REGULATION OF INFLAMMATORY ACTIVATION IN ENDOTHELIAL CELLS BY PIN1

DISSERTATION

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By

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ABSTRACT

Endothelial cells (EC) are a primary target for injury and cardiovascular risk factors. Endothelial inflammatory activation and dysfunction has been critically implicated in diverse cardiovascular diseases such as atherosclerosis, sepsis and heart failures. Inflammatory cytokines and bacterial products such as lipopolysaccharide (LPS) together with interferon-gamma (IFN-\(\gamma\)), can activate vascular endothelial cells, and induce expression of pro-inflammatory proteins, which essentially mediates inflammatory processes. Two prominent inflammatory processes are the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) and the formation of prostaglandins by cyclooxygenase-2 (COX-2), which may promote endothelial cell injury and play an important role in the pathogenesis of many cardiovascular diseases.

Protein phosphorylation regulates many cellular processes by causing changes in protein conformation. The prolyl isomerase PIN1 has been identified as a novel regulator of phosphorylation signaling that catalyses the conversion of specific phosphorylated motifs between the two completely distinct conformations in a subset of proteins. PIN1 regulates diverse cellular processes including
inflammation and immune responses. Various transcription factors and regulators have been identified as substrates for PIN1. For example, in response to cytokine signals, PIN1 acts on the phospho-p65 subunit of NF-κB and increases its nuclear accumulation and NF-κB activity. It enhances AP-1 activity via binding and isomerizing of both c-Jun and c-Fos. Such transcription factors are crucial for expression of inflammatory genes, including iNOS and COX-2. In addition, PIN1 can also regulate mRNA and protein turnover. However, the role of PIN1 in expression of these proteins in endothelial cells is unexplored.

Here, the effect of depletion of PIN1 on induction of iNOS by LPS and IFN-γ in murine aortic endothelial cells (MAEC) was determined. Knockdown (KD) of PIN1 by 85% with a shRNA-lentiviral system enhanced the induction of NO and iNOS protein by LPS/IFN-γ. Similar results were also observed in PIN1 knock out MAEC and cells transiently transfected with small interfering RNAs. There was no effect on induction of iNOS mRNA, suggesting a post-transcriptional effect. The enhanced levels of iNOS protein were functionally significant since LPS/IFN-γ was cytotoxic to MAEC lacking PIN1 but not MAEC harboring an inactive control construct, and because cytotoxicity was blocked by the NO synthase inhibitor Nω-nitro-L-arginine methyl ester. Knockdown of PIN1 increased the stability of iNOS protein in cycloheximide-treated cells. Furthermore, loss of iNOS
was blocked by the calpain inhibition, but not by the selective proteasome inhibition. Immunoprecipitation and GST-PIN1 pull-down results indicated that PIN1 can interact with iNOS. The results suggest that PIN1 associates with iNOS and can limit its induction by facilitating calpain-mediated degradation in MAEC (chapter 2).

In addition, regulation of PIN1 on COX-2 degradation was investigated. Similar to iNOS, knockdown of PIN1 in endothelial cells enhanced the induction of COX-2 and its production of prostaglandin E2 in response to LPS/IFN-γ. The additional increase in COX-2 protein due to PIN1 depletion was post-transcriptional, as induction of COX-2 mRNA by LPS/IFN-γ was the same in cells containing or lacking PIN1. Instead, the loss of COX-2 protein, after treatment with cycloheximide to block protein synthesis, was reduced in cells lacking PIN1 in comparison with control cells, indicating that degradation of the enzyme was reduced. Calpain inhibition also enhanced the induction of COX-2 by LPS/IFN-γ, and slowed its loss in after treatment with cycloheximide. In contrast to iNOS, physical interaction between COX-2 and PIN1 was not detected, suggesting that PIN1 may regulate COX-2 degradation by affecting calpain, rather than by an effect on COX-2 protein (chapter 3).
Since depletion of PIN1 reduced calpain-mediated turnover of iNOS and COX-2, we hypothesized that PIN1 regulated calpain activity in MAEC. Knockdown of PIN1 reduced the basal and LPS/IFN-γ stimulated activity of calpain as compared with MAEC harboring a control small hairpin RNA. However, the depletion of PIN1 did not affect the levels of the large or small subunits of ubiquitous, heterodimeric calpains. PIN1 did not interact with these proteins either. Instead, deletion of PIN1 increased the basal level of calpastatin (CAST), the endogenous protein inhibitor of heterodimeric calpains. The effect of PIN1 on CAST was post-transcriptional, as CAST mRNA was not affected by PIN1 depletion. Loss of CAST, after inhibiting protein synthesis with cycloheximide, was slower in KD than control MAEC, indicating that PIN1 regulated degradation of CAST. Inhibition of calpain increased CAST protein levels in control MAEC, whereas a pan-caspase inhibitor, did not, suggesting that calpain, not caspase, contributed to the turnover of CAST in MAEC. Extracts from KD MAEC treated with either vehicle or LPS/IFN-γ contained more inhibitory activity towards an exogenous calpain compared to control shRNA MAEC. Co-immunoprecipitation and glutathione-S-transferase-PIN1 fusion protein pull-down results indicated that CAST associated with PIN1. The PIN1 fusion protein also antagonized the ability of CAST to inhibit exogenous calpain in vitro. The results suggest that PIN1 associated with CAST, either facilitating degradation of CAST or
decreasing its inhibitory activity towards calpain in MAEC. The results suggest that PIN1 normally maintains calpain activity and consequently limits the induction of calpain substrates, such as iNOS and COX-2 in endothelial cells.

The regulation of calpain system by PIN1 in MAEC indicates that PIN1 may play an essential role in various calpain related physiological and pathological processes (chapter 4).

The role of PIN1 in the regulation of calpain activity in vivo was investigated as well. Here, calpain activity and CAST inhibitory activity in various organs from PIN1 wild type and knock out mice were measured. The regulation of the calpain system by PIN1 in vivo is more complicated and appeared tissue specific: lung and kidney from PIN1 knockout mice contained significantly lower calpain activity which may be due to the higher CAST inhibitory activity in them. However, higher calpain activity and lower CAST inhibitory activity were observed in brains from PIN1 knockout mice. Liver and heart from PIN1 knock out mice contained significantly lower calpain activity although the CAST inhibitory activity was similar to wildtype mice, indicating that mechanisms other than CAST were involved in PIN1-regulated calpain activity in these organs. PIN1 knockout influenced normal cardiac function, causing a significant decrease of stroke volume compared with wildtype (chapter 5).
Overall, the results presented here suggest that PIN1 regulates calpain/CAST system in endothelial cells and various organs, which further modulates the turnover of inflammatory calpain substrates, such as iNOS and COX-2. Thus, the work presented in this thesis will direct our further investigation of the important role of PIN1 in MAEC endothelial activation and dysfunction during inflammatory conditions. These mechanistic insights of endothelial biology will provide new therapy for the calpain/CAST related diseases, such as cardiovascular disease.
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ABBREVIATIONS

Ser/Thr-Pro: serine or threonine residues preceding proline

AD: Alzheimer’s diseases

CDKs: cyclin-dependent protein kinases

ERKs: extracellular signal-regulated kinases

SAPKs/JNKs: stress-activated protein kinases/c-Jun-N-terminal kinases

GSK3: glycogen synthase kinase-3

PPIases: peptidyl prolyl cis-trans isomerases

PIN1: protein interacting with NIMA (never in mitosis A)-1

GM-CSF: granulocyte macrophage colony stimulating factor

SOCS1: suppressor of cytokine signaling 1

MCL1: myeloid cell leukaemia sequence-1

APP: amyloid precursor protein

STAT1: signal transducer and activator of transcription-1

GAS: IFN-γ activation sites

NF-κB: nuclear factor-κB

IRF3: interferon-regulatory factor 3

AP-1: activating protein-1

ARE: adenine-uridine-rich elements
AUF1: AU-rich element-binding protein
APC: adenomatous polyposis coli protein complex
IGF-1: insulin-like growth factor 1
CAST: calpastatin
MAEC: murine aortic endothelial cells
IFN-γ: interferon gamma
LPS: lipopolysaccharide
iNOS: inducible nitric oxide synthase
COX-2: cyclooxygenase-2
PG: prostaglandins
shRNA: short hairpin RNA
KD: knock down
CHX: cycloheximide
GST: Glutathione S-transferase
NBT: Nitroblue Tetrazolium
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
CHAPTER 1
INTRODUCTION

A novel signaling regulatory mechanism:
PIN1-catalyzed prolyl isomerization after phosphorylation

Phosphorylation of proteins is a common reversible post-translational modification, which is controlled by kinases and phosphatases. Protein kinases add a phosphate group to Serine (Ser), Threonine (Thr) and Tyrosine (Tyr) residues in a protein and phosphotases remove it from proteins. Addition or removal of phosphate group can cause a dramatic conformational change in the structure of the protein and affect the properties of target proteins, which make it as one of the most important signaling mechanisms to regulate many cellular processes (1). For instance, phosphorylation on Tyr residues in certain proteins may function as specific binding sites for SH2 domain containing protein and allows the formation of signaling complex. Phosphorylation on Ser or Thr residues may also have similar functions (2-5). In addition, phosphorylation on some enzymes or receptors alters conformation, allowing cells to either turn on/off enzymes’ catalytic activity or increase/decreases receptor signaling intensity or duration (6, 7).
Protein kinases may phosphorylate their substrates in different consensus sequences. Some Ser/Thr kinases are called proline-directed kinases since they phosphorylate their substrate motifs (Ser/Thr-Pro motifs) in a proline directed manner. Cyclin-dependent protein kinases (CDKs), extracellular signal-regulated kinases (ERKs), stress-activated protein kinases/c-Jun-N-terminal kinases (SAPKs/JNKs), glycogen synthase kinase-3 (GSK3) and others are known as to be members of the proline-directed kinase family. Proline-directed phosphorylation is important for regulation of many cellular events (8) and have been implicated in the pathogenesis of many human diseases, such as cancer and Alzheimer’s disease (AD) (9-13).

Although phosphorylation is believed to regulate protein function via changing protein conformation, it remains largely unknown how conformational changes that occur after phosphorylation on Ser/Thr-Pro motifs alter protein’s function.

It is known that either trans or cis conformation can be adopted in any peptidyl bond. The trans conformation is much more energetically favored than cis and is more dominant in peptidyl bonds under most conditions. However, this is not the case in proline which contains a five membered ring and makes the free energy difference between cis and trans much smaller. Thus, proline adopts completely
distinct cis and trans conformations and is unique in providing an intrinsic backbone switch (11) (See Fig. 1.1). The spontaneous interconversion rate is very low without the assistance of a catalyst. Peptidyl prolyl cis-trans isomerases (PPIases) can dramatically speed up the conversion rates (14). So far, PPIases can be divided into several families: cyclophilins, FK506-binding protein and parvulins. These PPIases are structurally unrelated, have their own conserved amino acid sequence and substrate specificity. Cyclophilins and FK506-binding protein are cellular targets for immunosuppressive drugs cyclosporins and FK506/rapamycin respectively. Their actions involves facilitating protein folding or promoting in protein complex formation, while the PPIase activity is not necessary for immunosuppression (8). The biological significance of PPIase activity as an important regulatory mechanism was not known until the discovery of Protein Interacting with NIMA (never in mitosis A)-1 (PIN1).

Human PIN1 was originally identified by its ability to interact with NIMA, a mitotic kinase whose phosphorylation on multiple Ser/Thr-Pro motifs is critical for mitosis in Aspergillus nidulans (15). Although PIN1 belongs to parvulin subfamily, its substrate specificity is completely distinct from other parvulin members. PIN1 is the only identified mammalian prolyl isomerase that specifically acts on phosphorylated Ser/Thr-Pro peptide bonds and is highly conserved from yeast to
human. PIN1 contains a C-terminal PPIase domain and an N-terminal domain, characterized by two tryptophan residues separated by 22 amino acids that can bind to phosphorylated serine- or threonine-proline sequences in substrate proteins, known as a WW domain. PPIase domain and WW domain are coordinated to specifically act on phospho-substrates. The WW domain is responsible for binding to phospho-Ser/Thr-Pro-containing proteins. PPIase domain is sufficient to catalyze isomerization at phosphorylated Ser/Thr-Pro motifs and induce isomerization, which triggers various functional outcomes and regulates a wide range of cellular processes.

It is well-known that phosphorylation on Ser/Thr preceding Pro can dramatically reduce the isomerization rate between cis/trans conformation and make the peptide bond resistant to isomerization catalyzed by other known PPIase. Furthermore, phosphatases that modify pSer/Thr-Pro motifs and certain proteins that interact with these motifs are conformation-specific and only act on the trans conformation. Thus, PIN1 catalyzed isomerization of pSer/Thr-Pro motifs is extremely important in phosphorylation signaling. Restoration of the equilibrium between cis and trans would require minutes without a catalyst, while PIN1 can dramatically accelerate cis to trans or trans to cis isomerization rate and maintain equilibrium of cis and trans conformations on the millisecond timescale. Such an
acceleration in equilibrium from minutes to millisecond make it more relevant for the regulation of dynamic biological processes (16).

Fig 1.1 Conformational switch of Ser/Thr-Pro and p-Ser/Thr-Pro motifs catalyzed by PPIase.
Accumulating evidence indicates that PIN1-catalyzed isomerization after proline directed phosphorylation regulates a wide variety of key proteins, suggesting that the phosphorylation-specific, PIN1-catalyzed conformational regulation may be a common signaling mechanism. Isomerization of pSer/pThr-Pro motifs by PIN1 has been shown to play an important role in regulating diverse cellular processes, including immune responses, cellular stress responses, cell growth and proliferation and neuronal function. PIN1 is also critically involved in several human diseases including cancer, AD and microbial infections. Some of PIN1 functions in these cellular processes and related diseases are reviewed next.

**PIN1 and immune responses**

PIN1 may play an important role in immune by acting on Nuclear factor-κB (NF-κB) and interferon-regulatory factor 3 (IRF3) which are essential mediators in viral infection and other immune responses.

As a central mediator in the immune responses, NF-κB is important in the development of innate immune cells as well as B and T cells, which directly mediate immune responses (17). NF-κB also regulates expression of mediators of inflammation includes cytokines (for example, TNFα and IL-1β) and adhesion molecules (for example, ICAM-1). When exposed to cytokine signals, p65
subunit of NF-κB is phosphorylated in its Thr254-Pro motif and interacts with PIN1, which inhibits p65 interaction with endogenous inhibitory protein IκBα and increases its nuclear accumulation and activity (18). Furthermore, the association between PIN1 and p65 also inhibits its degradation by suppressor of cytokine signaling 1 (SOCS1). Deletion of PIN1 renders cells resistant to NF-κB activation by cytokine signals (18).

IRF3 has been identified as a PIN1 substrate recently (19). IRF3 controls the primary induction of interferon-β (IFN-β), which in turn controls expression of hundreds of genes and involved in viral infection. PIN1 interacts with phospho-IRF3 at pSer339–Pro340, facilitates IRF3 ubiquitination – proteasome mediated degradation and negatively regulates its activation. Therefore, knockdown or genetic deletion of PIN1 results in enhanced IRF-3-dependent production of interferon-β and reduces virus replication. These results also indicate the important role of PIN1 in the establishment of innate immunity against viral infection.

Recent studies have shown that PIN1 is important in the development of asthma (20-22). The activation of eosinophils in the lung triggers the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF), an essential survival factor for activated eosinophils. PIN1 isomerase activity is necessary for
GM-CSF mRNA stabilization and its secretion, which in turn increases the survival of activated eosinophils. The action of PIN1 on GM-CSF mRNA stability is through its association with the AU-rich element-binding protein, AUF1, which regulates the decay rate of GM-CSF mRNA. Similar results were also observed in human PBMC and CD4+ lymphocytes. PIN1 activity has been demonstrated to be enhanced in eosinophils obtained from the airways of patients with asthma, suggesting that PIN1 may be a potentially therapeutic target in allergic asthma.

**PIN1 and cellular stress**

In response to various stresses, many proline-directed kinases such as JNKs and p38 are activated and phosphorylate their substrate motifs (p-Ser/Thr-Pro), which are putative PIN1 binding sequences. Thus it is important to investigate whether PIN1 is involved in cellular stress. Several key proteins in cellular stress responses, such as p53 and p73 have been identified as PIN1 substrates, and PIN1-dependent isomerization regulates their function in cellular stress.

Genotoxic stress leads to p53 stabilization and accumulation, which is regulated by proline-directed phosphorylation and MDM-2-mediated degradation. PIN1 regulates p53 function by several different mechanisms (23, 24). Firstly, PIN1
acts on phosphorylated p53 and inhibits its binding to MDM2, therefore increasing p53 protein stability in response to DNA damage. Secondly, PIN1 may also be required for efficient binding of p53 on target promoters, enhancing the DNA-binding activity and transcriptional activity of p53. Thirdly, the association with PIN1 can stimulate binding of the p300 acetyltransferase to p53 and consequently increase p53 acetylation. Fourthly, PIN1 also mediates p53’s dissociation from the apoptosis inhibitor, iASPP, promoting cell death (25). The stability and activity of p73, a homologue to p53, can be up-regulated by PIN1 (26, 27). These results suggest that PIN1 is involved in the response to different cellular stresses by regulating substrate protein function or their interaction with other proteins. PIN1 itself can also be inactivated in oxidative stresses (28). Thus, it would be interesting to examine the significance of PIN1 in cellular stresses.

**PIN1, growth-signaling pathways and oncogenesis**

Proline-directed phosphorylation of proteins on certain Ser/Thr-Pro motifs is an essential signaling mechanism in cell proliferation and transformation in response to growth stimulation. By binding and isomerizing specific pSer/Thr-Pro motifs in many proteins central to tumorigenesis, including β-catenin, NF-kB, cyclinD1 and
p53, PIN1 regulates the duration and outcome of signaling after phosphorylation. The following section will describe how PIN1 plays a key role in the regulation of the Wnt/β-catenin, cytokine/ NF-κB and Neu-Raf-Ras-MAP kinase pathways (See Fig. 1.2).

As a key transcription activator in the Wnt pathway, β-catenin is negatively regulated by the adenomatous polyposis coli protein (APC) complex. APC interacts with β-catenin, holds it in the cytoplasm and triggers its degradation through proteasome pathway. PIN1 specifically binds to pSer 246-Pro of β-catenin and prevents it from binding to APC, which stabilizes β-catenin and increases its translocation into the nucleus (29). In the nucleus, β-catenin and its cofactors T-cell transcription factor activates targeted gene expression.

Mutation of the APC or β-catenin for example in their binding sites can disrupt the binding of APC to β-catenin, resulting in subsequent degradation and prolonged activation of β-catenin. However, in many other types of cancer where APC or β-catenin mutations are not common, PIN1 is a key regulator of β-catenin and strongly correlates with its expression levels and nuclear accumulation. For example, overexpression of PIN1 increases β-catenin level, while knockdown or genetic deletion of PIN1 decreases β-catenin level in breast cancer tissues. Thus
PIN1-mediated isomerization is an important mechanism in enhancing β-catenin activity and creates a positive growth signal for tumor cells.

Fig. 1.2 PIN1 regulates multiple pro-proliferative and pro-apoptotic pathways.
Considering the importance of NF-κB in oncogenesis and the fact that PIN1 critically regulates NF-κB signaling in response to cytokines (18), it is necessary to investigate the relationship between PIN1 overexpression and NF-κB activation in certain cancers. PIN1 interacts with the p65 subunit of NF-κB and inhibits its interaction with the endogenous inhibitory protein IκBα, which increases NF-κB nuclear accumulation and activity. Furthermore, PIN1 also inhibits p65 degradation by SOCS1 (18). Therefore, PIN1 overexpression would uncouple the inhibition of NF-κB by IκBα, which might explain why NF-κB signaling is overactivated even though IκBα is also upregulated in certain cancers.

Another well-known example is the Neu-Raf-Ras-MAP kinase pathway (30). Growth factor receptor Neu activates Raf and Ras in response to growth stimulation. The MAP kinase cascades are activated as a result. MAP kinases
phosphorylate c-Jun and c-Fos, thereby enhancing AP-1 transcriptional activity toward its downstream targets such as cyclin D1, which positively promotes cell cycle progression (30). Furthermore, MAP kinase-mediated phosphorylation of Raf inactivated Raf kinase, forming a negative feedback loop (31).

As an E2F downstream target gene, PIN1-catalyzed isomerization critically regulates Neu-Raf-Ras-MAPK signaling pathway by acting on multiple targets. First, PIN1 can directly act on c-Jun/c-Fos and increase AP-1 transcriptional activity and downstream target gene expression, including cyclin D1 (30, 32). Second, PIN1 directly interacts with cyclin D1 to enhance its stability and nuclear localization. The transcription factor, E2F, downstream target of cyclin D1, may be activated and increase PIN1 expression (33). Increased PIN1 expression in turn may enhance growth signaling by increasing cyclin D1 transcription and stabilization through a positive feedback loop (8). Third, the negative feedback loop can be blocked by PIN1 via promoting dephosphorylation of Raf kinase, prolonging the activation of Raf kinase (31).

PIN1 is overexpressed in many human cancers, such as malignancies of the breast and prostate (32). Ras signaling, ß-catenin, and NF-kB play critical roles in oncogenesis. Oncogenic signals, such as Neu activation, induce PIN1
overexpression, which greatly promotes cell transformation by enhancing Ras signaling, β-catenin expression, and NF-kB signaling. In contrast, knockdown of PIN1 induces apoptosis and inhibits tumorigenesis. PIN1 also enhances downstream signalling by amplifying positive feedback mechanisms (multiple targets: c-Jun, c-Fos, cyclin D1 and E2F) and suppressing negative feedback mechanisms (Raf), synergistically driving the cell in one direction, for instance, to growth promotion or transformation. Collectively, PIN1 plays an essential role in oncogenesis. PIN1 inhibition might be a potentially novel anticancer therapy target.

PIN1 in neuronal function and Alzheimer’s disease

Although it is expressed ubiquitously, PIN1 protein levels and activity vary significantly in various tissues. The PIN1 level in most neurons is relatively high and increases during neuronal differentiation (34, 35), suggesting that PIN1 may be involved in neuronal function. Several key proteins in neuronal function such as Tau (36), amyloid precursor protein (APP) (16), and myeloid cell leukaemia sequence-1 (MCL1) (37) have been reported as PIN1 substrates. Tau is able to bind to microtubules and promote microtubule assembly, which is abolished by
the phosphorylation of tau. PIN1-catalysed isomerization can restore the biological function of phosphorylated tau by different mechanisms (36). PIN1 directly interacts with phosphorylated tau and restores the ability of tau to bind to microtubules and promote microtubule assembly. PIN1 can also facilitate dephosphorylation of phosphorylated tau by Pro-directed phosphatase PP2A, which is conformation-specific and effectively dephosphorylates only the trans pSer/Thr-Pro isomer. In the case of APP, PIN1 binds to the phosphorylated Thr 668-Pro motif in APP and regulates its non-amyloidogenic processing, therefore producing neurotrophic α-APPs and reducing neurotoxic amyloid-β (Aβ) peptides. Amyloidogenic APP processing is favored in transgenic mice with PIN1 knockout alone or in combination with overexpression of mutant APP, which results in the accumulation of insoluble Abeta42, a major toxic species in brain, in an age-dependent manner. JNK3 induced MCL1 degradation and apoptosis after spinal cord injury. However, PIN1 binds and stabilizes MCL1, protecting oligodendrocytes from undergoing apoptosis after JNK3 activation (37).

