Function and Regulation of Pannexin 1 Channels in the Vascular Endothelium

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ABSTRACT

The nucleotide adenosine 5'-triphosphate (ATP) has classically been considered the cell's primary energy currency; however, a novel role for ATP as an extracellular autocrine/paracrine signaling molecule has evolved over the past century. Purinergic signaling is now known to regulate a plethora of physiological and pathophysiological processes in almost every organ system. In the vasculature, ATP and its metabolites elicit dual control over blood vessel tone and tissue perfusion, and purinergic signaling events have been implicated in vascular pathologies including atherosclerosis and inflammation. While the importance of extracellular ATP in the vascular system is well recognized, the mechanism(s) mediating the regulated release of the purine from vascular cells are less well understood and are a key target of current investigation. One such ATP-liberation mechanism has been ascribed to the recently identified pannexin (Panx) channels, namely Panx1. Initially, we characterized the expression and localization profiles of Panx isoforms across the systemic vasculature, identifying predominant representation by the Panx1 isoform in both smooth muscle (SMC) and endothelial cells (EC) comprising the blood vessel wall, with more heterogeneous expression profiles observed in specialized vascular systems including the heart, lung and kidney. In particular, the Panx1 isoform is highly expressed in ECs regardless of blood vessel type or size, while Panx1 expression is limited to SMCs of small arteries and arterioles. Panx1 forms hexameric channels in the plasma membrane of ECs, functioning to release ATP in response to a number of stimuli. However, prolonged Panx1 channel activity is extremely detrimental to cell viability. Here we report a novel negative regulatory mechanism that may govern the activity of Panx1 channels in ECs, by which the bioactive gas nitric oxide (NO) covalently modifies two cysteine (Cys) residues in the channel by a process termed S-

nitrosylation. Targeted S-nitrosylation of both Cys40 in the channel's predicted pore-lining region and Cys346 in the C-terminal tail are required for Panx1 channel inhibition by NO. Finally, we present new data placing EC Panx1 channels at the center of the acute vascular inflammatory response. Extracellular ATP is a potent pro-inflammatory mediator where it regulates interactions between circulating inflammatory cells and the vascular endothelium, primarily in the venous circulation. Venous EC activation by the pro-inflammatory cytokine Tumor Necrosis Factor alpha (TNFα) promotes the recruitment and infiltration of circulating leukocytes to local sites of tissue injury or infection. We report that activation of venous EC TNFα Receptor Type 1 promotes Panx1 channel activation and ATP release into the vessel lumen through a signaling cascade dependent on Src Family Kinases. Pharmacological inhibition and targeted genetic deletion of Panx1 in the venous endothelium essentially blocked leukocyte-EC interactions in a model of acute vascular inflammation. Taken together, we identify EC Panx1 channels as novel regulators of vascular inflammation, functionally integrating inflammatory and purinergic signaling to promote proper homing of inflammatory cells throughout the body.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
$ANAPP_3$	Arylazido aminopropionyl ATP
ApoE	Apolipoprotein E
ATP	Adenosine 5’-triphosphate
B2M	β2 microglobulin
BFA	Brefeldin A
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
Cdh5	Cadherin-5 (Vascular endothelial cadherin)
CD39	ecto-Apyrase
CD73	5’-ectonucleotidase
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
Cl^-	Chloride
Cx	Connexin
DTT	Dithiothreitol
EC	Endothelial cell
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
FFA	Flufenamic acid
fMLP	n-formyl-methionine-leucine-proline
GA	Glycyrrhetinic acid

GENC	Glomerular endothelial cell
GFP	Green fluorescent protein
GSH	Glutathione
GSNO	S-nitrosoglutathione
HUVEC	Human umbilical vein endothelial cell
HSaVEC	Human saphenous vein endothelial cell
HAoEC	Human aortic endothelial cell
HCoAEC	Human coronary artery endothelial cell
IEL	Internal elastic lamina
IP_3	Inositol 1,4,5-triphosphate
K^+	Potassium
K_{ATP}	ATP-sensitive potassium channel
KCl	Potassium chloride
LPS	Lipopolysaccharide
mAEC	Mouse aortic endothelial cell
MEJ	Myoendothelial junction
NA	Noradrenaline
NEM	N-ethylmaleimide
NO	Nitric oxide
ORCC	Outwardly rectifying chloride channel
Panx	Pannexin
PAR-1	Protease activated receptor type 1
PDG	Peptidoglycan
PECAM-1	Platelet endothelial cell adhesion molecule-1

PFA	Paraformaldehyde
PKA	Cyclic AMP-dependent protein kinase
PSGL-1	P-selectin glycoprotein ligand-1
RuR	Ruthenium red
SFK	Src family kinase
sGC	Soluble guanylate cyclase
SUR	Sulfonourea receptor
TDA	Thoracodorsal artery
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
VNUT	Vesicular nucleotide transporter
VSMC	Vascular smooth muscle cell

DEDICATION

This dissertation is dedicated to the most important people in my life: my family, my mentors, incredible friends and colleagues. Each of them has influenced my life in a very special way and it's their unwavering support that's made me the person and scientist I am today.

First, I would like to thank my mentor Brant. Over the past four years, he gave me freedom in the lab to pursue my own interests and think independently, while giving me a push when I needed it. He provided me the opportunity to explore not only new avenues of scientific interest but new parts of the world as well. The time I spent in Oxford was incredible and opened my eyes to a whole new world of international research. I will treasure that experience for the rest of my life. His continual encouragement and passion for science is contagious, a trait that I will try to carry forward with myself. Thank you for being a great mentor and friend.

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CHAPTER 1. GENERAL INTRODUCTION

Classically, the nucleotide adenosine 5'-triphosphate (ATP) has been considered the major energy source in the cell. Through the metabolic breakdown of ingested nutrients, subsequent production of the electron carriers NADH and $FADH_2$ in the citric acid cycle and the transfer of electrons through the electron transport chain, the mitochondrial ATP synthase synthesize ATP in the mitochondrial matrix, which is then transported to and accumulates in the cytosol at a concentration in the low mM range [1,2]. The cell utilizes the high-energy content of this molecule for a multitude of functions including its requirement as a cofactor for active transport of molecules across the plasma membrane, actin-myosin cross bridge cycling during muscle contraction and hydrolysis and transfer of its terminal phosphate group to proteins driven by protein kinases as a post-translational modification to modulate protein function. Through extensive characterization of the role of this nucleotide as an intracellular energy molecule, a novel role for ATP as a potent extracellular signaling molecule emerged nearly 50 years ago. Over the past 5 decades, extensive work has shown that ATP can be released from a number of different cell types and can exert effects via autocrine and/or paracrine signaling mechanisms. In 1971, Burnstock coined the term "purinergic", referring to nerves in the autonomic nervous system that released neither noradrenaline nor acetylcholine upon stimulation, but instead the purine nucleotide ATP [3]. This classification has since been expanded to encompass all aspects of purine signaling. While the effects of ATP as an extracellular signaling molecule have been extensively characterized, the mechanisms involved in its regulated release from different cell types remain an important topic of investigation [4-6].

1.1. Purinergic signaling in the vasculature

The effects of ATP signaling have been observed throughout the vasculature in both homeostatic and pathological conditions, including: reactive hyperemia [7,8], hypoxia-induced vasodilation [9,10], alpha-adrenergic receptor mediated vasoconstriction [11], hypertension [12], VSMC proliferation in atherosclerosis and vascular inflammation [13-17]. Elucidation of the mechanisms regulating purine nucleotide release in the cells comprising the vascular wall is critical for both understanding the pathogenesis of vascular diseases and the identification of novel drug interventions. In 1977, Forrester observed the release of ATP from cells in the cardiovascular system [18] and the release of ATP has since been shown in all major cell types of the vessel wall including endothelial cells [9,19-22], vascular smooth muscle cells [11,23], perivascular sympathetic nerves [24-27] and circulating erythrocytes [10,28-30].

The foundation for an active role of purinergic signaling in the vasculature was first observed by Drury and Szent-Gyorgyi in 1929, who demonstrated that the adenine nucleotides and nucleosides, when injected intravenously into the circulatory system of dogs, produced a potent sinus bradycardia and a fall in blood pressure [31]. This observation pioneered the concept that ATP may be an important signaling molecule in the cardiovascular system and could produce profound effects on cardiac output and blood pressure. Later, the identification and characterization of ATP-sensitive purinergic receptors at the plasma membrane of vascular smooth muscle and endothelial cells across vascular beds further suggested a role for ATP as an extracellular vasoactive signaling molecule which may contribute to blood pressure regulation at the level of the blood vessels themselves [32-35]. Purinergic receptors have since been classified into two

main families, designated as P1 and P2 receptors (with P annotating Purinergic). P1 receptors are further subclassified as A1, A2A, A2B or A3 receptors, and are G-protein coupled receptors that signal through either G_s or G_i and selectively bind the nucleoside adenosine to modulate levels of cAMP within the cell [36]. The P2 receptors have been subclassified as either ionotropic P2X receptors, of which 7 isoforms have been characterized to date ($P2X_{1\text{-}7}$ [37]), or metabotropic P2Y receptors, which contain eight isoforms ($P2Y_{1,\ 2,\ 4,\ 6,\ 11\text{-}14}$ [38]) (reviewed by Ralevic *et al* in [39]). The P2X receptors are ligand gated ion channels which exhibit a high binding affinity for ATP over its metabolic breakdown products. P2X receptor activation by ATP induces a conformational change in the transmembrane channel that allows the influx of extracellular cations including Na^+ and Ca^{2+} leading to cellular depolarization. This depolarization in turn can activate voltage-gated calcium channels to facilitate further Ca^{2+} influx strengthening the depolarization [40,41]. The P2Y family of receptors are G-protein coupled receptors that differentially bind to ATP, ADP or the uracil nucleotides UTP and UDP, depending on receptor subtype, and signal through G_q, G_i or G_s ultimately producing changes in the concentration of intracellular cAMP or Ca^{2+}. While numerous purinergic receptor isoforms have been identified in vascular cells, it has been shown that binding of ATP to $P2X_1$ receptors localized on VSMCs causes an influx of Ca^{2+} that results in constriction of the blood vessel [42,43]. Conversely, ATP in the blood vessel lumen can bind to $P2Y_1$ and $P2Y_2$ purinergic receptors localized to endothelial cells, which signals the production of inositol triphosphate (IP_3) and release of Ca^{2+} from the endoplasmic reticulum via activation of IP_3 receptors. This rise in intracellular calcium concentration leads to activation of endothelial cell nitric oxide synthase (eNOS),

resulting in the production of nitric oxide (NO) which feeds back on VSMCs to induce vasodilation [44-46]. Contrary to this traditional classification of differential P2 receptor effects in the vascular wall, luminal perfusion of ATP has also been shown to elicit vasodilation via activation of endothelial cell P2X receptors [47] and ATP activation of smooth muscle cell P2Y purinergic receptors has been shown to promote vasoconstriction in different vascular beds [11]. Altogether, these observations may suggest that purinergic signaling in the vasculature occurs at distinct micro-signaling domains where localized ATP release stimulates purinergic receptors based on their relative location to the site of release or that purinergic receptor expression in the vessel wall may vary depending on the vascular bed examined. Future studies are required to elucidate this observed difference. This dual control of vascular tone by the purine nucleotide ATP has been reviewed extensively by Burnstock [48].

Extracellular purine nucleotide concentration is tightly regulated in the vasculature. The presence and activity of metabolic enzymes that degrade ATP to ADP, AMP and adenosine, as well as expression of multiple P1 and P2 receptors across vascular beds has sparked the question as to which nucleotide or nucleoside is the primary vasoactive molecule. Berne *et al* first proposed an active role for adenosine in regulating vascular tone by showing that the nucleoside can induce coronary vasodilation in the isolated perfused heart [49]. Moreover, measurement of ATP levels in the venous effluent from the isolated rat hindlimb in response to hyperemia induced by muscle contraction were undetectable, while adenosine levels were significantly increased [50]. In a separate study, however, Hopwood and Burnstock observed that a significant portion of the dilation elicited by luminal ATP in the isolated perfused Langendorff heart preparation

was resistant to P1 receptor blockade with the non-selective adenosine receptor antagonist PACPX, supporting the role that ATP acting on P2 receptors on endothelial cells elicits the observed vasodilatory response [32]. Furthermore, the vasodilation observed in response to luminal ATP has been shown to be approximately 100 times more potent than that to adenosine [51]. Several years later, Bünger *et al* was able to carefully repeat these observations, but found the magnitude of difference significantly decreased compared to what was previously reported [52]. Altogether, these observations illustrate the dynamic regulation of purine nucleotide metabolism and support the idea that ATP can elicit potent effects on vascular cells before its metabolic breakdown.

In the blood vessel lumen, ATP concentration has been shown to increase during periods of hypoxia and ischemia. The source of this luminal ATP has been suggested to arise from liberation from circulating erythrocytes [10,53] as well as the endothelial cells that line the vessel lumen [9,19,20]. Locovei *et al* reported ATP release from erythrocytes in response to low oxygen tension and osmotic stress, providing a luminal source of ATP, presumably to act on endothelial cell purinergic receptors to induce vasodilation, an increase in blood flow and O_2 delivery to tissues [53]. Increased shear stress on endothelial cells has also been shown to induce ATP release into the vessel lumen [9,19,22]. At the smooth muscle cell-perivascular sympathetic nerve axis, ATP has been shown to be released as a co-transmitter along with noradrenaline from sympathetic nerves [24-26,54]. Release of ATP from perivascular nerve terminals and increases in concentration of the nucleotide at adjacent vascular smooth muscle cells induces vasoconstriction through activation of $P2X_1$ purinergic receptors [26,32].

Sneddon and Burnstock reported a portion of the excitatory junction potentials and constrictions elicited by perivascular nerve stimulation that is insensitive to α-adrenergic receptor blockade and is mimicked by application of exogenous ATP [26], suggesting an essential role for this nucleotide in sympathetic nerve induced contraction of blood vessels.

It has been suggested that chronic increases in ATP in the vasculature could potentiate pathological conditions such as hypertension and atherosclerosis; therefore, it is essential that the extracellular concentration of this nucleotide is tightly regulated. ATP is actively metabolized by a class of catalytic enzymes known as ecto-nucleotidases. These membrane-bound proteins present their catalytic core to the extracellular side of the plasma membrane, allowing selective degradation of extracellular nucleotides. Cells can also release soluble nucleotidases or exonucleotidases into the extracellular milieu, which can contribute to ATP metabolism outside of the cell [55]. The expression and activity of ecto-nucleotidases has been extensively characterized in the vasculature [56-59]. Of the ectoenzymes present in the vasculature, ectonucleotide triphosphate diphosphohydrolase 1 (E-NTPDase, also termed CD39 or ecto-apyrase) plays a substantial role in the breakdown of ATP and ADP to produce AMP [57]. The monophosphorylated nucleotide can then be metabolized further to adenosine by removal of its 5'-phosphate by the enzyme ecto - 5'-nucleotidase (CD73) [59]. CD39 localized to arterial smooth muscle cells reduces the concentration of ATP, ADP, UTP and UDP available for activation of P2 receptors and can reduce the extent and duration of constriction. It has been shown that injection of the purine nucleotide UDP into the circulation of mice lacking CD39 caused an initial vasodilation followed by a strong

constriction and a significant increase in mean arterial pressure. Also, endothelium-denuded aortic rings from these mice showed a significant increase in constriction to adenine and uridine nucleotides as compared to controls that express a functional enzyme [12]. In a separate study, Kauffenstein *et al* observed a marked decrease in blood pressure upon intravenous injection of UTP at a sub-threshold concentration for vasoconstriction, consistent with decreased nucleotide breakdown and increased signaling through P2Y receptors on the endothelium [60]. These studies indicate an essential role for ecto-nucleotidases in regulating the concentration of ATP both in the blood vessel lumen and in the extracellular space surrounding the vascular media and reinforce the dual effect of purines on peripheral resistance and blood pressure.

