., 1991; Tu et al., 2005a; Ionescu et al., 2006) and permeabilized cell models (Iino, 1990; Marshall & Taylor, 1993; Bootman et al., 1995; Marchant & Taylor, 1997) low concentrations of cytosolic Ca²⁺ are required for IP₃-mediated activation of the IP₃R, and have been shown to stimulate IP₃R activity at a fixed concentration of IP₃. Moreover, increasing [Ca²⁺]ᵢ has been shown to augment IP₃ binding to the IP₃R (Hilly et al., 1993; Cardy et al., 1997). However, despite a clear role for Ca²⁺ in the regulation of IP₃R activity, the effect of thiol-oxidants on the Ca²⁺ sensitivity of the IP₃R has not been investigated. We took advantage of the SERCA inhibitor TG to address whether changes in [Ca²⁺]ᵢ can influence IP₃R activity during an oxidative challenge in intact cells. TG-mediated inhibition of SERCA leads to a transient rise in [Ca²⁺]ᵢ due to passive leak of Ca²⁺ from the ER (Lytton et al., 1991). Our results reveal that both diamide and H₂O₂ increased the sensitivity of the IP₃R to a rise in [Ca²⁺]ᵢ induced by TG. This increased sensitivity toward cytosolic Ca²⁺ resulted in a rapid Ca²⁺ transient when cells were challenged with TG suggestive of CICR via the IP₃R. Moreover, regenerative Ca²⁺ oscillations, a well-established characteristic of IP₃-sensitive stores, were observed in response to near threshold concentrations of TG in diamide-treated cells. Concordant with a role for the
IP$_3$R in the Ca$^{2+}$ response to TG, low concentrations of HIST or BK produced a similar shift in the Ca$^{2+}$ profile following TG exposure. A role for CICR via the IP$_3$R in shaping the kinetics of the Ca$^{2+}$ transient induced by TG is consistent with the observations that TG-mediated changes [Ca$^{2+}$]$_i$ are dependent upon the basal level of IP$_3$ (Smith & Gallacher, 1994), and can be attenuated by the IP$_3$R blocker 2-APB (Luo et al., 2001). Taken together, our results suggest thiol-oxidizing agents increase the sensitivity of the IP$_3$R to activation by cytosolic Ca$^{2+}$. Additionally, our results demonstrate that the stimulatory effects of both diamide and H$_2$O$_2$ on IP$_3$R activation are downstream of receptor stimulation and are independent of PLC activity and IP$_3$ formation.

Oxidant-induced changes in IP$_3$R activity are thought to reflect a direct thiol modification of the receptor/channel. Functional IP$_3$Rs contain over 200 Cys residues (~60 Cys per monomer), and although a number of Cys have been identified as potential sites of redox modification (Joseph et al., 2006; Kang et al., 2008), a role for glutathionylation has not been defined. Our results demonstrate that, in addition to diamide (Lock et al., 2011), glutathionylation of native IP$_3$R$_1$ in cultured aortic ECs occurs in response to H$_2$O$_2$. The increase in IP$_3$R glutathionylation in response to thiol-oxidizing agents maybe indirect, and we cannot unequivocally exclude the possibility that glutathionylation of a tightly bound accessory/regulatory protein is responsible for the increase in IP$_3$R$_1$ detected by Western Blot following avidin pull-down. However, given the evidence that thiol-oxidizing agents can directly modify the IP$_3$R (Kaplin et al., 1994; Thrower et al., 1996; Poirier et al., 2001), a direct modification by glutathionylation seems plausible. In preliminary studies using BAECs permeabilized with saponin, we found that IP$_3$R$_1$ is not glutathionylated in its cytoplasmic domain (unpublished
observation) whereas robust glutathionylation of IP$_3$R$_1$ was observed in isolated BAEC microsomes under similar conditions (Lock et al., 2011). This would suggest that the IP$_3$R$_1$ is glutathionylated in an ER luminal domain. The IP$_3$R monomer has 6 membrane spanning segments; the NH$_2$- and COOH-termini are cytoplasmic and there are three luminal loops, L1-L3. L3 has four Cys residues that are conserved in type-1, -2, and -3 IP$_3$R. Previous studies by Mikoshiba’s group showed that the ER resident protein, ERp44, binds tightly to the type-1 receptor, but not to type-2 or type-3 (Higo et al., 2005). Binding of ERp44 to the IP$_3$R is sensitive to redox potential and is favored under reducing conditions and inhibited under oxidizing conditions. Binding of ERp44 to the IP$_3$R requires reduced Cys residues, and binding inhibits channel activity. Importantly, oxidized conditions will also favor protein S-glutathionylation. Thus, glutathionylation of one or more of these Cys residues may block interaction of ERp44 with IP$_3$R and hence prevent inhibition of the channel activity by ERp44. Further studies will be necessary to determine the role of these residues in IP$_3$R glutathionylation and the subsequent effect on IP$_3$R function.
3.5: FIGURES

3.5.1: Figure 3.1

A. HAECs in zero Ca\textsuperscript{2+}-ECS

![Graph showing time course of HAECs in zero Ca\textsuperscript{2+}-ECS with 100 \mu M diamide.]

B. BAECs in zero Ca\textsuperscript{2+}-ECS

![Graph showing time course of BAECs in zero Ca\textsuperscript{2+}-ECS with 100 \mu M diamide.]

C. Cells Oscillating (% of total)

![Bar graph showing percentage of cells oscillating in HAECs and BAECs with and without 100 \mu M diamide.]