The neuropathological hallmarks of Alzheimer’s disease are neurofibrillary tangles composed of tau and neuritic plaques comprising Abeta derived from amyloid precursor protein (APP). PIN1-catalysed prolyl isomerization regulates both tangle (phosphorylation and function of Tau) and plaque (APP processing
and Abeta production) pathologies. Notably, PIN1 is depleted and/or inhibited by oxidation in Alzheimer's disease neurons. PIN1 expression is inversely correlated with predicted neuronal vulnerability and neurofibrillary degeneration in Alzheimer's disease. PIN1 knockout in mice causes motor and behavioral deficits, tau hyperphosphorylation, tau filament formation and other progressive age-dependent neuropathies. Thus, PIN1 is pivotal in protecting against age-dependent neurodegeneration.

Regulation of PIN1

While PIN1 governs various aspects of cell signaling, PIN1 itself is tightly regulated at multiple levels under physiological conditions.

1. Protein level

Although present in all cells, PIN1 expression is strongly correlated with the proliferative activity of normal tissues. PIN1 is readily detectable in actively dividing cells, but is low in most terminally differentiated tissues, with the exception of neurons (38). Interestingly, PIN1 itself is also a target of several oncogenic pathways and its expression is significantly upregulated in
transformed cell lines and some human cancer tissues (32). For example, PIN1 is a target gene for the E2F family of transcription factors, which can be activated by Neu or Ras activation (33). Insulin-like growth factor 1 (IGF-1) induces PIN1 protein expression in human breast cancer cells, which is mediated via the phosphatidylinositol 3-kinase as well as the MAP kinase pathways (39). Expression of PIN1 was decreased in response to ischemia-reperfusion in liver (40).

2. Post-translational modification

Several post-translational modifications including phosphorylation, ubiquitination and oxidation, have been demonstrated to regulate PIN1 enzymatic activity or stability. Two phosphorylation sites have been identified in PIN1 (S16 and S65). S16 is located in PIN1 WW domain and its phosphorylation abolishes the binding ability of PIN1 to its substrate proteins (41). The S16A mutant of PIN1 binds to substrates constitutively, while S16D mutant of PIN1 is unable to bind to substrates. Given that PIN1 interacts with a large number of substrates, PIN1 appears to be predominantly phosphorylated on S16 in normal breast tissues and might prevent PIN1 from acting on unintended substrates. However, overexpressed PIN1 is mainly non-phosphorylated, is hyperactive in certain tumor tissues (32). PIN1 can be ubiquitinated and undergo proteasome-mediated
degradation. In addition, PIN1 has been shown to be phosphorylated on Ser65 by Polo-like kinase-1, which does not affect its isomerase activity, but rather contributes to its protein degradation (42). Inhibition of Polo-like kinase-1 activity enhances the ubiquitination of PIN1 and its degradation in human cancer cells. Importantly, PIN1 was found to be oxidatively modified and to have reduced activity in the hippocampus in mild cognitive impairment (MCI) and in the early stages of AD (28). However, oxidative modification sites on PIN1 and their effects on PIN1 activity are not known.

3. Subcellular localization

PIN1, a small 18-kDa protein, is able to diffuse easily across nuclear membrane, and its subcellular localization and function are dependent on the phospho-substrate availability. Thus, the localization of target proteins and related proline-directed kinases determine the distribution of PIN1 in either the nucleus or cytoplasm (43). For example, PIN1 is redistributed to cytoplasmic tangles by acting on hyperphosphorylated tau in AD neurons (34).
Summary

PIN1-mediated, phosphorylation-dependent prolyl isomerization has been identified as a novel regulatory mechanism. Such conformational regulation can have a profound impact on many key proteins in diverse cellular processes. Importantly, PIN1 levels and function are tightly regulated under physiological conditions and deregulation has a pivotal role in a growing number of pathological conditions. Thus, it is a potential new diagnostic marker of cancers or AD. It would be beneficial to develop drugs that target PIN1 for treating certain human diseases, such as cancer and immune disorders.
Endothelial inflammatory activation

My project is concerned with inflammatory activation of endothelial cells (EC). The thin monolayer of endothelial cells lines the interior surface of blood vessels in the entire circulatory system, which forms an interface between blood flow in the lumen and the underlying tissues. Rather than being a simple barrier between intravascular and interstitial compartments, the endothelium is a highly specialized and active tissue. Under resting conditions, the vascular endothelium is responsible for maintaining vascular homeostasis by modulating vascular tone and permeability and inhibiting platelet aggregation, leukocyte activation and adhesion (44). The endothelium also contributes to the local balance in pro-inflammatory and anti-inflammatory mediators and prevents the proliferation and migration of the underlying smooth muscle cells (44).

The role of endothelial cells is not confined to the regulation of the above functions. Because of its strategic anatomic position, EC are not only a primary target but also a mediator of injury. Multiple mechanisms, such as inflammation, oxidative stress, lipid abnormalities and genetic predisposition, can allow EC to participate in the inflammatory response, which is recognized as EC activation.
EC activation has been critically implicated in such as atherosclerosis, sepsis, vascular diabetic complications, and other cardiovascular diseases (45-48).

The growing understanding of intracellular signaling has led to the discovery that a common intracellular mechanism involving activation of transcription factors, including NF-κB, contributes to the diverse effects of EC activation (45). A wide range of agents such as certain bacteria and viruses, interleukin-1, tumour necrosis factor, oxidative stress, oxidized low density lipoproteins (49), and antiendothelial cell antibodies (50) are known to activate NF-κB and other important transcription factors. EC activation is not all or none but a graded response. Activation may occur locally, as in transplant rejection (51), or systemically, as in septic shock and the systemic inflammatory response.

Loss of vascular integrity, a shift to a pro-thrombotic state and expression of pro-inflammatory mediators are the core changes of endothelial cell activation. Loss of vascular integrity can expose the subendothelium and enhance permeability of the endothelium. The changes in EC integrity can be graded as well: local increases of permeability or major endothelial cell contraction, exposing large areas of subendothelium. The anti-thrombotic state in resting EC can be shifted
to the prothrombotic state with loss of the surface anticoagulant molecules thrombomodulin and heparan sulphate. Activation of EC also leads to the expression of many proinflammatory mediators. For example, induction of adhesion molecules such as E-selectin and ICAM-1 is critical to recruit macrophages and T lymphocytes to endothelium, which then infiltrate into the vessel wall, initiate cytokine synthesis, and modulate further inflammatory processes (52, 53). In addition, EC may also contribute to the production of inflammatory cytokines, chemoattractants and tissue factors, such as IL-6 and monocyte chemoattractant protein 1. In addition to secretion of cytokines, EC are also an important target of such cytokines. For example, inflammatory cytokines and bacterial products such as lipopolysaccharide (LPS) together with T-lymphocytes cytokine interferon-gamma (IFN-γ) can activate endothelial cells and induce expression of pro-inflammatory proteins, which essentially mediates inflammatory processes. Changes in expression of many proteins during EC activation of EC are often controlled by changes in transcription. Several transcription factors, such as NF-kB, AP-1 and β-catenin, are known substrates of PIN1. However, the role of PIN1 in EC has not been investigated. Two prominent in inflammatory processes are the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) and the formation of prostaglandins by cyclooxygenase-2 (COX-2). iNOS and COX-2 are induced by many inflammatory
stimuli, and may promote endothelial cell injury, which is believed to play a role in the pathogenesis of many cardiovascular diseases. Thus, the role of PIN1 in the induction of iNOS and COX-2 in EC is investigated in my study.

**Nitric Oxide Biology**

Diverse physiological and pathophysiological functions of EC are mediated by NO (54). In mammalian cells, NO can be produced from from l-arginine and molecular oxygen by 3 isoforms of NOS, two constitutive isoforms, neuronal (nNOS) and endothelial (eNOS), and one inducible isoform (iNOS). All three isoenzymes are homo-dimers and share similar cofactors FAD, FMN, BH4, and heme. nNOS and eNOS are constitutively expressed in neurons and EC, respectively. They are low output, Ca\(^{2+}\)-dependent enzymes producing NO in a pulsative manner. In contrast, iNOS is a high output, Ca\(^{2+}\)-independent enzyme whose expression can be induced in a wide range of cells by cytokines and other agents. After induction, iNOS produces NO continuously until the enzyme is degraded.
NO is a short-lived free radical and a very small compound that diffuses freely within cells from its site of formation to its site of action. NO regulates blood vessel tone in vascular system. NO derived from eNOS is a powerful vasodilator, which diffuses through cellular membranes from EC to smooth muscle cells (SMC) and activates guanylate cyclase leading to increased production of cyclic guanosine monophosphate (cGMP), which in turn modulates a wide variety of effectors such as ion channels, phosphodiesterases and protein kinases to decrease intracellular calcium and ultimately relax vascular smooth muscle (55, 56). Physiological concentrations of NO derived from eNOS also exert various vaso-protective effects, such as inhibition of platelet aggregation, suppression of integrin-mediated adhesion of leukocytes or monocytes to the endothelial surfaces, inhibition of proliferation and migration of SMC, and attenuation of proinflammatory-induced gene expression that control inflammation and oxidative injuries (57-61). Deregulation of NO derived from eNOS accompanies the development of atherosclerosis, vasospasm and thrombosis, and may contribute to some forms of hypertension and diabetic vascular diseases (54).
Inducible Nitric Oxide Synthesis (iNOS)

The role of nitric oxide (NO) generated by the inducible isoform of nitric oxide synthase (iNOS) is very complex. As an important host defense effector, NO derived from iNOS has been described to have beneficial anti-viral, anti-parasite, anti-microbe and anti-tumor effects (62-64). On the other hand, large amounts of NO derived from aberrant expression of iNOS is a marker of inflammation and a mediator of cell injury and activation. For example, induced at the wrong place or at the wrong time, iNOS has dramatic toxic effects on host cells and seems to be involved in the pathophysiology of different human diseases, including septic shock, myocardial ischemia-reperfusion injury, asthma, arthritis, tumor development, transplant rejection and neurodegenerative diseases (54, 64-67). Inhibition of iNOS may be beneficial for the treatment of inflammatory disease (64, 68, 69). Since modulation of iNOS expression is the most important component of iNOS regulation, it is essential to understand how iNOS is regulated normally and deregulated in pathological processes.

iNOS is low or undetectable in resting cell and can be induced by inflammatory cytokines, bacterial products or infection in many cell types, including EC (66).
The pathways regulating iNOS expression is complex and varies in different cells or species. For example, either LPS or IFN-γ is sufficient to induce iNOS expression in macrophages, while only the combination of them can induce iNOS in EC. iNOS induction can be regulated at both transcriptional and post-transcriptional levels, which will be reviewed next.

**Transcription**

Intense studies have been focused on transcription of iNOS. In general, activation of the transcription factors NF-kB and signal transducer and activator of transcription STAT-1α and thereby activation of the iNOS promoter seems to be an essential step in the regulation of iNOS transcription in most cells (63).

1. **Signal transducer and activator of transcription-1α (STAT-1α)**

As a well-known pro-inflammatory cytokine critically involved in the immune system and in inflammatory disorders, IFN-γ mediates biological responses mainly via activation of Janus kinase (JAK)-STAT pathway. IFN-γ binding causes its receptor to dimerize and activates the receptor-associated tyrosine kinase
JAKs, which in turn tyrosine phosphorylate the receptor, and recruit cytoplasmic STAT1 via SH2-phosphotyrosyl interactions. STAT1 is then phosphorylated on tyrosine 701 by the JAKs, which triggers STAT1 dimerization and translocation into the nucleus. Then, STAT1 dimers bind to IFN-γ activation sites (GAS) located in the promoters of target genes, inducing their transcription (70, 71).

The promoter region of the iNOS gene has been characterized in different species, including human, rat, and mouse (72-74). Sequence analysis of promoter revealed that several GAS are contained in mammalian iNOS promoter (75). Inhibition of JAK2 by pharmacological inhibitor or expression of dominant negative mutant JAK2 or STAT1 reduced STAT1α DNA binding activity and iNOS expression (76-79), suggesting the essential role of STAT-1α to directly bind the iNOS promoter and induce its expression in many cells including EC (80-82).

Activated STAT-1α also binds to the GAS site of the interferon regulatory factor-1 (IRF-1) promoter and induces its expression (83, 84). Effects of IRF-1 on iNOS mRNA induction depend on cell types. IRF-1 knock-out macrophages synthesized undetectable iNOS mRNA in response to stimulation, suggesting
that IRF-1 is essential for iNOS induction (85). The binding of IRF-1 to the iNOS promoter is required for the synergistic contribution of IFN-γ to transcription of iNOS, and mutation of IRF-1 binding sites blocked such synergy (86, 87). However, in certain cell types, such as hepatocytes, genetic deletion of IRF-1 does not influence iNOS induction in response to LPS/IFN-γ, suggesting that the role of IRF-1 in regulation of iNOS expression is cell or tissue specific (88, 89).

Phosphorylation of tyrosine701 regulates the dimerization of STAT1, which is an essential prerequisite for its transcription activity. In addition, phosphorylation within a PMS727P motif by MAPK, such as ERK1/2 and p38, can also regulate STAT1 activity, which may be required for its full transcriptional function in most of cases (80, 90, 91). Mice expressing a S727A mutation were more resistant to the LPS-induced septic shock, suggesting that this serine phosphorylation in STAT1 promotes inflammatory responses. Expression of IFN-γ-induced genes was strongly reduced in macrophages expressing STAT1 S727A mutant. Moreover, a loss of serine phosphorylation results in a decrease in association with the coactivator p300 acetyltransferase and histone acetylation at the interferon-responsive promoter. However, it is not clear how S727 phosphorylation regulates STAT1 transcription activity and protein-protein association.
Our results showed that STAT1 is crucial for iNOS induction since no detectable iNOS were induced in response to LPS/IFN-γ in STAT1 knock-out EC (Fig. 1.3). Transfection of wt STAT1 in the EC significantly induced iNOS expression. Y701A mutant had no influence on iNOS induction when compared to GF-2 vector plasmid, whereas S727A mutant partially induced iNOS. These results confirmed that Y701 is an essential prerequisite while S727 regulates its full transcription activity. Huang et al showed that STAT1 mediated the synergistic effect of LPS on IFN-γ in the induction of iNOS in EC. This was associated with p38 MAPK dependent phosphorylation of PMS727P. It is not known whether PIN1 affects STAT1α. Thus, I used the methods described in chapter 2 to investigate the potential for PIN1 to associate with STAT1. As shown in Fig. 1.4, STAT1 co-immunoprecipitated with PIN1 in LPS/IFN-γ treated MAEC, where phosphorylation of S727 is known to occur. A glutathione S-transferase PIN1 fusion protein also pulled down STAT1 from extracts of LPS/IFN-γ treated cells. Thus, it is essential to investigate whether the association of PIN1 regulates STAT1 function.
Fig 1.3 Role of STAT1 on iNOS induction in ECs.  A: 2 µg of vector plasmid (GF-2), wt STAT1 or its mutants Y701A and S727A are transfected in STAT1.
knockout murine aortic endothelial cells (MAEC). Cells were treated with LPS and IFN-γ for 24 h. Representative Western blots of iNOS and α-tubulin are shown. B. densitometric analysis of signal intensities of images was used to measured iNOS expression. Bars represent means + SE ratio of iNOS/α-tubulin from three cultures of each group. +p < 0.05 for comparison with GF-2, *p < 0.05 for comparison between STAT1 and S727A.
Fig 1.4 PIN1 interacts with STAT1. A: MAEC were treated with vehicle or LPS/IFN-γ for 24 h. Cell extracts were immunoprecipitated without (None) or with anti-PIN1 antibody (PIN1) and analyzed by Western blot analysis with anti-STAT1 and PIN1 antibodies.
STAT1 antibody. B: cell extracts after LPS/IFN-γ treatment for 24h were incubated with GST or GST-wild-type PIN1 fusion proteins, and glutathione sepharose, and analyzed by Western blotting with anti-STAT1 and anti-GST antibodies. Blots are representative of 3 independent cultures.

2. NF-κB

The family of NFκB transcription factors is a highly conserved protein complex from Drosophila to human. There are two structural classes of NF-κB proteins: class I (p50 and p52) and class II (RelA, RelB, and c-Rel). Both classes of proteins contain a highly conserved Rel homology domain (RH) in their N-termini, which serves as a DNA-binding domain and also as a dimerization interface to other NF-κB transcription factors, and the inhibitory IκB protein. Class II but not class I NF-κB proteins have a transactivation domain in their C-termini (92). NF-κB is found in almost all cell types and plays a pivotal role in regulating the immune responses (17). Deregulation of NF-κB has been implicated in many human diseases including to septic shock, viral infection, cancer inflammatory and autoimmune diseases (93).
NF-κB can be activated by exposure of cells to LPS or inflammatory cytokines such as TNF or IL-1, viral infection, UV irradiation, and by other physiological and nonphysiological stimuli (94). NF-κB proteins can form homodimers or heterodimers in vivo. The activity of NF-κB is tightly regulated by interaction with inhibitory protein IκB. Several different IκB proteins have been identified and each has different affinities for individual NF-κB dimers. In resting cells, NF-κB dimers are sequestered in the cytoplasm by interacting with IκB. In response to extracellular signals such as LPS, the IκB kinase (IKK) is activated, causes phosphorylation, inducing degradation of IκB, allowing the dissociation of IκB-NF-κB complex, resulting in NF-κB nuclear translocation. The activated NF-κB binds to κB sites in promoters and activate transcription of genes encoding proteins involved in immune or inflammatory responses and in cell growth control.

NF-κB binding sites in the iNOS promoter region have been demonstrated in human (95) and rodents (96, 97). Activation of NF-κB seems to be an essential mediator of regulation of iNOS transcription in most cells (63). Some of the anti-inflammatory agents such as glucocorticoids, TGF-β, aspirin and thiol compounds act through inhibition of NF-κB via either blocking its nuclear translocation (98), inhibiting its transactivation activity by direct protein-protein interaction (99) or enhancing expression of its specific inhibitor IκB (100, 101).
discussed earlier, PIN1 acts on p65 subunit of NF-κB, reduces p65 interaction with IκBα, increases its nuclear accumulation and activity and inhibits its degradation after ubiquitination by SOCS1. Based on this information, PIN1 may regulate iNOS induction by acting on NF-κB.

3. **AP-1 and other transcription factors**

The AP-1 transcription factor is a complex composed of proteins belonging to the c-Fos, c-Jun and other families, in which dimerization is required to promote binding of the complex to AP-1 recognition site. AP-1 induces target genes in response to many physiological and pathological stimuli, including cytokines, growth factors, stress signals, bacterial and viral infections, as well as oncogenic stimuli. The composition of AP-1 dimers, the quality of stimulus, the cell type and the cellular environment are important in determining the function of AP-1 (102).

Two copies of AP-1 binding sites have been identified in 5'-untranslated region of the human iNOS gene (103). These two sites differed greatly in their ability to induce iNOS in response to cytokine mixture (104). The upstream AP-1 site is critical for iNOS induction and mutations decreased gene activation by 90%. However, the downstream AP-1 site is less important and its mutation only
reduced promoter activity by 45%. Functional AP-1 regulatory regions are required for IFN-γ induced activation of the human NOS2 promoter, suggesting a role for AP-1 activation/binding in the IFN-γ induction of genes. c-Fos and STAT-1 associated after IFN-γ treatment and bind to GAS element in close proximity to AP-1 sites to regulate transcription of iNOS (105). As indicated earlier, PIN1 is known to regulate AP-1, but the role of potential effects of PIN1 on AP-1 in iNOS expression is not known.

Several other transcription factors such as cAMP-responsive element binding protein (CREB) (106), CCAAT-enhancer box binding protein (C/EBP) (97), β-catenin (107), peroxisome proliferator activated receptor (PPAR) (108), hypoxia-induced factor-1 (HIF-1) (109), STAT3 (99) and p53 (110) are involved in regulating iNOS expression in different cells.

**mRNA stability and translation**

Post-transcriptional mechanisms, such as mRNA stability and translation are also critically involved in the regulation of iNOS expression. The steady-state levels of many mRNAs are determined in part by their decay rates. The interaction between cis-acting sequence elements in these mRNAs and certain
ribonucleoproteins controlled mRNA stability. In 3’-untranslated region (3’-UTR) of mRNAs encoding cytokine and other inducible proteins, there is one type of cis-acting instability determinant called adenine-uridine–rich elements (AREs). AREs stabilize or destabilize mRNA by interacting with different RNA binding proteins such as AU binding factor 1 (AUF-1), Hu antigen R (HuR) and other heterogeneous nuclear ribonucleoproteins (hnRNPs) (111-115).

Regulation of iNOS mRNA stability also plays a key role in iNOS induction in mammalian cells. For example, significant basal activity of the human iNOS promoter was shown in some human cell lines such as AKN hepatocytes, while no detectable iNOS mRNA was observed (101). TGF-β and other agents have been demonstrated to reduce iNOS mRNA by decreasing iNOS mRNA stability (116).

Several AREs in 3’-UTR have been identified in human (117) and rodent (118) iNOS mRNA, which may interact with several different RNA binding proteins and regulate its stability. AUF1 interacts with AREs and overexpression of AUF1 in DLD1 cell destabilizes iNOS mRNA (63). In contrast, binding of HuR to the 3’-UTR of iNOS mRNA seems to stabilize this mRNA. Inhibition of HuR reduced the induction of iNOS mRNA by cytokine, whereas overexpression of HuR
potentiated cytokine-induced iNOS expression (119). In addition to HuR and AUF1, other RNA binding proteins also are involved in the regulation of iNOS. Two hnRNP family members, L and I, were demonstrated to interact with the 3’-UTR of murine iNOS mRNA, which is modulated by inflammation, indicating their function in the post-transcriptional regulation of murine iNOS expression (120). As we mentioned earlier, PIN1 regulates GM-CSF mRNA stability by acting on AUF1. Thus, the possibility of PIN1 to regulate iNOS mRNA stability can not be excluded.

In addition, mRNA translation is a regulated step. In human primary cardiomyocytes, cytokine mixtures induced high and persistent levels of iNOS mRNA, but no iNOS protein (121). TGF-β can also reduce iNOS expression by decreasing iNOS mRNA translation in primary murine macrophages (116). Lee’s results showed that decreasing l-arginine blocked iNOS protein expression and NO production without affecting induction of iNOS mRNA, or the stability of iNOS protein (122, 123). In fact, depletion of l-arginine inhibits the translation of iNOS mRNA, in which eIF2α phosphorylation and eIF2 alpha kinase are critically involved (122, 123).
Posttranslational regulation and degradation

Posttranslational regulation of iNOS protein includes dimerization, phosphorylation, and protein degradation.