1.2. Purinergic Control of Vascular Inflammation

While the foundation for purinergic control of vascular functions was initially characterized extensively in the context of vascular reactivity and overall blood flow and pressure regulation, it has become increasingly clear that extracellular ATP also plays important regulatory roles in the vascular inflammatory response. Vascular inflammation can be characterized as acute (physiological) or chronic (pathological) in nature.

The acute vascular inflammatory response is an innate process of inflammatory cell homing to infected or damaged tissues resulting from integrated cross-talk between circulating leukocytes and the vascular endothelium, primarily at the level of post-capillary venules [61]. This process has been highly studied and is now known to occur in a step-wise manner beginning with local recruitment, rolling (fast and slow), adhesion and final extravasation into the surrounding tissue (reviewed in [62]). Extravasated leukocytes (primarily neutrophils) function as phagocytes to sequester and engulf

infectious agents and cell debris and aid in the repair of damaged tissue. The molecular mechanisms for leukocyte rolling, adhesion and extravasation involve the upregulation of adhesion molecules at the luminal surface of endothelial cells and the secretion of chemoattractants and cytokines. In particular, P-selectin (CD62P), which is present in Weibel-Palade bodies positioned just under the EC plasma membrane, is initially mobilized to the cell surface where it binds to a glycoprotein ligand present on the surface of circulating leukocytes called P-selectin glycoprotein ligand-1 (PSGL-1) [63,64]. P-selectin participates in the tethering of these cells to the EC membrane initiating the rolling stage. E-selectin (CD62E) also plays important rolls in rolling, but its expression is induced downstream of EC activation by mechanisms involving NFkB-dependent gene transcription [65,66]. Recently, new evidence has distinguished a tethering mechanism by which rolling leukocytes utilize a catch-bond system to crawl along the endothelium [67]. As circulating leukocytes make contact with ECs, they form tethers at the trailing edge that are weight-bearing and consist of P-sel/PSGL-1 bonds. As the leukocyte is carried downstream by the shear force of flow, these bonds break, rolling with the cell as a "sling" until they make contact with the EC on the next revolution. These slings lay down an adhesion substrate at the front of the cell that will eventually become weight-bearing as the cell rolls forward [67]. As rolling persists, other adhesion molecules, namely vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), in addition to several integrins, are upregulated at the EC surface where they participate in slow rolling and firm adhesion between ECs and rolling leukocytes [68-70]. The firmly adhered leukocytes also become activated during this process by cytokines that are sequestered near the EC membrane

and migrate through the blood vessel wall by chemoattraction [71]. Following extravasation, primed neutrophils actively engulf harmful microbes and cell debris.

In contrast to the acute inflammatory response, chronic inflammation occurs in response to persistent infection or injury, or can manifest as an auto-immune disorder. During this pathological state, there is an over-recruitment of blood mononuclear cells, primarily monocytes and lymphocytes, into the inflamed tissue where these cells can cause further tissue injury and fibrosis (for extensive review see [72]). Following infiltration, monocytes become activated by local cytokines and toxins, where they secrete a number of damaging factors. Chronic inflammation is now recognized as a central mediator of atherosclerosis, Alzheimer's disease, type 1 diabetes, multiple sclerosis and inflammatory bowel syndromes [73-75].

As our understanding of the molecular mechanisms governing leukocyte interactions with the endothelial cell wall has advanced, purinergic signaling has emerged as a prominent regulatory pathway in both acute and chronic inflammatory conditions. Specifically, purinergic receptors have been implicated in regulating vascular inflammation, airway inflammation and chronic inflammatory bowel syndrome [15,16,76,77]. Cellular necrosis and apoptosis (prevalent in inflamed tissues) results in cellular ATP release to serve as a "find-me/eat-me" signal for infiltrating phagocytes [78]. In addition, neutrophils themselves can release ATP to modulate the relative permeability of the vascular endothelium supplying inflamed tissues [79]. At the level of the vascular endothelium, purinergic P2Y6 and P2Y1 receptor signaling has been directly linked to the acute inflammatory response [16,17]. Genetic deletion of endothelial cell P2Y6 receptors potently inhibited neutrophil interactions with the venous endothelium in

response to a lipopolysaccharide (LPS) model of systemic inflammation. In this study, both LPS and the pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα) increased the expression of EC adhesion molecules through a P2Y6 receptor-dependent mechanism. In addition, TNFα upregulated P2Y6 receptor expression in endothelial cells, further suggesting a direct role for purinergic signaling in this inflammation model [16]. In a separate study, endothelial cell P2Y1 receptors were shown to regulate TNFα- and IL-1β-induced adhesion molecule expression on cultured ECs and genetic ablation of P2Y1 decreased leukocyte rolling and adhesion in response to infusion of the cytokines [17]. Together, these studies point towards a direct role for purinergic receptors in promoting an inflammatory endothelial cell phenotype. While it is now widely accepted that ATP in the vascular system plays important roles, the mechanisms of its release into the extracellular milieu are a topic of current investigation with much effort placed on identification of the channels or transporters responsible for its release from the different vascular cell types. The following sections examine the evidence for ATP release from vascular cells with an emphasis placed on vesicular exocytosis, ATP-binding cassette (ABC) transporters, the plasma membrane F_1/F_0-ATP synthase, connexin hemichannels and the recently identified pannexin channels.

1.3. Mechanisms of ATP Release

1.3.1. Vesicular Exocytosis

Proteins synthesized in the endoplasmic reticulum and processed through the Golgi apparatus can be packaged into intracellular vesicles that traffic and fuse with the plasma membrane in a process termed vesicular exocytosis. This event can result in incorporation of membrane bound proteins into the plasma membrane or release of

soluble proteins and intracellular molecules into the extracellular space. Vesicular exocytosis occurs on a second-to-second basis in secretory cell types to release soluble proteins, signaling molecules and neurotransmitters important in maintaining homeostasis. In the vascular system, perivascular sympathetic nerve stimulation leads to the vesicular release of noradrenaline (NA) and produces an excitatory junction potential at the post-junctional membrane of innervated vascular smooth muscle cells and subsequent vasoconstriction. This response has been well characterized by the activation of α-adrenergic receptors localized at the post-junctional membrane of innervated smooth muscle cells and can be mimicked by application of exogenous NA. However, α-adrenergic receptor blockade at the post-junctional membrane has been shown to completely inhibit the response to exogenously-applied NA but not that seen by sympathetic nerve stimulation, suggesting the action of a second signaling molecule, notably ATP [26]. The observation by Westfall *et al* that tritiated [H^3] purine nucleotides are taken up by sympathetic nerves, along with NA, and released upon field stimulation of the guinea pig vas deferens suggests a key role for vesicular release of ATP from these nerves [80]. The contraction following sympathetic nerve stimulation in the guinea pig vas deferens as well as the rat tail artery is biphasic, characterized by a fast initial contraction followed by a slower sustained contraction. Application of the P2 receptor antagonist arylazido aminopropionyl ATP ($ANAPP_3$) inhibits the initial constriction, but has no effect on the secondary sustained contraction, indicating the presence of an ATP mediated component. The opposite is seen during α-adrenergic receptor blockade with the α_1-adrenergic receptor antagonist prazosin. The ATP-mediated contractile response is also eliminated by application of tetrodotoxin, a potent neurotoxin that inhibits voltage-

gated sodium channels important for vesicular exocytosis at nerve terminals, further supporting release of ATP from sympathetic nerves by vesicular exocytosis [24].

Endothelial cells lining the vessel lumen are constantly subjected to conditions of varying blood flow and shear stress. The release of vasoactive substances including ATP during conditions of increased shear stress has been extensively documented [9,19,81]. The mechanism responsible for this event, however, is currently a topic of investigation. It has been proposed that similar to ATP release from sympathetic perivascular nerve terminals, endothelial cells may release ATP in a vesicular manner. Labeling of intracellular ATP with quinacrine, a quinolone-acridine derivative that has a high binding affinity for ATP [82,83], in cultured human umbilical vein endothelial cells (HUVEC) revealed a punctate staining pattern within the cell consistent with intracellular vesicles [84]. Moreover, pretreatment of HUVECs with monensin, an inhibitor of vesicle formation at the Golgi, resulted in a significant decrease in quinacrine fluorescence as compared to untreated cells, consistent with localization of ATP to intracellular vesicles in these cells. Stimulation of quinacrine labeled endothelial cells by increasing the shear stress at 10 or 25 dynes/cm^2 lead to a rapid decrease in quinacrine fluorescence and increase in ATP concentration in the extracellular media, suggesting release of ATP stores via an exocytotic mechanism. In contrast, unstimulated cells showed little detectable ATP in the extracellular medium. Also, monensin was shown to abolish the release of ATP from cultured HUVECs exposed to a shear stress of 25 dynes/cm^2. These observations suggest that vesicular exocytosis of ATP from endothelial cells could occur during changes in vessel blood flow and shear stress.

While evidence has surfaced suggesting the vesicular release of ATP from different tissues, the question has remained how ATP from the cytosol is packaged into secretory vesicles. Recently, a vesicular nucleotide transporter, termed VNUT, was identified in a number of secretory cell types [85]. This transporter was found to be localized to intracellular vesicles and was active in pumping ATP into the vesicle lumen, utilizing a proton gradient established by the vacuolar-ATPase (v-ATPase). Hydrolysis of ATP by v-ATPase drives the translocation of protons across the vesicular membrane, concentrating them in the vesicle lumen [86]. Although the establishment of a proton gradient creates both an electrochemical potential difference as well as pH gradient across the vesicular membrane, the activity of VNUT has been shown to be solely dependent on the electrochemical difference [85]. While VNUT fulfills the role of a transporter for concentrating ATP into intracellular vesicles, it remains to be established whether this transporter is expressed and active in perivascular sympathetic nerves and endothelial cells.

1.3.2. ABC transporters

The ATP-binding cassette transporters are a class of integral membrane proteins that utilize the energy of ATP hydrolysis to facilitate the movement of a large array molecules across the plasma membrane of cells, including cholesterol, lipids and many hydrophobic drugs [87]. Each member of an ABC transporter has two conserved intracellular ATP binding domains that bind and hydrolyze ATP. Three of these proteins, the cystic fibrosis transmembrane conductance regulator (CFTR), the multidrug resistance gene product *mdr* (also known as P-glycoprotein) and the sulfonylurea receptor (SUR) have been suggested to not only utilize ATP as an energy source for active transport, but to

physically transport the purine nucleotide out of the cell for autocrine/paracrine purinergic signaling [88-90]. With the search for a channel or transporter that is responsible for ATP release in a multitude of cell types, the ABC transporters have become a potential candidate to fill this role. To date, however, much work is focused on determining whether these membrane transporters are capable of transporting ATP out of the cell themselves, or whether they regulate the activity of another channel or transporter responsible for the event.

In 1989 the gene responsible for cystic fibrosis (CF) was identified. Characterization of its gene product, CFTR, revealed a transmembrane transport protein belonging to the ABC transporter family that is involved in cellular chloride homeostasis [91-93]. CFTR has been shown to produce a small Cl^- current itself, and it is thought that activation of CFTR by cAMP-dependent protein kinase A (PKA) regulates the activity of a large-conductance outwardly rectifying chloride channel (ORCC), whose activity is also absent in cystic fibrosis patients lacking the functional CFTR gene. Elucidation of the mechanism mediating this event in epithelial cells has revealed that cAMP-dependent activation of CFTR results in the release of ATP, which can then bind and activate P2 purinergic receptors in an autocrine/paracrine signaling mechanism and stimulate Cl^- efflux from the cell by activation of ORCCs [94]. In agreement with these findings, whole-cell and inside-out patch clamp recordings of cells transfected with CFTR revealed ATP currents that were dependent on cAMP and PKA activation, which were absent in cells lacking the ABC transporter [89]. Related studies show ATP currents in cells expressing the multiple drug resistance gene product P-glycoprotein, another member of the ABC transporter family, further supporting a functional role for this family of

proteins in the release of ATP [88]. While the major focus of ATP release from CFTR has been in epithelial cell physiology, this transporter has been identified in vascular smooth muscle cells [95], endothelial cells [96] and circulating erythrocytes [28] and platelets [97] providing a potential conduit for ATP release into from these cells.

Erythrocytes from CF patients with mutations in CFTR show marked reductions in ATP release upon membrane deformation, a stimulus known to induce ATP release from these cells. Also, incubation of erythrocytes from healthy donors with glibenclamide, a sulfonylurea drug shown to inhibit ABC-transporters and the ATP-sensitive K^+ channel (K_{ATP}), or niflumic acid, an inhibitor of cyclooxygenase-2 that has been proposed to inhibit CFTR, results in a significant decrease in deformation-induced ATP release [28]. However, it should be noted that the pharmacology associated with these studies is complicated by non-specific drug interactions with targets other than CFTR. Nonetheless, the role for CFTR in ATP release from erythrocytes is further supported by studies showing that constitutive activation of PKA, a mechanism known to regulate CFTR activity, by incubation with the active S-stereoisomer of cAMP causes increased ATP release whereas incubation with the inactive R-stereoisomer does not [98].

The sulfonylurea receptor (SUR), recently identified as a member of the ABC transporter family, has been shown to form a functional complex with the K_{ATP} channel [99]. Buildup of intracellular ATP causes membrane depolarization by directly inhibiting potassium efflux through the constitutively active K_{ATP} channel. The sulfonylurea drug glibenclamide is known to inhibit potassium currents from the K_{ATP} channel. This event has been suggested to occur through direct action on SUR to inhibit ATP efflux from the cell and increase the intracellular concentration of ATP. Indeed, activation of K_{ATP}

currents by diazoxide, a sulfonylurea known to activate K_{ATP} channels in pancreatic beta cells [100], is dependent on the presence of releasable intracellular ATP [101]. In the rat pulmonary vasculature, flow-induced release of ATP by the endothelium and the resultant vasodilation can be attenuated by luminal perfusion with glibenclamide [102]. This observation might be explained by inhibition of ATP release from SUR on endothelial cells, or by inhibition of ATP release from CFTR localized to these cells. Ultimately, inhibition of ATP release from either of these transporters could result in decreased autocrine/paracrine signaling through P2Y purinergic receptors on the endothelium which may explain the attenuated vasodilation. The release of ATP through CFTR in the vascular endothelium has not been directly examined, but the expression and Cl^- channel activity of CFTR have been observed in these cells [96].

While the studies presented above have suggested a role for ABC-transporters in directly releasing ATP from cells, studies have emerged opposing this idea. Importantly, it has been shown that CFTR reconstituted in planar lipid bilayers failed to conduct ATP, and CFTR in stably transfected mammalian cell lines, as well as endogenous CFTR in intact organs and human lung cell lines was not involved in the release of ATP from these cells [103]. These studies had a large impact in silencing the notion that ABC transporters may be involved in releasing ATP from cells, however, more recently, a novel study implicating CFTR in acidosis-induced ATP release from skeletal muscle cells has re-ignited the potential for ABC transporters in releasing ATP. In this study, the authors noted a significant decrease in the amount of ATP release from acidotic skeletal muscle cells upon blockade of CFTR with the selective CFTR inhibitor $CFTR_{inh}172$ and by selective knockdown of CFTR with siRNA [104]. These studies suggest a functional

role for CFTR and other ABC transporters as ATP release channels. Expression of these proteins in the vascular endothelium as well as in vascular smooth muscle cells [95,105] may provide a potential mechanism for ATP release from these cells, warranting further investigation into their functional role in the vascular system.