* N.S.
Figure 3.1: *Effect of diamide on [Ca\(^{2+}\)]_i in HAECs and BAECs.*

Fluorescence ratio (340/380) was recorded from single fura-2-loaded HAECs (A) and BAECs (B) bathed in zero Ca\(^{2+}\)-ECS during treatment with 100 µM diamide, as indicated at the top of each panel. In this and all subsequent figures, a representative experiment (i.e., a single coverslip) is shown with individual cells depicted as different gray scale traces. C) The number of cells oscillating in response to diamide was quantified for each individual experiment as the percent of the total cells monitored exhibiting at least one oscillation in [Ca\(^{2+}\)]_i during the 10 min treatment period. A total of 118 (untreated) and 133 (100 µM diamide) HAECs, and 231 (untreated) and 202 (100 µM diamide) BAECs were analyzed per experimental condition. Values represent the mean ± SE of 3-4 experiments (coverslips) for each experimental condition. (*) P < 0.05 compared to paired-untreated controls.
3.5.2: Figure 3.2

A.

zero Ca\textsuperscript{2+}-ECS

\[ \text{30 nM HIST} \]

340/380

Time (min)

0 2 4 6 8 10 12

B.

100 \( \mu \text{M diamide in zero Ca}^{2+}-\text{ECS} \)

\[ \text{30 nM HIST} \]

340/380

Time (min)

0 2 4 6 8 10 12

C.

Cells Oscillating (% of total)

[0 0.001 0.01 0.1 1 10]

[HIST] (\( \mu \text{M} \))
Figure 3.2: *Diamide sensitizes HAECs to histamine-stimulated Ca^{2+} oscillations.*

Fura-2-loaded HAECs in zero Ca^{2+}-ECS were left untreated (A) or treated for 5 min with 100 µM diamide (B) prior to challenging with 30 nM histamine (HIST), as indicated by the bars at the top of each panel. (C) HAECs were left untreated (solid circles) or treated with 100 µM diamide (open circles) for 5 min prior to stimulation with the indicated [HIST] for an additional 5 min in the continued absence or presence of 100 µM diamide. The number of cells oscillating was quantified as the percent of the total cells monitored exhibiting at least one oscillation in [Ca^{2+}]_i during the 5 min exposure to HIST. Values represent the mean ± se of 3-5 experiments for each experimental condition. In untreated controls, and total of 156, 129, 153, 177, 218, 165, 137, and 115 cells were analyzed for each [HIST] (0, 0.001, 0.01, 0.03, 0.1, 0.3, 1.0, and 10 µM, respectively), whereas 155, 133, 165, 172, 206, 143, 124, and 109 cells were analyzed for each [HIST] in diamide-treated monolayers.
3.5.3: Figure 3.3

A. No pretreatment (CTL)

B. Xestospong in C (XeC)

C. Ryanodine (Ryn)

D. Cells Oscillating (% of total)

Graphs showing the effects of different treatments on cell oscillations.
Figure 3.3: Effects of diamide are attenuated by xestospongin C but not ryanodine.

Fura-2-loaded HAECs were left untreated (A), or pretreated with either 10 µM Xestospongin C (XeC) for 30 min (B), or 20 µM ryanodine (Ryn) for 10 min (C) immediately prior to sequential exposure, in zero Ca$^{2+}$-ECs, to 70 nM HIST for 5 min followed by 100 µM diamide for an additional 5 min in the continued presence of HIST, as indicated by the bars at the top of each panel. All recordings in panels B and C were made in the continued presence of the indicated inhibitor. D) The number of cells oscillating in response to diamide was quantified as described in the legend of Fig 3.1. A total of 139 (untreated), 132 (XeC pretreated), and 118 (Ryn pretreated) cells were analyzed per experimental condition. Values represents the mean ± SE of 3 experiments for each condition. (*) P < 0.01 compared to paired-untreated controls.
3.5.4: Figure 3.4

A. zero Ca\textsuperscript{2+}-ECS

B. 20 μM diamide in zero Ca\textsuperscript{2+}-ECS

C. Cells Oscillating (% of total) vs. [BK] (nM)
Figure 3.4: Diamide sensitizes BAECs to bradykinin-stimulated Ca$^{2+}$ oscillations.

Fura-2-loaded BAECs in zero Ca$^{2+}$-ECS were left untreated (A) or treated for 5 min with 20 µM diamide (B) prior to challenging with 100 pM bradykinin (BK), as indicated by the bars at the top of each panel. C) BAECs were left untreated (solid circles) or treated with 20 µM diamide (open circles) for 5 min prior to stimulation with the indicated [BK] for an additional 5 min in the continued absence or presence of diamide. Values represents the mean ± SE of 3-8 experiments for each experimental condition. In untreated controls, a total of 401, 371, 530, 468, 345, 193, and 196 cells were analyzed for each [BK] (0, 0.03, 0.07, 0.1, 0.3, 1.0, and 10 nM, respectively), whereas 340, 329, 436, 471, 316, 225, 180, cells were analyzed for each [BK] in diamide-treated monolayers.
3.5.5: Figure 3.5

A. zero Ca^{2+}\text{-ECS}

B. 100 \mu M diamide in zero Ca^{2+}\text{-ECS}

C. 30 nM HIST in zero Ca^{2+}\text{-ECS}

D. Cumulative Frequency (%)

E. Cumulative Frequency (%)

Latency To Peak (sec)
Figure 3.5: Diamide enhances thapsigargin-mediated changes in $[Ca^{2+}]_i$ of HAECs.