Newly synthesized iNOS has to form a homodimer to be functionally active in NO production in the presence of co-factors and substrates. The regulation of iNOS activity through effects on its dimerization has been demonstrated. For example, kalirin associates with iNOS and inhibits iNOS activity by preventing the formation of iNOS homodimers (124). Also, NOS-associated protein-110 kDa (NAP-110) directly interacts with N-terminus of iNOS, and inhibits iNOS catalytic activity by preventing formation of iNOS homodimers (125). A series of potent and selective inducible nitric-oxide synthase (iNOS) inhibitors, such as antifungal imidazoles, was shown to prevent iNOS dimerization in cells and inhibit iNOS in vivo (126).

iNOS protein has also been shown to undergo phosphorylation in human and mouse cells (127-130). The role of phosphorylation under physiological conditions for iNOS is controversial. In murine macrophages, enhancement of
iNOS tyrosine phosphorylation by a protein tyrosine phosphatase inhibitor is associated with increased iNOS activity in both soluble and insoluble subcellular fractions (130). However, Src-mediated tyrosine151 phosphorylation in HEK293 cells redistributed iNOS to detergent-insoluble fraction and decreased activity of human iNOS (129). Salh et al also reported that iNOS protein was phosphorylated in concordance with the production of NO, and abolition of iNOS phosphorylation by both rapamycin and Ly294002 resulted in almost complete inhibition of NO production (127).

iNOS is ubiquitined and degraded by proteasome activity in some cell types stimulated in different ways (131-137) and by calpain in other experimental situations (134-137). Cell type, stimulus, or other factors may affect the iNOS turnover path that operates in different systems. Before my study, it was not known whether PIN1 regulated iNOS degradation by either pathway.
Prostaglandin biology and COX-2 regulation

The prostaglandins (PGs) play an essential role in the maintenance of vascular homeostasis. Deregulation of prostaglandins synthesis has been implicated in many cardiovascular diseases, inflammatory disorder, and cancers. For example, PGI₂ is a vasodilator and inhibits platelet aggregation, whereas TXA₂ is a vasoconstrictor and enhances platelet aggregation. Thus, it is important to understand factors and conditions that might affect the synthesis of PGs.

Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) catalyze the rate-limiting step in the formation of PGs from arachidonic acid. COX-1 is expressed in most cells all the time, making it a housekeeping molecule since COX-1 promoter lacks TATA and CAAT boxes but is rich in GC content, a feature of a housekeeping genes. COX-1 leads to production of PGs that maintain integrity of the gastric mucosa, mediate normal platelet function, and regulate renal blood flow (138).

In contrast, COX-2 is undetectable in most tissues until a stimulus induces it. Several extra-cellular signals such as bacterial endotoxin, cytokines, or growth factors rapidly induce COX-2 in many cell types including endothelial cells. COX-
2 appears responsible for synthesis of PGs involved in diseases, particularly those related to acute and chronic inflammation.

Similarly to iNOS, COX-2 is controlled at transcriptional and post-transcriptional levels. (i) Transcription of COX-2 is induced rapidly and transiently in response to many stimuli; (ii) COX-2 mRNA stability and translational efficiency are governed by several regulatory elements in the 3'–UTR; (iii) COX-2 activity and turnover are also regulated (139).

Transcription

The human COX-2 gene promoter contains several regulatory elements that stimulate COX-2 transcription in different cell types (140). Transcription factors, including NF-kB (140-142), AP-1 (142-144), STAT1 (142), CREB (145), C/EBP (146), β-catenin-T cell factor-4 (TCF-4) (147), nuclear factor activated T cells (NFAT) (142, 148) and nuclear factor for IL-6 expression (NF-IL6) (141, 142), regulate COX-2 transcription either alone or synergistically. Transcriptional regulation of the COX-2 gene is very complex in that it can involve numerous signaling pathways, and the mechanism varies depending on the specific stimulus and the cell type. Most of the transcriptional signaling pathways require
activation of some proline-directed kinases including ERK1/2 and p38 (149), PKC and GSK-3 (148, 150).

mRNA stability

COX-2 expression can also be critically regulated at post-transcriptional level, which is mediated by AREs present within the 3'-UTR of COX-2 mRNA. The COX-2 ARE associates with different ARE RNA-binding proteins to target the mRNA for either stabilization, rapid decay, or inhibition of translation (151). For example, AREs in the 3'-UTR of COX-2 mRNA cause rapid mRNA decay by interacting with different RNA binding proteins (152). HuR and tristetraprolin (TTP) binds to the COX-2 AREs and exert opposite effects on COX-2 expression. In normal colon cells, low levels of nuclear HuR and higher levels of TTP were responsible for rapid decay of COX-2 mRNA. Increased HuR and decreased TTP, as detected in colon cancer cells, results in elevated expression of COX-2 (152, 153). COX-2 mRNA was also stabilized by activating β-catenin in NIH3T3 and 293T cells, which is mediated by the interaction between β-catenin with the COX-2 ARE in vitro and in vivo. In addition, β-catenin induced the cytoplasmic localization of HuR, which may bind to β-catenin and facilitate β-catenin-
dependent stabilization of COX-2 mRNA (154). The translational repressor protein, TIA-1, can also interact with COX-2 AREs and inhibit mRNA translation without affecting COX-2 transcription or mRNA turnover (155). Collectively, rapid mRNA decay and translational inhibition play a significant role in controlling COX-2 gene expression and if deregulated, allow for overexpression of COX-2, contributing to tumorigenesis. Although PIN1 regulates ARE binding proteins and several transcription factors mentioned here, the effect of PIN1 on COX-2 mRNA has not been described.

**Activity and protein degradation**

COX-2 enzymatic activity is affected directly by nitric oxide (156). NO has high affinity for the iron heme center in the catalytic domain of COX-2 and stimulates COX-2. In addition, iNOS specifically binds to COX-2 and the iNOS-derived NO can S-nitrosylate it, enhancing COX-2 activity. Disruption of iNOS-COX-2 binding prevented NO-mediated activation of COX-2 (157).

COX-2 degradation limits the amount of COX-2 in normal cells. The deregulation of COX-2 degradation leads to its overexpression in tumors and contributes to various pathologies such as colon cancer (158, 159). Several different
mechanisms have been reported to regulate COX-2 degradation. Proteasome-mediated degradation is initiated by post-translational N-glycosylation at Asn-594. COX-2 is embedded in the luminal membrane of the endoplasmic reticulum (ER) and the N-glycosyl group is then processed and unfolded, presumably by ER chaperones. Then COX-2 is translocated to the cytoplasm and undergoes ubiquitination and proteolysis by 26S proteasome \((160-167)\). Substrate-dependent suicide inactivation is the second pathway for COX-2 protein degradation. COX-2 inhibitors retard COX-2 protein degradation in HEK293 cells expressing COX-2. Although the mechanism of substrate-dependent degradation is not clear, proteasome activity seemed not be involved \((162, 168, 169)\). The results from our lab, and from others, also showed that COX-2 is a calpain substrate \((170, 171)\). In human synovial fibroblasts, cleavage of COX-2 was reduced by a calpain inhibitor, E64d, indicating a role for calpain and/or cathepsin. In chapter 3, I describe the effects of other selective calpain inhibitors on COX-2 induction in MAEC. Although PIN1 regulates the susceptibility of several proteins (for example, cyclin E and c-myc) to ubiquitination and proteasomal degradation, its effect on COX-2 turnover has not been investigated. In summary, induction of iNOS and COX-2 can be regulated at multiple mechanisms: transcription, post-transcription (mRNA stability), translation and post-translation (phosphorylation and degradation). PIN-1 is the only identified
peptidyl-prolyl isomerase that can catalyze the isomerization of pSer/pThr-Pro motifs and regulate the substrate protein function in a phosphorylation-dependent manner. PIN1 interacts and increases the level or activity of several transcription factors that are critical for iNOS and COX-2 transcription. These transcription factors include NF-κB (18), c-Jun (32), c-Fos (30), β-catenin (29), STAT-1α (Fig. 1.4), STAT3 (172), C/EBP (173), HIF-1α (174) and p53 (23, 24). Furthermore, mRNA stability is regulated by interaction between AREs in 3’UTR and RNA binding protein including AUF-1, which is a PIN1 substrate (20, 21). PIN1 also regulates the turnover of substrates by modulating ubiquitin-dependent protein degradation (29, 32, 175, 176). Phosphorylation in Ser/Thr residues has been observed in human and mouse iNOS (127-130), which contains several Ser/Thr-Pro motifs and may be putative PIN1 substrates. Considering these significant results and the critical role of iNOS and COX-2 in vascular diseases, it is necessary to investigate whether, and how, PIN1 regulates iNOS and COX-2 expression in EC.
Calpain and Calpastatin (CAST)

In chapter 2 and 3, I describe that PIN1 depletion has no effect on iNOS or COX-2 mRNA. Instead, depletion of PIN1, or calpain inhibitors increased the induction of iNOS and COX-2. Depletion of PIN1 also reduced calpain activity in MAEC (chapter 4). Given the potential role for calpain suggested by these studies, I now provide a review of calpain and its regulation.

Calpains are calcium-dependent neutral cysteine proteases. Some isoforms are widely expressed, whereas others are only expressed in specific tissues. In mammals, the two best-characterized members of the calpain family, μ-calpain and m-calpain are ubiquitously expressed and play crucial roles in many different physiological processes. Transient influx of calcium into cells can activate calpains locally (for example, those close to Ca2+ channels). The activated calpains play a physiological role by catalyzing the controlled proteolysis of its target proteins. Overactivation of μ- and m-calpain has been implicated in a number of pathologies associated with altered calcium homeostasis such as AD, and cataract formation, as well as secondary degeneration resulting from myocardial ischemia, cerebral ischemia, traumatic brain injury and spinal cord injury (177). Calcium overload excessively activated calpain in these cases,
which leads to unregulated proteolysis of target protein with consequent irreversible tissue damages.

So far, more than one hundred proteins have been identified as calpain substrates. Several structural proteins such as spectrin (178) and talin (179) can be cleaved by calpains, indicating the important role of calpains in remodeling the cytoskeletal structures and regulating cell spreading and mobility. Functional cleavage of kinases (protein kinase C (180), focal adhesion kinase (181) and GSK-3 (182)), phosphatases (183) and receptors (β-integrin (184), and epidermal growth factor receptor (185) and androgen receptor (186)) allows calpain to regulate signal transduction. Calpains may also regulate gene expression by cleavage of several transcription factors, such as c-Jun, c-Fos (187, 188), c-myc (189, 190), β-catenin (191, 192), NF-kB (193) and p53 (194). Calpain can also trigger apoptosis by acting on Bcl-2 proteins (195).

These substrates may contain one or several calpain cutting sites and undergo limited proteolysis by calpains, generating protein fragments with different functions. For example, m-calpain cleaved GSK-3 at the N-terminus and generated two fragments of 40 kDa and 30kDa. Such truncation increased GSK-
3 activity because of the removal of N-terminal inhibitory domain (182). Calpain cleaved β-catenin at the N-terminus in neurons and the truncated fragments lost the sensitivity to GSK-3β-directed proteolysis and became more stable (196). Tompa’s study showed calpain substrates have high frequency of Ser/Thr-Pro motifs (197), which is the consensus site of proline directed kinases and the phosphorylated forms may associate with PIN1. It is known that phosphorylation in Ser/Thr-Pro motifs is involved in the susceptibility to proteases (12, 198). Thus, it is necessary to investigate whether PIN1-catalyzed isomerization after phosphorylation regulates the susceptibility of substrates to calpains.

**Domain structure**

µ-calpain is a heterodimer of calpain 1 and 4, while m-calpain is a heterodimer of calpain 2 and 4. Several domains are contained in calpain 1 and 2: 1) a NH2-terminal sequence; 2) the active domain IIA and IIB; 3) domain III; 4) domain IV. The amino-terminal sequence may interact with small subunit and be involved in calpain stability. Domain IIA and IIB contain a catalytic triad formed by Cys-His-Asn. Domain III is also involved in binding to phospholipids (199). Calpain 4, the common small subunit in µ and m, contains two functional domains: a NH2-terminal hydrophobic domain V and C-terminal domain VI. Both Domain IV from calpain 1/2 and Domain VI from calpain 4 contain penta-EF-hand (PEF) domains.
The heterodimeric formation between the two subunits of µ- and m-calpain is through a pairing of the fifth EF-hand of Domain IV and Domain VI.

In the absence of calcium, µ- and m-calpain are inactive since Cys residue is distant from His and Asn residues within the active site. This crystal structure reveals that binding of calcium to domain IV in calpain 1/2 and domain VI in calpain 4 induces conformational changes, producing a more compact structure. Critical residues in domain IIa and IIb can move together to form a catalytically active complex (200).

Fig 1.5 Domain structure of µ- and m-calpain.
Regulation of calpain activity

Calpain activity is regulated at multiple levels: expression of the subunits (201, 202), calcium requirement (203), phosphorylation (204, 205) and, most importantly, by the endogenous inhibitor, calpastatin (CAST) (201-209).

Expression of calpains

The 5'-upstream region of both large and small subunit gene lacks a TATA or CAAT box sequences and is GC rich. As mentioned earlier, this is a feature of housekeeping genes. However, recent studies showed that calpain 2 can also be induced by different cellular stimuli. For example, the COX-2 selective inhibitor, NS-398, decreased mRNA level of calpain 2 in IEC-6 cells (202). The mRNA level of calpain 2, but not calpain 1, can be upregulated by treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) in HeLa cells (210) or by eccentric exercise in skeletal muscles (201).
Calcium requirement

The physiological concentration of calcium in living cells is 50-500 nM (206, 211, 212), which is sufficient to activate μ-calpain, but much less than the 400-800 μM required to activate m-calpain in vitro. These calcium concentrations may not apply to calpains in vivo since biomolecules in cell that interact with calpains can reduce their calcium requirement. For example, certain phospholipids including phosphatidylinositol 4,5-bisphosphate (PIP2) lower the calcium concentration required for activation of μ- and m-calpain (213, 214). Similarly, protein-protein interactions, such as acyl-CoA-binding proteins, may also change the calcium requirement of calpains (215, 216).

Phosphorylation

Both calpain 1 and calpain 2 can be phosphorylated at Ser, Thr and Tyr residues. The phosphorylated residues are mainly located in two areas: at the domain I/II junction (IIa) and at the N-terminus of domain II (IIb) (206). Phosphorylation at several sites controls the activity of calpain. For example, m-calpain is activated by phosphorylation of calpain 2 at Ser50 by ERK during migration of fibroblasts and in keratinocytes stimulated with EGF (204, 205, 217-220). Calpain activity can also be inhibited by phosphorylation. Phosphorylation of calpain 2 by protein
kinase A reverts m-calpain to an inactive conformation and blocks EGF-induced activation of m-calpain during fibroblast migration (205). Activated protein kinase C iota (PKCiota) by nicotine induces phosphorylation of both µ- and m-calpains and is involved in accelerated migration and invasion of human lung cancer cells (221).

CAST

The activity of calpains is tightly controlled by the endogenous inhibitor CAST. CAST was originally identified when no calpain activity could be detected in crude porcine skeletal muscle homogenates until precipitation of calpain, indicating its efficiency in inhibiting the heterodimeric calpains (222). CAST is an intrinsically unstructured protein, which is converted into a defined structure only on binding to active calpain in the presence of calcium (200). CAST contains an N-terminal non-inhibitory leader L domain and four conserved inhibitory domain I-IV. Although the L domain is not inhibitory, it binds to calcium-free calpain, and may affect the distribution of calpains before activation (223). Each inhibitory domain is able to efficiently and specifically interact with one calpain molecule and block its activity although the affinity and inhibitory efficiency may be different (222). The specificity of inhibition of heterodimeric µ- and m-calpain and not other
cysteine protease is determined by the unique interaction between CAST and calcium-bound calpain. Two recent crystallographic studies demonstrated how CAST blocks the activity of the calpain by using a previously undiscovered mechanism. Three subdomains, A, B, and C are contained in each inhibitory domain of CAST. Subdomain A and C is response to binds the first EF-hand sequence of Domain IV (calpain 1/2) and Domain VI (calpain 4) respectively, which increase the affinity of interaction and the inhibitory activity of subdomain B. B contacts DI-DIII region of calpain 1/2 and blocks the active site. The highly conserved sequence of B generates a well-organized local structural conformation, which allows it wrap to around calpain’s active site and escape from the cleavage by calpains (200, 224, 225).

Fig. 1.6 Domain structure of CAST
Calpains are a major ubiquitous cellular proteolytic system. Disruption of the calpain–CAST balance occurs in several pathological conditions where calpain is over-activated. These diseases include AD, cancer, cataract formation, traumatic...
brain and spinal cord injury, ischemia/reperfusion injury, and atherosclerosis (177). Thus maintaining the balance by increasing the expression of CAST protein or its inhibitory activity is crucial to protect from pathological disorders associated with excessive calpain activity.

Deregulations of both PIN1 and the calpain system have each been implicated in certain human diseases. PIN1 may negatively correlate with calpain activity in human diseases, including AD and spinal cord injury. For example, deletion of PIN1 and enhanced calpain activity contributes to pathological process of AD (8, 28, 37, 226). PIN1 binds and stabilizes phospho-MCL-1, thus protecting oligodendrocytes from apoptosis after spinal cord injury (37). Upregulation of calpain, has been implicated in apoptosis and tissue degeneration in spinal cord injury, while overexpression of CAST and calpain inhibitor prevented apoptosis in spinal cord injury (227-235). Collectively, PIN1 appears to protect, while calpain may contribute to such diseases. In early studies, I found that PIN1 deletion did not affect iNOS and COX-2 mRNA. Therefore, I investigated the effects of PIN1 on expression and turnover of iNOS and COX-2, and on calpain activity in MAEC.
CHAPTER 2
PIN1 REGULATES DEGRADATION OF iNOS
IN ENDOTHELIAL CELLS

Abstract
The peptidyl-proline isomerase Protein Never in Mitosis Gene A Interacting-1 (PIN1) increases the level or activity of several transcription factors that can induce iNOS. PIN1 can also regulate mRNA and protein turnover. Here, the effect of depletion of PIN1 on induction of iNOS by LPS/IFN-γ in murine aortic endothelial cells (MAEC) was determined. Suppression of PIN1 by 85% with small hairpin RNA enhanced the induction of NO and iNOS protein by LPS/IFN-γ. There was no effect on induction of iNOS mRNA, suggesting a posttranscriptional effect. The enhanced levels of iNOS protein were functionally significant since LPS/IFN-γ was cytotoxic to MAEC lacking PIN1 but not MAEC harboring an inactive control construct, and because cytotoxicity was blocked by the NO synthase inhibitor  \( N^\omega \)-nitro-L-arginine methyl ester. Consistent with posttranscriptional action, knockdown of PIN1 increased the stability of iNOS protein in cycloheximide-treated cells. Furthermore, loss of iNOS was blocked by the calpain inhibitor carbobenzoxy-valinyl-phenylalaninal but not by the selective
proteasome inhibitor epoxomicin. Immunoprecipitation indicated that PIN1 can interact with iNOS. Pull down of iNOS with a wild-type glutathione-S-transferase-PIN1 fusion protein, but not with a mutant of the amino terminal phospho-(serine/threonine)-proline binding WW domain of PIN1, indicated that this domain mediates interaction. The results suggest that PIN1 associates with iNOS and can limit its induction by facilitating calpain-mediated degradation in MAEC.

**Introduction**

*Cis-trans isomerization* about the bond between an amino acid and proline regulates protein conformation, interactions, and susceptibility to enzymes, including kinases, phosphatases, and proteases. Interconversion between the *cis* and *trans* forms is slow, and proline (P), preceded by phosphorylated (p) serine (S) or threonine (T), isomerizes even more slowly than unphosphorylated sequences. Protein Never in Mitosis Gene A Interacting-1 (PIN1) is the only known *cis-trans* peptidyl-prolyl isomerase that acts on p(Ser/Thr)-Pro (236, 237). PIN1 binds to p(S/T)-P via its amino terminal WW domain. It also catalyzes proline isomerization with its carboxyl terminal catalytic domain (237, 238).
The fundamental effect of PIN1 on protein conformation may account for its diverse roles in cell cycle progression, proliferation, and transcription (18, 239). PIN1 increases the activity of β-catenin, NF-κB, and AP-1. PIN1 also inhibits degradation of β-catenin and NF-κB p65, increasing their protein level and nuclear accumulation (18, 29, 30). These transcription factors are associated with induction of inflammatory gene products. PIN1 is also known to regulate degradation of other proteins and mRNA stability (8, 21, 240).

iNOS is one of the inflammatory genes activated by β-catenin, NF-κB, and AP-1 (63, 72, 107, 241, 242). After induction, iNOS can produce large amounts of NO from l-arginine in many cell types, including endothelial cells (80, 242, 243). Whether induction of iNOS is beneficial or pathogenic depends on additional factors, such as the concentration of l-arginine, cofactors, inhibitors, and oxidants (244-248). A role for PIN1 in the induction of iNOS has not been determined.

Although PIN1 regulates protein conformation and turnover of many of its identified substrates, its role in NO production has not been investigated. Since PIN1 regulates factors that could affect iNOS, the effect of PIN1 knockdown in murine aortic endothelial cells (MAEC) stimulated with *Escherichia coli* endotoxin LPS and IFN-γ was determined.
Materials and Methods

Endothelial cell growth supplement, heparin, phenylmethylsulfonyl fluoride, Bradford reagent, E. coli LPS, serotype 0111:B4, Nω-nitro-L-arginine methyl ester (L-NAME), and polybrene were purchased from Sigma Chemical (St. Louis, MO). Recombinant mouse IFN-γ was from R&D Systems (Minneapolis, MN). Carbobenzoxy-valinyl-phenylalaninal (zVF), also known as calpain inhibitor III or MDL-28170, PD150606, epoxomicin, and cycloheximide (CHX) were obtained from Calbiochem (La Jolla, CA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Tris-base, ethylenediamine tetraacetic acid, NaCl, Na3VO4, NaF, Tween 20, sodium dodecyl sulfate, ethidium bromide, and agarose were obtained from Fisher Scientific (Fair Lawn, NJ). Triton X-100 was purchased from Pierce (Rockford, IL). HEK293T cells were obtained from American Type Culture Collection (Manassas, VA). Silica columns were purchased from Quiagen (Valencia, CA). Fugene 6 transfection reagent was obtained from Roche (Indianapolis, IN). Glutathione-sepharose was purchased from Amersham Biosciences (Uppsala, Sweden). Quickchange mutagenesis and RNAMaxx in vitro transcription reagents were obtained from Stratagene (La Jolla, CA). Recombinant dicer was from Ambion (Austin, TX). Dulbecco's minimum essential medium, trypsin, isopropyl-β-d-thiogalactopyranoside, TRIzol,
Superscript Reverse Transcriptase, Platinum PFX and Taq DNA polymerases, RNAse-free DNAse, deoxynucleotides, recombinant protein G agarose, and plasmid pcDNA 3.1 CTGFP TOPO were purchased from Invitrogen (Carlsbad, CA). Plasmid pGEX4T-3 was from Pharmacia (Piscataway, NJ). Plasmids for lentivirus production were purchased from Addgene (www.addgene.org), where they were deposited by Drs. Robert Weinberg (pCMV-dR8.2 dvpr and pCMV-VSV-G; Whitehead Institute for Biomedical Research) and David Root (pLKO.1; Massachusetts Institute of Technology).