1.3.3. Cell surface F_1/F_0-ATP Sythanse

The process of oxidative phosphorylation in the mitochondria sets up an electrochemical proton gradient across the inner mitochondrial membrane that is utilized by the mitochondrial F_1/F_0-ATP synthase (referred to as ATP synthase) to drive the synthesis of ATP in the mitochondrial matrix. This enzyme is composed of the F_0 membrane bound subunit, which anchors the protein in the inner mitochondrial membrane and houses the pore where proton transport takes place, and the catalytic F_1 subunit, which extends into the mitochondrial matrix and catalyzes the formation of ATP from ADP and inorganic phosphate (for review see [106]). While it was originally thought that ATP synthase was localized and active only in the mitochondria, recent evidence has detected the enzyme at the plasma membrane of a number of cell types, including vascular endothelial cells [107-110]. Angiostatin, a product of plasminogen cleavage in the fibrinolytic system, exhibits high affinity binding to the alpha and beta subunits of the F_1 subunit of ATP synthase in membrane fractions from HUVECs and has been identified as a potent inhibitor of angiogenesis and tumor development [107,111,112]. The anti-angiogenic effects of angiostatin are thought to be mediated by inhibition of ATP release as extracellular ATP is suggested to be a pro-angiogenic factor for endothelial cells [113]. Binding of angiostatin to the F_1 subunit of plasma membrane ATP synthase in endothelial cells inhibits its activity and the formation of extracellular

ATP [114]. In these cells, immunocytochemistry performed under non-permeablizing conditions revealed surface expression of the F_1 subunit further indicating an extracellular localization of the ATP synthase catalytic head. In the vasculature, endothelial cells exposed to increases in blood flow and shear stress release ATP into the lumen to promote vasodilation. Measurement of ATP in the media from cultured human pulmonary artery endothelial cells exposed to increased flow conditions revealed a flow-dependent increase in ATP release that could be inhibited by angiostatin as well as by an antibody directed against the beta subunit of the F_1 catalytic head [21]. These observations present a potential mechanism by which endothelial cells in the blood vessel lumen can release ATP in response to hemodynamic forces. As ATP synthase utilizes a proton motive force for ATP generation, the mechanism of its activation by increased flow remains to be elucidated. In order to synthesize ATP at the extracellular surface of the endothelial cell plasma membrane, a proton gradient must be established across the cell membrane local to the ATP synthase, which could potentially affect the intracellular pH and have detrimental effects on the activity of a number of intracellular proteins by acidifying the regions of the cytosol. Importantly, as this enzyme catalyzes the formation of ATP from ADP and inorganic phosphate, these metabolites must be present in the blood vessel lumen in concentrations sufficient to promote ATP synthase activity. The activity of ecto-nucleotidases and soluble nucleotidases in the plasma may also potentiate degradation of these precursor molecules. Nonetheless, the ectopic expression of the ATP synthase at the plasma membrane of vascular endothelial cells suggests that this protein may be involved in regulating vascular purinergic signaling events. Future studies may provide insights into this novel mechanism of ATP release from cells.

1.3.4. Connexin Hemichannels

Connexins (Cx) are vertebrate gap junction proteins that form hexameric oligomers (called connexons) in the endoplasmic reticulum and Golgi, which are then trafficked to and inserted into the plasma membrane of cells [115]. When a connexon from one cell is in close apposition to a connexon expressed on an adjacent cell, the two proteins can form non-covalent linkages between their extracellular loops, creating an intercellular gap junction channel that connects the cytosol of the two cells. Gap junctions mediate cell-to-cell communication by the transport of intracellular signaling molecules and second messengers less than 1 kD, as well as propagation of membrane potential currents between joined cells [116-118]. To date, about 20 mammalian connexin isoforms have be identified, and the role of these proteins in gap junctional communication has been extensively characterized. However, there have been reports of undocked connexons or hemichannels at the surface of a number of cell types including astrocytes and glia in the central nervous system [119-121], circulating polymorphonuclear granulocytes [79] and monocytes [122], vascular endothelial cells [22,123-125] and vascular smooth muscle cells [126]. It has been proposed that these hemichannels may function as conduits for the transport of molecules, including ATP, between the cytosol of a cell and the extracellular space [127,128]. Under resting physiological conditions, connexin hemichannels remain in a closed impermeable state but it has been suggested that stimuli including decreases in extracellular Ca^{2+} ($[Ca^{2+}]_e$) [129,130], strong membrane depolarizations [131,132], mechanical stimulation [124,125] and metabolic inhibition [133] are capable of opening these hemichannels. Dye uptake experiments are a commonly used experimental technique to evaluate the permeability of membrane

channels to dyes of varying molecular weights. Li *et al* demonstrated that a decrease in $[Ca^{2+}]_e$ resulted in cellular uptake of multiple low-molecular weight dyes in HeLa cells transfected to overexpress Cx43 and normal rat kidney cells. Moreover, dye uptake in a Novakoff hepatoma cell line was markedly reduced upon expression of an antisense Cx43 vector and significantly increased upon over expression of the protein, indicating activity of Cx43 hemichannels in mediating this event [128]. Analysis of the biophysical properties of hemichannels composed of Cx43 revealed an ionic conductance of ~220 pS during electrophysiological patch clamp studies in HeLa cells overexpressing Cx43. This measured conductance was approximately twice that observed for gap junctions, indicating that the measured currents were produced by opening of connexin hemichannels and not gap junctions [131]. Moreover, ATP liberation by C6 glioma cells overexpressing Cx43 was detected in both whole-cell and inside-out patch clamp studies coupled to bioluminescence assays, whereas no detectable ATP release was measured in cells expressing a truncation mutant or GFP tagged Cx43. Both of these modifications have been shown to eliminate channel activity. Also, ATP release from Cx43 expressing cells was attenuated by the addition of two different gap junction blockers, gadolinium^{3+} and carbenoxolone [134]. In addition, cultured astrocytes have been reported to release ATP through connexin hemichannels inducing calcium wave propagation [127]. Application of the gap junction inhibitor flufenamic acid attenuated both dye uptake and ATP release from these cells, suggesting a mechanism involving transport through connexin hemichannels. In a separate study, inflammatory insults such as TNFα and IL-1β in the central nervous system induced ATP and glutamate liberation from astrocytes via Cx43 hemichannels [119]. Altogether, these observations provide evidence for a role

of connexin hemichannels in mediating ATP release from a number of different cell types.

To date, four connexin isoforms are abundantly expressed in the vascular smooth muscle and endothelial cells that comprise the blood vessel wall: Cx37, Cx40, Cx43 and Cx45 [135,136]. Gap junctions formed by these isoforms have been shown to have important roles in regulating cross talk between adjacent VSMCs and ECs, as well as between the two cell types at the myoendothelial junction (for review see [137]). In particular, gap junctions comprised of Cx40 in the vascular endothelium are important for calcium wave propagation in conducted vasodilation [138]. At the level of the myoendothelial junction, the point at which endothelial cells and smooth muscle cells come into contact through the internal elastic lamina, Cx40 and Cx43 form gap junctions that allow for the transfer of second messengers and ions between the two cell types to coordinate vasomotor responses [139]. As these vascular cells highly express connexin proteins, it is possible that there exists a population of undocked connexin hemichannels that could provide a conduit for ATP release into the vessel lumen or extracellular milieu surrounding the vascular media.

Several recent studies have examined the potential for endothelial cells and smooth muscle cells to release ATP from connexin hemichannels. It has been shown that propagation of calcium waves in confluent monolayers of bovine corneal endothelial cells occurs in response to mechanical stimulation in which a glass micropipette is used to mechanically perturb the plasma membrane of a cell [125]. In these studies, application of the gap junction blocker flufenamic acid and the connexin mimetic peptide Gap26 significantly reduced calcium wave propagation. Dye transfer was not affected,

indicating that release of a paracrine signaling molecule from connexin hemichannels elicits a calcium response in an adjacent cell and not direct transfer of calcium from one cell to another in this system. Further investigation revealed the release of ATP from these cells upon mechanical stimulation, as well as increased calcium wave propagation in the presence of the CD39 inhibitor ARL67156. The results of this study suggest a role for a distinct pool of connexin hemichannels at the non-junctional membrane of endothelial cells in the cornea that are capable of releasing ATP to promote calcium wave propagation through a paracrine signaling mechanism during mechanical stress. In the juxtaglomerular vasculature of the renal system, glomerular endothelial cells (GENCs) propagate calcium waves to regulate renal blood flow and the glomerular filtration rate through the release of ATP and purinergic signaling mechanisms [140]. Mechanical stimulation of cultured GENC resulted in the formation of calcium waves that were inhibited by the gap junction uncoupler 18α-glycyrrhetinic acid (18α-GA) and by siRNA knockdown of Cx40 in these cells. As Cx40 is highly expressed and is capable of coupling GENCs through gap junctions, the contributions of ATP release and purinergic signaling mechanisms to the observed calcium wave were evaluated by application of an ATP scavenging cocktail consisting of hexokinase, a glycolytic enzyme that utilizes high amounts of ATP, and apyrase to degrade extracellular ATP. During mechanical stimulation in the presence of the cocktail, calcium wave propagation between adjacent glomerular endothelial cells was greatly attenuated with no significant effect on dye transfer between cells, indicating a role for extracellular ATP in mediating calcium wave propagation. The authors utilized an ATP biosensor assay to measure ATP release from cultured GENCs. The biosensor system consisted of loading PC12 cells, which express a

variety of purinergic receptors, with the calcium indicator dye Fluo-4 and applying them to a confluent monolayer of GENCs. In response to the mechanical stimuli, GENCs released ATP, which bound to purinergic receptors on the PC12 cells, thereby eliciting an increase in intracellular calcium as detected by Fluo-4 fluorescence, which enabled direct quantification of ATP release from the endothelial cells. This response was attenuated by inhibiting the purinergic receptors on PC12 cells with the non-selective P2 purinergic receptor antagonist suramin, which validated the specificity of their biosensor system to extracellular ATP. The mechanically induced release of ATP from GENCs was abolished by siRNA knockdown of Cx40 and could be restored by addition of exogenous ATP to the system [124]. Altogether, these different studies suggest that these endothelial cells express undocked connexin hemichannels at the plasma membrane, which are capable of releasing ATP in response to mechanical stimulation.

While hypoxia has been suggested to increase flow-induced ATP release from endothelial cells [9], it has also been suggested that hypoxia inhibits ATP liberation by Cx43 hemichannels in the endothelium [22]. The authors of the latter study concluded that hypoxia decreased ATP release from cultured endothelial cells by down regulation of Cx43 mRNA and hemichannel expression at the plasma membrane, as well as an upregulation of Cx43 phosphorylation at Ser368 [141], which has been shown to inhibit channel activity. This observation suggests that connexin hemichannels may be implicated in ATP release under certain conditions; however, they do not play a role in hypoxia induced ATP release from endothelial cells.

Connexin hemichannels may play an important role in the liberation of ATP during pathologies such as acute infection, inflammation and atherosclerosis. Endothelial cells

exposed to the gram-positive bacterial wall component peptidoglycan (PDG) derived from *Staphylococcus epidermidis* have been shown to release ATP through Cx43 hemichannels [123]. This study revealed an increase in Cx43 mRNA as well as induction of the pro-inflammatory cytokine interleukin-6 and Toll like receptor 2 (TLR2) in endothelial cells in response to PDG. The induction of this inflammatory cascade was coupled to increased Cx43 hemichannel activity at the plasma membrane of these cells and release of ATP into the media.

During atherosclerosis, circulating monocytes adhere to and migrate across the vascular endothelium where they differentiate into macrophages and foam cells in the subintimal space, eventually leading to atherosclerotic plaque formation and narrowing of the vessel lumen (for review see [142]). A novel study by Wong *et al* indicated a protective role for ATP released from circulating monocytes via Cx37 hemichannels during atherosclerosis [122]. Complete genetic knockout of Cx37 in the proatherogenic $ApoE^{-/-}$ mouse line resulted in a marked increase in atherosclerotic lesion formation and area. Further investigation revealed that monocytes from the $Cx37^{-/-}/ApoE^{-/-}$ mice had a significant decrease in basal ATP release as compared to those from mice expressing functional Cx37. This reduction in ATP liberation from monocytes lacking Cx37 resulted in a significant increase in monocyte adhesion to cultured endothelial cells, supporting their *in vivo* evidence for increased plaque formation and size. Monocyte adhesion was also increased in response to connexin hemichannel blockade by 18α-GA, and the connexin mimetic peptides Gap26 and Gap27. In a separate study, activation of neutrophils with fMLP (N-formyl Met-Leu-Pro) induced ATP release that resulted in decreased endothelial cell permeability [79]. Release of ATP from these cells was

significantly reduced upon addition of the Cx43 specific mimetic peptide ^{43}Gap26 as well as from Cx43$^{-/-}$ neutrophils, indicating a release mechanism involving Cx43 hemichannels. The released ATP was actively degraded to adenosine by the activity of CD39 expressed on the neutrophils themselves and CD73 expressed on endothelial cells. Binding of adenosine to A2a adenosine receptors on the endothelial cells resulted in decreased paracellular permeability. The mechanisms surrounding A2a adenosine receptor mediated changes in endothelial cell permeability have also been investigated [143]. While the data presented above suggest a role for ATP release from Cx hemichannels in circulating inflammatory cells as a protective mechanism against infiltration into the vascular wall, a host of literature suggests that ATP promotes vascular smooth muscle cell migration and proliferation through purinergic signaling mechanisms [13,14,144]. It has also recently been shown that fibroblasts release ATP from connexin hemichannels promoting profibrotic responses in the heart [145] and these observations may suggest a role for resident vascular fibroblasts to release ATP via this mechanism to promote purinergic signaling in the blood vessel wall. Taken together, connexin hemichannels may be important for regulating ATP release in the blood vessel lumen to modulate endothelial cell permeability, while differential ATP release mechanisms in the vascular media may be involved in modulating vascular smooth muscle cell phenotype, such as ATP released from parasympathetic nerves [146].

Altogether, the data presented above support a potential role for connexin hemichannels in ATP release under pathological (i.e. inflammatory insults, atherosclerosis, decreased $[Ca^{2+}]_e$) and physiological (i.e. mechanical stimulation) conditions; however, it remains to be elucidated as to whether these hemichannels play a

role in ATP release from vascular cells for the regulation of vascular tone and blood pressure.

1.3.5. Pannexin Channels

In 2000, a novel protein family was identified termed pannexins (Panx) that are orthologs of the invertebrate gap junction proteins, the innexins, and share a similar membrane topology to mammalian gap junction proteins, connexins [147]. To date, three pannexin isoforms have been identified (Panx1, Panx2 and Panx3), and the distribution of each across different tissues and cell types is a current topic of investigation. Panx1 and Panx3 are believed to assemble into hexamers, while Panx2 may form heptamers or octomers [148,149]. The pannexins are assembled in the ER and Golgi and are transported to the plasma membrane in a fashion similar to the connexins; however, the pannexins do not form gap junctions and therefore their nomenclature is designated as pannexin channels and not hemichannels [150]. This key difference is likely due to the fact that the pannexins are highly glycosylated on their extracellular loops, which may impede docking with pannexin channels on neighboring cells [151,152]. Therefore, it has been suggested that pannexins form membrane channels that allow transport of molecules between the intra- and extracellular space. Upon characterization of the properties of the channels formed by pannexins in a heterologous expression system, it was observed that unlike connexin hemichannels, pannexin channels are not regulated by $[Ca^{2+}]_e$, allowing these channels to open under physiological $[Ca^{2+}]_e$ [153]. Expression of Panx1 in *xenopus* oocytes revealed maxi anion currents upon membrane depolarization with KCl, and patch clamp studies demonstrated the permeability of these channels to ATP [154]. This observation has since prompted multiple studies investigating the role

of Panx1 channels in ATP release from a number of cell types including: astrocytes, glial cells and neurons in the central nervous system [155-160], T-cells [161-163], airway epithelia [164,165], taste cells [166], keratinocytes [167], circulating erythrocytes [30,53], and vascular smooth muscle cells and endothelial cells [11,168]. While Panx1 channels constitute ATP release channels that function under physiological conditions, there have also been reports implicating these proteins in apoptosis and cell death [78], as well as in activation of T-cells during inflammation [161-163]. Therefore, much effort is currently devoted to identifying the mechanisms that regulate pannexin channel permeability to purines and other intracellular molecules, as well as potential binding partners that may regulate the release of ATP through these channels.