Fura-2-loaded HAECs in zero $Ca^{2+}$-ECS were left untreated (A), or treated with either 100 µM diamide (B) or 30 nM HIST (C) for 5 min immediately prior to challenging with 300 nM thapsigargin (TG). A-C) Cells were segregated into two phenotypic profiles based on both the rate and magnitude of the TG-mediated change in $[Ca^{2+}]_i$. Cells represented as *black lines* displayed a slow rate of rise and a small peak change in $[Ca^{2+}]_i$, whereas cells represented as gray scale lines exhibited an enhanced response characterized by a rapid rate of rise and a large change in $[Ca^{2+}]_i$. D) Cumulative frequency of the maximal change in fura-2 fluorescence (*Peak Ratio*) observed in response to TG in control (*solid circles*), diamide (*open squares*) and HIST (*shaded triangles*) treated cells. E) Cumulative frequency of the time from TG exposure to the maximal change in fura-2 fluorescence (*Latency to Peak*) for control (*solid circles*), diamide (*open squares*), and HIST (*shaded triangles*) treated cells. A total of 121 (control), 135 (diamide), and 149 (HIST) cells were analyzed from 4 experiments for each experimental condition. D, E) The TG-induced *Peak Ratio* and *Latency to Peak* for both diamide- and HIST-treated cells were significantly different from untreated controls; $P < 0.001$. 

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3.5.6: Figure 3.6

A. zero Ca\textsuperscript{2+}-ECS

B. 20 μM diamide in zero Ca\textsuperscript{2+}-ECS

C. 100 pM BK in zero Ca\textsuperscript{2+}-ECS

D. Cumulative Frequency (%)

E. Cumulative Frequency (%)

- CTL
- 20 μM diamide
- 100 pM BK
Figure 3.6: Diamide enhances TG-mediated changes in [Ca$^{2+}$]$_i$ of BAECs.

Fura-2-loaded BAECs in zero Ca$^{2+}$-ECS were left untreated (A), or treated with either 20 µM diamide (B) or 100 pM BK (C) for 5 min immediately prior to challenging with 300 nM TG. A-C) Individual cell traces are segregated into two phenotypic profiles as described in the legend of Fig 5. Inset in (A) shows a single-cell trace exhibiting a delayed CICR response to TG that was sometimes observed. D) Cumulative frequency of the Peak Ratio (D) and the Latency to Peak (E) observed in response to TG in control (solid circles), diamide- (open squares) and BK-treated (shaded triangles) cells. A total of 294 (control), 281 (diamide) and 276 (BK) cells were analyzed from 4 experiments for each experimental condition. D, E) The TG-induced Peak Ratio and Latency to Peak of both diamide- and BK-treated cells were significantly different from untreated controls; P < 0.001.
3.5.7: Figure 3.7

A. HAECs
   10 μM HIST in zero Ca^{2+}-ECS
   5 min treatment
   • CTL
   • 100 μM diamide

B. BAECs
   10 nM BK in zero Ca^{2+}-ECS
   5 min treatment
   • CTL
   • 20 μM diamide

C. HAECs
   1 μM ionomycin in zero Ca^{2+}-ECS
   5 min treatment
   • CTL
   • 100 μM diamide

D. BAECs
   1 μM ionomycin in zero Ca^{2+}-ECS
   5 min treatment
   • CTL
   • 20 μM diamide
Figure 3.7: Diamide has no effect on the ionomycin- or maximal agonist-releasable Ca$^{2+}$ stores in HAECs and BAECs.

Fura-2-loaded HAECs (A, C) and BAECs (B, D) bathed in zero Ca$^{2+}$-ECS were challenged with 100 µM diamide (HAECs) or 20 µM diamide (BAECs) for 5 min immediately prior to stimulation with a maximal conc. of receptor agonist (A, B) or 1 µM ionomycin (C, D) for an additional 5 min in the continued presence of diamide. A-D) For clarity 4 minutes of the treatment period is omitted by the breaks. Values represent the mean ± SE of 3 experiments for each experimental condition. Where error bars are absent the SE is smaller than the symbol. A, B) The total number of cells analyzed for each condition is given in the legends of Figs. 3.2 and 3.4. C, D) A total of 103 (untreated) and 104 (diamide) HAECs, and 228 (untreated) and 254 (diamide) BAECs were analyzed for each condition.
3.5.8: Figure 3.8

A. Cumulative Frequency (%) vs. Peak Ratio (340/380)

B. Cumulative Frequency (%) vs. Latency to Peak (sec)

Legend:
- None - CTL
- None - diamide
- XeC - diamide
- Ryn - diamide
Figure 3.8: Effect of xestospongin C and ryanodine on the diamide-enhanced TG response in HAECs.