Anti-iNOS antibody was from Transduction Laboratories (Lexington, KY). Anti-PIN1 was purchased from R&D Systems (Minneapolis, MN). Anti-NFKB p65 antibody is from Rockland Immunochemicals (Gilbertsville, PA). Anti-glutathione-S transferase (GST) was prepared as described previously (249). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Renaissance Enhanced Chemiluminescence Reagent was purchased from New England Nuclear Life Sciences (Boston, MA). DETA NONOate was from Caymen Chemical (Ann Arbor, MI).
Cells

MAEC were cultured from aortas as described previously (243). The protocol for cell isolation was carried out under approval of the Ohio State University Animal Care and Use Committee.

Short hairpin RNA

The oligomer 5’-CCG GCC GGG TGT ACT ACT TCA ATC ACT CGA GTG ATT GAA GTA GTA CAC CCG GTT TTT G-3’ was designed from murine pin1 (Genbank accession NM 023371) to produce a hairpin structure (self-complimentary sequences underlined) with an intervening loop CTCGAG and targeting the 5’ underlined sequence in the PIN1 mRNA. This oligomer was annealed to a complimentary oligomer 5’-AAT TCA AAA ACC GGG TGT ACT ACT TCA ATC ACT CGA GTG ATT GAA GTA GTA CAC CCG G-3’ to produce AgeI- and EcoRI-compatible overhangs. The annealed DNA was ligated into AgeI- and EcoRI-restricted pLKO.1 vector with T4 ligase, adjacent to a U6 RNA polymerase promoter to drive transcription the shRNA (250), as described by Moffat et al (251). A mutated sequence was also produced as a control short hairpin RNA (shRNA).
Lentiviral particles were produced as described by Stewart et al. (252). In brief, a mixture of 6 µg pLKO.1 vector, 5 µg pCMV-dR8.2 dvpr, and 2 µg pCMV-VSV-G, complexed with Fugene 6 in Dulbecco's minimum essential medium, was added to HEK293T cells in a 75-cm² flask. Two days after cotransfection, the virus containing supernatant was collected and filtered (0.45 µm) to remove cell debris. The supernatant was stored at –80 °C until used to treat MAEC.

Stable PIN1 knockdown (KD) or control (C) shRNA cells were generated using described methods (253). MAEC were cultured for 24 h and then infected with 500 µl of lentiviral supernatants and 8 µg polybrene/ml. After 24 h the viral solution was removed and fresh medium was added. Cells were then grown with 9 µg puromycin/ml. After cell killing and regrowth, cells were transferred to a new flask with puromycin. This selection was repeated four times. The entire process was repeated with two additional MAEC preparations, resulting in three independent stable KD and control shRNA cell cultures.

Small inhibitory RNA

PIN1 small inhibitory RNA (siRNA) was generated by in vitro transcription from a double-stranded DNA template made by reverse transcription-polymerase chain reaction of MAEC RNA with platinum PFX polymerase and primers containing the T7 RNA polymerase promoter sequence at their 5' ends, as described
previously (254). PIN1 cDNA was amplified with sense primer 5'-GCG TAA TAC GAC TCA CTA TAG GGA GAA CGA GGA GAA GCT GCC ACC A and antisense primer 5'-GCG TAA TAC GAC TCA CTA TAG GGA GAT CTG TGC GCA GGA TGA TAT G based on Genbank accession number NM 023371 (underlined bases). A control sequence from green fluorescent protein was also produced as described previously (254). After in vitro transcription of the PCR products with T7 RNA polymerase, long double-stranded RNA was digested with recombinant Dicer. The siRNA was purified and MAEC were transfected by electroporation with 150 nM siRNA, as described (254). Treatments of these MAEC were initiated 24 h after transfection.

Treatments

MAEC were cultured in Dulbecco’s minimum essential medium-0.5% fetal bovine serum for 18 h and then treated with this medium alone, which contained 0.54 mM L-arginine, or this medium containing 10 µg LPS and 20 ng IFN-γ per milliliter, and other agents for various times. Where necessary, protein synthesis was inhibited with 90 µg cycloheximide/ml. Some cells were treated with DETA NONOate.
NO accumulation

Medium was collected, and nitrite, reflecting NO, in medium was measured by the Griess reaction, as described previously (243).

Cytotoxicity

To assess cell injury, MAEC were cultured in 96-well plates and treated as described. Cells were rinsed with PBS and fixed with 3% formaldehyde, and adherent cells were stained with 0.5% crystal violet, solubilized, and A590 measured (255).

Superoxide anion assay

Nitroblue tetrazolium (NBT) reduction assay is used to determine the production of superoxide anion (O2(-)) (256). In brief, KD and Control shRNA MAEC were treated with vehicle or LPS/IFN-γ for 23h, then treated with NBT (0.25 mg/ml) for 1h to allow the formation of NBT formazan deposits, a product of NBT-intracellular O2(-) reaction. Then cells were rinsed with PBS twice and methanol once to remove extracellular NBT. Intracellular NBT formazan deposits were dissolved by potassium hydroxide (2M) and dimethylsulfoxide and then measured its absorbance using a microplate reader at 620 nm.
mRNA levels

Cells were rinsed with phosphate-buffered saline and lysed with TRIzol reagent, and RNA was extracted and recovered by ethanol precipitation. RNA was dissolved in water, and its concentration was determined by absorbance of an aliquot at 260 nm. Three micrograms of RNA were reverse transcribed, and cDNA was subjected to polymerase chain reactions for murine iNOS, VCAM-1, and β-actin as described previously (80, 257). Fifty percent of each reaction was subjected to agarose electrophoresis and ethidium bromide staining. Gels were digitally photographed.

GST-PIN1 fusion proteins. Murine PIN1 was cloned by reverse transcription and polymerase chain reaction with Platinum PFX Polymerase followed by addition of Platinum Taq polymerase to add A to the 3’ ends of the expected 504 base reaction product. Sense (5’-GAA GAT GGC GGA CGA GGA GA-3’) and antisense (5’-CCT CAT TCT GTG CGC AGG A-3’) primers were designed from Genbank accession number NM 023371. A sample of the reaction was incubated with pcDNA 3.1 CTGFP TOPO as instructed by the manufacturer and then used to transform E. coli DH5α. Colonies were selected and clones confirmed by complete sequencing. The wild-type plasmid was used for polymerase chain reaction mutagenesis. Primers to generate the lysine-64 to alanine (K64A)
mutation were 5'-CTC ACA TCT GCT GGT GGC GCA CAG CCA GTC TCG G-3' and 5'-CCG AGA CTG GCT GTG CGC CAC CAG CAG ATG TGA G-3'. Primers to generate the tryptophan-33 to alanine (W33A) mutation were 5'-CAA CGC CAG CCA GGC GGA GCG GCC CAG C and 5'-GCT GGG CCG CTC CGC CTG GCT GGC GTT G-3'. After digestion of the wild-type template with DpnI and bacterial transformation, positive clones were selected and sequenced to confirm mutagenesis.

To produce GST fusions of these PIN1 proteins, wild-type W33A and K64A plasmids were used as templates in polymerase chain reactions with Platinum PFX polymerase, plasmid-specific sense primer, 5'-GAT CGG ATC CAT GGC GGA CGA GGA GAA GC-3' containing a BamHI restriction site, and antisense primer, 5'-GCA TGC CTG CTA TTG TCT-3', located 3' of the PIN1 stop codon in each plasmid. Each reaction product was digested with BamHI and NotI, isolated from an agarose gel, and ligated into pGEX-4T3 with T4 ligase in frame to the carboxyl terminal of the GST sequence. Ligation reactions were used to transform E. coli, and clones were selected and confirmed by sequencing. GST-PIN1 fusion proteins were produced from transformed E. coli BL21 cells induced with isopropyl-β-d-thiogalactopyranoside. Bacteria were sonicated in phosphate-buffered saline and extracted with 1% Triton X100, and debris was removed by
centrifugation (16,000 g x 10 min). The extract was incubated with glutathione-
sepharose, washed with 0.1 M Tris pH 7 and stored at 4°C (258).

Immunoprecipitation, GST pulldown, and Western blot analysis
Cells were washed with phosphate-buffered saline and lysed and sonicated in
lysis buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 250 mM NaCl, 5
mM ethylenediamine tetraacetic acid, 4 mM Na3VO4, 20 mM NaF, 1 mM
phenylarsine oxide, 30 µg/ml aprotinin, and 30 µg/ml leupeptin. Nuclei were first
isolated from some cells by centrifugation, as described previously (80), and
treated with lysis buffer before processing for Western blot analysis. Protein
concentration was determined by the method of Bradford (259).

For immunoprecipitation, 500 µg of cell lysate protein were incubated with 5 µg
anti-PIN1 antibody for 2 h at 4°C with rocking. Recombinant protein G agarose
was added, and samples were incubated for 1 h. For GST pulldowns, 500 µg of
MAEC lysate protein were precleared by incubation with glutathione-sepharose
at 4°C for 2 h. The supernatant was then incubated with the specific glutathione-
sepharose-GST-fusion proteins at 4°C for 2 h.
All samples were then centrifuged 16,000 g x 1 min, and the supernatant was discarded. The agarose or sepharose pellets were washed three times with lysis buffer by resuspension and centrifugation.

For Western blot analysis, 12 µg proteins were mixed with 20 µl denaturing sample buffer (62.5 mM Tris, pH 6.7, 10% glycerol, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 0.003% bromphenol blue). Rinsed agarose or sepharose pellets from immunoprecipitation or GST-pulldown were resuspended in 30 or 100 µl denaturing sample buffer, respectively. Samples were heated for 10 min at 95°C (260). Proteins were separated on 4–20% Tris-glycine polyacrylamide gels and transferred to nitrocellulose.

After transfer, the blots were blocked with 5% nonfat dry milk in Tris-buffered saline buffer and Tween 20 (0.1% Tween 20, 10 mM Tris, pH 7.5, and 150 mM NaCl) and probed with primary antibody. After blots were washed, appropriate horseradish peroxidase-conjugated secondary antibody was added and incubated for 1 h. Proteins were visualized by using enhanced chemiluminescent reagents, captured on X-ray film, and scanned to produce digital images.
Data analysis

Protein levels in Western blot analyses and polymerase chain reaction products in images were quantified with Image J 1.22d (NIH). Data were analyzed by Student's t-test or analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons.

Results

PIN1 targeting shRNA and siRNA efficiently knockdown PIN1 protein

PIN1 was reduced more than 85% with the specifically targeted PIN1 knockdown shRNA vector and not with the inactive control shRNA construct (Fig. 2.1A/B). PIN1 protein level in control shRNA cells was similar to that in primary MAEC, indicating that the control shRNA was inactive (Fig. 2.1B). PIN1 was also suppressed by transient transfection with a siRNA pool produced from a 538-bp reverse transcription/polymerase chain reaction product of PIN1 mRNA that was digested with recombinant Dicer, in comparison with control RNA produced from a green fluorescent protein cDNA (Fig. 2.1C).
A.

5’- ccggCCGGGTGTACTTTCAATCActc

KD

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Control

5’- ccggCCGGGTGTACTTCAATCActc

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B.

- PIN1

- α-tubulin

PIN1/α-tubulin Signal Ratio

KD  Control  None

Fig 2.1 Suppression of PIN1 in MAEC
Fig 2.1 continued

Fig 2.1 Suppression of PIN1 in MAEC. A: sequences encoding PIN1 knockdown (KD) short hairpin RNA (shRNA) and inactive control shRNA (control) in the lentiviral system. B: KD or control were used to generate stable MAEC clones as described in the MATERIALS AND METHODS. Proteins were extracted from KD shRNA, control shRNA, or uninfected MAEC (None), and Western blotted with antibodies specific for PIN1 and α-tubulin. C: PIN1 was also suppressed using an small inhibitor RNA (siRNA) pool produced by in vitro transcription of long
double-stranded RNA and digestion with recombinant Dicer. Representative blots are shown. Densitometric analysis of signal intensities of images was used to measured PIN1 expression. Bars represent means + SE of 3–4 independent cultures of each group. +p < 0.001 for comparison with between KD and control shRNA.

*PIN1 suppression increases iNOS induction in response to LPS and IFN-γ*

Previously, it was found that cotreatment with LPS and IFN-γ was required to induce iNOS in MAEC (80, 243). Here, the effect of PIN1 shRNA on iNOS expression was measured in KD and control shRNA cells stimulated with 10 µg LPS and 20 ng IFN-γ per ml. Western blot analysis demonstrated that iNOS was induced more in PIN1 KD than in control shRNA cells 24 h after treatment with LPS/IFN-γ (Fig. 2.2A). Similar results were obtained in two other independent pairs of cells selected for KD and control shRNA (data not shown). Suppression of PIN1 by transient transfection with siRNA also increased the induction of iNOS (Fig. 2.2B). Genetic deletion of PIN1 also increased iNOS induction, as shown in Fig. 2.2C. Reconstitution of PIN1 plasmid in KD cells decreased iNOS induction in response to LPS/IFN-γ (Fig. 2.2D). Therefore, induction of iNOS protein was increased by two different methods of PIN1 suppression and PIN1 genetic deletion.
Fig. 2.2 Effect of PIN1 knockdown or genetic deletion on iNOS continued
Fig. 2.2 Effect of PIN1 knockdown or genetic deletion on iNOS. KD and control shRNA MAEC (A), uninfected MAEC electroporated with diced control or PIN1
siRNA (B) or MAEC from PIN1 knockout (ko) and wildtype (wt) mice were treated with LPS and IFN-γ for 24 h. Representative Western blots are shown in C. Bars represent means ± SE ratio of iNOS/α-tubulin from densitometric analysis of three cultures of each group. +p < 0.05 for comparison between KD and control shRNA. D. 2 µg of empty vector (GF-2) and PIN1 plasmids were transfected in PIN1 KD MAEC. Cells were starved for 16h and treated with LPS/IFN-γ for 24h. Representative Western blots are shown.

Knockdown of PIN1 does not influence iNOS and VCAM-1 mRNA

Despite the increases in iNOS protein there was no difference in iNOS mRNA between KD and control shRNA cells from 1 to 48 h after treatment with LPS/IFN-γ (Fig. 2.3, A and B). LPS-IFN-γ also induced VCAM-1 mRNA as expected, and, as with iNOS, PIN1 depletion did not increase the levels of this message (Fig. 2.3C). Nuclear NF-κB p65, which may contribute to transcription of both iNOS and VCAM-1 mRNAs, rose slightly more rapidly in KD MAEC when compared with that of control, but the peak level was similar in KD and control shRNA cells (Fig. 2.3D). These results are consistent with post-transcriptional regulation of iNOS by PIN1 in MAEC.
Fig. 2.3 Effect of PIN1 knockdown on iNOS and VCAM-1 mRNA and NF-κB
Fig. 2.3 Effect of PIN1 knockdown on iNOS and VCAM-1 mRNA and NF-κB. KD and control (C) were treated with LPS/IFN-γ. Total RNA or nuclear protein was isolated at the indicated times. mRNAs encoding iNOS, VCAM-1, and β-actin were determined by reverse transcription-polymerase chain reaction and agarose
gel electrophoresis. A: representative images of iNOS and β-actin products in ethidium-stained gels. Quantitation of iNOS (B) and VCAM-1 (C) mRNA-to-β-actin signal ratios. Bars represent means + SE ratio of iNOS to β-actin mRNA from densitometric analysis of images from three independent cultures. *p < 0.05 for comparison with 0 h. +p < 0.05 for comparison between KD and control shRNA. Nuclear NF-κB p65 was assessed by Western blotting (D) and (E).

Knockdown of PIN1 increases nitric oxide production and causes NO synthase-dependent cytotoxicity

The Griess reaction demonstrated a low basal level of nitrite in medium of all unstimulated MAEC. LPS/IFN-γ caused significant elevation of nitrite in the medium in both KD and control shRNA cells after 48 h. However, the nitrite level was significantly higher in KD when compared with that of control shRNA MAEC (Fig. 2.4A). To determine the functional significance of the enhanced iNOS levels, KD and control shRNA cells were treated with 1 mM L-NAME, a general NOS inhibitor, and with LPS/IFN-γ. Cytotoxicity was measured by crystal violet staining of adherent cells remaining in culture after 48 h and 72h. LPS/IFN-γ caused no significant detachment of MAEC harboring the control construct that does not suppress PIN1 (Fig. 2.4B/C). In contrast, LPS/IFN-γ caused significant loss of KD
cells, which was prevented by L-NAME. Thus depletion of PIN1 enhanced the induction of iNOS by LPS/IFN-γ and promoted NO synthase-dependent cytotoxicity. Interestingly, DETA NONOate, an NO donor, induced similar detachment in both KD and control shRNA MAEC only at high millimolar concentrations (Fig. 2.4D), suggesting other factors were required in LPS/IFN-γ induced cell detachment. Superoxide anion can interact with NO to form more toxic agents. PIN1 KD contained significantly higher level of superoxide anion than that in control shRNA MAEC (Fig. 2.4E), indicating its possible involvement in cell detachment in response to LPS/IFN-γ.
Fig. 2.4 Effect of PIN1 knockdown on NO accumulation and cytotoxicity

continued
Fig. 2.4 continued

C.

D. continued
Fig. 2.4 continued

E.

Fig. 2.4 Effect of PIN1 knockdown on NO accumulation and cytotoxicity. A: KD and control MAEC were incubated in Dulbecco’s minimum essential medium-0.5% fetal bovine serum overnight and treated with vehicle or 10 µg LPS/ml and 20 ng IFN-γ/ml (L/I) for 48 h. The concentration of nitrite in cell culture supernatant was measured by the Griess reaction. Bars represent means + SE of 7 separate determinations. *p < 0.05 for comparison between vehicle and L/I-treated cells. +p < 0.05 for comparison between KD and control shRNA cells. PIN1 KD and control shRNA MAEC were pretreated without or with 1 mM L-
NAME for 1 h and then treated vehicle or LPS/IFN-γ for 48 h (B) and 72h (C), or treated with an NO donor, DETA NONOate, at various concentrations for 72h (D). After crystal violet staining was completed, the percent viability, with vehicle set to 100%, was determined. E. PIN1 KD and control shRNA MAEC were treated with vehicle or LPS/IFN-γ for 23h and were cultured in media containing NBT (0.25 mg/ml) for 1h. Superoxide anion generation was measured by NBT reduction assay as described in Material and Methods. Bars represent means + SE of of 6–8 separate determinations. *p < 0.05 for comparison between vehicle and L/I-treated cells. +p < 0.05 for comparison between KD and control shRNA cells.

**Knockdown of PIN1 stabilizes iNOS**

Since PIN1 knockdown did not affect the induction of iNOS mRNA, degradation of iNOS was investigated. KD and control shRNA cells were treated with 90 µg CHX/ml 24 h after induction with LPS/IFN-γ to block translation. Proteins were extracted 0, 8, and 24 h after the addition of CHX and subjected to Western blot analysis. The level of iNOS fell to less than 50% of the initial value by 8 h after CHX in the control shRNA cells. In KD cells, a similar decrease in iNOS was delayed until 24 h after CHX treatment (Fig. 2.5). The half-life of iNOS protein
was about 7 h in control shRNA cells, but it was prolonged to about 24 h in PIN1 KD MAEC.

Fig 2.5 Effect of PIN1 knockdown on iNOS protein stability. KD and control shRNA MAEC were treated with LPS and IFN-γ for 24 h. The protein synthesis
inhibitor cycloheximide (CHX) 90 µg/ml was added, and cell extracts were collected at the indicated times. A: iNOS and α-tubulin were measured by Western blot analysis. Representative blots are shown. B: iNOS and α-tubulin levels in each sample were determined by image analysis of Western blots. Ratios of iNOS to α-tubulin were determined, and the average ratio ± SE of 4 independent cultures for each point, as a percentage of the value at 0 h after CHX treatment, was calculated. The dashed line marks the 50% value. +p < 0.05 for comparison between KD and control shRNA at the indicated time.

*Calpain inhibition stabilizes iNOS in MAEC*

Others found that calpain degraded iNOS in RAW 264.7 cells and in vitro (137). Here, KD and control shRNA MAEC were treated with vehicle or the calpain inhibitor zVF (25 µM) for 1 h and then stimulated with LPS/IFN-γ for 24 h. zVF significantly increased induction of iNOS by LPS/IFN-γ in KD and control shRNA cells. Induction of iNOS was again greater in KD when compared with control shRNA cells (Fig. 2.6, A and B). The effect of zVF on degradation of iNOS was next examined by treating control shRNA MAEC with CHX 24 h after induction with LPS/IFN-γ. The drop in iNOS seen after 8 h in vehicle-treated cells was
prevented by zVF. The value only decreased to 59% of the starting value 24 h after CHX in cells exposed to zVF compared with 14% in cells incubated without the calpain inhibitor (Fig. 2.6C).

A.

<table>
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<tr>
<th>shRNA:</th>
<th>Vehicle</th>
<th>zVF</th>
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<tbody>
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<td>Control</td>
<td>KD</td>
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<tr>
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<td>α-Tubulin</td>
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Fig. 2.6 Effect of calpain inhibition on iNOS
Fig. 2.6 continued

B

Fig. 2.6 Effect of calpain inhibition on iNOS. A: PIN1 KD and control shRNA MAEC were pretreated with vehicle or zVF (25 µM) for 1 h and then treated with
LPS and IFN-γ for 24 h. Representative Western blot analyses of iNOS are shown. B: densitometric analysis of signal intensities of images from three samples per group for the ratio of iNOS to α-tubulin. Bars represent the means + SE of three independent cultures per group. *p < 0.05 for comparison between vehicle and zVF. +p < 0.05 for comparison between KD and control shRNA. C: control shRNA MAEC were treated as in A and then treated with CHX (90 µg/ml). Cell extracts were collected at the indicated times after CHX and analyzed by Western blot analysis for iNOS and α-tubulin. Ratios of iNOS to α-tubulin were determined. The mean percentage of the value at 0 h after CHX treatment ± SE of 2–4 independent cultures per group is shown. The dashed line marks the 50% value. +p = 0.018 and *p = 0.066 for one-tail comparison between vehicle and zVF at the indicated time.

_The proteasome does not contribute to iNOS degradation in MAEC_

The ubiquitin-proteasome pathway can target iNOS for degradation, and it affects iNOS transcription by degrading inhibitor of κB (IκB) α/β. The latter effect increases levels of active NF-κB, which can induce iNOS mRNA (131). When KD and control shRNA MAEC were initially treated with 2 µM epoxomicin, a selective
proteasome inhibitor (261), for 1 h and then stimulated with LPS/IFN-γ, induction of iNOS was prevented (Fig. 2.7A). As expected the blockage of iNOS induction by epoxomicin was associated with increased levels of IκBα and β in LPS/IFN-γ treated cells (Fig. 2.7B). IκBα/β levels were similar in KD and control shRNA MAEC. MAEC were then treated with LPS/IFN-γ for 24 h to induce iNOS, followed with epoxomicin and 90 µg CHX/ml for 0, 8, or 24 h. Epoxomicin had no effect on the loss of the induced iNOS protein over time after CHX in control shRNA MAEC (Fig. 2.7C).