During cellular apoptosis, dying cells release nucleotides into the extracellular space to promote phagocyte chemotaxis, engulfment and clearance. Recent evidence has implicated Panx1 channels in mediating ATP release during this process. Chekeni *et al* observed activation of Panx1 channels in apoptotic cells induced by caspase cleavage of the intracellular carboxy-tail of Panx1. More recent evidence has characterized the importance of the Panx1 C-tail in regulation of channel permeability [169]. This event resulted in ATP release into the extracellular milieu and recruitment of phagocytes. The release of ATP as well as monocyte and leukocyte recruitment to cells stimulated with anti-Fas or UV light, both of which are known pro-apoptotic stimuli, were significantly attenuated upon knockdown of Panx1 with Panx1 siRNA, indicating that these channels are the conduit for ATP release and promotion of apoptotic cell clearance [78]. While this study indicates a role for ATP release from Panx1 in cell death, ATP release from Panx1 has been shown to be protective during pathological insults such as ischemia. In

astrocytes, ischemic stress has been shown to suppress the release of ATP to protect against cellular death and promote survival. This protection from ischemic insult was suggested to occur through inhibition of ATP release from Panx1 channels, by a negative feedback mechanism involving the $P2X_7$ purinergic receptor [156]. Prolonged activation of $P2X_7$ receptors results in the opening of a large pore permeable to divalent cations such as Ca^{2+} that eventually leads to cell death [170-172]. This study revealed that ischemic insult to cultured cortical astrocytes resulted in initial ATP release from Panx1 channels that in turn activated $P2X_7$ receptors, inducing closure of Panx1 channels, which prevented loss of integral intracellular constituents and averted cell death. Together, these studies identify differential roles for Panx1 in pathological states.

While evidence is accumulating that supports pannexins as ATP release channels across cell types, their expression and activity in the vasculature are a current topic of investigation. Initial studies have identified key roles for Panx1 channels in regulating ATP release from circulating erythrocytes during hypoxia and during membrane deformation [30,53], from vascular smooth muscle cells upon α_{1D}-adrenergic receptor activation [11] and from endothelial cells in response to thrombin stimulation [168]. Liberation of ATP from these cells occurs under physiological calcium concentrations and may play pivotal roles in regulating vessel tone and peripheral resistance. Initial evidence has suggested that ATP release from circulating erythrocytes in response to low oxygen tension occurs through the ABC-transporter CFTR [28] (see *ATP binding cassette transporters* section); however, recent studies have presented conflicting evidence for the ability of ABC transporters to release ATP [173,174]. A recent study by Locovei *et al* demonstrated that ATP release during depolarization of erythrocytes by

high extracellular K^+, as well as decreased oxygen tension, was inhibited by the gap junction blocker carbenoxolone. The authors noted that the erythrocytes in this study had no detectable Cx43 expression, further suggesting that carbenoxolone inhibited Panx1 channels and not Cx hemichannels. While carbenoxolone has been shown to inhibit connexins as well as pannexins, the EC50 for pannexin inhibition is ~5μM, whereas incubation of *xenopus* oocytes expressing Cx46 (the model connexin isoform for examining properties of connexin hemichannels) with >10μM carbenoxolone had no effect on hemichannel current [153]. In a separate study, inhibition of ATP release from erythrocytes exposed to low oxygen tension was evaluated in the presence of three separate inhibitors of pannexin channels, carbenoxolone, probenecid and the blocking peptide ^{10}Panx1 [30]. This study revealed that treatment of human erythrocytes with any of these three inhibitors significantly attenuated low oxygen tension-induced ATP release, further supporting a functional role for Panx1 channels in ATP release from circulating erythrocytes under these conditions.

Vascular smooth muscle cells in small arteries that are involved in regulation of blood flow and blood pressure have been shown to contract in response to α-adrenergic receptor stimulation. Sympathetic nervous impulses elicit release of the catecholamine norepinephrine from nerve terminals innervating vascular beds resulting in activation of the α_{1D}-adrenergic receptor, an increase in smooth muscle cell $[Ca^{2+}]_i$ and vasoconstriction, ultimately producing an increase in peripheral resistance and blood pressure [175]. The Isakson laboratory recently identified a novel role for Panx1 channels in regulating the α_1-adrenergic receptor mediated response [11]. In this study, stimulation of isolated pressurized thoracodorsal arteries with the α_1-adrenergic receptor

agonist phenylephrine (PE) resulted in strong vasoconstriction, consistent with previous observations. Recent characterization of the thoracodorsal artery has shown that it is highly innervated by adrenergic nerves, develops spontaneous tone and responds to various vasoactive agents similar to other small resistance artery models [176]. In the presence of three different pannexin inhibitors probenecid, mefloquine and ^{10}Panx1 blocking peptide, the constriction to PE was significantly reduced. Also, application of apyrase to the superfusion bath surrounding the blood vessel resulted in a similar inhibition of contraction. Finally, stimulation of cultured human coronary arterial vascular smooth muscle cells with PE induced ATP release from these cells that was significantly attenuated by pretreatment of the cells with the ^{10}Panx1 blocking peptide. Together, these results identify a novel role for vascular smooth muscle cell Panx1 channels in releasing ATP during α_1-adrenergic receptor activation, a process that is essential for the regulation of vascular tone and blood pressure. These results indicate a potential therapeutic approach for blood pressure regulation. It was also observed that expression of Panx1 in endothelial cells of the blood vessel wall was significantly enhanced. In addition, Goedecke *et al* recently demonstrated that HUVECs release ATP in response to thrombin, which was sensitive to carbenoxolone and knockdown of endogenous Panx1 [168]. Furthermore, analysis of the expression patterns for the different pannexin isoforms in the smooth muscle and endothelial cells across the arterial tree suggests increased expression in smooth muscle cells as vessel diameter decreases, supporting the idea that ATP release from Panx1 channels in vascular smooth muscle cells of small arteries and arterioles may play an important role in regulating blood flow [177].

To date little attention has been paid to the role of pannexin channels in the vascular endothelium, in particular those in the regulation of inflammation. However, characterization of the pharmacological properties of a number of gap junction inhibitors has suggested that some of the previous studies identifying connexin hemichannels as the cellular pathway for ATP release may potentially be due to activity of Panx channels [178]. In fact, differentiating between pannexin channels and connexins hemichannels has increasingly become an issue in the study of these respective proteins. These issues have been primarily associated with the lack of specificity of pharmacological blockers of the two channels, overlapping expression in a number of cell types and tissues, the use of different experimental approaches to assess channel function and the potential issue of compensation by other isoforms in studies employing genetic manipulation of Cx and Panx expression. Due to these limitations in segregating Cx- and Panx-dependent activity in relation to ATP release, it has become increasingly difficult to ascribe unequivocal function to one channel over the other in a number of physiological and pathological contexts. These limitations are discussed below in detail.

1.4. Limitations in pharmacological selectivity for Cx hemichannels and Panx channels.

The study of Cx hemichannel and Panx channel function has relied intently on the use of pharmacological inhibitors of channel currents, dye uptake and ATP release. This review will focus on the most commonly used pharmacological inhibitors of Cxs and Panxs; for a comprehensive list of Panx and Cx inhibitors refer to [178-180]. Notably, a number of gap junction blockers including flufenamic acid (FFA), the long-chain alcohols octonol and heptanol, glycyrrhetinic acid (GA) derivatives (18α-GA and

carbenoxolone (CBX)) and mimetic peptides have been employed to inhibit Cxs. Many of these substances have been reported to block both Cx hemichannel and gap junction activity, which has limited the interpretation of specific contribution of hemichannels vs. gap junctions in cell-to-cell communication. For example, FFA, a potent inhibitor of Cx based gap junctions, strongly reduces hemichannel currents in unpaired *Xenopus oocytes* overexpressing Cx50 or Cx46 [181]. In addition, the GA derivative 18α-GA which prevents dye coupling between cells paired by gap junctions, inhibits dye uptake by Cx43 hemichannels in astrocytes [182]. Nonetheless, several of these inhibitors have been assessed in their relative potency for inhibition of Cx hemichannels and Panx channels. In terms of Cx hemichannels, FFA, octonol and heptanol have been reported to block gap junction and Cx hemichannel activity with minimal effect on Panx1 channels [153,181,183,184]. Comparatively, FFA has been reported to inhibit Cx hemichannel activity at low micromolar concentrations, while analysis of the effects of this compound on Panx1 channels expressed in oocytes showed only modest inhibition of Panx1 currents at 300μM [153]. As Panxs have emerged as possible candidate channels for ATP release, a number of these inhibitors that were originally thought to selectively block Cx-based gap junctions and hemichannels are now known to also block Panx channels, in some cases to a much higher degree than Cx hemichannels. This is best exemplified by the GA derivative CBX which inhibits both Cx hemichannels and Panx1 channels. Pharmacological assessment of CBX potency has revealed a substantially greater affinity for Panx1 channel inhibition than Cx hemichannel inhibition (EC_{50} = ~5μM for Panx1 vs 10-100μM for Cx43 hemichannels [153,182]). Based on these observations, many previous studies employing CBX to block Cx hemichannel function likely also blocked

Panx1 function and as a result, it has now become common to employ CBX as a Panx1 blocker. At the other end of the spectrum, a few compounds have been identified that are more specific for Panx channel inhibition than Cx hemichannel inhibition, notably the uricosuric drug probenecid. Probenecid has been reported to block Panx1 currents and dye uptake in oocytes expressing the channel, with no effect on currents carried by Cx46 or $Cx32_1 43$ (a chimera of Cx32 containing the first extracellular loop of Cx43) [185]. In addition, probenecid reduced $[Ca^{2+}]_i$ in response to histamine in subcutaneous fibroblasts which was dependent on Panx1-mediated ATP release [186].

As the problem of pharmacological selectivity for Cx hemichannels and Panx channels has grown, novel methods for targeting each respective channel have been developed with the mimetic peptides receiving major focus. Cx mimetic peptides were first developed to block gap junction formation and subsequent intercellular communication and were widely used in model systems where cellular conduction is essential including the heart [187], lung epithelium [188], and vascular smooth muscle and endothelial cells [189] [190]. These peptides have been designed to mimic the primary amino acid sequence of varying regions of Cx isoforms, with the majority mimicking the extracellular loop regions. Following the observation that Cx mimetic peptides could inhibit gap-junctional communication in coupled cells, presumably by preventing docking of Cx hemichannels between cells, their ability to selectively block Cx hemichannels was assessed. Of particular note, the Cx mimetics Gap26 and Gap27 which mimic regions of the first and second extracellular domains of Cx43, respectively, can inhibit currents carried by Cx43 hemichannels expressed in HeLa cells [191,192]. Moreover, Gap26 has been reported to inhibit ATP release from corneal endothelial cells

in response to mechanical stress and reduce basal ATP release from vascular endothelial cells in culture [22,125]. While these studies initially advanced the repertoire of pharmacological agents to selectively block Cx hemichannels and gap junctions, recent evidence has emerged indicating cross-inhibition of Panx1 channels by several Cx mimetic peptides. Most notably, the Gap26 and Gap27 peptides were shown to inhibit Panx1 currents in oocytes as well as those carried by Cx43 [193]. Moreover, the Cx mimetic peptide ^{32}Gap24, which mimics a sequence in the Cx32 intracellular loop domain, had no effect on hemichannel currents from cells expressing the $Cx32_1 43$ chimera but drastically reduced Panx1 currents [193]. These observations may suggest that the mode of action of these mimetic peptides is not dependent on the mimicked sequence, as Panx1 does not share sequence homology in either its extracellular loop or intracellular domains with Cxs. Based on the cross-inhibition observed with many of the currently employed connexins mimetic peptides, new efforts have been made to develop novel peptide antagonists that may specifically inhibit Cx hemichannels without affecting gap junction channels or Panx channels. Recently, a novel Cx mimetic peptide termed Gap19 was developed, mimicking a sequence in the intracellular loop of Cx43. Gap19 has been reported to specifically prevent Cx43 hemichannel activity in HeLa cell expressing exogenous Cx43 as well as endogenous Cx43 currents in isolated cardiac myocytes through a mechanism that disrupts intramolecular interactions between the intracellular loop and C-tail of Cx43 [194]. This inhibition was selective for Cx43 hemichannels without affecting Panx1 channel currents or Cx40 hemichannel currents. In addition to Gap19, a second mimetic peptide designed to interrupt intracellular loop:C-tail interactions called TAT-L2 has been reported to specifically inhibit endogenous Cx43

hemichannel activity in endothelial cells and hemichannel activity in ectopic expression systems [195]. These new peptides have shed light on the gating of Cx43 hemichannels which appears to be tightly regulated by interactions between the intracellular domains of Cx43. Due to the increasing success of mimetic peptides in targeting Cx hemichannels, a mimetic peptide of the first extracellular loop of Panx1 termed ^{10}panx1 was developed and has subsequently been shown to significantly inhibits channel currents, dye uptake and ATP release mediated by Panx1 in a number of cell types [164,196,197]. The Panx mimetic peptide has not escaped the issue of cross-inhibition, however, as it was recently shown that the ^{10}panx1 peptide also blocks Cx46 hemichannel currents [193].

Based on the sum of these observations, the study of Cx hemichannel and Panx channel function remains plagued by the overlapping pharmacology. As we continue to accumulate new information on the relative potency of current and new drugs for blockade of Panxs and Cxs, it will be important to not rely solely on one inhibitor but to utilize a gamut of blockers at appropriate concentrations to define the contributions of Cx hemichannels and Panx channels to purinergic signaling events.

1.5. Experimental methodologies in the study of Cx and Panx function.

In addition to the limitations in segregating Cx hemichannel and Panx channel function pharmacologically, there is a discourse in the methods utilized to interrogate each channel's function as it relates to ATP release. Currently, three techniques are commonly employed to measure Cx and Panx activity: dye uptake or release, electrophysiological preparations to measure channel currents and strategies aimed at directly quantifying ATP liberation from cells. Dye uptake assays have been used as a parameter of cell permeability in response to a range of stimuli that evoke Cx

hemichannel or Panx channel opening. These assays rely on the ability of a dye present in the extracellular milieu to cross the plasma membrane and become concentrated inside of the cell and quantified by fluorescence microscopy. Some commonly used fluorescent molecules include Lucifer yellow, ethidium bromide, propidium iodide, DAPI, YoPro-1 and ToPro-1 (e.g., [78,198,199]). Each of these molecules has very distinct characteristics including differences in charge, size and conformation which may specifically affect their respective permeabilities through Cx hemichannels or Panx channels. In agreement, differences in the permeability of several of these dyes through gap junctions and Cx hemichannels have been assessed in HeLa cells expressing a number of different Cx isoforms (Cx26, Cx31, Cx32, Cx40, Cx43 and Cx45) [200,201]. The results of these studies indicate that specific Cx isoforms harbor differential permeabilities to specific fluorescent molecules, likely based on the relative charge or size of the permeant and conductance properties of the gap junction or hemichannel. Based on these observations, the selection of molecules for dye uptake experiments should be considered carefully depending on the particular isoform being studied [200]. Notwithstanding, dye uptake experiments have provided a strong output for quantifying the permeability of cells as it relates to hemichannel or channel activation but this methodology should not be solely relied on for the assessment of ATP release as one is not indicative of the other.