Cumulative frequency analysis of the Peak Ratio (A) and Latency to Peak (B) in response to TG of fura-2-loaded HAECs in zero Ca\(^{2+}\)-ECS. The experimental protocol was the same as in Fig 3.5, except cells were left untreated (open squares) or pretreated with either 10 µM xestospongin C (XeC) for 30 min (light shaded triangles) or 20 µM ryanodine (Ryn) for 10 min (dark shaded diamonds) immediately prior to sequential challenge with 100 µM diamide for 5 min followed by 300 nM TG for an additional 5 min in the continued presence of diamide. Where indicated all recordings were made in the continued presence of the inhibitor. Control cells (solid circles) were left untreated and were not challenged with diamide prior to TG exposure. A total of 147 (control), 168 (diamide), 114 (XeC pretreated), 105 (Ryn pretreated) cells were analyzed from 3-4 experiments for each experimental condition. A, B) The Peak Ratio and Latency to Peak of untreated cells or cells pretreated with XeC or Ryn prior to sequential challenge with diamide and TG were significantly different from untreated controls challenged with only TG; P < 0.001. The Peak Ratio of XeC pretreated cells was significantly different from untreated cells sequentially challenged with diamide and TG; P < 0.001.
3.5.9: Figure 3.9

A. Cumulative Frequency (%) vs. Peak Ratio (340/380)

B. Cumulative Frequency (%) vs. Latency to Peak (sec)

Legend:
- Pretreatment
- Vehicle
- Baflomycin
- CTL*
**Figure 3.9:** Bafilomycin had no effect on the diamide-enhanced TG response in HAECs.

Cumulative frequency of the *Peak Ratio* (A) and the *Latency to Peak* (B) in response to TG of fura-2-loaded HAECs in zero Ca\(^{2+}\)-ECS. Experimental protocol was the same as in Fig 3.5, except HAECs were pretreated with vehicle (*open square*) or 100 nM bafilomycin for 1 hr (*shaded circles*) immediately prior to sequential challenge with 100 µM diamide for 5 min followed by 300 nM TG for an additional 5 min in the continued presence of diamide. **A, B** For reference, the dotted lines illustrate the cumulative frequency analysis of the TG response in controls cells (data set from Fig 5). A total of 185 (vehicle), and 122 (bafilomycin) cells were analyzed from 3-5 experiments for each condition. The diamide-enhanced *Peak Ratio* and *Latency to Peak* in response to TG of bafilomycin-treated cells was not significantly different from vehicle-treated controls.
Figure 3.10

A. zero Ca\(^{2+}\)-ECS
3 nM TG

B. 100 μM diamide in zero Ca\(^{2+}\)-ECS
3 nM TG

C. 100 μM diamide in zero Ca\(^{2+}\)-ECS
3 nM TG

D. Cells Oscillating (% of total)

0 20 40 60 80

CTL Diamide

*
Figure 3.10: *Diamide stimulates Ca\(^{2+}\) oscillations in response to 3 nM TG in HAECs.*

Fura-2 loaded HAECs in zero Ca\(^{2+}\)-ECS were left untreated (A) or treated for 5 min with 100 µM diamide (B) prior to stimulation with 3 nM TG in the continued absence or presence of diamide, as indicated by the bars at the top of each panel. **C** A representative single-cell trace demonstrating multiple Ca\(^{2+}\) oscillations in response to 3 nM TG in the presence of diamide. **D** The number of cells oscillating in response to TG was quantified for each individual experiment as the percent of the total cells monitored exhibiting at least one oscillation in [Ca\(^{2+}\)], during the 13 min treatment period. A total of 101 (control) and 112 (diamide) cells were analyzed for each experimental condition. Values represents the mean ± SE of 3 experiments per condition. (*) P < 0.001 compared to paired controls.
3.5.11: Figure 3.11

A. HAECs in zero Ca\(^{2+}\)-ECS

B. BAECs in zero Ca\(^{2+}\)-ECS

C. Cells Oscillating (% of total)

- No treatment
- 100 μM H\(_2\)O\(_2\)

HAECs | BAECs
--- | ---
N.O. | *
**Figure 3.11:** Effect of H$_2$O$_2$ on [Ca$^{2+}$]$_i$ in HAECs and BAECs.

Fura-2 fluorescence ratio was recorded from single HAECs (A) and BAECs (B) in zero Ca$^{2+}$-ECS during treatment with 100 μM H$_2$O$_2$, as indicated at the top of each panel. C) The number of cells oscillating in response to H$_2$O$_2$ was quantified as described in the legend of Fig 3.1. A total of 111 (untreated) and 151 (H$_2$O$_2$) HAECs, and 217 (untreated) and 248 (H$_2$O$_2$) BAECs were analyzed. Values represent the mean ± SE of 3-4 experiments for each experimental condition. (*) P < 0.001 compared to paired-untreated controls (paired Student’s t-test); Not observed (N.O.).
3.5.12: Figure 3.12

A.

zero Ca\textsuperscript{2+}-ECS

30 nM HIST

B.

100 \mu M \text{H}_2\text{O}_2\text{ in zero Ca}\textsuperscript{2+}-ECS

30 nM HIST

C.

Cells oscillating [% of total]

\text{[HIST]} (\mu M)

0 0.001 0.01 0.1 1 10
Figure 3.12: $H_2O_2$ sensitizes HAECs to histamine-stimulated $Ca^{2+}$ oscillations.