A.

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Fig. 2.7 Effect of proteasome inhibition on iNOS. continued
Fig. 2.7 continued

**B**

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<tr>
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**C.**

![Graph](image13.png)

**Fig. 2.7** Effect of proteasome inhibition on iNOS. **A:** PIN1 KD and control MAEC were pretreated with vehicle or epoxomicin (EPO, 2 µM) for 1 h and then treated with LPS and IFN-γ for 24 h. Representative Western blot analyses of iNOS and
α-tubulin in KD and control shRNA. B: MAEC were pretreated with vehicle or epoxomicin for 1 h and then without or with LPS and IFN-γ (L/I) for 15 min. Extracts were analyzed by Western blot analysis using anti-IκBα/β antibody. C: control shRNA MAEC were treated LPS and IFN-γ for 24 h, then with epoxomicin for 1 h, and finally with CHX (90 µg/ml). Cell extracts were collected at the indicated times after CHX and analyzed by Western blot analysis for iNOS and α-tubulin. Ratios of iNOS to α-tubulin were determined. The mean percentage of the value at 0 h after CHX treatment ± SE of 2–4 independent cultures per group is shown.

**PIN1 interacts with iNOS**

The potential for interaction between iNOS and PIN1 was assessed. iNOS was coimmunoprecipitated with PIN1 in control shRNA MAEC treated with LPS/IFN-γ (Fig. 2.8A). To investigate requirements for PIN1 domains in interaction with iNOS, a WW domain mutant, W33A, which does not bind p(S/T)-P sites in substrates, and a peptidyl-prolyl isomerase catalytic mutant, K64A, were generated. Samples were incubated with glutathione-sepharose and GST, GST-PIN1, GST-W33A PIN1, or GST-K64A PIN1. GST failed to bind iNOS, but iNOS
was pulled down by GST-PIN1. GST-W33A PIN1 interacted poorly with iNOS, whereas the K64A catalytic mutant pulled down a detectable level of iNOS (Fig. 2.8B).

Fig. 2.8. PIN1 interacts with iNOS. A: control shRNA MAEC were treated with LPS/IFN-γ for 24 h. Cell extracts were immunoprecipitated without (None) or with
anti-PIN1 antibody (PIN1) and analyzed by Western blot analysis with anti-iNOS antibody. B: cell extracts were incubated with GST or GST-wild-type PIN1, -W33A or -K64A fusion proteins, and glutathione sepharose, and analyzed by Western blotting with anti-iNOS and anti-GST antibodies. Blots are representative of 3 independent cultures.

**DISCUSSION**

The role of PIN1 in the inflammatory response of endothelial cells has not been described. PIN1 is known to increase the level and/or activity of several transcription factors that mediate induction of iNOS (18, 30, 32, 72, 80, 107). It was expected that suppression of PIN1 would limit the induction of iNOS by LPS/IFN-γ in MAEC. However, depletion of PIN1 by 85% increased induction of iNOS protein, whereas increases in iNOS mRNA caused by LPS/IFN-γ were not affected (Fig. 2.3, A and B). Experiments in cytokine-treated PIN1 knockout murine embryonic fibroblasts and in unstimulated siRNA-treated cancer cells indicated that NF-κB activation of a reporter gene was reduced. In cytokine-stimulated knockout embryonic fibroblasts, NF-κB activity was impaired, probably as a result of increased proteasomal degradation of p65 in the complete absence
of PIN1 (18). Partial suppression of PIN1 in MAEC here did not affect the peak level of nuclear NF-κB p65, induction of endogenous iNOS mRNA, or of another p65-regulated mRNA, VCAM-1, in MAEC (Fig. 2.3). We speculate that different transcription factors and proteins may be significantly affected at different levels of PIN1, and the level of PIN1 required for expression, function, and turnover of different proteins may vary with cell type and stimulus. The results here demonstrate that PIN1 depletion with shRNA increased iNOS by a posttranscriptional mechanism in LPS/IFN-γ-treated MAEC.

Consistent with the increased level of iNOS, knockdown of PIN1 increased the accumulation of nitrite in the medium (Fig. 2.4A). The increases in NO production and/or iNOS were functionally significant since LPS/IFN-γ caused cell injury in KD MAEC, which was antagonized by a NO synthase inhibitor L-NAME (Fig. 2.4B/C). The loss of cell adherence is a delayed response, since none was seen at 24 h and was greater after 48 and 72 h. DETA NONOate, an NO donor, induced detachment only at high millimolar concentrations, and KD and control shRNA cells were equally susceptible (Fig. 2.4D). This suggests that additional factors are required for detachment induced by LPS/IFN-γ. For example, NO from iNOS may combine with superoxide from iNOS or other sources, or with other molecules, generating increased levels of agents more toxic than NO in KD cells.
(262-265). In fact, along with NO, superoxide was increased more in KD than control shRNA cells (Fig. 2.4E). Whereas other mediators of cell detachment and their sources remain to be determined, the results indicate that PIN1 suppression increased the induction of iNOS and NO synthase-dependent effects in MAEC.

Calpain and proteasomes degrade iNOS in various cell types (131, 134). Here, depletion of PIN1 stabilized iNOS protein in CHX-treated MAEC (Fig. 2.5). Furthermore, the calpain inhibitor zVF enhanced the induction of iNOS by LPS/IFN-γ and antagonized the loss of iNOS after inhibition of protein synthesis (Fig. 2.6). In contrast, the selective proteasome inhibitor epoxomicin (261) did not raise iNOS levels or slow its loss after CHX. Epoxomicin at 2 µM was effective against proteasome activity, since acute loss of IκB α/β caused by LPS/IFN-γ and induction of iNOS was partially blocked when it was applied before LPS/IFN-γ (Fig. 2.7). The degree of prevention of the loss of IκB, however, was apparently sufficient to prevent iNOS induction in epoxomicin-treated cells. The overall results indicate that depletion of PIN1 inhibited iNOS degradation and that degradation was mainly due to calpain, as opposed to proteasomal activity, in MAEC treated with LPS/IFN-γ.
iNOS was ubiquitinated in A549 and bronchial epithelial cells treated with cytokines, in RAW 264.7 cells treated with LPS-IFN-γ, and in HEK293 cells transfected with the human enzyme (131, 132). Degradation of iNOS was antagonized by proteasome inhibitors in HEK293 cells transfected with iNOS, and in RAW 264.7 cells and RT4 epithelial cells treated with LPS or a cytokine mixture for 48 h (131, 132). On the other hand, a glucocorticoid agonist reduced iNOS levels in IFN-γ-treated RAW 264.7 cells via calpain activation (134). Interestingly, exogenous treatment of HEK293 cells with the endogenous calpain inhibitor protein CAST (206) did not affect degradation of transfected iNOS (131). This suggests that proteasomes, but not calpain, contributed to turnover of transfected iNOS in HEK293 cells. Thus cell type, stimulus, level, or duration of induction, and other factors may affect the iNOS turnover path that operates in different systems.

In vitro proteolysis of iNOS by calpain I is modulated by protein interactions and conformation. Monomers of iNOS were more sensitive to calpain compared with dimers. Degradation was also antagonized by calmodulin, which associates with murine iNOS at a well-characterized site between amino acids 503 and 532 (136, 137). Conformation outside this domain is another factor since heating of an iNOS mutant, lacking the calmodulin-binding sequence, reduced its susceptibility
to calpain I, whereas degradation of wild-type iNOS was insensitive to heat treatment \((137)\). PIN1 may regulate iNOS conformation or protein interactions that affect degradation by calpain.

PIN1 might affect iNOS turnover by association with the enzyme, which is phosphorylated \((129)\), and contains several S/T-P motifs that could be WW ligands. iNOS was coimmunoprecipitated with PIN1 using an anti-PIN1 antibody (Fig. 2.8A). In vitro pulldowns with GST-PIN1 and domain mutants indicated that the WW domain was critical, whereas the K64A catalytic mutant was able to precipitate detectable iNOS (Fig. 2.8B). Interaction between PIN1 and iNOS could be required for effects of PIN1 on iNOS turnover. However, PIN1 could mediate, or regulate, the association of iNOS with itself or other cofactors, like calmodulin or heat shock protein 90 \((137, 266)\). PIN1 could also stimulate calpain or reduce inhibition by endogenous CAST \((206)\). These possibilities are under investigation.

PIN1 is known to affect the expression and function of its various substrates by many mechanisms \((236)\). The range of mechanisms include regulation of transcription, mRNA turnover, enzyme activity, sensitivity to phosphatases, and unbiquitination \((20, 21, 29, 30, 175, 267)\). The findings presented here suggest that PIN1 significantly affects iNOS protein turnover in MAEC that apparently rely
on calpain for its degradation. Thus PIN1 may play a significant role in NO synthase-mediated disease or drug actions depending on cell type and stimulus.

In conclusion, suppression of PIN1 increased the induction of iNOS without affecting the mRNA. Instead, PIN1 knockdown antagonized the degradation of iNOS in MAEC, which was sensitive to a calpain inhibitor but not a proteasome inhibitor. WW interactions with p(Ser/Thr)-Pro motifs and proline isomerization may control the sensitivity of iNOS to calpain or the function of the calpain/CAST system in MAEC.
CHAPTER 3
PIN1 REGULATES DEGRADATION OF COX-2
IN ENDOTHELIAL CELLS

Abstract
The peptidyl-proline isomerase, Protein Never in Mitosis Gene A Interacting-1 (PIN1), regulates turnover of inducible nitric oxide synthase (iNOS) in murine aortic endothelial cells (MAEC) stimulated with E. coli endotoxin (LPS) and interferon-γ (IFN-γ). Degradation of iNOS was reduced by a calpain inhibitor, suggesting that PIN1 may affect induction of other calpain-sensitive inflammatory proteins, such as cyclooxygenase (COX)-2, in MAEC. MAEC, transduced with lentivirus encoding an inactive control short hairpin (sh) RNA or one targeting PIN1 that reduced PIN1 by 85%, were used. Cells were treated with LPS/IFN-γ, calpain inhibitor (carbobenzoxy-valinyl-phenylalaninal, zVF), cycloheximide and COX inhibitors to determine the effect of PIN1 depletion on COX-2. LPS or IFN-γ alone did not induce COX-2. However, treatment with 10 µg LPS plus 20 ng IFN-γ per ml induced the COX-2 signal on western blots 10-fold in control shRNA MAEC. Induction was significantly greater (47-fold) in PIN1 shRNA cells. COX-2-dependent prostaglandin E2 production was also 3 times greater in cells lacking PIN1 compared to the control cells. The additional increase in COX-2 protein due
to PIN1 depletion was post-transcriptional, as induction of COX-2 mRNA by LPS/IFN-γ was the same in cells containing or lacking PIN1. Instead, the loss of COX-2 protein, after treatment with cycloheximide to block protein synthesis, was reduced in cells lacking PIN1 in comparison with control cells, indicating that degradation of the enzyme was reduced. zVF also enhanced the induction of COX-2 by LPS/IFN-γ, and slowed its loss in after treatment with cycloheximide. In contrast to iNOS, physical interaction between COX-2 and PIN1 was not detected, suggesting that PIN1 may regulate COX-2 degradation by affecting calpain, rather than by an effect on COX-2 protein. The results suggest that PIN1 can limit COX-2 induction by facilitating calpain-mediated degradation in MAEC.

**Introduction**

Protein Never in Mitosis Gene A Interacting-1 (PIN1) is an enzyme that regulates transcription, and turnover of mRNA and proteins. PIN1 is a cis-trans peptidyl-prolyl isomerase that binds to phosphorylated serine- or threonine-proline sequences in substrate proteins via an amino-terminal WW domain, and isomerizes the site with its carboxyl-terminal catalytic domain (8). Isomerization of the phosphorylated serine- or threonine-proline motif has a significant effect on
conformation of many phospho-proteins. The conformational switching catalyzed by PIN1 allows it to regulate transcription factors, mRNA stabilization factors, and the susceptibility of a growing list of proteins to post-translational modifications and proteases (8, 18, 20, 21, 29, 30, 268). Previously, we found that depletion of PIN1 and treatment with a calpain inhibitor each reduced the degradation of iNOS in MAEC stimulated with *E. coli* endotoxin LPS and IFN-γ. PIN1 bound to iNOS suggesting that it might directly regulate the sensitivity of iNOS to calpain (135). PIN1 may also regulate expression of inflammatory proteins by an effect on calpain.

COX-2 is induced by LPS, IFN-γ, and other factors in endothelial cells cultured from various organs and species (141, 269-275). Elevated endothelial COX-2 may contribute to vascular pathogenesis (276, 277). This enzyme is also significant for endotoxin action as COX-2 knockout mice are resistant to LPS-induced inflammation and death (278). COX-2 has a relatively short half-life, indicating that turnover may effectively control its expression (141). While COX-2 and iNOS can be degraded by several processes, calpain inhibitors are known to suppress their cleavage (135, 141, 163, 170, 279). Thus, the purpose of this investigation was to determine whether PIN1 regulates the expression of COX-2, which is induced by LPS and IFN-γ in MAEC. It was hypothesized that PIN1
would associate with COX-2 and that depletion of PIN1 would enhance its induction in MAEC. The impact of PIN1 depletion on calpain activity was also determined.

**Materials and Methods**

Endothelial cell growth supplement, heparin, phenylmethylsulfonyl fluoride, Bradford reagent, *E. coli* LPS, serotype 0111:B4, arachidonic acid and polybrene were obtained from Sigma Chemical Co. (St Louis, MO). Recombinant mouse IFN-γ was from R&D Systems (Minneapolis, MN). Cycloheximide, carbobenzoxy-valinyl-phenylalaninal (zVF, MDL-28170 or calpain inhibitor III), PD150606, porcine µ-calpain and carboxybenzyl-phenylalanine-arginine-7-amido-4-methylcoumarin were obtained from Calbiochem (La Jolla, CA). Fetal bovine serum was from Hyclone Laboratories (Logan, UT). Agarose, ethidium bromide, ethylenediamine tetraacetic acid, sodium dodecyl sulfate, NaCl, Na3VO4, NaF, tris-base and tween 20 were obtained from Fisher Scientific (Fair Lawn, NJ). Triton X-100 was from Pierce (Rockford, IL). Dulbecco’s minimum essential medium, trypsin, Trizol, Superscript Reverse Transcriptase Taq DNA polymerase, RNase-free DNase, deoxynucleotides, and protein G agarose were purchased from Invitrogen (Carlsbad, CA). Glutathione-sepharose was purchased from
Amersham Biosciences (Uppsala, Sweden). A prostaglandin E2 competition enzyme-linked immunosorbent assay kit was obtained from R and D Systems, Minneapolis, MN. Anti-COX-2 antibody directed against a 16 amino acid sequence, ending 7 residues from the C-terminus of the protein, SC-560 and NS-398 were purchased from Cayman Chemical (Ann Arbor, MI). Anti-PIN1 was from R&D Systems (Minneapolis, MN). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were from Jackson immunoresearch Laboratories, Inc. (West Grove, PA). Renaissance Enhanced Chemiluminescence Reagent was purchased from New England Nuclear Life Sciences (Boston, MA).

**Cells**

MAEC were cultured from aortas of mice in accordance with the Guide for the Care and Use of Laboratory Animals from the U.S. National Institutes of Health (280). As described previously, cells were transduced with short hairpin RNA (shRNA) to knockdown (KD) PIN1 or with an inactive mutant sequence (control), and selected for stable modification. This produced KD MAEC with approximately 15% of the level of PIN1 protein found in control and non-transduced MAEC (135).
Treatments

KD and control MAEC were incubated in Dulbecco’s minimum essential medium/0.5% fetal bovine serum for 18 h, and then treated with medium or 10 µg LPS and 20 ng IFN-γ per ml, and other agents for various times. zVF was added 1 h before LPS/IFN-γ to inhibit calpain (281). Ninety µg cycloheximide/ml was used to inhibit protein synthesis after induction of COX-2 with LPS/IFN-γ (135). COX-2-dependent prostaglandin E2 production was measured after incubating cells with LPS/IFN-γ for 24 h. Cells were then incubated in fresh medium containing 20 µM arachidonic acid, LPS/IFN-γ and the COX-1 selective antagonist, SC-560 (1 µM) (282), with or without the COX-2 selective antagonist, NS-398 (10 µM) (283). The medium was collected after 2 h and stored at -80 degrees C. Prostaglandin E2 was measured by competition enzyme-linked immunosorbent assay in comparison with prostaglandin E2 standard by the manufacturer’s instructions.

mRNA levels

RNA was extracted with Trizol, precipitated, and dissolved in water. cDNA was produced from 3 µg of RNA. cDNA was amplified by polymerase chain reaction for β-actin as described previously (284), and for COX-2. COX-2 primers were
sense, 5'-CCG GAC TGG ATT CTA TGG TG, and antisense, 5'-AGG AGA GGT TGG AGA AGG CT from Genbank accession BC052900, producing a 263 base pair product. Half of each reaction was electrophoresed in 1% agarose. Gels were imaged and analyzed after ethidium bromide staining.

*In vitro cleavage by calpain.*

After culture, KD and control cells were treated with LPS/IFN-γ for 24h, then were scraped in calpain assay buffer (50 mmol/L Hepes buffer (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% Triton X-100). Cell lysates were kept in ice for 20min and clarified by centrifugation at 16000g for 20min. In vitro cleavage by calpain was performed by incubating 44ul of cell soluble fraction with various amounts of purified porcine µ-calmain and CaCl2 (5mM) at 30 degree for 1h. The reaction was stopped by adding 2 × SDS sample buffer. COX-2 was detected by western blotting with anti-COX-2 antibody. COX-2 signal intensity was calculated as a percent of the value with no added calpain are shown.

*Immunoprecipitation, Glutathione S-transferase Pulldown, and Western Blotting*

As previously described (135), cells were washed, sonicated in lysis buffer, and protein concentration was measured. For western blotting, 12 µg of sample protein were denatured and separated on 4-20% Trisglycine, SDS-
polyacrylamide gels and transferred to nitrocellulose. For immunoprecipitation, 500 µg of cell lysate protein was incubated with 5 µg anti-PIN1 antibody and protein G agarose. For pulldown, glutathione S-transferase or glutathione S-transferase-PIN1 fusion protein was added to 500 µg of cell lysate protein and glutathione-sepharose. Samples were then denatured for electrophoresis and western blotting. Blots were immunostained and imaged on X-ray film by enhanced chemiluminescence. Films were scanned and digital images of proteins were analyzed.

*Cathepsin activity assay*

Confluent cells were washed three times with PBS and scraped in 1ml of ice-cold PBS. Cells were collected by centrifugation at 1500 x g for 2 min at 4 °C. Collected cells were resuspended in a reaction buffer (50 mM Na-acetate, 1 mM EDTA and 2 mM dithioerythritol pH 5.5), and were sonicated four times for 10 s with 1-min breaks. After sonication, the lysate was centrifuged at 1500 x g for 5 min at 4 °C to remove the cell debris, and the supernatant was used for cathepsin activity measurements. The reaction was started by mixing 20 µM cathepsin substrate carboxybenzyl-phenylalanine-arginine-7-amido-4-methylcoumarin, in the reaction containing 2 µg supernatant. 7-amido-4-methylcoumarin release was monitored at 37°C for 30 min by fluorescence, with
excitation at 380 nm and emission at 460 nm, and the initial velocity was determined (285, 286).

**Data Analysis**

Bands in images of polymerase chain reaction gels and scanned western blots were measured with Image J 1.34s (NIH). Prostaglandin E2 concentrations were estimated from a standard curve and calpain activity was indicated by the fluorescence increase per minute. Data were analyzed by Student’s t test or analysis of variance with Bonferroni correction for multiple comparisons (287).
**Results**

*PIN1 suppression or genetic deletion increases COX-2 induction in response to LPS and IFN-γ.*

First, COX-2 induction in control MAEC was tested when exposed to vehicle, LPS (10 µg/mL), IFN-γ (20 ng/mL) and combination of LPS/IFN-γ for 24h. Similarly to iNOS, COX-2 protein was very low in vehicle-treated control MAEC and incubation with either LPS or IFN-γ alone did not induce it. However, stimulation with 10 µg LPS plus 20 ng IFN-γ per ml for 24 h dramatically increased induction of COX-2 protein in control shRNA MAEC (Fig. 3.1A). COX-2 was induced more in PIN1 KD than in control shRNA cells 24 h after treatment with LPS/IFN-γ (Fig. 3.1B). However, no significant difference of COX-1 signal was detected in either vehicle or LPS/IFN-γ treatment (Fig. 3.1B). Genetic deletion of PIN1 also increased the induction of COX-2 (Fig. 3.1D). Therefore, induction of COX-2 protein was increased by both PIN1 suppression and deletion. COX-2- mediated prostaglandin E2 production increased 3-fold in KD MAEC, but not in the control cells (Fig 3.1E).
Fig. 3.1 Effect of PIN1 knockdown on COX-2 protein and activity

continued
Fig. 3.1 continued

C.

D.
Fig. 3.1 Effect of PIN1 knockdown on COX-2 protein and activity. A. control (C) shRNA MAEC were treated with vehicle (culture medium), LPS, IFN-γ or LPS and IFN-γ (L/I) for 24 h. Representative western blots and analysis of COX-2 expression are shown. B. KD and control (C) shRNA MAEC were treated with LPS and IFN-γ (L/I) for 24 h and representative western blots of COX-2 and COX-1 expression are shown in B and analysis of COX-2 and COX-1 expression are shown in C. D. MAEC from PIN1 knockout (ko) and wildtype (wt) mice were treated with LPS and IFN-γ (L/I) for 24 h. In C and D, bars represent mean ± SE
ratio of COX-2/α-tubulin from densitometric analysis of 3 cultures of each group. +, p<0.05 for comparison between KD and control cells or between knockout and wildtype cells treated in the same manner. E. COX-2-dependent prostaglandin E2 production was measured in cells treated with LPS/IFN-γ, 20 µM arachidonic acid and 1 µM SC-560, with or without 10 µM NS-398. Bars represent mean + SE concentration of prostaglandin E2 in medium for 5 cultures in each group. +, p<0.05 for between KD and control cells treated in the same way.

Knockdown of PIN1 does not influence COX-2 mRNA

LPS/IFN-γ induced COX-2 mRNA in KD and control shRNA cells were measured by RT-PCR. Despite the increases in iNOS protein, there was no difference in COX-2 mRNA between KD and control shRNA cells at various periods after treatment with LPS/IFN-γ (Fig. 3.2A/B), suggesting post-transcriptional regulation of COX-2 by PIN1 in MAEC.
Fig. 3.2 Effect of PIN1 knockdown on COX-2 mRNA. KD and control (C) shRNA MAEC were treated with LPS and IFN-γ (L/I) for 0-8 h. mRNAs encoding COX-2 and β-actin were determined by RTPCR and agarose gel electrophoresis. A representative image of COX-2 and β-actin PCR products from a single ethidium-
stained gel is shown. Bars represent mean + S.E. ratio of COX-2: β-actin products from densitometric analysis of images from 3 independent cultures run in a single gel. *:p<0.05 for comparison with cells treated for 0 h.