Electrophysiology has been the workhorse for assessing Cx hemichannel and Panx channel activity. These methods, coupled with ectopic expression systems to isolate the channel of interest, have provided great insight into the properties of both Cxs and Panxs and aided in discerning the relative selectivity of pharmacological blockers for

the two channels as discussed above. Whole-cell and singe-channel recordings have identified a number of unique properties between Cx hemichannels and Panx channels. Cx43 hemichannels expressed in oocytes show a conductance of ~220pS whereas Panx1 conductance has been initially reported to be ~500pS [154,202]. Cx hemichannels have been shown to be tightly regulated by membrane potential, with strong depolarizations (~+60mV) activating the channel while Panx1 channels can open under hyperpolarizing potentials in the physiological range (>-20mV) [202,203]. Moreover, electrophysiological analyses have revealed that both Cx43 hemichannels and Panx1 channels are permeable to ATP. Utilizing an ATP gradient between the patch pipette and bath solution, Bao *et al*. demonstrated the direct permeability of ATP through single Panx1 channels in excised patches [154]. Similarly, whole cell and excised patch clamp techniques have indicated conductance of ATP through hemichannels composed of Cx43 [134]. These latter studies also employed dual electrophysiological recordings and bioluminescence for simultaneous photo-detection of ATP in inside-out patches from Cx43 expressing oocytes, directly coupling the electrophysiological recording of hemichannel activity to ATP permeability. While the implement of electrophysiology in the study of Cx hemichannel and Panx channel function has significantly advanced our knowledge on the unique properties of these two families of channels, there remain limitations in extrapolating these functional studies to the physiological setting. As mentioned above, many of the observations implicating Cx hemichannels and Panx channels activity in ATP release have relied on ectopic expression systems to isolate the channel from contaminating signals. Thus, these channels are placed in an artificial setting that may not reflect their endogenous environment. Given these considerations,

future studies aimed at assigning Cx hemichannel or Panx channel activity to ATP release should make effort to analyze these channel properties in native cells. Nonetheless, the knowledge we have gained on the biophysical properties of Cxs and Panxs through the use of electrophysiological techniques has made great strides in differentiating between the two families of proteins.

Assessment of the contribution of Cx hemichannels and Panx channels to cellular ATP release should not depend on dye uptake and electrophysiological recordings alone, but employ assays for the direct detection of ATP liberated from the cell. The current gold-standard for ATP quantification relies on luciferin:luciferase chemistry where the enzyme luciferase catalyzes the oxidation of ATP and luciferin ultimately resulting in the generation of a photon which can be readily quantified by luminometry. Luciferin:luciferase bioluminescence has subsequently been implemented in a number of models to directly test the accumulation of extracellular ATP in response to Cx hemichannel and Panx channel activation [30,125,134,165,204,205].

The aforementioned methods currently used for assessing Panx channel and Cx hemichannel activity as it relates to ATP release and purinergic signaling have shed light on the properties of these two channels and have provided direct evidence that both Cx hemichannels and Panx channels are permeable to ATP. It is important to note that not all studies are created equally, with experimental conditions and outputs differing between groups. Based on these points, future studies geared towards assigning function to one channel over another should strive to implement a combination of these methods.

The release of intracellular ATP into the extracellular space promotes signaling through purinergic receptors that has been extensively characterized in multiple cell types

and experimental systems. Importantly, evidence for purinergic signaling in the regulation of vascular tone at both vascular smooth muscle and endothelial cells warrants investigation into how this purine nucleotide is released from the cells. Current studies have proposed mechanisms of ATP release by vesicular exocytosis, transport through ATP-binding cassette transporters, a plasma membrane F_1/F_0-ATP synthase and liberation via connexin hemichannels or pannexin channels **(Figure 1)**. The stimuli used to evoke ATP release from vascular cells in these studies have varied and importantly it must be noted that quantification of ATP in cell supernatants can easily be confounded by stimuli that induce cell lysis. Because cells contain millimolar amounts of cytosolic ATP, rupture of even a single cell in a given preparation can release a sufficient amount of ATP to elicit physiological effects (i.e. Ca^{2+} wave propagation [206]) and may dramatically skew the quantification and interpretation of ATP released from cells. In particular, it has recently been noted that increases in shear stress on endothelial cells can induce cell lysis, which may be the rational for the noted increase extracellular ATP [207]. It is also not known whether the different pharmacological agents employed to evaluate the mechanisms of ATP release have effects on lytic-ATP release. It is therefore essential that experiments evaluating ATP release by quantification in cell supernatants be controlled for lytic-ATP release. Nonetheless, the studies reviewed above have examined ATP release from cells in the vasculature by multiple mechanisms and promote the idea that differential release mechanisms may be employed during specific physiological and pathological situations. However, the recently identified Panx1 channels have been shown to be closed at resting membrane potentials and are capable of opening in the presence of physiological extracellular calcium, in response to α_1-

adrenergic receptor activation and hypoxia and are ubiquitously expressed throughout the vasculature. These results support the notion that Panx channels may be the prominent ATP release channels in the vascular system. The identification of Panx1 in the vascular endothelium raises the possibility that ATP released from these cells during periods of increased shear stress and hypoxia, or in response to other signaling mediator such as thrombin or histamine, may occur by activation of these channels. Based on these points, we hypothesize that Panx1 channels function in the vascular endothelium to integrate inflammatory and purinergic signaling cascades imparting a change to an inflammatory EC type. We propose that Panx1 channels release ATP downstream of TNFα receptors in venous ECs and that this process may be negatively regulated by nitric oxide. The following chapters detail the examination of these hypotheses.

FIGURE 1: MECHANISMS OF ATP RELEASE FROM CELLS OF THE BLOOD VESSEL WALL

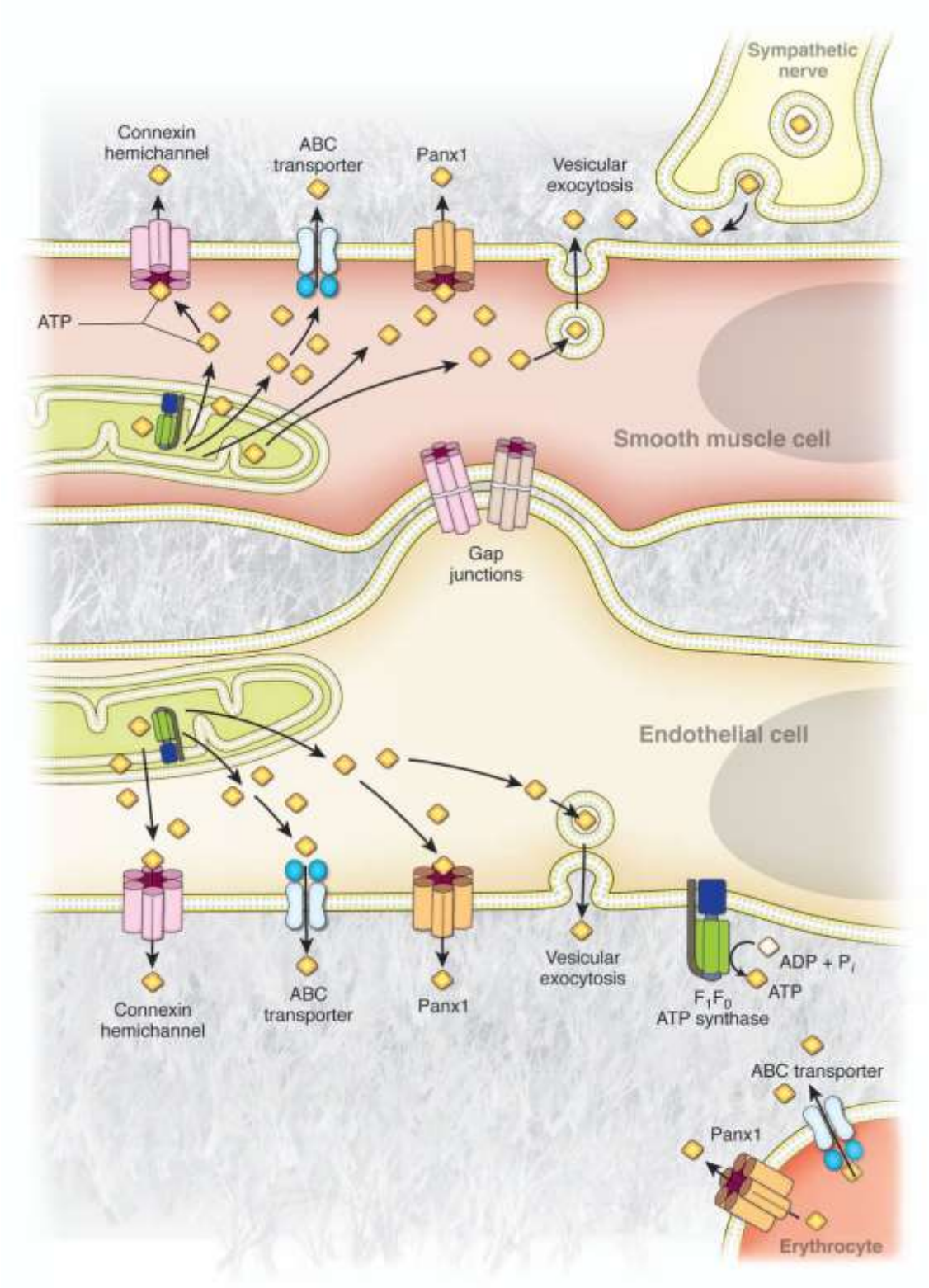

Figure 1. Mechanisms of ATP release from cells of the blood vessel wall. Representative illustration of the currently proposed mechanisms of ATP release from endothelial cells, vascular smooth muscle cells, perivascular sympathetic nerves and circulating erythrocytes in the vascular system. Cytosolic ATP available for release from these cells is generated by glycolysis in the cytosol and oxidative phosphorylation in the mitochondria. In sympathetic nerves, ATP release has been shown to occur via vesicular exocytosis. Vascular smooth muscle cells have been suggested to release ATP through membrane transporters and channels including connexin hemichannels and pannexin channels, as well as potentially by ATP-binding cassette (ABC) transporters. Vascular endothelial cells have been proposed to release ATP via vesicular exocytosis, ABC-transporters, connexin hemichannels, pannexin channels and by direct synthesis at the extracellular plasma membrane by a cell surface F1/F0-ATP synthase. Circulating erythrocytes have been suggested to release ATP through ABC-transporters and via pannexin channels. Image prepared by Anita Impagliazzo.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials and Methods for Chapter 3:

HEK293T Cells: Human embryonic kidney cells 293T (HEK293T) were used under passage 20 and grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 1% non-essential amino acids. Cells were transfected with mouse Panx1 (pcDNA3.1), mouse Panx2 (pEGFP-N1 [151]) or mouse Panx3 (pEGFP-N1 [151]) plasmids at 750 ng/mL using 8 µg Lipofectamine 2000 on $6x10^5$ cells for 15 hours. Cells were washed with PBS and were either fixed with 4% paraformaldehyde before being processed for immunocytochemistry (as previously described [208]) or protein was extracted for Western blot analysis.

Western blot: Western blots were performed as described previously [208]. For all experiments, 50 µg of protein was loaded into each well.

qRT-PCR: qRT-PCR was performed as described previously [209] with primers for mouse and human Panx1, Panx2 and Panx3 **(Table 1).** Transcripts were quantified by comparing to the housekeeping gene β2 microglobulin (B2M) [210].

Animals: All mice were male, 10-14 weeks of age, on a C57Bl/6 genetic background and were cared for under the provisions of the University of Virginia Animal Care and Use Committee. The $Panx1^{-/-}$ mice were kind gifts of Genentech Corporation and were cared for under provisions of the University of Western Ontario Animal Care and Use Committee. The $Panx1^{-/-}$ mouse tissue samples were harvested at the University of Western Ontario and sent to the University of Virginia for analysis. All experiments were performed on a minimum of n=3 mice.

Table 1. Primer sequences for mouse and human Panx1, Panx3 and Panx3

Mouse	**Panx1**	**Forward: 5'-TAAGCTGCTTCTCCCCGAGT-3'**
		Reverse: 5'-TGGCAAACAGCAGTAGGATG-3'
	Panx2	**Forward: 5'AAGCATACCCGCCACTTCTC-3'**
		Reverse: 5'-GGGGTACGGGATTTCCTTCT-3'
	Panx3	**Forward: 5'-CCCATTCTCAGCAGCATCAT-3'**
		Reverse: 5'-ACTCCTGGGCGAAAGCTAGA-3'
Human	**Panx1**	**Forward: 5'-TGCAGAGCGAGTCTGGAAAC-3'**
		Reverse: 5'-CAGCCTTAATTGCACGGTTG-3'
	Panx2	**Forward: 5'-GTCACCCTGGTCTTCACCAA-3'**
		Reverse: 5'-GCAGGAACTTGTGCTCAAACA-3'
	Panx3	**Forward: 5'-CTCAAAGGACTGCGTCTGGA-3'**
		Reverse: 5'-CTGCCGGATGCTGAAGTTAC-3'

Embedding and immunocytochemistry: Prior to tissue harvesting, fixation was performed by perfusing room temperature 4% paraformaldehyde (PFA) made in PBS through the heart. The specific tissues were immediately removed from the animal and placed in 4% PFA for 30 minutes before being placed in 70% ethanol for paraffin embedding. Paraffin sections (4-5 μm in thickness) were de-paraffinized and processed for immunocytochemistry as previously described [11].

Imaging: All images were obtained with an Olympus Fluoview 1000. For each vessel bed, a minimum of 5 sections from each mouse, with a minimum of 3 mice, were evaluated. Figures are representative of composite z-stacks. For z-stacks, a minimum of 5 images at a depth of 0.5 μm/image were compiled. Although each of the different pannexin isoforms was scanned at different settings, the individual pannexin isoforms were scanned at the same settings across tissue beds.

Antibodies: Each of the pannexin antibodies were made in rabbit and initially described in [151]. The Panx1 antibodies were made against amino acids 247-265 (SIKSGVLKNDSTIPDRFQC) in the extracellular loop (anti-Panx1 EL [151]) or amino acids 395-409 (QRVEFKDLDLSSEAA) in the carboxyl tail (anti-Panx1 CT [151]) The Panx2 antibody was made against amino acids 494-508 (ASEKKHTRHFSLDVH) and the Panx3 antibody was made against amino acids 379-392 (KPKHLTQHTYDEHA). AlexaFluor 594 donkey anti-rabbit secondary antibodies were used to observe all primary antibodies. Nuclei were stained with DAPI (Invitrogen). At least five observations were made per mouse, per experiment.

Electron Microscopy: All tissues were processed and stained for immunogold-transmission electron microscopy as previously described [211]. Quantification of gold

beads in endothelium (EC), at the myoendothelial junction (MEJ), or vascular smooth muscle cells (VSMC) was performed as previously described [211] on five TEM images per artery that were sectioned 10 μm apart. Using Metamorph analysis software, areas of EC, MEJ and VSMC were determined in μm^2 and the number of gold beads in each area was counted. Measurements are representative of the average number of gold beads per $\mu m^2 \pm$ SE. For each vessel bed, a minimum of 4 observations from each mouse, with a minimum of 3 mice, were evaluated.