Fura-2-loaded HAECs in zero $Ca^{2+}$-ECS were left untreated (A) or treated for 5 minutes with 100 µM $H_2O_2$ (B) prior to challenging with 30 nM histamine (HIST) for an additional 5 min, as indicated by the bars at the top of each panel. C) HAECs were left untreated (solid circles) or treated with 100 µM $H_2O_2$ (open circles) for 5 min prior to stimulation with the indicated [HIST] for an additional 5 min in the continued absence or presence of $H_2O_2$. The number of cells oscillating in response to HIST stimulation was quantified as described in the legend of Fig 3.2. Values represent the mean ± SE of 3-6 experiments for each experimental condition. In untreated controls, a total of 117, 157, 155, 133, 208, 225, 94, and 104 cells were analyzed for each [HIST] (0, 0.001, 0.01, 0.03, 0.1, 0.3, 1.0, and 10 µM, respectively), whereas 116, 95, 141, 135, 225, 171, 97, and 104 cells were analyzed for each [HIST] in $H_2O_2$-treated monolayers.
3.5.13: Figure 3.13

A. zero Ca\(^{2+}\)-ECS

B. 100 \(\mu\)M \(H_2O_2\) in zero Ca\(^{2+}\)-ECS

C. Cumulative Frequency (%)

D. Cumulative Frequency (%)
Figure 3.13: $H_2O_2$ enhanced TG-mediated changes in $[Ca^{2+}]_i$ of HAECs.

Fura-2-loaded HAECs in zero Ca$^{2+}$-ECS were left untreated (A), or treated with 100 µM $H_2O_2$ (B) for 5 min immediately prior to challenging with 300 nM TG. A, B) Individual cell traces are segregated into two phenotypic profiles as described in the legend of Fig 3.5. C, D) Cumulative frequency analysis of the Peak Ratio (C) and the Latency to Peak (D) in response to TG in control (solid circles) and $H_2O_2$ (open squares) treated cells. A total of 118 (control) and 168 ($H_2O_2$) cells were analyzed from 3-4 experiments for each experimental condition. C, D) The TG-induced Peak Ratio and Latency to Peak of $H_2O_2$-treated cells was significantly different from untreated controls; $P < 0.001$. 


3.5.14: Figure 3.14
**Figure 3.14:** $H_2O_2$ increases glutathionylation of native $IP_3R_1$ in vivo.

BioGEE-loaded BAECs were suspended in $Ca^{2+}$-ECS and cells were divided equally into aliquots as indicated above each lane. Cells were left untreated (control) or incubated for 10 min with 0.1 mM or 1.0 mM $H_2O_2$. Following treatment, the membrane lysates from one sample of each of the indicated conditions tested was incubated with 20 mM DTT prior to pull-down. Biotinylated proteins were extracted from cleared lysates with avidin-agarose beads and probed for the $IP_3R_1$ by Western Blot. Aliquots of cell lysates prior to pull-down are shown as *input* controls. Result shown is representative of 3 independent experiments.
### 3.6: TABLE 3.1

A) HAECs

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B) BAECs

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Table 3.1: Characteristics of HIST- and BK- stimulated Ca\(^{2+}\) oscillations in control and diamide pretreated HAECs (A) and BAECs (B), respectively. For every cell oscillating the fluorescence ratio of fura-2 (340/380) at the peak of the Ca\(^{2+}\) oscillation (Amplitude) and the length of time from receptor stimulation to the peak change in [Ca\(^{2+}\)]\(_i\) (Latency) was recorded. A) The number of cells listed is the total HAECs oscillating in response to the indicated concentration of HIST. The total number of HAECs monitored at each HIST concentration tested, in both the absence and presence of diamide, is listed in the legend of Fig 3.2. B) The number cells listed is the total BAECs oscillating in response to the indicated concentration of BK. The total number of BAECs monitored at each BK concentration tested, in both the absence and presence of diamide, is listed in the legend of Fig 3.4. A-B) Values represent MEAN ± SD. Not applicable (NA).
CHAPTER 4

Discussion & Future Directions
4.1: SUMMARY

The major finding of this Dissertation is that oxidative stress increases the sensitivity of the IP$_3$R to activation by cytosolic Ca$^{2+}$, i.e. enhanced CICR. Since IP$_3$Rs are primarily regulated by IP$_3$ and Ca$^{2+}$, enhancing the Ca$^{2+}$ sensitivity of the IP$_3$R by the conversion to a more oxidizing cellular environment may provide a novel way for regulating IP$_3$R activity independent of a rise in IP$_3$. Importantly, enhanced CICR correlated with a reduction in cellular GSH, and an increase in P-SSG formation, suggesting glutathionylation, possibly of the IP$_3$R itself, is the underlying mechanism governing this response. Changes in cellular thiol-disulfide balance, in particular the GSH/GSSG ratio, may therefore provide a common way for regulating IP$_3$R activity during physiological redox signaling processes and during the pathological response to oxidative stress. Further studies are necessary to fully elucidate role of protein S-glutathionylation on IP$_3$R function and its consequence on cellular behavior.