**Knockdown of PIN1 stabilizes COX-2**

Since PIN1 knockdown did not affect the induction of COX-2 mRNA, the effect of PIN1 depletion on degradation of COX-2 after induction with LPS/IFN-γ was assessed. Cells were induced with LPS/IFN-γ and then treated with 90 µg cycloheximide/ml to block translation. Proteins were extracted 0, 2, and 4 h after the addition of CHX and subjected to Western blot analysis. The level of 72 kDa COX-2 protein fell to less than 50% of the initial value by 2 h after addition of cycloheximide to control shRNA cells. A decrease in COX-2 by approximately 50% was delayed until 4 h after CHX treatment in KD MAEC (Fig. 3.3)
Fig. 3.3 Effect of PIN1 knockdown on COX-2 stability. KD and Control shRNA MAEC were treated with LPS and IFN-γ for 24 h. Cycloheximide (90 µg/ml) was added, and cell extracts were collected at the indicated times and western blotted for COX-2 and α-tubulin. The average COX-2/α-tubulin signal intensity ratio + SE of 4 independent cultures for each point, as a percent of the value at 0
h after cycloheximide treatment is shown. The dashed line marks the 50% value. +, p<0.05 for comparison between similarly treated KD and control cells at the indicated time.

Calpain inhibition stabilizes COX-2 in MAEC

Similarly to iNOS, COX-2 is also known as a substrate of calpains (170, 171). In human synovial fibroblasts, cleavage of COX-2 was reduced by E64d indicating a role for calpain and/or cathepsin. Thus, effects of calpain inhibition on COX-2 degradation were measured. PIN1 KD and control shRNA MAEC were pretreated with vehicle and different doses of a calpain inhibitor, zVF for 1 h, and then treated with LPS and IFN-γ for 24 h. zVF increased the induction of the 72 kDa COX-2 protein in both KD and control cells in a dose dependent manner (Fig. 3.4 A). Induction of COX-2 was greater in KD when compared with control shRNA cells (Fig. 3.4B). Similar results were also observed by the pretreatment of a calpain/cathepsin inhibitor, E64d (Fig. 3.4C). Calpain can regulate certain gene transcriptions, for example, eNOS in pulmonary artery endothelial cells (288), which may be due to its action on transcription factor substrates, such as β-catenin, c-Fos, c-Jun and p53. Thus, the effect of calpain inhibition on COX-2...
transcription was examined here. Such an increased induction of COX-2 by calpain inhibition was not due to transcription, since zVF pretreatment did not affect induction of COX-2 mRNA in the MAEC (Fig. 3.4D). The effect of zVF on degradation of COX-2 was next examined by treating control shRNA MAEC with CHX 24 h after induction with LPS-IFN-γ. zVF reduced the degradation of COX-2 in control MAEC by an amount similar to PIN1 KD shRNA. COX-2 protein decreased to 63% of its initial value after 4 h in zVF-treated control MAEC, and to 61% in zVF-treated KD MAEC (Fig. 3.4E).

Fig. 3.4. Effect of calpain inhibition on COX-2 protein

A.
Fig. 3.4 continued

B.

| shRNA: KD | Treatment: Vehicle | zVF | Control |
| COX-2 | | |
| α-Tubulin | | |

| KD | Vehicle | zVF |
| COX-2 | | |
| α-Tubulin | | |

C.

| KD | Vehicle | E64d | Control | Vehicle | E64d |
| COX-2 | | |
| α-Tubulin | | |

continued
Fig. 3.4 continued

D.

<table>
<thead>
<tr>
<th>Treatment:</th>
<th>Vehicle</th>
<th>zVF</th>
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<tbody>
<tr>
<td>shRNA:</td>
<td>KD</td>
<td>C</td>
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<td>KD</td>
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COX-2

β-Actin

COX-2/β-actin mRNA Signal Intensity Ratio

KD
Control

Vehicle  zVF

Continued
Fig. 3.4 continued

E.

Fig. 3.4 Effect of calpain inhibition on COX-2 protein. A: PIN1 KD and control (C) shRNA MAEC were treated with vehicle, different doses of zVF for 1 h, and then treated with LPS and IFN-γ for 24 h. B. Bars represent mean + SE ratio of COX-2 to α-tubulin from densitometric analysis of images from 3 independent cultures in
each group pretreated with vehicle or 25 µM zVF. *: p <0.05 for comparison between vehicle and zVF. +, p <0.05 for comparison between KD and control shRNA. 

C. Representative western blots of COX-2 and α-tubulin were shown when KD and control MAEC were treated with vehicle or 6 µM E64d for 1h and followed by LPS/IFN-γ for 24h. 

D. Effect of zVF on COX-2 mRNA. KD and control (C) shRNA MAEC were treated with vehicle (DMSO) or 25 µM zVF for 1 h, then with LPS/IFN-γ for 1 h. mRNA for COX-2 and β-actin were assessed as in figure 3. Representative agarose electrophoresis of PCR products is shown. Bars represent the mean + SE of ratio of COX-2/β-actin signal intensity + SE of 4 independent cultures. 

E. KD and control shRNA MAEC were treated with 25 µM zVF for 1 h, then with LPS and IFN-γ for 24 h. Cycloheximide (90 µg/ml) was added, and cell extracts were collected at the indicated times and western blotted for COX-2 and α-tubulin. The average COX-2/α-tubulin signal intensity ratio + SE of 4 independent cultures for each point, as a percent of the value at 0 h after cycloheximide treatment is shown. The dashed line marks the 50% value.

**COX-2 is cleaved by purified porcine µ-calpain in vitro**

The above results indicate that calpain contributes the degradation of COX-2 in MAEC. Thus, purified calpain was used to test whether COX-2 was an in vitro substrate for calpain. The incubation of COX-2 with purified porcine µ-calpain
resulted in the loss of COX-2 in a dose dependent manner (Fig. 3.5). COX-2 from control extracts appeared to more susceptible to porcine µ-calpain than that from KD, which may be due to different PIN1 level in KD and control shRNA cells.

Fig. 3.5 Degradation of COX-2 by purified porcine µ-calpain in vitro. PIN1 KD and control shRNA MAEC were treated with LPS/IFN-γ for 24 h. Cell extracts from
KD and control were collected and incubated with various amounts of purified porcine \( \mu \)-calpain for 1h in the presence of \( \text{Ca}^{2+} \) (5 mM). COX-2 was detected by western blotting with anti-COX-2 antibody. Representative western blots of COX-2 and \( \alpha \)-tubulin are shown. The average COX-2 signal intensity + SE of 3 independent reactions for each point, as a percent of the value with no added calpain are shown. +, p <0.05 for comparison between KD and C.

*Calpain, but not cathepsin, contributes to PIN1 regulated COX-2 degradation in MAEC*

It was known that E64d and zVF also inhibit cathepsins. Therefore, it is essential to determine whether cathepsins contribute to PIN1 regulated COX-2 degradation. A more specific calpain inhibitor, PD150606 is able to increase COX-2 induction in both KD and control cells in a dose dependent manner (Fig. 3.6A). Also cathepsin activity was assayed by using fluorescent substrate Z-Phe-Arg-AMC in KD and control MAEC. No significant difference was observed between KD and control in either vehicle or LPS/IFN-\( \gamma \) treatment group, suggesting that PIN1 regulates COX-2 degradation mainly via calpain but not cathepsins (Fig. 3.6B).
Fig. 3.6 Cathepsin does not contribute to PIN1-regulated COX-2 degradation.

A. PIN1 KD and control shRNA MAEC were treated with vehicle, different doses of calpain inhibitor, PD150606, for 1 h, and then treated with LPS and IFN-γ (L/I)
for 24 h. Representative western blots of COX-2 and α-tubulin are shown. B.
Effect of PIN1 knockdown on cathepsin activity. Initial rate of cleavage of the
fluorogenic calpain substrate was measured in extracts of KD and control cells
treated with LPS/IFN-γ for 24 h. Bars represent mean + SE fluorescence
increase/min for 4 independent cultures in each group.

PIN1 does not interact with COX-2

Since PIN1 is known to bind its substrate proteins, its interaction with COX-2 was
investigated. COX-2 from extracts of LPS/IFN-γ -treated control cells was not
pulled down with glutathione-S-transferase-PIN1 fusion protein or glutathione-S-
transferase. Immunoprecipitation of PIN1 from extracts of LPS/IFN-γ -treated
control cells did not produce any COX-2 detectable on western blots (not shown).
Therefore, PIN1 may regulate COX-2 degradation via affecting calpain activity.
Fig. 3.7 PIN1 does not interact with COX-2 in MAEC. Control shRNA MAEC were treated with LPS-IFN-γ for 24 h. Cell extracts were incubated with GST or GST-wild-type PIN1 fusion proteins, and glutathione sepharose, and analyzed by Western blotting with anti-COX-2 and anti-GST antibodies.

*Proteasome contributes to COX-2 degradation but is not regulated by PIN1 in MAEC*

COX-2 can also undergoes ubiquitination and proteolysis by 26S proteasome (160-167). Pretreatment of a selective proteasome inhibitor, epoxomicin (2µM)
inhibited degradation of IκB α/β, blocked activation of NF-κB and prevent iNOS induction (135). Differently from iNOS, COX-2 induction was not prevented but increased by epoxomicin pretreatment (Fig. 3.8A). Epoxomicin does not affect COX-2 mRNA level either, suggesting no involvement of NF-κB in COX-2 transcription in MAEC (Fig. 3.8B). Effects of proteasome inhibition on COX-2 degradation was also examined. COX-2 degradation was severely reduced by epoxomicin in both control and KD MAEC (Fig. 3.8B). After 4 h, the level of COX-2 was unchanged in control shRNA cells treated with epoxomicin, compared with a drop to 31% of its initial level in the absence of epoxomicin. COX-2 decreased only to 90% of its initial level in epoxomicin-treated KD MAEC, compared with the decrease to 47% of the initial value in vehicle-treated KD cells (Fig. 3.8C).
Fig. 3.8 Effect of proteasome inhibition on COX-2 mRNA and protein

continued
Fig. 3.8 continued

B.

![Image of gel blots showing COX2 and β-Actin signal intensity ratios](image)

![Graph showing COX2/β-Actin signal intensity ratio](graph)

continued
Fig. 3.8 continued

C.

**Fig. 3.8 Effect of proteasome inhibition on COX-2 mRNA and protein.** A. PIN1 KD and control (C) shRNA MAEC were pretreated with vehicle or a proteasome...
inhibitor EPO (2 µM) for 1 h, and then treated with LPS and IFN-γ for 24 h. COX-2 and α-tubulin are shown and analyzed. Bars represent mean + SE ratio of COX-2 to α-tubulin from densitometric analysis of images from 3 independent cultures in each group. *: p <0.05 for comparison between 0 and 24 h. +, p <0.05 for comparison between KD and control shRNA. B. mRNA for COX-2 and β-actin were assessed. Representative agarose electrophoresis of PCR products is shown. Bars represent the mean + SE of ratio of COX-2/β-actin signal intensity + SE of 4 independent cultures. C. KD and control shRNA MAEC were treated with 2 µM EPO for 1 h, then with LPS and IFN-γ for 24 h. Cycloheximide (90 µg/ml) was added, and cell extracts were collected at the indicated times and western blotted for COX-2 and α-tubulin. The average COX-2/α-tubulin signal intensity ratio + SE of 4 independent cultures for each point, as a percent of the value at 0 h after cycloheximide treatment is shown. The dashed line marks the 50% value.

*Calpain and proteasome differently contribute COX-2 degradation*

Epoxomicin also increased the levels of several bands smaller than 72 kDa COX-2 in LPS/IFN-γ treated cells (Fig. 3.9, zones II-IV). The pattern of these C-terminal immunoreactive bands was identical in control and KD shRNA cells.
treated with epoxomicin or the combination of epoxomicin and zVF. zVF or E64d alone caused the accumulation of several C-terminal COX-2 fragments smaller than 72 kDa that were of different sizes than the bands in epoxomicin-treated cells. These bands in zones II and III also appeared identical in vehicle-, zVF- and E64d-treated KD cells. Although lower in intensity, bands visible in zones II and III in control cells treated with zVF or E64d also appeared similar to bands seen in KD cells treated with vehicle-, zVF- or E64d. The results with zVF and epoxomicin, alone and in combination, were replicated in 3 independently treated cultures of KD and control cells for each condition (not shown). Thus, fragments of COX-2 that accumulated in KD cell extracts were qualitatively similar to those from cells treated with calpain inhibitors, and different from those seen after epoxomicin.
Fig. 3.9 Effect of PIN1 knockdown, and calpain and proteasome inhibition on COX-2 fragments. KD and control shRNA MAEC were treated for 24 h with LPS/IFN-γ and Vehicle, zVF (zVF, 25 µM), E64d (6 or 25 µM), epoxomicin (EPO, 2 µM), or EPO plus zVF. Samples were processed for western blotting for COX-2. The blots were immunostained and developed together in the same reagents. The 72 kDa full length COX-2 is marked with an arrow. Regions I, II III and IV are marked for discussion in the text.
**Discussion**

PIN1 regulates the levels and activity of factors that can affect COX-2 synthesis in various cell types\(^1\). Here, suppression of PIN1 in endothelial cells did not affect COX-1 but increased the induction of COX-2, and COX-2-dependent production of prostaglandin E2 by LPS/IFN-γ. Despite a nearly 5-fold greater induction of COX-2 protein in KD compared with control MAEC, there was no difference in the induction of COX-2 mRNA. This suggests that PIN1 regulates COX-2 by a post-transcriptional mechanism. Previously, it was found that PIN1 knockdown reduced calpain-mediated degradation of iNOS in MAEC. As with iNOS, the loss of 72 kDa COX-2 in cycloheximide-treated MAEC was reduced by PIN1 depletion, consistent with post-transcriptional action. Results from cycloheximide experiments showed that the half life of COX-2 was 4h and 2h in KD and control MAEC respectively.

Since COX-2 has a relatively short half-life, inhibition of turnover could lead to large, cumulative, post-transcriptional increases after induction with LPS/IFN-γ\(^1\). Others found that cleavage of COX-2 was reduced by the calpain inhibitor, E64d, in human synovial fibroblasts indicating a role for calpain and/or cathepsin\(^1\). In agreement with this, induction of 72 kDa COX-2 in KD and control

135
MAEC was increased by E64d, and by the calpain inhibitor, zVF. E64d and zVF may also inhibit cathepsin, which appears not to contribute PIN1-regulated COX-2 degradation since no significant difference in cathepsin activity was observed in KD and control in basal and LPS/IFN-γ stimulated condition. An even more selective calpain inhibitor PD150606 was able to increase the induction of COX-2 efficiently, suggesting the essential role of calpain but not cathepsins in COX-2 degradation. Calpain inhibition also reduced the rate of loss of 72 kDa COX-2 in cycloheximide-treated control cells to a level similar to that in KD MAEC. These results suggest that PIN1 depletion reduces calpain-mediated turnover of COX-2. Consistent with this idea, zVF and PIN1 KD caused the appearance of similar C-terminal COX-2 fragments smaller than 72 kDa on western blots, using a COX-2 antibody directed to the C-terminal of COX-2. E64d also induced the accumulation of these COX-2 fragments, further suggesting that they are a consequence of inhibiting calpain. However, a simple interpretation is that inhibition of calpain by zVF and E64d shifted processing of COX-2 to enzymes other than calpain, and that depletion of PIN1 had a similar effect.

COX-2 is also a substrate for proteasomes and PIN1 regulates proteasomal degradation of several other proteins (cyclin E, c-myc and p65). Here, inhibition of proteasomal activity with epoxomicin increased induction of COX-2 by
LPS/IFN-γ, with no effect on COX-2 mRNA. This contrasts with the strong inhibition of the induction of iNOS by epoxomicin when it was present before LPS/IFN-γ. The inhibition was associated with prevention of acute loss of inhibitors of kappa B α and β (see chapter 2). This suggests that in MAEC, induction of COX-2 is not regulated primarily by inhibitors of kappa B/NFkB.

Epoxomicin severely inhibited the loss of COX-2 after cycloheximide treatment in KD and control cells, indicating that the proteasome inhibitor increased COX-2 by inhibiting its degradation in MAEC. This contrasts with the lesser reduction of COX-2 degradation caused by calpain inhibitor or depletion of PIN1. A striking difference between epoxomicin-treated cells and PIN1 KD, zVF- or E64d-treated cells was the accumulation of a distinct set of C-terminal COX-2 fragments smaller than 72 kDa (zones II and III). Blocking the proteasome probably leads to processing of COX-2 by calpain-independent pathways, since the fragments were similar in cells treated with epoxomicin alone and with epoxomicin plus zVF. We speculate that products of proteasomal action on COX-2 are subsequently processed by calpain in MAEC. Although the sequence of steps yielding various COX-2 fragments is not completely delineated by these inhibitor studies, the qualitatively similar effect of PIN1 KD and calpain inhibitors on COX-2 suggests that PIN1 regulates calpain-dependent processing of the enzyme.
Previously, we observed that PIN1 depletion and zVF each increased the induction of iNOS, and reduced its degradation. PIN1 physically interacted with iNOS. The WW and catalytic domains of PIN1 appeared to contribute to the association (135). This suggested that PIN1 depletion might alter the susceptibility of its targets to digestion by calpain. For example, PIN1 could associate with these substrates and catalyze proline isomerization, affecting protease sensitivity. In contrast to iNOS, however, interaction between COX-2 and PIN1 was not detected here. A role for direct interaction between COX-2 and PIN1 cannot be completely excluded, however, since association of the proline isomerase with its putative substrate may be weak or transient. PIN1 could also affect association of COX-2, or iNOS, with other proteins that may indirectly regulate proteolysis. It is also possible that PIN1 regulates the induction of COX-2 and iNOS, by affecting calpain activity in MAEC. This possibility is addressed in chapter 4.

The effects of COX-2 in acute and chronic inflammatory responses in the vasculature are complicated by multiple primary and secondary stimuli that may be present, and by cellular factors, such as supply of arachidonic acid, complement of various prostaglandin synthases, and expression of prostaglandin
receptors (277). Thus, it remains to be determined whether selective manipulation of PIN1 in endothelial cells will be therapeutically useful. In any case, the results suggest that COX-2 is degraded by calpain, which is regulated by PIN1 in MAEC.

In summary, suppression of PIN1 increased induction of COX-2 by LPS/IFN-γ by a post-transcriptional mechanism. Consistent with the short lifespan of COX-2 in MAEC, suppression of PIN1 and calpain inhibitor increased its induction. The similarity in COX-2 degradation in KD MAEC and cells treated with calpain inhibitors, and differences from cells treated with epoxomicin, indicate that PIN1 selectively affects a component of COX-2 turnover mediated by calpain. This previously unknown connection suggests that PIN1 may normally function to restrain the induction COX-2, iNOS, and perhaps other substrates in MAEC in calpain dependent manner. PIN1 is likely to regulate a range of calpain-dependent endothelial activities.
CHAPTER 4
PIN1 REGULATES THE CALPAIN/CAST SYSTEM
IN ENDOTHELIAL CELLS

Abstract
Depletion of the peptidyl-proline isomerase, Protein Interacting with Never in Mitosis Gene A -1 (PIN1), increased the induction of iNOS and COX-2 in MAEC treated with E. coli endotoxin LPS plus IFN-γ. The enhanced induction was by a post-transcriptional effect associated with reduced degradation of iNOS and COX-2. Degradation of iNOS and COX-2 was inhibited by the calpain inhibitor, carbobenzoxy-valinyl-phenylalaninal (zVF). We hypothesized that PIN1 regulated calpain activation or CAST in MAEC. Knockdown of PIN1 with small hairpin RNA reduced the basal and LPS/IFN-γ stimulated activity of calpain as compared with MAEC harboring a control small hairpin RNA. However, the depletion of PIN1 did not affect the levels of the large or small subunits of ubiquitous, heterodimeric calpains. PIN1 did not interact with these proteins either. Instead, deletion of PIN1 increased the basal level of CAST, the endogenous protein inhibitor of heterodimeric calpains. The effect of PIN1 on CAST was post-transcriptional, as CAST mRNA was not affected by PIN1
depletion. Loss of calpastatin, after inhibiting protein synthesis with
cycloheximide, was slower in KD than control MAEC, indicating that PIN1
regulates degradation of CAST. Inhibition of calpain with zVF increased CAST
protein levels in control MAEC, whereas a pan-caspase inhibitor, did not,
suggesting that calpain, not caspase, contributed to the turnover of basal CAST
in MAEC. Extracts from KD treated with either vehicle or LPS/IFN-γ contained
more inhibitory activity towards an exogenous calpain compared to control
shRNA MAEC. Co-immunoprecipitation and glutathione-S-transferase-PIN1
fusion protein pull-down results indicated that CAST associates with PIN1. The
PIN1 fusion protein also antagonized the ability of CAST to inhibit exogenous
calpain in vitro. The results suggest that PIN1 associates with CAST, either
facilitating degradation of CAST or regulating its inhibitory activity towards
calpain in MAEC. PIN1 may normally maintain calpain activity and consequently
limit the induction of calpain substrates, such as iNOS and COX-2 in endothelial
cells. The regulation of calpain system by PIN1 in MAEC indicates that PIN1 may
play an essential role in various calpain related physiological and pathological
processes.
Introduction

The peptidyl-prolyl isomerase, PIN1, acts on proline (P) following phosphorylated (p) serine (S) or threonine (T) (p(S/T)-P) (236, 237). PIN1 contains an N-terminal domain, characterized by two tryptophan residues separated by 22 amino acids that can bind to phosphorylated serine- or threonine-proline sequences in substrate proteins, known as a WW domain. PIN1 also isomerizes p(S/T)-P with its carboxyl-terminal catalytic domain (237, 238). This conformational change may have profound effects on protein-protein interactions, subcellular location and the susceptibility to enzymes (18, 21, 29, 30, 236, 237, 239, 240, 293).

Previously, we found that depletion of PIN1 with small hairpin RNA enhanced the induction of iNOS and COX-2 in MAEC treated with LPS/IFN-γ. Increased expression of iNOS and COX-2 was due to reduced degradation of each protein, which was also antagonized by a calpain inhibitor. Although PIN1 associated with iNOS, no association was detected with COX-2. PIN1 KD and calpain-inhibitors caused production of similar COX-2 fragments, suggesting that PIN1 may regulate calpain and then modulate COX-2 degradation in MAEC.

Calpain is a family of calcium-activated neutral cysteine proteases that have been implicated in many physiological processes such as cytoskeletal
remodeling, cell cycle progression, gene expression, apoptosis and tissue injury (186, 294, 295). The major ubiquitous isoforms of the enzyme, micro (µ) and milli (m), are heterodimers between calpain 1 and 2 and the common small subunit, calpain 4. Heterodimeric calpain activity is regulated at multiple levels: expression of the subunits, requirement for calcium, phosphorylation, autoproteolysis, intracellular distribution, and, most importantly, by the endogenous inhibitor CAST (201-209). Since depletion of PIN1 and calpain inhibition reduced the turnover of iNOS and COX-2 in MAEC, we hypothesized that PIN1 either regulates calpain or CAST in these cells (206, 296).