En face imaging of MEJs: The thoracodorsal artery (TDA) was maximally dilated by whole-mouse perfusion with Krebs-HEPES buffer (in mM: 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 4 $NaHCO_3$, 1.2 KH_2PO_4, 2 $CaCl_2$, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonicacid (HEPES) and 6 D-glucose, pH 7.4 with NaOH) containing sodium nitroprusside followed by fixation with 4% PFA. The arteries were removed, cut longitudinally and conventional immunocytochemistry was performed (as described above). The arteries were mounted in DAPI and coverslip sealed to provide a flat surface for confocal imaging. Autofluorescence of the internal elastic lamina between the endothelium and smooth muscle was readily apparent with "holes" between the two layers of cells which has been extensively documented (e.g., [212-214]). In our study, the detection of punctate fluorescence in >25% of the "holes" was considered indicative of protein localization to MEJs.

2.2. Materials and Methods for Chapter 4:

Chemicals and reagents: Reduced L- glutathione (GSH), DL-Dithiothreitol (DTT), N-ethylmaleimide (NEM) and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were purchased from Sigma. Diethylammonium (Z)-1-1(N, N-diethylamino)diazen-1-

ium-1,2-diolate (DEA-NONOate) was purchased from Cayman chemicals and prepared by dissolving in 10 mM NaOH (all DEA solutions were adjusted to pH 7.4). S-nitrosoglutathione (GSNO) was prepared by incubating reduced GSH with sodium nitrate. Carbenoxolone (CBX) was purchased from Fisher. Biotin-HPDP and EZ-link sulfo-NHS-LC-biotin were purchased from Thermo Scientific.

Cell culture and transfections: Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified eagle medium (DMEM) High Glucose (Gibco) supplemented with 10% fetal bovine serum (Life Technologies), 1% penicillin/streptomycin, 1% L-glutamine and 1% non-essential amino acids and maintained at 37°C in a humidified 5% CO_2 incubator. All cells were used for experiments at passage $\leq$ 20. Primary mouse aortic endothelial cells (mAECs) were purchased from Cell Biologics, cultured in ECM-MV media and maintained at 37°C in a humidified 5% CO_2 incubator. All mAECs were used for experiments at passage $\leq$ 8 as per the manufacturer's recommendations.

Cells at 80-90% confluency were transfected with plasmids or siRNAs using the Lipofectamine 2000 reagent (Invitrogen) following the manufacturers protocol. For electrophysiology experiments, cells were co-transfected with the Panx1 pcDNA3.1 plasmid and green fluorescent protein (2.5 µg of Panx1 plasmid with 0.5 µg of pEGFP) and plated onto poly-L-lysine-coated glass coverslips 24 hours later. The cells were returned to the incubator and allowed to adhere for at least 1 hour prior to use. Recordings were conducted within 1 day of plating.

Plasmid generation and site-directed mutagenesis: Full length murine Panx1 coding region was amplified by PCR from a Panx1-EGFP-N1 plasmid, previously described in

[152], using the following primers: forward – 5'caaatgggcggtaggcgtgt 3', reverse – 5' cttgtggccgtttacgtcgc 3'. The PCR product was digested (*HindIII* and *BamHI*) and ligated into pcDNA3.1. The final construct was sequenced to confirm proper insertion. Single cysteine-to-alanine mutations were performed in the Panx1 pcDNA3.1 construct using the QuikChangeII Site-Directed Mutagenesis Kit (Agilent Technologies) and double cysteine-to-alanine mutations were constructed using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer's protocol.

Biotin Switch assay: The biotin switch assay was performed as previously described [215,216]. Briefly, cell monolayers were treated with 100 µM GSNO, 50 µM DEA-NONOate or vehicle for 10 minutes at 37°C. Monolayers were then washed with PBS, cells lysed in RIPA buffer containing protease inhibitors and protein quantified using the Bradford technique. Proteins were precipitated with acetone and pelleted by centrifugation for 5 minutes at 10,000xg. Pellets were resuspended and free cysteine thiols were blocked with NEM for 20 minutes at 50°C. Proteins were precipitated as described above to remove excess NEM and S-nitrosylated cysteines were reduced with 1 mM ascorbate in the presence of 1mM $Cu^{2+}$and biotinylated with biotin-HPDP for 1 hour at room temperature. Biotinylated proteins were then pulled down with streptavidin-agarose beads for 1 hour at room temperature and subjected to SDS PAGE and Western blotting for detection of S-nitrosylated Panx1. For negative controls, ascorbate was omitted from the assay which prevented reduction of S-nitrosothiols and subsequent biotinylation.

Electrophysiology: Whole cell voltage-clamp recordings were performed as described [169]. Recordings were obtained at room temperature with 3-5 MΩ Sylgard-

coated borosilicate glass patch pipettes and an Axopatch 200B amplifier (Molecular Devices). The internal solution contained 30 mM tetraethylammonium chloride, 100 mM $CsMeSO_4$, 4 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM HEPES, 10 mM EGTA, 3 mM ATP-Mg and 0.3 mM GTP-Tris (pH 7.3). The bath solution was composed of 140 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.3). Bath solutions containing 50 µM CBX, 1 mM DTT, or 100 µM DEA NONOate (bubbled with 100% O_2) were flowed over the cells at a rate of approximately 2 ml/min while 100 µM GSNO, 100 µM GSH, or 100 µM GSH with 100 µM H_2O_2 were pipetted directly into the bath under stop-flow conditions. Ramp voltage clamp commands were applied at 5-s intervals using pCLAMP software and a Digidata 1322A digitizer (Molecular Devices). Peak currents were taken at +80 mV and percent inhibition was calculated by dividing the decrease in peak current by the total Panx1 current (defined by its CBX sensitivity). We quantified the DTT-reversible component of GSNO/DEA-inhibited current to define the fraction of current inhibition that was due to S-nitrosylation.

ATP release assay: HEK293T cells or mAECs were plated in 24 well plates coated with 0.01% poly-L-lysine or 0.2% gelatin, respectively. HEK293T cells at 80-90% confluency were transfected with plasmids encoding $Panx1^{WT}$ or Panx1 cysteine mutants for 24 hours. mAECs, cells were either allowed to grow to confluency or transfected at 80-90% confluency with two siRNAs targeting Panx1 (Ambion)[11] for 48 hours to knockdown endogenous Panx1. The media was removed from each well and cells were carefully washed 2 times with warm Krebs-HEPES buffer containing 2 mM Ca^{2+} and 1% BSA. Cells were then incubated in fresh Krebs-HEPES buffer containing 2 mM Ca^{2+} and

1% BSA for 30 minutes at 37°C to allow degradation of any ATP released as a result of mechanical stimulation imparted by changing the media. Ecto-nucleotidases were inhibited by incubating cell monolayers with 300 μM ARL 67156 (Tocris) for 30 minutes at 37°C. HEK293T cells were stimulated by depolarizing the cells with 100 mM KCl for 1 minute or from mAECs by treatment with 1 U/mL mouse thrombin for 5 minutes (as previously described [205]). To evaluate the effect of NO donors on ATP release, cells were pretreated with 100 μM GSNO or 50 μM DEA NONOate for 10 minutes. For controls, cells were pretreated with 100 μM GSH or 50 μM CBX. Following stimulation of the cells, 75 μL of the cell supernatant was collected and placed immediately on ice. All samples were centrifuged at 5000xg for 2 minutes and 50 μL of each sample was transferred to a 96-well plate. Using a FluoStar Omega luminometer, 50 μL of luciferin:luciferase reagent (ATP bioluminescence assay kit HSII; Roche) was injected into each well and luminescence was immediately recorded. ATP concentration in each sample was calculated from a standard curve for all experiments. Data are expressed as a % change in ATP release from control conditions (i.e., unstimulated cells) or % inhibition of ATP release by GSNO for experiments on HEK293T cells expressing Panx1 cysteine mutants.

Cell surface protein biotinylation: HEK293T cells were transfected to express $Panx1^{WT}$ or Panx1 cysteine mutants as described above and grown to confluency in 6-well plates. For experiments examining the effect of NO donors on Panx1 membrane expression, confluent monolayers were treated for various times (0-10 minutes) with GSNO. Cells were washed once with cold PBS then incubated with cold DMEM (without FBS) and 50 μM CBX at 4°C for 30 minutes. CBX was added to prevent biotin

from passing through Panx1 channels, which would label intracellular proteins. Cells were then washed with PBS and incubated at 4°C for 1 hour in cold PBS (1.5 mL/dish) containing EZ-link-sulfo-NHS-LC-biotin (1 mg/mL) and CBX (50 μM). The cells were washed again with PBS and lysed in PBST (PBS + 0.5-1% Triton-X 100) containing protease inhibitors. Total protein was quantified using the Bradford technique and equal amounts of protein were incubated with Streptavidin-agarose beads for 2 hours at 4°C to pull down biotinylated proteins. Beads were washed 5 times with PBST, spun down and bound proteins eluted by incubation with laemmli buffer. Eluted proteins were subjected to SDS-PAGE and Western blotting for detection of Panx1.

Immunofluorescence microscopy: Transfected HEK cells were fixed in 4% PFA for 15 minutes and subjected to standard immunocytochemistry as previously described [11]. Images were obtained with an Olympus Fluoview 1000 laser scanning confocal microscope.

cGMP assay: Transfected HEK cells were incubated with ODQ (10 μM) for 20 minutes prior to treatment with GSNO (100 μM) or vehicle for 10 minutes at 37°C. Cells were then isolated and lysed in buffer provided in the cGMP XP® Assay kit (Cell Signaling) and the assay was performed according to manufacturer's protocol. A standard curve of known cGMP concentrations was constructed and used to calculate cGMP concentrations in the experimental samples.

Data Analysis: Results are presented as means ± SEM. Statistical significance was determined by $p < 0.05$ using a Mann-Whitney U-test or Kruskal-Wallis test followed by Dunn's test for multiple comparisons.

2.3. Materials and Methods for Chapter 5:

Cell culture: Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza. Primary human saphenous vein (HSaVEC), human aortic endothelial cells (HAoEC) and human coronary artery endothelial cells (HCoAEC) were purchased from PromoCell. All ECs were maintained under standard cell culture conditions in endothelial growth medium (EGM) supplied by Lonza. For siRNA knockout down Panx1, HUVEC and HSaVECs were plated in 6-well (expression) or 24-well plates (ATP release) and grown to 70-80% confluence. Non-targeting or siRNAs targeting the 3rd and 4th exons of the human *PANX1* gene were transfected into ECs using Lipofectamine RNAiMAX reagent and knockdown was achieved following a 72 hour incubation.

Mice: All mice were male, 10-14 weeks of age, on a C57Bl/6 genetic background and were cared for under the provisions of the University of Virginia Animal Care and Use Committee and the LSU Health Sciences Center-Shreveport Animal Care and Use Committee and followed the National Institute of Health guidelines for the care and use of laboratory animals. The inducible, endothelial cell-specific Panx1 mice (VECadER^{T2}/Panx1$^{fl/fl}$) mice were generated by crossing VECadER^{T2+}/Panx1$^{wt/wt}$ mice (a kind gift from Dr. Ralf Adams, Max Plank Institute, Germany) with VECadER^{T2-}/Panx1$^{fl/fl}$ mice (a kind gift from Dr. Kodi Ravichandran, University of Virginia). To selectively induce Panx1 deletion in the vascular endothelium, VECadER^{T2}/Panx1$^{fl/fl}$ mice received intraparitoneal injections of 100 μL of Tamoxifen (10 mg/mL) for 10 consecutive days. A subset VECadER^{T2}/Panx1$^{fl/fl}$ mice were injected with peanut oil (the vehicle for tamoxifen) and served as littermate controls.

ATP release assays: Human arterial and venous endothelial cells were plated in 24 well plates coated with pre-coated with 0.2% gelatin and grown to confluency. On the day of experiment, the media was removed from each well and cells were carefully washed 2 times with warm serum free basal EC medium supplemented with 1% BSA. Cells were then incubated in 300 µL of fresh media for 30 minutes at 37°C to allow degradation of mechanically stimulated ATP release. Endogenous ecto-nucleotidases were inhibited by incubating cell monolayers with 300 µM ARL 67156 (Tocris) for 30 minutes at 37°C. Cells were then stimulated with recombinant human TNFα (R&D Systems). For dose response experiments, ECs were incubated with 0.5, 1, 5, 10, 50 or 100 ng/mL TNFα for 30 minutes at 37°C. For time-course experiments, ECs were stimulated with 10 ng/mL recombinant TNFα. In experiments where pharmacological inhibitors were employed, ECs were incubated with antagonists in parallel with ARL 67156 for 30 minutes. To inhibit TNFR1, ECs were incubated with the peptide antagonist of the receptor WP9QY (10 µM). Vesicular ATP release was inhibited with brefeldin A (BFA; 10 µg/mL) and CALHM1 channels were pharmacologically blocked with ruthenium red (RuR; 20 µM). Panx1 channels were inhibited with carbenoxolone (CBX; 50 µM) and the inhibitory peptide ^{10}panx1 (200 µM). Following stimulation with TNFα or vehicle, 150 µL of the cell supernatant was collected and immediately placed into pre-chilled 1.5 mL Eppendorf tubes on ice. All samples were centrifuged at 5000xg for 3 minutes and 50 µL of each sample was transferred to an opaque 96-well plate. Using a FluoStar Omega luminometer, 50 µL of luciferin:luciferase reagent (ATP bioluminescence assay kit HSII; Roche) was injected into each well and luminescence was immediately recorded. ATP concentration in each sample was calculated from a

standard curve for all experiments. Data are expressed as a % change in ATP release from control conditions (i.e., unstimulated cells). For isolated vessel experiments, 2nd order mesenteric venules or arterioles were cannulated on glass micropipettes and perfused luminally with a MOPS buffered PSS solution containing TNFα. ATP was quantified in the perfusate as described above.

Cx hemichannel depletion/cell surface protein biotinylation: Cell surface biotinylation was performed as described previously[217]. To selectively deplete Cx43 hemichannels from the EC plasma membrane, venous and arterial ECs were grown to confluency in 6-well plates (Western blotting) or 24-well plates (ATP release) and treated with the vesicular exocytosis inhibitor brefeldin A (BFA; 5 µg/mL) for 5 hours at 37°C. Cells were washed once with cold 1x PBS and incubated with cold DMEM (without FBS) and 50 µM CBX at 4°C for 30 minutes. CBX was added to prevent biotin from passing through Panx1 channels, which may label intracellular proteins. Cells were washed with PBS and incubated at 4°C for 1 hour in cold PBS (1.5 mL/dish) containing EZ-link-sulfo-NHS-LC-biotin (1 mg/mL) and CBX (50 µM). The cells were washed again with PBS and lysed in PBS-T (PBS + 0.5-1% Triton-X 100) containing protease inhibitors. Total protein was quantified using the Bradford technique and equal amounts of protein were incubated with Streptavidin-agarose beads for 2 hours at 4°C to pull down biotinylated proteins. Beads were washed 5 times with PBS-T, spun down and bound proteins eluted by incubation with laemmli buffer. Eluted proteins were subjected to SDS-PAGE and Western blotting for detection of Panx1.

Immunofluorescence microscopy: Male VECadER^{T2}/Panx1$^{fl/fl}$ mice (Tamoxifen or Peanut oil injected), were euthanized by CO_2 asphyxiation and subsequent cervical

dislocation. Prior to tissue harvesting, fixation was performed by perfusing room temperature 4% paraformaldehyde (PFA) made in PBS through the heart. .The mesentery was immediately excised and 2nd order venules dissected free of surrounding fat and connective tissue and place in 4% PFA for 30 minutes before transfer to 70% ethanol for paraffin embedding. Paraffin sections (4-5 μm in thickness) were de-paraffinized and processed for immunocytochemistry as previously described For detection of Panx1 knockout in the endothelium, vessel sections were processed for conventional immunolabeling and incubated overnight at 4°C with a primary antibody directed against the murine Panx1 C-tail [152].