4.2: EFFECT OF OXIDATIVE STRESS ON Ca$^{2+}$ HOMEOSTASIS & SIGNALING

The disruption of cellular Ca$^{2+}$ homeostasis and Ca$^{2+}$ signaling mechanisms is a hallmark of oxidative stress. Oxidative stress is known to affect many of the major ion channels, transporters, and accessory factors essential to the orchestration of intracellular Ca$^{2+}$ dynamics (Kourie, 1998). In non-excitable cells, often the first detectable signs of oxidative stress on intracellular Ca$^{2+}$ dynamics is a rise in basal [Ca$^{2+}$], and the emergence of global oscillations in [Ca$^{2+}$]. As discussed in Chapter 1 section 1.1,
cellular Ca$^{2+}$ homeostasis is a balance between the mechanisms regulating the efflux of Ca$^{2+}$ out of the cytosol and the influx of Ca$^{2+}$ into the cytosol. Thus, oxidative stress can produce a rise in [Ca$^{2+}$], by either inhibiting pathways of Ca$^{2+}$ efflux or activating pathways of Ca$^{2+}$ influx. Both SERCA and PMCA pumps, the primary pathways of Ca$^{2+}$ efflux, are inhibited by oxidative stress (Bellomo et al., 1983; Scherer & Deamer, 1986; Viner et al., 1999; Zaidi & Michaelis, 1999; Zaidi et al., 2009), and a reduction in the pumping activity of either one will lead to a rise in [Ca$^{2+}$]. However, inhibition of SERCA will only cause a sustained rise in [Ca$^{2+}$], when coupled with Ca$^{2+}$ influx into the cytosol by SOCE. This is because the inability to replenish ER Ca$^{2+}$ stores constitutively activates Ca$^{2+}$ influx, which eventually overwhelms the capacity of PMCA pumps to remove cytosolic Ca$^{2+}$. In the absence of Ca$^{2+}$ influx, inhibition of SERCA only leads to a transient rise in [Ca$^{2+}$], as elevated Ca$^{2+}$ is rapidly removed from the cytosol by PMCA pumps. In contrast, inhibition of PMCA pumps will lead to a sustained rise in [Ca$^{2+}$], in both the presence and absence of Ca$^{2+}$ influx mechanisms, since PMCA pumps represent the last possible route of Ca$^{2+}$ exit from the cytosol. Thus, in the absence of Ca$^{2+}$ influx, a sustained rise in basal [Ca$^{2+}$] can only be explained by inhibition of Ca$^{2+}$ clearance from the cell via PMCA pumps.

The influx of Ca$^{2+}$ into the cytosol following the activation of Ca$^{2+}$-permeable ion channels can also lead to a rise in [Ca$^{2+}$]. In this regard, oxidative stress has been shown to activate SOCE in the absence of a reduction in ER [Ca$^{2+}$] (Hawkins et al., 2010), and to stimulate Ca$^{2+}$ influx through other redox sensitive channels in the plasma membrane including members of the canonical and melastatin families of transient receptor potential (TRP) channels (Bogeski et al., 2011). Alternatively, oxidative stress can cause a rise in
[Ca\(^{2+}\),] by stimulating the release of Ca\(^{2+}\) from intracellular organelles. Potential release pathways include: IP\(_3\)Rs (see below) and Ryn receptors located in the ER/SR; the translocon complex in the ER/SR following disruption of protein synthesis; NAADP-sensitive two pore channels (TPC) found in endolysosomes; and the permeability transition pore formed in the inner mitochondrial membrane during the transition to cellular death pathways. Oxidative stress can also produce a rise in [Ca\(^{2+}\),] by reducing the Ca\(^{2+}\) buffering capacity of the cytosol. These, and many other mechanisms are potentially capable of elevating basal [Ca\(^{2+}\),] during oxidative stress. However, in non-excitable cells such as vascular ECs, global oscillations in [Ca\(^{2+}\),] are almost exclusively driven by the release of Ca\(^{2+}\) from internal stores via IP\(_3\)Rs.

4.3: EFFECT OF OXIDATIVE STRESS ON IP\(_3\)R FUNCTION

As discussed previously, IP\(_3\)Rs are primarily regulated by IP\(_3\) and Ca\(^{2+}\), and the integrated action of these molecules on the receptor/channel are intimately involved in the initiation, propagation, and termination of global oscillations in [Ca\(^{2+}\),]. During oxidative stress the prevailing theory is that Ca\(^{2+}\) oscillations are due to an increased affinity of the IP\(_3\)R for IP\(_3\). In contrast, our present results argue that the primary basis for elevated IP\(_3\)R activity during oxidative stress is actually due to an enhanced sensitivity of the IP\(_3\)R to activation by cytosolic Ca\(^{2+}\).

The fundamental basis for how oxidative stress and Ca\(^{2+}\) regulates IP\(_3\)R activity can be understood in a manner analogous to the mechanism by which IP\(_3\) and Ca\(^{2+}\) are thought to regulate the IP\(_3\)R. Typically IP\(_3\) binds to and activates the IP\(_3\)R in a graded
fashion, and as more IP₃Rs are activated, the release of Ca²⁺ from individual channels activates adjacent channels and clusters of channels by CICR. Thus, IP₃ essentially increases the susceptibility of the IP₃R to activation by cytosolic Ca²⁺. In this regard, it is thought that IP₃ binding produces a conformational change in the tertiary structure of the IP₃R which exposes stimulatory Ca²⁺ binding sites, and the binding of Ca²⁺ to these putative site/s is the actual stimulus responsible for channel activation (Marchant & Taylor, 1997). Similarly, we found oxidative stress exhibits a graded effect on IP₃R activity, and like IP₃, appears to primarily increase the sensitivity of IP₃Rs to activation by cytosolic Ca²⁺. However, this response occurred in the absence of increased PIP₂ hydrolysis, suggesting oxidant-induced changes in IP₃R activity are independent of a rise in IP₃. Instead, by elevating [Ca²⁺], using TG to inhibit SERCA, we found that IP₃R-mediated Ca²⁺ oscillations during oxidative stress were dependent upon the rate and magnitude of the rise in [Ca²⁺]. Low [TG], which produced a slow rate and small rise in [Ca²⁺], (presumably due to Ca²⁺ clearance via the PMCA) elicited Ca²⁺ oscillations after an initial latency period, suggesting that a critical threshold of [Ca²⁺] must be exceeded in order to trigger this “all-or-nothing” oscillatory response. Consistent with a “Ca²⁺ threshold” to activation, high [TG], which produced a faster and larger rise in [Ca²⁺], evoked a more rapid and robust oscillatory response in sensitized cells (i.e. diamide- or H₂O₂-treated cells). When taken together, these finding demonstrate cytosolic Ca²⁺ activates the IP₃R in a concentration-dependent manner during oxidative stress, and suggest Ca²⁺ is the trigger that initiates the oscillation, whereas oxidative stress primes the system by sensitizing the IP₃R. Ultimately, when a critical number of IP₃Rs are activated, or a critical threshold of cytosolic Ca²⁺ is reached, oxidative stress can evoke
Ca\(^{2+}\) oscillations by increasing the susceptibility of IP\(_3\)Rs to activation by CICR. Thus, any of the mechanisms described above, or any mechanism for that matter which produces a rise in basal [Ca\(^{2+}\)], could potentially activate IP\(_3\)R-mediated Ca\(^{2+}\) oscillations during increased oxidative stress.