**Material and Methods**

Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT). Bradford reagent, endothelial cell growth supplement, serotype 0111:B4, heparin, phenylmethylsulfonyl fluoride, polybrene, and antibodies against calpain 1, calpain 2 and CAST subdomain 4A were purchased from Sigma Chemical Co. (St Louis, MO). Anti-CAST antibody was purchased from cell signaling Technology. Dulbecco’s minimum essential medium, trypsin, isopropyl-β-d-thiogalactopyranoside, Trizol, Superscript Reverse Transcriptase Taq DNA polymerase, RNAse-free DNase, deoxynucleotides, pcDNA 3 plasmids were from Invitrogen (Carlsbad, CA). Cycloheximide, carbobenzoxy-valinyl-
phenylalaninal (zVF, or MDL-28170 or calpain inhibitor III) and [4-((4-(dimethylamino)phenyl)azo)benzoic acid, succinimidyl ester]-threonine-proline-leucine-lysine-serine-proline-proline-serine-proline-arginine-[5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid] and porcine erythrocyte µ-calpain were obtained from Calbiochem (La Jolla, CA). Triton X-100 and enhanced chemiluminescence reagent were obtained from Pierce (Rockford, IL). Tween 20, tris-base, sodium dodecyl sulfate, NaCl, Na3VO4, NaF, ethidium bromide, ethylenediamine tetraacetic acid and agarose were from Fisher Scientific (Fair Lawn, NJ). Carbobenzoxy-valinyl-alanyl-aspartyl fluormethyl ketone (zVAD) and anti-PIN1 were from R&D Systems (Minneapolis, MN). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

Cells

MAEC, cultured from aortas of mice in accordance with the Guide for the Care and Use of Laboratory Animals from the U.S. National Institutes of Health, selected for stable knockdown (KD) of PIN1 by 85%, and MAEC transduced with an inactive control vector were used. These cells were were cultured and treated with zVF and cycloheximide as described previously (135).
Calpain activity and CAST inhibitory activity

Cells were washed and scraped in 1 ml of ice-cold PBS, centrifuged (2 min at 1500 x g, 4°C), and resuspended in 100 mM Tris-HCl, 5 mM EDTA, 5 mM benzamidine, 1 mM dithiothreitol, 10 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride. Cell extracts were sonicated with four 10 s pulses. Debris was removed by centrifuging at 15,000 x g for 20 min. Calpain activity was measured using [4-((4-(dimethylamino)phenyl)azo)benzoic acid, succinimidyl ester]-threonine-proline-leucine-lysine~serine-proline-proline-proline-serine-proline-arginine-[5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid as a substrate. The reaction contained 15 mM calcium in 50 µl of calpain buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10mM β-mercaptoethanol, pH 7.5) and 40 µg extracted protein. Substrate was added to produce 100 µM, and reaction was monitored at 30°C by fluorescence (320 nm excitation and 480 nm emission).

CAST activity was measured by determining the inhibitory effect of cell extracts towards degradation of the calpain substrate by exogenous porcine µ-calpain. Cell extracts, isolated as described above, were incubated at 37 degrees for 1 h in the presence of 15 mM Ca^{2+} to inactivate endogenous calpain (206). 0.8 units of porcine µ-calpain and 60 µg cell extract proteins were combined and adjusted to 50 µl with calpain buffer. The reaction was started by adding 100 µM calpain
substrate and monitored as indicated above. CAST inhibitory activity was expressed as % inhibition of the exogenous calpain (100 x [calpain activity in the absence of extracts minus calpain activity in the presence of extracts] / [calpain activity in the absence of extracts]). Glutathione S-Transferase (GST)-PIN1 fusion protein (1 µg) was added to some reactions to assess its effect on CAST inhibitory activity (135).

mRNA Levels
RNA was extracted and analyzed by RT-PCR as described previously (135). CAST primers were designed from Genbank Accession NM 009817 (sense: 5’-CTT GTC TCA GGA CTT CTC CA, antisense: 5’-TGA GGT CTC TGT AGT TGA GG). β-actin was measured as described previously (284). PCR products were electrophoresed in agarose gels and stained with ethidium bromide for digital imaging.

GST-pull down and Western Blotting
For GST pulldown, 500 µg of MAEC lysate protein were pre-cleared by incubation with glutathione-sepharose at 4 ºC for 2 h. The supernatant was then incubated with a specific GST-PIN1 fusion proteins at 4 ºC for 2 h. All samples were then centrifuged 16,000 g x 1 min, and supernatants were discarded. The
sepharose pellets were washed three times with lysis buffer by resuspension and centrifugation. Western blots were produced and analyzed as described previously (135).

**Data Analysis**

Digital images of proteins and PCR products were measured with Image J 1.34 (NIH). Data were analyzed by Student’s t test or analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons (297).

**Results**

*Knockdown of PIN1 decreases Calpain activity in MAEC*

Previous results showed that PIN1 regulated calpain mediated iNOS and COX-2 degradation. For example, PIN1 may associate with iNOS and catalyze proline isomerization, affecting its susceptibility to calpain cleavage. In contrast to iNOS, we have not detected interaction between COX-2 and PIN1 with similar conditions of immunoprecipitation, suggesting that PIN1 may have multiple mechanisms to regulated calpain mediated substrate degradation. The
similarities between the effects of PIN1 KD and calpain-inhibitors on COX-2 fragments suggested that PIN1 may regulate calpain.

Here, the fluorogenic calpain substrate, DABCYL-TPLKSPSPPSPR-EDANS was used to measure basal and LPS/IFN-γ stimulated calpain activity in KD and control MAEC as described by Tompa et al. (197). The fluorogenic calpain substrate is cleaved by porcine µ-calpain, which is blocked by a calpain inhibitor, zVF but not by a proteasome inhibitor epoxomicin (EPO) (Fig. 4.1A). Basal calpain activity was 2.2-fold greater in control cells than in KD (Fig. 4.1B). LPS/IFN-γ stimulation dramatically increased calpain activity in both KD and control cells while calpain activity was still about 2-fold greater in control cells than in KD.
Fig. 4.1 Effect of PIN1 knockdown on calpain activity. A. Purified porcine μ-calpain was added into reaction mixture containing DMSO, zVF (25 μM) or
epoxomicin (EPO, 2 µM). Calpain activity was measured as initial rate of cleavage of the calpain substrate (DABCYL)-TPLK~SPPPSPR-(EDANS). B. KD and control shRNA MAEC were treated with vehicle or LPS/IFN-γ for 24h and calpain activity were measured. Bars represent mean + SE from 4 independent cultures in each group. *, p <0.05 for comparison between KD and control shRNA; +, p <0.05 for comparison between vehicle and LPS/IFN-γ treatment.

Suppression of PIN1 does not affect protein level of calpain 1, 2 and 4 in MAEC

The ubiquitous μ- and m-calpains are heterodimers of calpain small subunit (calpain 4) with calpain 1 and 2 respectively (206). Since a reduction in calpain activity could be due to reduced expression of the major subunits calpain 1, 2 or 4, expression was assessed by western blotting. Suppression of PIN did not affect the protein level of these calpain subunits either in basal and LPS/IFN-γ stimulated condition (Fig. 4.2A/B). Even though both calpain 1 and calpain 2 can be phosphorylated in Ser and Thr residues (204-206, 217-220), neither of them could be pull down by GST-PIN1 fusion protein, suggesting that PIN1 regulates calpain activity indirectly rather than direct association with calpains (Fig. 4.2C).
Fig. 4.2 Effect of PIN1 knockdown on calpain 1, 2 and 4 protein levels

continued
**Fig. 4.2** Effect of PIN1 knockdown on calpain 1, 2 and 4 protein levels. PIN1 KD and control (C) MAEC were starved overnight and cell lysates were collected.
Suppression of PIN1 affects basal CAST protein level of in MAEC

The activity of heterodimeric calpains is tightly controlled by CAST (200). Thus the level of CAST in PIN1 KD and control MAEC was examined. The reduced calpain activity of KD cells was associated here with a 2.7-fold increase in CAST protein signal compared with control MAEC (Fig. 4.3A). LPS/IFN-γ treatment for 24h decreased CAST in KD to a similar level to control, which may be due to decreased CAST mRNA level (Fig. 4.3B) or increased calpain activity (Fig. 4.1B).
Fig. 4.3 Effect of PIN1 knockdown on CAST protein and mRNA

continued
Fig. 4.3 continued

B.

![Graph showing CAST/β-actin signal intensity ratio over time with KD and C shRNA samples.](image)

Fig. 4.3 Effect of PIN1 knockdown on CAST protein and mRNA. A. KD and control (C) shRNA MAEC were treated with vehicle or LPS/IFN-γ for 24h. Cell extracts of were collected and CAST and α-tubulin were measured by western blotting. Representative blots and analysis are shown. Bars represent mean ± S.E. ratio of CAST/α-tubulin from densitometric analysis of 3 cultures of each.
group. #: p <0.05 for comparison between KD and control shRNA. B. KD and control shRNA MAEC were treated with LPS/IFN-γ and mRNAs were collected at indicated time periods. mRNA encoding CAST and β-actin were determined by RT-PCR and agarose gel electrophoresis. Representative images of CAST and β-actin PCR products in ethidium-stained gels are shown. Bars represent mean + S.E. ratio of CAST:β-actin mRNA from densitometric analysis of images from 3 independent cultures. + and *: p <0.05 for comparison between 2h, 4h, 8h and 0h in KD and control respectively.

*Suppression of PIN1 affects basal CAST stability in MAEC*

PIN1 knockdown did not affect CAST mRNA, suggesting a post-transcriptional effect of PIN1 on CAST expression. Increased expression of CAST could result from reduced degradation of the protein. The loss of CAST after inhibition of protein synthesis was therefore investigated. KD and control shRNA cells were treated with 90 µg CHX/ml to block translation. Proteins were extracted at 0, 7, 24, and 48 h after the addition of CHX and subjected to western blot analysis. Regression analysis indicated that the rate of loss of CAST was significantly different between KD and control MAEC. CAST level fell to less than 50% of the
initial value by 24 h after CHX in the control shRNA cells. In KD cells, a similar
decrease in CAST was not detected even 48 h after CHX treatment. The half-life
of CAST protein was about 17 h in control shRNA cells, but it was prolonged to
more than 48 h in PIN1 KD MAEC (Fig 4.4A). CAST is susceptible to degradation
by calpain \((298, 299)\) and by caspases \((300-303)\). Thus, the effects of calpain
inhibitor, zVF and the general caspase inhibitor, zVAD, on CAST were
determined. zVF significantly increased CAST protein in control shRNA cells to
an extent in PIN1 KD (Fig 4.4B), while CAST protein level did not differ between
vehicle and zVAD treatment in either PIN1 KD or control (Fig 4.4C).
Fig. 4.4 Effect of PIN1 knockdown on CAST protein stability

continued
Fig. 4.4 continued

B.

C.

Fig. 4.4 Effect of PIN1 knockdown on CAST protein stability. A. The protein synthesis inhibitor cycloheximide (90 µg/ml) was added to KD and control (C)
shRNA MAEC, and cell extracts were collected at the indicated times. CAST and α-tubulin in each sample were determined by image analysis of western blots. Representative blots are shown. Ratios of CAST to α-tubulin were determined, and the average ratio ± SE of 3 independent cultures for each point, as a percent of the value at 0 h after cycloheximide treatment, was calculated. The dashed line marks the 50% value. The solid lines are linear regressions for KD and control cells. The slopes were significantly different (p<0.05). PIN1 KD and control shRNA MAEC were treated with vehicle, zVF (25 µM) (B) or a general caspase inhibitor zVAD (25 µM) (C) for 48h. CAST and α-tubulin were determined by image analysis of western blots. Bars represent the mean ± S.E. of three independent cultures per group. *, p<0.05 for comparison between vehicle and zVF or zVAD. #, p<0.05 for comparison between KD and control shRNA.

*Suppression of PIN1 affects both basal and LPS/IFN-γ-stimulated CAST inhibitory activity in MAEC*

The inhibitory efficiency of CAST on calpain can also be regulated by post-translational modifications, including phosphorylation (208, 209). Therefore, the inhibitory activity of CAST towards exogenous porcine μ-calpain was examined in
PIN1 KD and control cells. Cell extracts from untreated PIN1 KD contained significantly higher CAST inhibitory activity than that in control MAEC (Fig. 4.5A). Interestingly, even though the protein level of CAST in KD was similar to that in control after LPS/IFN-γ treatment, KD still contained significantly higher CAST inhibitory activity than control shRNA MAEC (Fig. 4.5B). This may explain the lower calpain activity in KD after LPS/IFN-γ treatment. PIN1 might affect CAST inhibitory activity by association with CAST, which is phosphorylated (208, 209) and contains several S/T-P motifs. Thus, the potential for interaction between CAST and PIN1 was assessed. GST-PIN1 but not GST pulled down CAST (Fig. 4.5C), and CAST was coimmunoprecipitated with PIN1 from extracts of control shRNA MAEC (Fig. 4.5D).
Fig. 4.5 Effect of PIN1 knockdown on CAST inhibitory activity

continued
Fig. 4.5 Effect of PIN1 knockdown on CAST inhibitory activity. Cell extracts of KD and control (C) shRNA MAEC were treated with vehicle (A) or LPS/IFN-γ (B) for 24h and CAST inhibitory activities were measured by using porcine µ-calpain.
#: p <0.05 for comparison between KD and control cells. Cell extracts from control shRNA MAEC were incubated with GST or GST-wild-type and glutathione sepharose (C), and immunoprecipitated without or with anti-PIN1 antibody (D). Western blotting with anti-CAST and anti-PIN1 was shown.

**Purified GST-PIN1 decreases CAST inhibitory activity**

From the above results, PIN1 may directly associate with CAST and regulate its inhibitory activity. Thus, the effect of direct addition of purified GST, GST-PIN1 and its mutants, W33A and K64A, in the CAST inhibitory activity assay was examined. GST-PIN1 significantly decreased CAST inhibitory activity of KD extracts treated with vehicle or LPS/IFN-γ compared with GST (Fig. 4.6A/B). Both binding activity and catalytic activity were required for this effect, since W33A and K64A were unable to decrease CAST activity compared to GST.
Fig. 4.6 Purified GST-PIN1 decreases CAST inhibitory activity. KD MAECs were treated with vehicle (A) or LPS/IFN-γ (B) for 24h and cell extracts were collected. Purified GST, GST-PIN1 and its mutants W33A, K64A (1 µg) were added into
CAST inhibitory activity reaction mixture. Bars represent mean + S.E. ratio of percentage of CAST inhibitory activity of 5 cultures of each group +: p <0.05 for comparison between GST-PIN1 and GST, W33A, K64A.

Discussion

We previously found that PIN1 depletion and the calpain inhibitor, zVF, increased the induction of iNOS and COX-2, and reduced their degradation. PIN1 associated with iNOS, suggesting that it might regulate its susceptibility to digestion by calpain. Thus, binding of PIN1 to iNOS, or proline isomerization in iNOS, might regulate the sensitivity of the enzyme to calpain. However, we did not detect any association between PIN1 and COX-2, indicating PIN1 may also regulate COX-2 turnover via activating calpain. Consistent with these observations, depletion of PIN1 by 85% in MAEC decreased calpain activity in vehicle and LPS/IFN-γ stimulated MAEC (Fig 4.1). Decreased calpain activity could result from decreases in levels of calpain subunits in cells lacking PIN1. However, depletion of PIN1 did not reduce the expression of subunits of ubiquitous heterodimeric μ- or m-calpains, calpain 1, 2 and 4 here (Fig 4.2). Calpain activity can also be regulated by phosphorylation. For example, phosphorylation on calpain 2 by protein kinase A (PKA) decreased its activity.
while ERK directly phosphorylates and activates calpain 2 both in vitro and in vivo (204, 205). It is possible that PIN1 binds phosphorylated calpains and regulates its activity directly. However, this was not the case in MAEC since association of heterodimeric calpain subunits with PIN1 was not observed.

Calpain activity is controlled by the endogenous protein inhibitor, CAST, which inhibits heterodimeric µ- and m-calpain. Increases in CAST protein are associated with reduced calpain activity in several experimental settings (304, 305). Here, depletion of PIN1 increased the basal level of CAST in MAEC (Fig. 4.3A), which may not result from transcription since no significant differences of mRNA were observed in KD and control shRNA cells (Fig. 4.3B). This suggests that PIN1 regulates translation or degradation of CAST. Here, the loss of CAST after treating MAEC with cycloheximide was measured. Although CAST was longer-lived than iNOS or COX-2 as expected, depletion of PIN1 greatly stabilized it (Fig. 4A). Although CAST is susceptible to degradation by calpain and caspases, inhibition of calpains, but not caspases, increased CAST level in control (Fig. 4.4B/C). The results suggest that CAST is normally degraded by calpain in MAEC. In parallel with the CAST protein level, PIN1 KD contained significantly higher inhibitory activity (Fig. 4.5A). Interestingly, CAST inhibitory activity was higher in KD compared with control after LPS/IFN-γ treatment. This
may explain lower calpain activity in KD, even though the CAST protein level was similar to that in control after LPS/IFN-γ treatment (Fig. 4.5B). Furthermore, purified GST-PIN1 but not mutant W33A and K64A significantly decreased CAST inhibitory activity in both extracts of vehicle and LPS/IFN-γ treated cells (Fig. 4.6). Phosphorylation of the active CAST by protein kinase C and protein kinase A decreases its inhibitory activity (208, 209). Thus, PIN1 may directly associate with phosphorylated CAST and reduce its inhibitory activity against calpain in MAEC (Fig. 4.4C/D).

The calpain-CAST system is in a loop, in which heterodimeric calpain is a protease and CAST is its specific endogenous inhibitor. CAST is also a substrate of the enzyme. Two crystallographic studies uncover a novel mechanism of protease inhibition (200, 224, 225). Subdomain A and C in CAST inhibitory domain binds to two separate domains, IV and VI, in calpain 2 and calpain 4 respectively, which allows inhibitory subdomain B to wrap around the active site region of the calpain heterodimer and inhibit its activity. In both of these studies, several amino acids adjacent to the active site of calpain in subdomain B form a loop, which is held away from the catalytic cysteine, avoiding proteolysis by calpain. Deletion of one or 2 amino acids in the loop led to cleavage by the calpain. Previous studies have shown that the disordered regions between
inhibitory domains can be cleaved by calpain even at relatively high concentrations of CAST (225). Thus its binding sequence (subdomain A/C), inhibitory sequence (subdomain B) and cleavage sequence (region between inhibitory domains) are located separately in CAST. Ten S/T-P motifs are located in mouse CAST protein (close to subdomain A, B, C and in region between Inhibitory domains), some of which are highly conserved in different species, and might bind PIN1. PIN1 may therefore regulate calpain activity by affecting the conformation of CAST bound to calpain, for example, modulating the binding affinity of CAST to calpain (subdomain A/C), inhibitory efficiency against calpain (subdomain B) or susceptibility to calpain (region between inhibitory domains). The effects of PIN1 on CAST may require their interaction which was observed in (Fig. 4.4C/D). Association between PIN1 and CAST is consistent with the known serine/threonine phosphorylation of CAST (208, 306), although the specific phosphorylated residues have not been identified. Phosphorylation of CAST at unknown residues also reduces its interaction with calpain (223). PIN1 could indirectly regulate the phosphorylation state of CAST by modulating kinases or phosphatases. Altered phosphorylation could then affect the inhibitory activity of CAST. It is not clear whether one or more than one are phosphorylated and responsible for the association with PIN1. Thus, to identify S/T-P sites necessary for direct interaction between PIN1 and CAST is essential for understanding how
PIN1 regulates calpain system in endothelial cells. Related signaling pathways and the responsible kinases in basal and LPS/IFN-γ stimulated conditions are equally important to be investigated.

Collectively, PIN1 significantly affects calpain activity by regulating CAST in MAEC. PIN1 associates with CAST, reducing its protein stability and efficiency to inhibit calpains. The elevation of basal CAST in cells lacking PIN1 is due to reduced degradation, which appears to be mediated by calpain. Consistent with the known phosphorylation of CAST, PIN1 binds to the inhibitor *in vitro* and antagonizes its ability to inhibit calpain. Regulation of calpain via CAST by PIN1 may account for its effect on induction of iNOS and COX-2 in MAEC, and it may affect other calpain substrates and activities of endothelial cells. Alterations in PIN1 expression or function may significantly affect a broad range of calpain-dependent processes in EC. Further investigations should be performed to understand how PIN1 interacts and regulates CAST.
CHAPTER 5
PIN1 REGULATES CALPAIN/CAST SYSTEM
IN VIVO

Abstract

Previous studies showed that PIN1 regulated calpain activity by acting on calpastatin (CAST) in MAEC. PIN1 may either facilitate degradation of CAST or reduce its inhibitory activity to maintain calpain activity, and consequently limit the induction of calpain substrates, such as iNOS and COX-2, in endothelial cells. Whether PIN1 regulates calpain/CAST system in vivo, however, is not clear. In the present study, this role of PIN1 was examined by studying PIN1 knockout and wildtype mice. The regulation of calpain system by PIN1 in vivo is tissue specific: lung and kidney from knockout mice contained significantly lower calpain activity which may be due to the higher CAST inhibitory activity in them. However, higher calpain activity and less CAST inhibitory activity were observed in brains from PIN1 knockout mice. Liver and heart from PIN1 knockout mice contained significantly lower calpain activity although the CAST inhibitory activity is similar to wildtype mice, indicating mechanisms other than CAST were involved in PIN1-regulated calpain activity in these organs. PIN1 knockout
influenced normal cardiac function, causing a significant decrease in stroke volume compared with wildtype.

**Introduction**

Depletion of the peptidyl-proline isomerase, PIN1, increased the induction of iNOS and COX-2 in MAEC treated with E. coli endotoxin LPS plus IFN-γ (135, 171). The enhanced induction was a posttranscriptional effect associated with reduced degradation of iNOS and COX-2. Degradation of iNOS and COX-2 was inhibited by the calpain inhibitor, carbobenzoxy-valinyl-phenylalaninal (zVF). Knockdown of PIN1 with small hairpin RNA reduced the basal and LPS/IFN-γ stimulated activity of calpain as compared with control shRNA MAEC. PIN1 may either facilitate degradation of CAST or regulate its inhibitory activity to maintain calpain activity, and consequently limit the induction of calpain substrates, such as iNOS and COX-2, in endothelial cells. However, the role of PIN1 in the regulation of calpain activity in vivo has not been investigated yet. Here, calpain activity and CAST inhibitory activity of different organs including lung, kidney, brain, liver and heart from PIN1 wildtype and knockout mice were measured.
Materials and Methods

Animals
All aspects of animal use were performed in accordance with the guidelines of
the National Institutes of Health and under approval of the Ohio State University
Animal Care and Use Committee. Animals were housed in a sterile cage rack
system with HEPA-filtered air circulation (approximately 50 air changes/hour,
Allentown Caging Inc., Allentown, PA). Mice were sacrificed with an overdose of
pentobarbital sodium.