Western blotting for SFK and Panx1 phosphorylation: Confluent EC monolayers were homogenized in ice cold Triton extraction buffer (50 mmol/L Tris-HCL, 150 mmol/L NaCl , 5 mmol/L EDTA, 1% deoxycholate, 1% NP-40 and 1% Triton-X100 in phosphate buffered saline and pH adjusted to 7.4) containing protease and phosphatase inhibitors. Samples were incubated with rotation at 4°C for 20 minutes to solubilize proteins then centrifuged for 5 minutes at 13,000xg to pellet cell debris. Protein concentration was quantified using the BCA method. 15-20μg of total protein was subjected to SDS gel electrophoresis using 4-12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose for Western blotting. Blots were incubated overnight at 4ºC with primary antibodies against pY416 SFKs or pY198 Panx1 followed by washing. For loading controls, blots were incubated with primary antibodies for GAPDH (pSFK normalization) or the non-phosphorylated epitope spanning Y198 in Panx1 (pPanx1 normalization). Protein was detected using Licor secondary antibodies visualized and quantitated using Licor Odyssey.

Intravital Microscopy for leukocyte rolling, adhesion and emigration: Mice were prepared for intravital microscopy of the cremaster muscle. Briefly, mice were anesthetized with ketamine hydrochloride (150 mg/kg, IP) and xylazine (7.5 mg/kg, IP). The cremaster was isolated, laid over a viewing pedestal, superfused with bicarbonate-buffered saline and covered with saran wrap. After 30 min equilibration, a venule with a wall shear rate (WSR) of ≥500/s, diameter between 20 μm and 40 μm, and the least number of adherent and emigrated leukocytes was chosen for further study. A 1 min baseline recording was made, after which 50 ml TNFα (1.7 ng/ml in 0.1% BSA) or vehicle control was added under the saran wrap every 30 min. 1 min recordings were made just prior to each addition, for 3 hrs. The TNFα was initially reconstituted in PBS at 3.4 ng/ml and 150 ml aliquots were frozen. An aliquot was mixed 1:1 with BBS containing 0.2% BSA at the time of the experiment. Leukocyte rolling, adhesion and emigration were determined by off-line analysis. Rolling leukocytes were identified as moving slower than the V_{RBC}, and the # leukocytes crossing the midline of the section were counted and expressed as #/min. Rolling velocity was calculated for up to 10 cells per time point, and the average expressed as mm/s. Leukocytes were considered adherent if they stopped for at least 30 s (expressed as #/mm^2 vessel wall), and emigrated leukocytes were leukocytes identified in the interstitium (expressed as # cells/mm^2 tissue).

Statistics: All statistics were performed using GraphPad Prizm software. For multiple comparisons, statistics were performed using a one-way or two ANOVA followed by pairwise analysis. A student's *t*-test was used for individual comparisons if normally distributed.

CHAPTER 3. EXPRESSION OF PANNEXIN ISOFORMS IN THE MURINE SYSTEMIC ARTERIAL NETWORK

3.1. Abstract

Pannexins (Panx) form ATP-release channels and have been proposed to play an important role in the regulation of vascular tone. However, distribution of Panx across the arterial vasculature is not documented. We tested antibodies against Panx1, Panx2 and Panx3 on HEK cells (which do not endogenously express Panx proteins) transfected with plasmids encoding each pannexin isoform and $Panx1^{-/-}$ mice. Each of the Panx antibodies was found to be specific and was tested on isolated arteries using immunocytochemistry. We demonstrate that Panx1 is the primary isoform detected in the arterial network. In large arteries, Panx1 was primarily in endothelial cells, whereas in small arteries and arterioles Panx1 localizes primarily to the smooth muscle cells. In coronary arteries Panx1 was the predominant isoform expressed, except in arteries less than 100 μm Panx3 became detectable. Only Panx3 was expressed in the juxtaglomerular apparatus and cortical arterioles. The pulmonary artery and alveoli had expression of all three Panx isoforms. No Panx isoforms were detected at the myoendothelial junctions. We conclude that the specific localized expression of Panx channels throughout the vasculature points towards an important role for these channels in regulating the release of ATP throughout the arterial network.

3.2. Introduction

Pannexins (Panx) are a class of glycoproteins that oligomerize to form channels at the plasma membrane [154,218,219]. Pannexin proteins are found in three different isoforms (Panx1, Panx2 and Panx3) in cultured cells and *in vivo*. To date, Panx1 has been characterized extensively and has been shown to be ubiquitously expressed. Conversely, Panx2 expression has been found primarily in the central nervous system [203,220,221]

and Panx3 is mainly expressed in the skin, osteoblast and chondrocytes [222-224]. While Panx proteins have been shown to possess a similar membrane topology to the vertebrate gap junction proteins, the connexins, there are key differences in their respective functions within the cell. One of the most important differences is that connexins allow for a direct communication between two cells by forming gap junction channels through the docking of two connexin hemichannels, or connexons, whereas the formation of gap junctions by the docking of two Panx channels has never been demonstrated *in vivo* [225]. Another key difference is the ability of Panx channels to open and release ATP into the extracellular space under physiological extracellular calcium concentration, whereas hexameric connexin hemichannels present at the plasma membrane have been shown to be closed under these conditions and open when extracellular calcium concentration is reduced [128,129,226]. Therefore, since their first description in 2000, Panx channels have been suggested to act as paracrine release channels that are strongly implicated in the release of purine nucleotides from cells [30,154,162,165,227].

The role of extracellular purines, including ATP, in the systemic circulation has been shown to be important for several vascular functions including the regulation of vascular tone [12,60], reactive hyperemia during contraction of skeletal muscle [7,8] and hypoxia-induced vasodilation [9,10,19]. Although there are well-described reports of ATP release occurring from both circulating erythrocytes and sympathetic nerves innervating vascular smooth muscle cells [10,24-30,54], there is accumulating evidence indicating that endothelial and smooth muscle cells of the vascular wall can also release ATP [11,19-21,84,114,168]. The conduit for ATP release from these cells continues to be investigated, but several reports suggest that Panx channels may be an important

candidate. Indeed, we have recently demonstrated that both smooth muscle and endothelial cells in small arteries express Panx1 and our results showed that vascular smooth muscle cells can release ATP through Panx1 channels [11]. However, the distribution of Panx isoforms across the vasculature is not yet known. This is an important omission considering the potential role these channels may have in the vasculature, where ATP and its breakdown products have been documented to have tremendous physiological importance for decades [8,29,31,48,49,51,140,228-232]. Importantly, Panx channels in endothelial cells could play essential roles in ATP signaling in the blood vessel lumen which could potentially include vasodilation and monocyte recruitment. Alternatively, expression of Panx channels in smooth muscle cells may be involved in purinergic signaling such as the regulation of vasoconstriction or vascular smooth muscle cell proliferation [13,14]. Together, the expression of these channels in vascular cells could play important roles in regulating a number of physiological processes.

As blood vessels across the arterial tree experience a variety of different environments, identification of how ATP is released from the endothelial and smooth muscle cells of different arterial beds is essential for understanding how purinergic signaling is regulated in the control of vascular tone in both blood pressure regulation and maintenance of proper organ physiology. Therefore, as Panx proteins have been strongly implicated in ATP release from cells, we sought to characterize the expression of the different Panx isoforms across the vasculature to help provide a more detailed understanding of the potential ATP release mechanisms in these cells.

3.3. Results

Initially HEK293T cells were tested for endogenous Panx expression by quantitative RT-PCR for detection of mRNA **(Figure 2a)** or by Western blot for detection of protein **(Figure 2b).** There was no detectable Panx3 mRNA, with minimal amounts of Panx1 and Panx2 mRNA as compared to the housekeeping gene B2M [210]; however, no endogenous protein was detected for any of the Panx isoforms in these cells. We therefore transfected the HEK293T cells with plasmids encoding murine Panx1, Panx2 or Panx3. Western blot analysis of HEK293T cells transfected with the Panx plasmids revealed specific bands for each Panx isoform when probed with the respective Panx antibody **(Figure 2b).** Using immunocytochemistry, we found expression of each transfected Panx isoform at the plasma membrane **(Figure 2c-e)**, as well as intracellular staining for Panx2 which has been shown in a number of cell types, again demonstrating the specificity of the Panx antibodies.

FIGURE 2. SPECIFICITY OF PANX ANTIBODIES IN CULTURED CELLS.

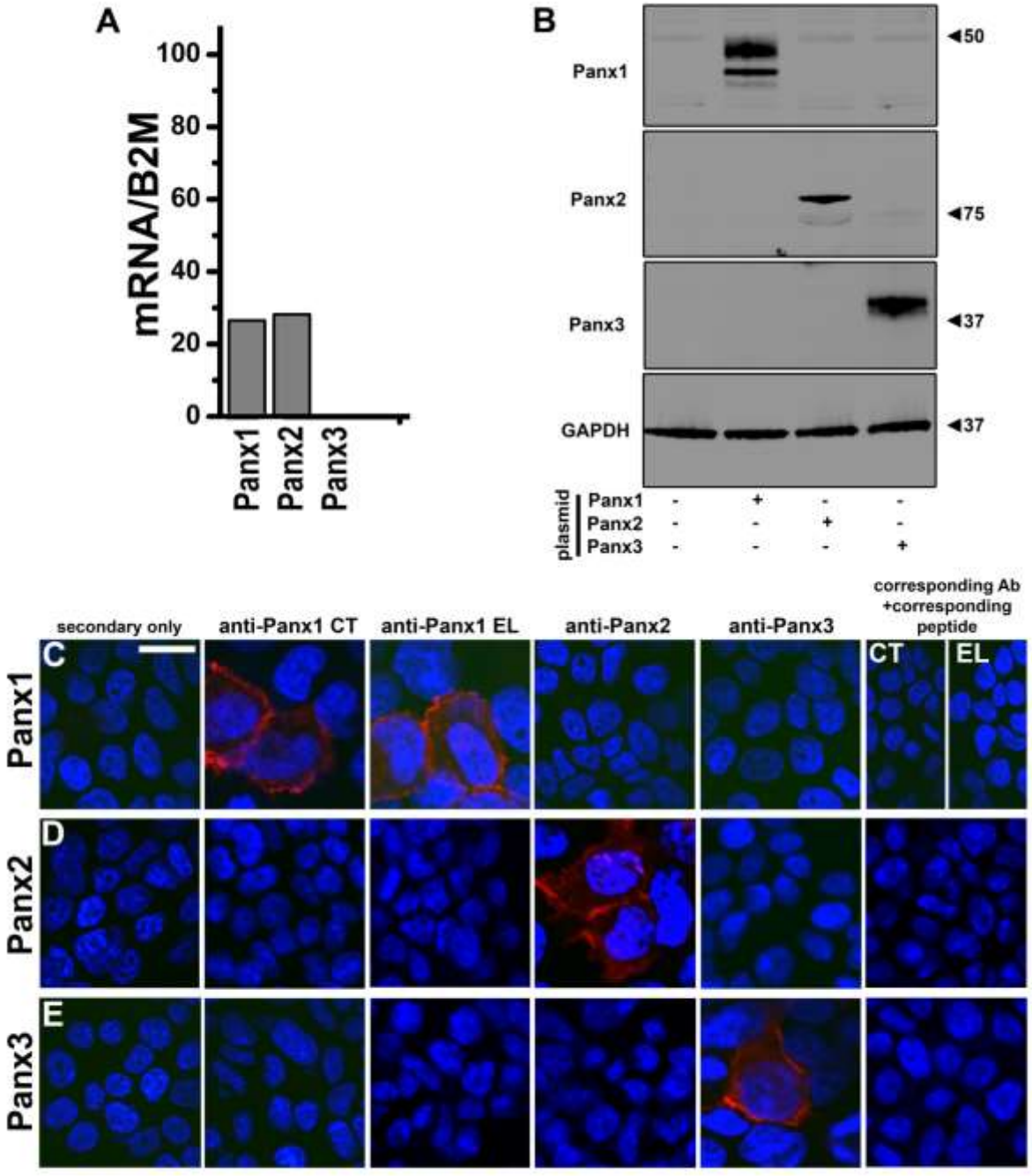

Figure 2. Specificity of pannexin antibodies in cultured cells. HEK293T cells were tested for endogenous pannexin expression by mRNA via RT-PCR **(a).** and by protein via Western blot **(b)**. In A, mRNA expression is normalized to the housekeeping gene β2 microglobulin (B2M). The HEK cells were transfected with Panx1, Panx2 or Panx3 plasmids, stained using anti-Panx1 CT antibody, anti-Panx1 EL antibody, anti-Panx2 antibody or anti-Panx3 antibody and detected with anti-rabbit IRDye 800CW (LiCOR) for Western blots **(b)** or anti-rabbit Alexa 594 for immunofluorescence **(c-e).** In addition, for immunofluorescence the antibody corresponding to the transfected pannexin isoform was incubated with its respective blocking peptide for negative controls. In **C-E**, blue are DAPI stained nuclei and red is the expression of each pannexin; scale bar is 10 μm in unstained images and 5 μm in stained images.

Next we tested the specificity of the Panx1 antibodies on thoracodorsal arteries (TDA) as we previously reported that this is the only isoform expressed in the TDA wall [11]. Using TDA harvested from C57Bl/6 mice, we found consistent staining in both endothelial cells and vascular smooth muscle cells using both the anti-Panx1 CT and anti-Panx1 EL antibodies **(Figure 3a).** We could not detect any Panx1 in TDAs isolated from the $Panx1^{-/-}$ mouse **(Figure 3b)**, again demonstrating the specificity of the Panx1 antibodies.

FIGURE 3. SPECIFICITY OF PANX1 ANTIBODIES IN ARTERIES.

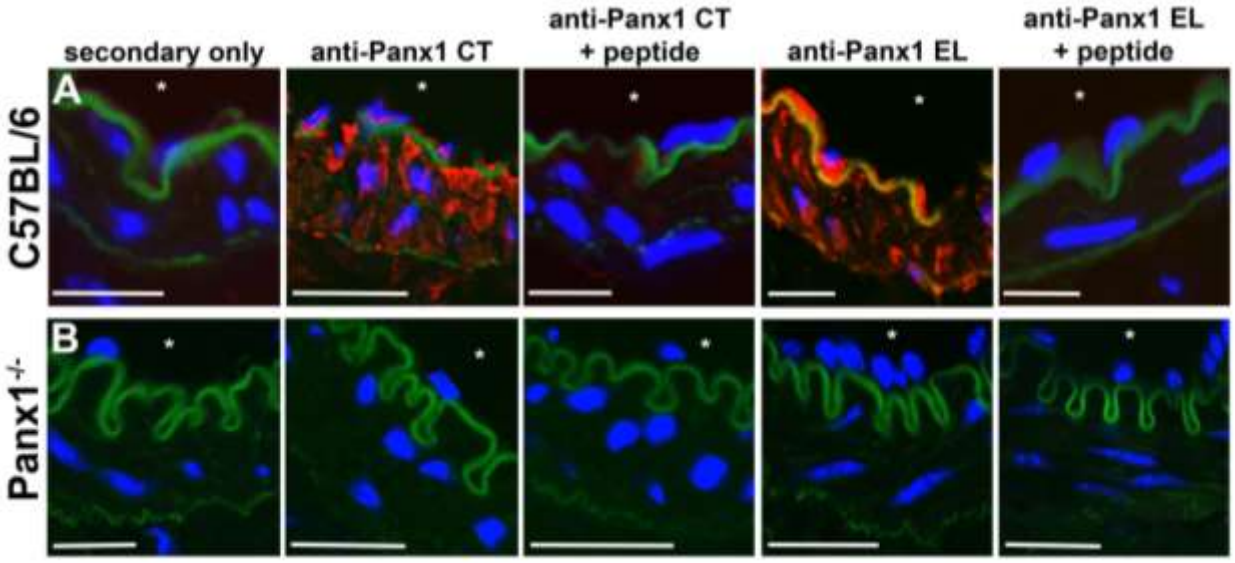

Figure 3. Specificity of Panx1 antibodies in arteries. The anti-Panx1 antibodies were tested on mouse TDA from C57Bl/6 **(a)** and Panx1$^{-/-}$ mice **(b)**. Blue are DAPI stained nuclei, green is autofluorescence of internal elastic lamina and red is pannexin staining. Scale bar in each image is 20 μm and asterisks indicate the lumen of the artery.