Although our results indicate oxidative stress increases CICR via IP\(_3\)Rs in the absence of a rise in IP\(_3\), we cannot unequivocally exclude a role for IP\(_3\) in this response since most cells exhibit some basal level of IP\(_3\). The cellular concentration of IP\(_3\) is a consequence of both its rates of generation and degradation, thus it is possible that impaired IP\(_3\) degradation could elevate IP\(_3\) independent of phosphoinositide hydrolysis. However, it seems unlikely in our studies that either diamide or H\(_2\)O\(_2\) is impeding IP\(_3\) degradation since neither oxidant elicited Ca\(^{2+}\) oscillation in naïve HAECs. Instead, it appears that cells with a high level of basal IP\(_3\) are more likely to oscillate in response to oxidative stress (i.e. BAECs), whereas cells with low basal IP\(_3\) levels are more resistant to the induction of oscillations (i.e. HAECs). Further studies are necessary to confirm this hypothesis, and to gain a more complete understanding of the complex relationship between cellular redox status, IP\(_3\), and Ca\(^{2+}\) on IP\(_3\)R behavior.

### 4.4: THE ROLE OF GLUTATHIONYLATION ON IP\(_3\)R FUNCTION

Glutathionylation has emerged as a biologically important, redox-sensitive, post-translation protein modification. Our present results suggest that oxidant-induced changes in IP\(_3\)R function may be due to a direct modification of the receptor/channel by GSH. However, a large and growing list of proteins and molecules modulate IP\(_3\)R
activity, and it is possible that glutathionylation of regulatory protein/s or molecule/s is responsible for the changes in IP$_3$R activity. Essentially, glutathionylation could enhance the interaction or modification of the IP$_3$R with stimulatory proteins/molecules or reduce the interaction with or modification by inhibitory proteins/molecules. Although a number of indirect mechanisms by which glutathionylation could be modulating IP$_3$R activity can be envisioned, the simplest explanation is that a direct modification of the IP$_3$R by GSH is responsible for its enhanced activity during oxidative stress.

IP$_3$Rs are structurally and functionally similar to Ryn receptors, the predominant intracellular Ca$^{2+}$ release channel in excitable cells (i.e. striated muscle and neuronal cell types). It is well established that Ryn receptors are constitutively glutathionylated at a number of Cys residues \textit{in vivo} (Aracena-Parks \textit{et al.}, 2006), and \textit{in vitro} glutathionylation of the Ryn receptor increased following exposure to either H$_2$O$_2$ plus GSH or GSNO (Aracena \textit{et al.}, 2005). Moreover, increased glutathionylation correlated with increase in the single channel activity of Ryn receptors reconstituted into artificial lipid bilayers (Aracena \textit{et al.}, 2003; Bull \textit{et al.}, 2008). Likewise, we found that; IP$_3$R$_1$ exhibited endogenous glutathionylation \textit{in vivo}, chemical oxidants increased glutathionylation of the IP$_3$R$_1$ both \textit{in vivo} and \textit{in vitro}, and glutathionylation of IP$_3$R$_1$ correlated with enhanced activity during an oxidative challenge. However, in order to confirm that the direct modification of IP$_3$R$_1$ by glutathionylation is responsible for its enhanced activity, a site directed-mutagenesis approach converting Cys residues to alanine (Ala) residues before systematically reintroducing the Cys residues at their appropriate locations is required. The functional effect of glutathionylation on IP$_3$R$_1$ mutants can be studied in whole cells, permeabilized cells, and by electrophysiology,
whereas a biochemical approach similar to that described in the present *Dissertation* can be used to probe the susceptibility of IP$_3$R$_1$ mutants to be glutathionylated in response to oxidative insults both *in vivo* and *in vitro*. Importantly, these experiments must be performed using the genetically modified chicken B cell line (DT40) devoid of functional IP$_3$Rs in order to avoid confounding effects due to endogenous IP$_3$Rs.