Calpain activity and CAST inhibitory activity
For calpain assay in vivo, different organs were collected and homogenized in
lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 5 mM benzamidine, 1 mM
dithiothreitol, 10 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl
fluoride). Tissue homogenates were sonicated with four 10 s pulses. Debris was
removed by centrifuging at 15,000 x g for 20 min. Calpain activity was measured
using [4-((4-(dimethylamino)phenyl)azo)benzoic acid, succinimidyl ester]-
threonine-proline-leucine-lysine-azoserine-proline-proline-proline-serine-proline-
arginine-[5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid as a substrate.
The reaction contained 15 mM calcium in 50 µl of calpain buffer (10 mM HEPES,
150 mM NaCl, 1 mM EDTA, 5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10mM β-mercaptoethanol, pH 7.5) and certain amount of extracted protein. Substrate was added to produce 100 µM, and reaction was monitored at 30 °C by fluorescence (320 nm excitation and 480 nm emission). CAST activity was measured by determining the inhibitory effect of tissue homogenates towards degradation of the calpain substrate by exogenous μ-calpain. Clarified tissue homogenates were incubated at 95 degrees for 10 min to inactivate endogenous calpain. 0.8 units of porcine μ-calpain and tissue homogenates were combined and adjusted to 50 µl with calpain buffer. The reaction was started by adding 100 µM calpain substrate and monitored as indicated above. CAST inhibitory activity was expressed as % inhibition of the exogenous calpain (100 x [calpain activity in the absence of extracts minus calpain activity in the presence of extracts] / [calpain activity in the absence of extracts]).

Assessment of cardiac function

Comprehensive cardiovascular performance measures were conducted on male PIN1 wildtype and knockout mice (C57BL/6) (age 8 weeks, n=6) using echocardiography at rest and following acute isoproterenol treatment (ISO, 2.5mg/kg i.p.). At 8 weeks, in vivo cardiovascular function was determined using a Sonos 1000 echocardiography unit (Hewlett-Packard, Andover, MA). Mice
were anesthetized by isoflurane inhalation (0.25–1.5% isoflurane in 95/5% O2/CO2) and normothermia was maintained by a heating pad. A 7.5 MHz pediatric probe (optimized and dedicated to rodent studies) placed in the parasternal, short axis orientation recorded LV systolic and diastolic internal dimensions. Three loops of M-mode data were captured for each animal, and data were averaged from at least 5 beat cycles/loop. Parameters were determined using the American Society for Echocardiography leading-edge technique in a blinded fashion.

**Results**

*Effect of PIN1 on body weight and organ weight*

Different organs were collected from five PIN1 wildtype and knockout mice. Interestingly, PIN1 knockout mice are smaller with decreased body weight and organ weight when compared to wildtype mice.
Table 5.1 Effect of PIN1 on body weight and organ weight. Five female PIN1 knockout (-/-) and wildtype (+/+) mice were sacrificed at the age of 6 months. The whole body and organs (right lung, right kidney, right brain and whole heart) were weighted. * p<0.05 for comparison between PIN1 -/- and +/- samples.

PIN1 knockout decreases calpain activity in lung and kidney

Calpain and CAST inhibitory activity was measured in lung and kidney from PIN1 wildtype and knockout mice. Consistently to lower calpain activity in PIN1 KD MAEC, lung and kidney from knockout mice contained significantly less calpain activity (Fig. 5.1.A/C), which may be due to the higher inhibitory activity of CAST in PIN1 knockout. No significant difference of CAST protein was observed in lung from PIN1 knockout and wildtype mice, indicating that PIN1 may regulate CAST
activity (Fig. 5.1.B). CAST activity in kidney may result from other isoforms of CAST since the long form was not detected (Fig. 5.1.D).

A. 

![Graph showing the effect of PIN1 knock out on calpain and CAST inhibitory activity in lung and kidney.]

Fig. 5.1 Effect of PIN1 knock out on calpain and CAST inhibitory activity in lung and kidney

continued
Fig. 5.1 continued

B. PIN1: -/- +/+ MAEC CAST

C.

![Graph showing CAST Signal Intensity and Initial Reaction Velocity](image)

continued
Fig. 5.1 continued

D.

<table>
<thead>
<tr>
<th>PIN1:</th>
<th>-/-</th>
<th>+/+</th>
<th>MAEC</th>
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<td>CAST</td>
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Fig. 5.1 Effect of PIN1 knock out on calpain and CAST inhibitory activity in lung and kidney. Lung (A) and kidney (C) from PIN1 knockout (-/-) and wildtype (+/+)
mice (n=5) were collected and homogenized. Calpain (left panel) and CAST activity (right panel) were assayed as described in Materials and Methods. #: p
<0.05 for comparison between PIN1 -/- and +/+ samples. Lung (B) and kidney (D) samples were homogenized and CAST was measured by western blotting. Representative blots and analysis are shown. Bars represent mean + S.E. of CAST signal from densitometric analysis of 5 cultures of each group.
PIN1 knockout increases calpain activity in brain

Opposite to lung and kidney, brains from PIN1 knockout contained higher calpain activity (Fig. 5.2A) with lower CAST inhibitory activity compared with wildtype. The lower CAST inhibitory activity may be due to lower CAST protein level in PIN1 knockout (Fig. 5.2B).

A.

Fig. 5.2 Effect of PIN1 knockout on calpain and CAST inhibitory activity in brain

continued
Fig. 5.2 Effect of PIN1 knockout on calpain and CAST inhibitory activity in brain. Brains from PIN1 knockout (-/-) and wildtype (+/+ ) mice (n=5) were collected and homogenized. Calpain (left panel) and CAST activity (right panel) were assayed as described in Materials and Methods. #: p <0.05 for comparison between PIN1 -/- and +/+ samples. B. Brain samples were homogenized and CAST was measured by western blotting. Representative blots and analysis are shown.
Bars represent mean + S.E. of CAST signal from densitometric analysis of 5 cultures of each group. +: p <0.05 for comparison between PIN1 -/- and +/+ samples.

PIN1 knockout decreases calpain activity without influencing CAST activity in liver and heart

Liver and heart from PIN1 knockout mice contained significantly less calpain activity although the CAST inhibitory activity is similar to wildtype (Fig. 5.3 D/E), indicating mechanisms other than CAST were involved in PIN1-regulated calpain activity in these organs.
Fig. 5.3 Effect of PIN1 knockout on calpain and CAST inhibitory activity in liver and heart.

continued
Fig. 5.3 continued

C.

Fig. 5.3  Effect of PIN1 knockout on calpain and CAST inhibitory activity in liver and heart. Liver (A) and heart (C) from PIN1 knockout (-/-) and wildtype (+/+) mice (n=5) were collected and homogenized. Calpain (left panel) and CAST activity (right panel) were assayed as described in Materials and Methods. #: p <0.05 for comparison between PIN1 -/- and +/- samples. Liver (B) samples were homogenized and CAST was measured by western blotting. Representative blots and analysis are shown. Bars represent mean + S.E. of CAST signal from densitometric analysis of 5 cultures of each group.
PIN1 knockout influences cardiac function

High quality cardiac ultrasound imaging was achieved under light inhalation anesthesia as described in the methods section. Although no difference in heart rate was detected, PIN1 knockout mice showed decrease in SV at rest and after acute cardiac stress compared with wildtype mice (Fig. 5.4).

Fig. 5.4 Effect of PIN1 knockout on cardiac function
Fig. 5.4. Effect of PIN1 knockout on cardiac function. Comprehensive cardiovascular performance measures were conducted on male PIN1 wildtype (+/+) and knockout (-/-) mice (age 8 weeks, n=6) using echocardiography at rest and following acute isoproterenol treatment (ISO, 2.5mg/kg i.p.). Cardiac performance parameters heart rate (HR) and stroke volume (SV) were shown in A and B respectively. Bars represent mean ± S.E. of values of 6 animals. #: p
<0.05 for comparison between basal and isoproterenol groups, *: p <0.05 for comparison between PIN1 -/- and +/+.

**Discussions**

Since PIN1 function may be tissue specific, the much more complicated regulation of calpain-CAST system by PIN1 status in vivo may be expected. PIN1 deficiency decreased calpain activity and increased calpastatin activity in lung and kidney, which may be due to the regulation of CAST by PIN1 (Fig. 5.1). Calpain and CAST levels were highest in the lung (307), thus it is necessary to study how calpain/CAST system is involved in various aspects of lung function. For example, in pulmonary artery endothelial cells, calpain activity is necessary to maintain angiogenesis and eNOS gene transcription, protein content and its activity, while calpain inhibition by increased CAST protein level in response to cigarette smoke extracts impaired angiogenesis, decreased eNOS gene transcription, and acetylcholine-induced endothelium-dependent relaxation of the pulmonary artery (288, 308, 309). PIN1 might regulate calpain-mediated angiogenesis and eNOS expression and activity in pulmonary artery endothelial cells.
Excessive activation of calpains or loss of CAST and deregulation of PIN1 have been implicated in neurodegenerative diseases, traumatic brain and spinal cord injuries (295). For example, genetic deletion of PIN1 and enhanced calpain activity contributes to the pathological process of AD (8, 28, 37, 226). PIN1 binds and stabilizes phospho-MCL-1, thus protecting oligodendrocytes from apoptosis after spinal cord injury (37). Upregulation of calpain, has been implicated in apoptosis and tissue degeneration in spinal cord injury, while overexpression of CAST and calpain inhibitor prevented apoptosis in spinal cord injury (227-235). Collectively, PIN1 protects, and calpain contributes to the process of these diseases. Therefore, it is essential to find out how calpain-CAST system is regulated by PIN1 in brain. The effect of PIN1 on calpain-CAST system in brain is quite different from that in lung and kidney: PIN1 knockout mice contained more calpain activity, while lower CAST inhibitory activity was observed (Fig. 5.2). This result appears to verify the negative correlation between PIN1 and calpain activity. Lower CAST activity in brains from PIN1 knockout mice may be due to its decreased protein level (Fig. 5.2).

Although excessive activation of calpain contributes to cardiovascular remodeling induced by angentensin II (310), myocardial dysfunction during sepsis (311) and
during myocardial ischemia and reperfusion \((312)\), the physiological function of calpain activity in normal, unstressed hearts was not clear until recently. Galvez et al demonstrated that loss of calpain activity in mouse heart results in progressive dilated cardiomyopathy with decreased ventricular ejection performance \((313)\). Basal cardiac calpain activity is necessary for ubiquitin-dependent degradation of specific cardiac proteins, whose abnormal accumulation causes cardiomyocyte degeneration and heart failure \((313)\).

Interestingly, PIN1 knockout mice showed significant decrease in stroke volume, both at rest and after acute cardiac stress with isoproterenol (Fig. 5.4), indicating a potential role of PIN1 in normal cardiac function. PIN1 may regulate cardiac function by modulating calpain activity in heart (Fig. 5.3). However, no difference of CAST level and its activity was observed in hearts from PIN1 knockout and wildtype mice, suggesting that PIN1 may act on calpain interacting proteins other than CAST, or directly on calpains, to regulate its activity in the heart. PIN1 may also regulate other calpains, such as calpain 3, which is found in skeletal and cardiac muscles, but which is not inhibited by CAST \((206, 313)\).

Regulation of calpain/CAST by PIN1 is more complicated in vivo than in MAEC. CAST activity is controlled by phosphorylation. For example, two forms of CAST are present in brains. One form is efficient to inhibit calpain, while the second
form is almost completely inactive. Dephosphorylation of the second form by phosphatases can convert it into an active form. Phosphorylation of the active form by PKC and PKA decreased its inhibitory efficiency (208). Different phosphorylation patterns or interconversion between two forms may be involved in the distinct regulation of CAST activity by PIN1 in lung/kidney and brain. Also CAST expression patterns are different in various organs. For example, western blotting results showed that CAST in brain samples contained two bands. The band with low molecular weight was predominant. It was not the case in lung, since the band with high molecular weight was predominant. These two bands were not detected in kidney samples, and CAST in livers showed three bands. These different bands may represent various isoforms of CAST or degradation fragments of the long isoform. Thus phosphorylation in various CAST isoforms and their association with PIN1 should be investigated in MAEC and various organs.
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Endothelial inflammatory activation and dysfunction have been critically implicated in diverse cardiovascular diseases such as atherosclerosis, sepsis and heart failure. Inflammatory cytokines and bacterial products such as LPS and IFN-γ, can activate vascular endothelial cells, induce the expression of iNOS and COX-2. They are similarly induced by many inflammatory stimuli, produce NO and various prostaglandins and are believed to play a role in the pathogenesis of many cardiovascular diseases.

Both iNOS and COX-2 are regulated by multiple mechanisms including transcription, mRNA stability, translation and post-translation. Many key proteins in these steps are regulated in a phosphorylation and/or conformation dependent manner. Although PIN1-mediated conformational change of pSer/Thr-Pro motifs is extremely important in phosphorylation signaling, the role of PIN1 on iNOS and COX-2 induction had been unexplored. Considering the importance of iNOS and COX-2 in endothelial activation, our investigations were carried out to understand
the effect of PIN1 suppression on iNOS and COX-2 induction and to elucidate the underlying molecular mechanisms in endothelial cells.

Stable suppression of PIN1 was generated by using shRNA-lentiviral based knock-down technique, which efficiently decreased PIN1 protein level by 85%. Here we reported for the first time, the critical involvement of PIN1 in the induction of these proteins. Suppression of PIN1 enhanced the induction iNOS and COX-2 protein by LPS-IFN-γ, which were confirmed by knock-down cells by small interfering RNAs and MAEC isolated from PIN1 wildtype and knockout mice. Both iNOS and COX-2 were functional in producing NO and proglandin E2. There was no effect on induction of iNOS and COX-2 mRNA, suggesting a posttranscriptional effect. Next, we began using cycloheximide-treated cells to study PIN1 function on their protein stability. It turned out that knockdown of PIN1 increased the stability of iNOS and COX-2 protein. Since both iNOS and COX-2 undergo calpain and proteasome dependent degradation (131-137, 160-167, 170, 171), Pharmacological inhibitors of calpain and proteasome were used to elucidate the underlying molecular mechanisms. My results showed that calpain inhibition blocked the degradation of iNOS and COX-2, which is regulated by PIN1 status. Immunoprecipitation and GST-PIN1 pull-down results indicated that PIN1 may directly interact with iNOS and regulate its susceptibility to calpain
cleavage. However, no direct interaction were detected between PIN1 and COX-2, suggesting that PIN1 may regulate calpain mediated degradation of COX-2 by different mechanisms, for example, influencing calpain activity. Accordingly, we investigated whether PIN1 regulated calpain activity in endothelial cells. Calpains are Ca2+-regulated cysteine proteases. $\mu$- and m-calpain, two well characterized isoforms, are ubiquitously expressed, catalyze limited proteolysis of substrates and control various cellular functions. Although calpains have been intensely studied for more than 30 years, it is still not very clear how calpain activity is regulated in living cells. Our results demonstrated for the first time PIN1 can regulate calpain activity. In MAEC, knockdown of PIN1 decreased calpain activity in both basal and LPS/IFN-$\gamma$-stimulated conditions. Furthermore, PIN1 neither affected calpain 1, 2 and 4 protein levels nor directly associated with them.

CAST, an endogenous protein inhibitor, is capable of specifically and reversibly binding and inhibiting calpain activity. The rapidly accumulating structural information makes it possible to understand how m-calpain is activated by calcium and how CAST inhibits its activity specifically and potently, as described in chapter 1 and 4 (200, 224, 225). My results demonstrated that the inhibition of calpain by CAST is critically regulated by PIN1 in endothelial cells. PIN1 knockdown enhanced CAST inhibitory activity in both basal and LPS/IFN-
stimulated condition, while purified GST-PIN1, but not its mutants, W33A and K64A, significantly decreased it compared to GST. Furthermore, PIN1 knockdown could stabilize basal CAST in a calpain-dependent manner. Immunoprecipitation and GST-PIN1 pull-down results indicated that PIN1 directly interacted with CAST and regulated either its susceptibility to calpain cleavage or its inhibitory activity on calpain. Furthermore, the regulation of calpain-CAST activity was demonstrated in vivo. In lung, kidney, liver and heart, PIN1 knockout may decrease calpain activity compared with PIN1 wildtype mice, by acting on CAST or other calpain interacting proteins. In brain, PIN1 knockout increased calpain activity with decreased CAST protein level and activity compared with PIN1 wildtype.
Figure 6.1 PIN1 regulates calpain mediated proteolysis. Calpains catalyze limited cleavage of substrates, which can be regulated by PIN1 via several different mechanisms. First, PIN1 may directly interact with calpain substrates (for example, iNOS) and influence the susceptibility to calpains. Second, PIN1 may regulate calpain activity by acting on its endogenous inhibitor, CAST as in MAEC, lung and kidney. PIN associates with CAST, either regulating CAST protein...
stability or the inhibitory activity. Third, calpain interacting proteins other than
CAST may be PIN1 targets, as in liver and heart.

Arrows indicate interaction or stimulation

symbols indicate inhibition

“p” indicates phosphorylation

Question mark indicates unknown factors or components

**Future directions**

Our studies indicated that PIN1 normally limits iNOS and COX-2 induction by
facilitating calpain-mediated degradation in MAEC. In addition, many PIN1
substrates, such as β-catenin, c-Fos, c-Jun and p53, are also calpain substrates.
Thus, it would be interesting to investigate whether it is a common mechanism
that PIN1-catalyzed conformational changes regulate calpain mediated
degradation.

Our studies indicated that PIN1 may bind to its substrate, iNOS, and regulate its
calpain-mediated degradation in EC. Although in vitro studies can provide useful
information, it is important that they are verified by in vivo investigations. Thus, induction of iNOS and COX-2 in vivo, especially in EC should be examined in PIN1 wildtype and knockout mice. PIN1 functions may be cell or tissue specific. Thus different cell types may have different responses to PIN1 knockdown in vitro and in vivo. For example, induction of iNOS and COX-2 in various cell types other than EC (for example, neural and immune cells), should be investigated since both PIN1 and iNOS are critically involved in Alzheimer’s disease. Considering the involvement of iNOS and COX-2 in various diseases including cardiovascular, neurodegenerative diseases and cancers, different disease models should be investigated PIN1 wildtype and knockout mice.

COX-2 degradation in MAEC is controlled by calpain and proteasome pathways. Epoxomicin completely inhibited the rate of loss of 72 kDa COX-2 in cycloheximide treated KD and control cells, while calpain inhibition only partially reduced it. COX-2 fragments were similar in cells treated with epoxomicin alone and with epoxomicin plus zVF, suggesting proteasome inhibition may also block the processing of COX-2 by calpains. All these results indicated that proteasome and calpain pathways are not completely separated and may sequentially contribute to the degradation of COX-2. Further investigations are needed to test this idea.
Our investigations also identified PIN1 as a key regulator of the calpain system and subsequent calpain-mediated substrate degradation. However, it leaves several questions that need to be resolved to better understand the function of PIN1 in calpain related physiological and pathological processes. Although PIN1 regulated calpain activity, and CAST protein level or inhibitory activity in MAEC, further study is necessary to investigate whether regulation of calpain by PIN1 is caused by its action on CAST. In this context, the effect of PIN1 on calpain activity should be determined when CAST is overexpressed or deleted in EC.

Although the direct interaction between PIN1 and CAST is demonstrated in MAEC, we have not yet characterized phosphorylation sites which mediated the association. CAST in brain tissues can be phosphorylated by PKC and PKA, which promotes a decrease in its inhibitory efficiency (208, 209). Several S/T-P motifs are located close to subdomain A/C (binding to calpains), subdomain B (inhibiting calpains), and regions between Inhibitory domains (calpain cleavage sites). Thus it is essential to identify S/T-P sites which are necessary to form direct interaction between PIN1 and CAST. Signaling pathways and the responsible kinases are equally important to be identified. Only the long isoform of CAST was investigated in my study. More than 10 isoforms of CAST have been reported. All of them contain inhibitory domain and are able to efficiently
inhibit calpains. Thus phosphorylation in various CAST isoforms and their association with PIN1 should be investigated in MAEC and various organs.

Regulation of calpain activity in vivo is more complicated than in MAEC. PIN1 knockout may decrease calpain activity in lung, kidney, liver and heart, by acting on CAST or other calpain interacting proteins. Calpains are important to regulate physiological processes, for example, protecting against cardiomyocyte degeneration and heart failure induced by abnormal accumulation of specific cardiac proteins \( (313) \). Overactivation of calpains or loss of CAST and deregulation of PIN1 have been implicated in neurodegenerative diseases, traumatic brain and spinal cord injuries \( (8, 28, 37, 226-235) \). Therefore, it will be necessary to investigate how calpain-CAST system is regulated by PIN1 and how this interaction contributes to these diseases, which will provide valuable information to develop drugs that target PIN1 and calpains. For example, does PIN1 regulate normal cardiac function by modulating calpain activity? PIN1 protects, and calpain contributes to the process of neurodegenerative diseases. My study showed that PIN1 knockout increased calpain activity with decreased CAST protein level and activity in brain compared with wildtype, which may indicate that PIN1 negatively correlates with calpain activity in these diseases. Compared with wildtype mice, PIN1 knockout decreased calpain activity in liver
and heart without influencing CAST inhibitory activity, suggesting calpain interacting proteins other than CAST are regulated by PIN1. In this context, it would be interesting to identify these factors in liver and heart.

Although we found that PIN1 interacted with STAT1 in MAEC, the functional consequence was not known. Expression of SOCS-1 or STAT1-driven luciferase in a reporter plasmid in response to different stimuli should be examined in either PIN1 knockdown or overexpression condition. Other members of STAT family, especially STAT3 and STAT5 contain similar PMSP site in their transactivation domain. Thus, we can not exclude the possibility that PIN1 regulates their activity, especially STAT3. Mice lacking STAT3 Ser727 phosphorylation are smaller in size with less weight in many organs (314), which is similar to PIN1 knockout mice in my study and from others’ results (38). Mice lacking STAT3 Ser727 phosphorylation had lower serum levels of insulin-like growth factor 1 (IGF-1) (314), whereas deletion of STAT5 a/b in skeletal muscle reduced IGF-1 level locally (315). Insulin-like growth factor 1 (IGF-1) signaling crucially control organ size and weight (316, 317), which is decreased by aging and implicated in degenerative diseases including AD (318-320). Considering the role of PIN1 in aging related disease, it would be interesting to study whether PIN1 regulates IGF-1 signaling by acting on STAT3, 5 or certain signaling components. It is still
possible that IGF-1 signaling regulates PIN1 expression or activity and contributes to organ control and development of degenerative diseases.

Although PIN1 is ubiquitous, and may act on many proteins to regulate transcription, mRNA stability, translation, protein turnover and protein function, my thesis shows that the calpain/CAST system is regulated by PIN1. Furthermore, inflammatory activation in endothelial cells appears to be greatly affected by calpains. This work suggests that PIN1, calpain and CAST might be targeted to manipulate endothelial function in diseases.
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