The redox potential of both the cytosol and ER are important parameters to consider when designing experiments to investigate the functional effect of oxidative stress on IP$_3$R behavior. Since IP$_3$R are located intracellularly, the channel properties of the IP$_3$R can only be examined in broken cells or by the incorporation of IP$_3$Rs into artificial lipid bilayers. Although the mechanism/s remains unknown, changes in the redox potential of the cytosol can directly impact the redox potential of the ER, and permeabilization of the plasma membrane has been shown to increase oxidation of ER proteins (Molteni *et al.*, 2004). The vast majority of previous studies examining IP$_3$R channel properties did not account for changes in the redox environment following disruption of the plasma membrane. Thus, the IP$_3$R in these studies may have been at least partially oxidized before any measurements were made. In light of this, studies on IP$_3$R channel properties should be revisited in experiments where the redox potential of the cytosol and ER are tightly controlled to mimic the redox environment *in vivo*. To this end, we can obtain qualitative measures of the cytosolic and ER redox potentials of intact cells using heterologous expression of targeted fluorescent indicators, such as Ro-GFP (Dooley *et al.*, 2004) or HyPer (Belousov *et al.*, 2006), which have been designed to measure cellular redox. These measurements can be used as an index to ensure the redox status of the cytosol and ER reflect that found *in vivo* during future studies on IP$_3$R...
function. We can also use these probes to explore how and where ROS/RNS production occurs inside the cell. In this manner we can investigate changes in the redox environment of the cytosol and the ER during physiological signaling processes and during the pathological response to oxidative stress.

4.5: POTENTIAL ROLE OF IP$_3$R GLUTATHIONYLATION DURING PHYSIOLOGICAL SIGNALING

It is well recognized that the activation of cell surface receptors are coupled to the production of ROS/RNS (Thannickal & Fanburg, 2000), and in ECs, the generation of ROS/RNS has been observed in response to a number of receptor agonists including BK and HIST. BK is reported to increase the production of H$_2$O$_2$, (Larsen et al., 2009), O$_2^{•−}$ (Holland et al., 1990), NO, and, due to the reaction of O$_2^{•−}$ with NO, ONOO$^−$ (Kooy & Royall, 1994). More recently, studies in cultured HAECs have shown HIST stimulates NOX activity (Hu et al., 1999), and NOX activation enhances the release of Ca$^{2+}$ from IP$_3$-sensitive stores (Hu et al., 2000). In this regard, the production of ROS in response to HIST-stimulated Ca$^{2+}$ oscillations is essential to NF-kB mediated induction of vascular cell adhesion molecule 1 (VCAM1) gene expression (Zhu et al., 2008); a finding consistent with previous studies reporting NF-kB transcriptional activity is regulated by Ca$^{2+}$ oscillation frequency (Dolmetsch et al., 1998; Hu et al., 1999). Ca$^{2+}$ oscillations necessary to drive changes in gene transcription persist long after the initial activation of cell surface receptors, whereas the generation of IP$_3$ is transient, and IP$_3$ is reverted back to basal levels within minutes following receptor stimulation. Thus, the production of
ROS/RNS following receptor stimulation may tune IP$_3$R sensitivity to CICR in order to produce sustained changes in [Ca$^{2+}$]$_i$ in the absence of elevated IP$_3$.

Protein synthesis in the ER is also associated with the generation of luminal ROS; produced during the formation of inter- and intra-molecular disulfide bonds by oxidoreductases and PDIs during oxidative protein folding and maturation processes. This rise in ROS must be buffered or extruded from the ER in order to maintain the appropriate luminal redox environment. The GSH/GSSG ratio sets the redox status of the ER lumen, and a shift to more oxidizing conditions may increase the formation of P-SSG mixed disulfides in the ER. Our preliminary studies suggest the IP$_3$R is glutathionylated in an ER luminal domain. Thus a shift to more oxidizing conditions may stimulate glutathionylation of the IP$_3$R. If this is true, cells undergoing extensive protein synthesis may be more sensitive to receptor agonists coupled to the activation of IP$_3$Rs; this hypothesis requires further testing. Moreover, ROS production following stimulation of gene transcription and the resulting protein synthesis could provide a feed-forward mechanism for regulating IP$_3$R activity in order to improve protein processing and secretory events. Alternatively, disruption of protein synthesis will also lead to a rise in ROS in the ER. During conditions of ER stress associated with the unfolded protein response an increase in IP$_3$R-mediated Ca$^{2+}$ release is necessary to induce apoptosis (Molteni et al., 2004). Thus, the production of ROS in the ER may serve dual roles in regulating IP$_3$R function during physiological signaling and the pathological response to oxidative stress.
4.6: POTENTIAL ROLE OF IP₃R GLUTATHIONYLATION DURING PATHOLOGICAL SIGNALING

In addition to increasing IP₃R function during oxidative stress, glutathionylation may serve a role in protecting the IP₃R from irreversible thiol-modification. As discussed previously, the time frame of glutathionylation is dependent upon both the activity of glutaredoxins and the availability of GSH. The conversion from physiological to pathological signaling during oxidative stress may depend upon the cellular capacity to reverse the changes in P-SSG mixed disulfide formation. Whereas, our present results suggest that glutathionylation increases IP₃R activity, the inability to revert back to a reduced state might inhibit the IP₃R. Thus, it is possible that once IP₃Rs are activated and release Ca²⁺, de-glutathionylation is necessary to reset the protein to an activatable conformation. Alternatively, a loss of IP₃R activity during an oxidative challenge may be due to irreversible protein oxidation when GSH can no longer buffer the harmful rise in reactive species. In as much as the primary focus of our present studies entailed reversible changes in IP₃R behavior, the effects of glutathionylation associated with irreversible loss of IP₃R function remains virtually unexplored and additional studies will be required to address these possibilities.

4.7: CONCLUDING REMARKS

Since IP₃Rs are expressed in virtually all mammalian cells, and IP₃R-mediated Ca²⁺ signaling governs a diverse array of cellular processes, changes in IP₃R function by glutathionylation may have broad implications in human physiology. A future challenge
will be to identify how glutathionylation impacts IP3R-dependent signaling processes in health and disease.
APPENDIX

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