Because we determined the Panx antibodies to be specific and Panx1 to be endogenously expressed in vascular cells of the TDA, we tested for Panx isoform expression through the systemic arterial tree, beginning in the aorta **(Figure 4a)**, then carotid artery **(Figure 4b)**, femoral artery **(Figure 4c)**, renal artery **(Figure 4d)**, TDA **(Figure 4e)**, abdominal arteries **(Figure 4f)**, arterioles in the spinotrapezius muscle **(Figure 4g)**, and finally cremasteric arterioles **(Figure 4h)**. Throughout the arterial tree, Panx1 was the only protein detected and was consistently expressed in endothelium regardless of artery size. However, the expression of Panx1 in smooth muscle cells was poorly detectable in larger conduit arteries (aorta/carotid/femoral). Some expression of Panx1 in smooth muscle cells was detected in renal arteries, whereas in smaller arteries including TDA, abdominal artery, spinotrapezius arterioles and cremasteric arterioles, Panx1 was found throughout the smooth muscle. Similar to the smaller systemic arteries, both small (luminal diameter of 20-90 μm) and large (luminal diameter of 100-250 μm) coronary arteries also expressed Panx1 in endothelium and smooth muscle cells **(Figure 5a-b)** and no Panx2 expression **(Figure 5c-d)**. Interestingly, in coronary arteries with a luminal diameter less than 100 μm, Panx3 was detected in endothelium and smooth muscle **(Figure 5e-f)**.

FIGURE 4. PANX EXPRESSION IN THE MURINE SYSTEMIC ARTERIAL NETWORK.

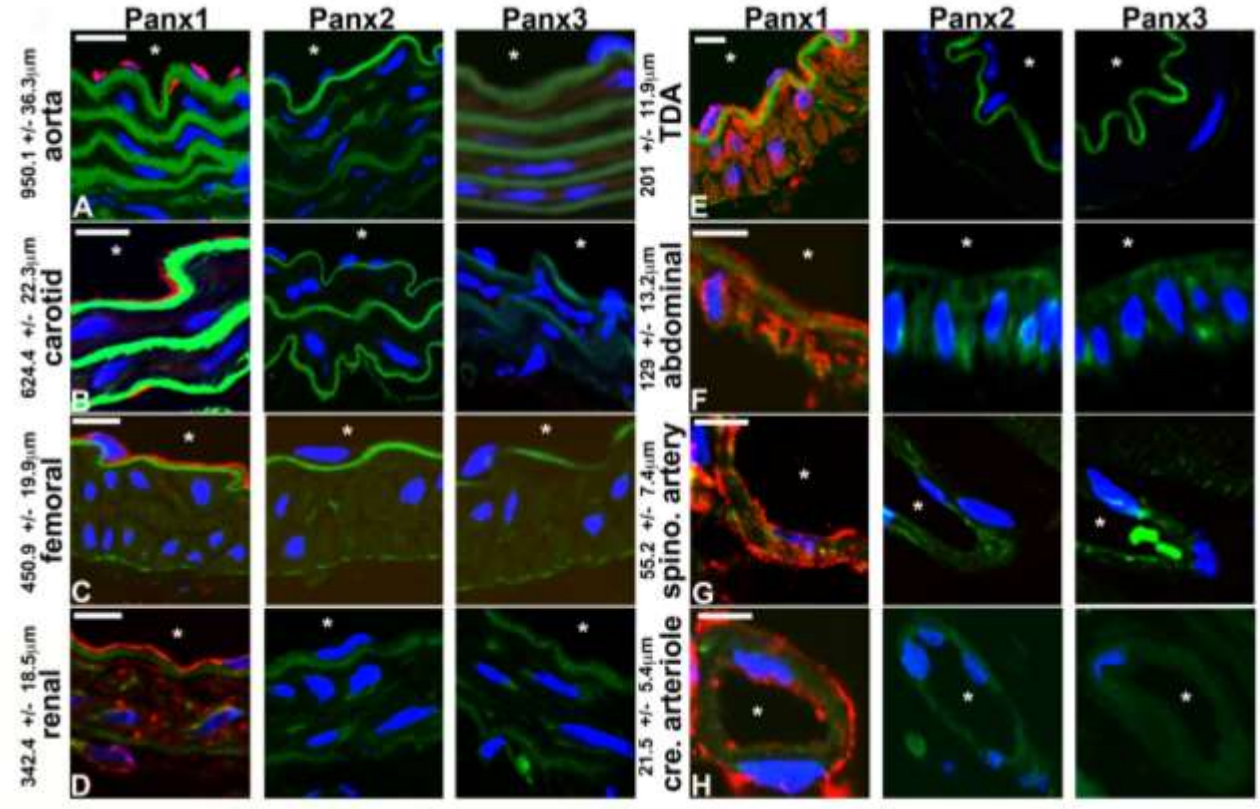

Figure 4. Pannexin expression in the murine systemic arterial network. In A-H, each Panx antibody was tested on arteries of progressively decreasing size starting with the aorta **(A)**, then carotid artery **(B)**, femoral artery **(C)**, renal artery **(D)**, TDA **(E)**, abdominal artery **(F)**, an arteriole from the spinotrapezius muscle **(G)** and finally a cremasteric arteriole **(H)**. Blue are DAPI stained nuclei, green is autofluorescence of internal elastic lamina and red is pannexin staining. Scale bar in each image is 10 μm and is representative for the row of staining; asterisks indicate the lumen of the artery.

FIGURE 5. PANX EXPRESSION IN THE CORONARY ARTERIES

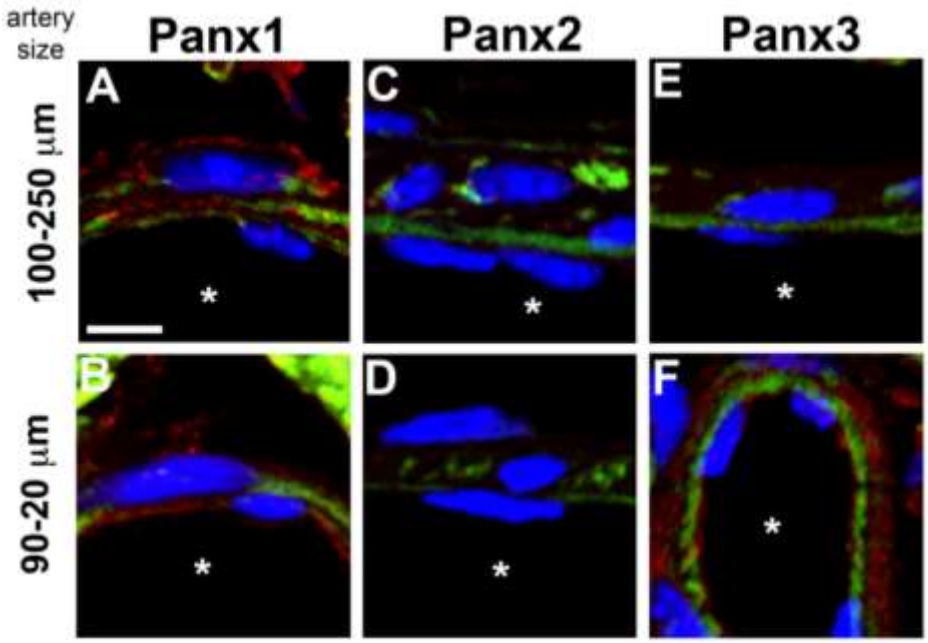

Figure 5. Pannexin expression in the coronary arteries. Coronary arteries that were between 100-250 μm in diameter **(A, C, E)** or 20-90 μm in diameter **(B, D, F)** were assessed for expression of Panx1 **(A-B)**, Panx2 **(C-D)** or Panx3 **(E-F)**. In each image, blue is DAPI stained nuclei, green is autofluorescence of the internal elastic lamina and red is pannexin staining. Scale bar is 5 μm and representative for all images; asterisks indicate the artery lumen.

Next we tested whether Panx were expressed at MEJs, where endothelium and smooth muscle make contact in small arteries and arterioles [233,234]. Using immuno-TEM, we quantified the amount of each Panx isoform expressed in the coronary, cremasteric and TDA and found little detectable Panx expression at myoendothelial junctions in selected arteries **(Figure 6a-d)**. While immuno-TEM can be a valuable method for quantifying the distribution of proteins at distinct subcellular locations within tissues at high resolution, this method has its limitations and results can potentially be confounded by non-uniform antigen distribution across sections. Therefore, we also analyzed Panx isoform expression at the MEJ by performing whole-mount immunocytochemistry on isolated TDA that were fixed in a maximally dilated state to facilitate visualization of MEJs at IEL holes. This method allows for visualization of large areas of antigen distribution. In agreement with our immuno-TEM data, there were no pannexin isoforms detectable; however, Cx43, as previously found to be located at MEJs via quantified immuno-TEM [215]; as used above), was localized to these holes **(Figure 6e)**.

FIGURE 6. MINIMAL PANX1 EXPRESSION AT MYOENDOTHELIAL JUNCTIONS

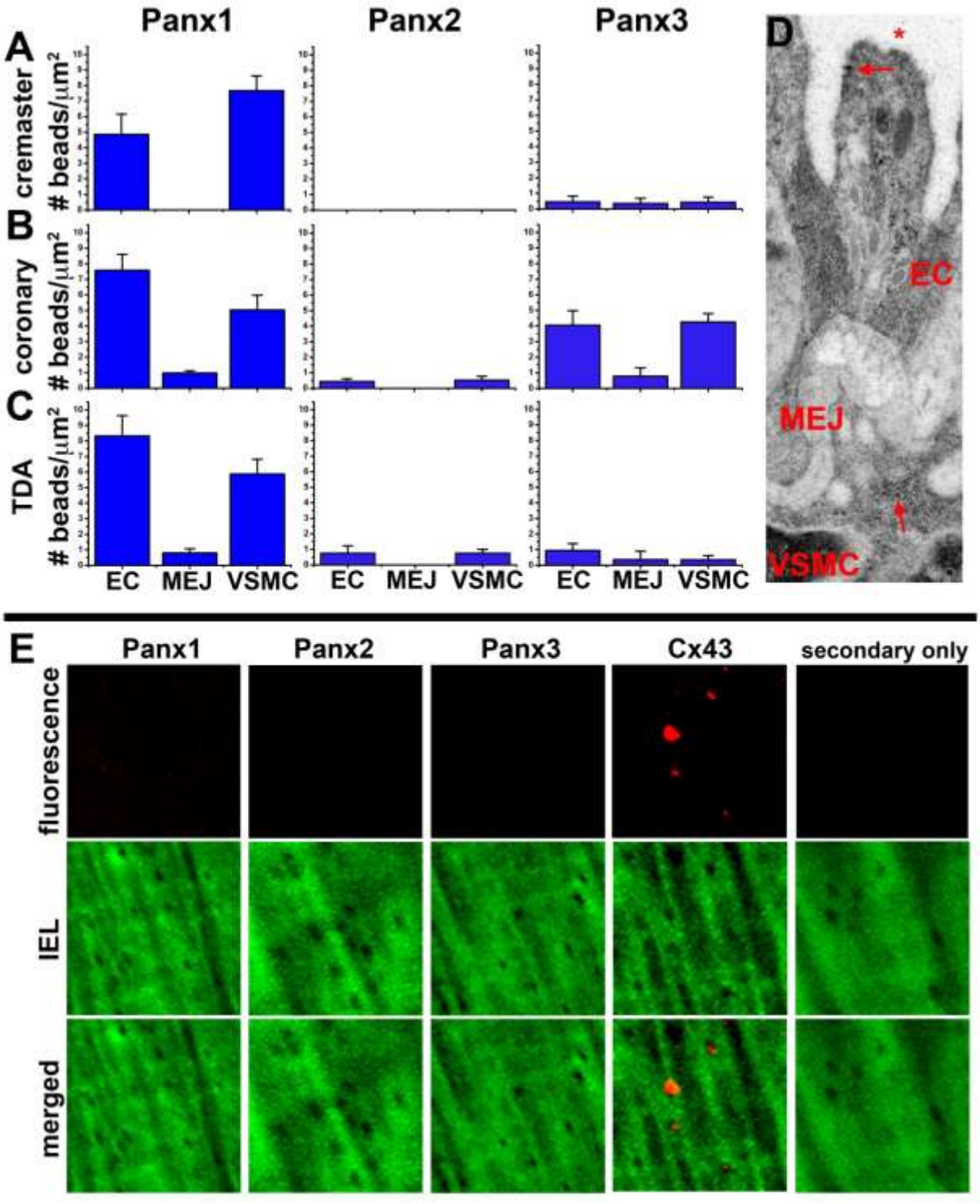

Figure 6. Minimal pannexin expression at myoendothelial junctions. In **A-C,** the number of gold beads after staining with Panx1, Panx2 or Panx3 per μm² in endothelial cells (EC), MEJ and vascular smooth muscle (VSMC) was quantified in cremaster arterioles (20-40 μm; **A**), coronary arteries (50-100 μm; **B**), and TDAs (200 μm; **C**). In **D**, representative immuno-TEM from cremasteric arterioles is shown with gold beads representing Panx1. Asterisks indicate the artery lumen and arrows indicate representative location of gold beads. Scale bar is 0.5 μm. In **E**, holes in the internal elastic lamina from TDAs, corresponding to possible MEJs, were examined for protein expression. Green is autofluorescence of the internal elastic lamina and red is the protein of interest. Only when Cx43 antibody was used punctate fluorescence could be detected in the holes. Each image is 15 μm x 15 μm.

We also examined Panx expression in the arterial network of the kidney **(Figure 7)**. We did not detect any Panx1 or Panx2 in the juxtaglomerular apparatus, **(Figure 7a-b)**, but Panx3 was found throughout the unit in very distinct punctate stains **(Figure 7c)**, which was not detectable when incubated with the Panx3 peptide **(Figure 7d)**. This same pattern of expression was found in arterioles of the cortical kidney with only Panx3 being detectable in the endothelium, with more limited expression in smooth muscle cells **(Figure 7e-g)**, which was again absent when incubated with the Panx3 peptide **(Figure 7h)**.

Lastly, as a contrast to the systemic circulation, we examined Panx expression in the pulmonary circulation **(Figure 8)**. The first order intrapulmonary artery had positive staining for Panx1 in the endothelium and smooth muscle, although there was some Panx1 staining in the IEL. It is not clear why staining would be in the IEL but the most likely reason is Z-stack compression. Panx2 was expressed in the smooth muscle cells with some detectable Panx2 in endothelial cells **(Figure 8a-b)**. Peptide competition eliminated the Panx2 staining **(Figure 8c)**. The expression of Panx3 was predominantly in the endothelium, but there remained some detectable expression in the smooth muscle cells **(Figure 8d)**. In the distal lung, Panx1 and Panx3 were the dominant isoforms expressed, with some detectable Panx2 **(Figure 8e-h)**. Of particular note in the alveoli, it appeared that the pannexins could also be localized around the nuclei.

FIGURE 7. PANX EXPRESSION IN THE KIDNEY

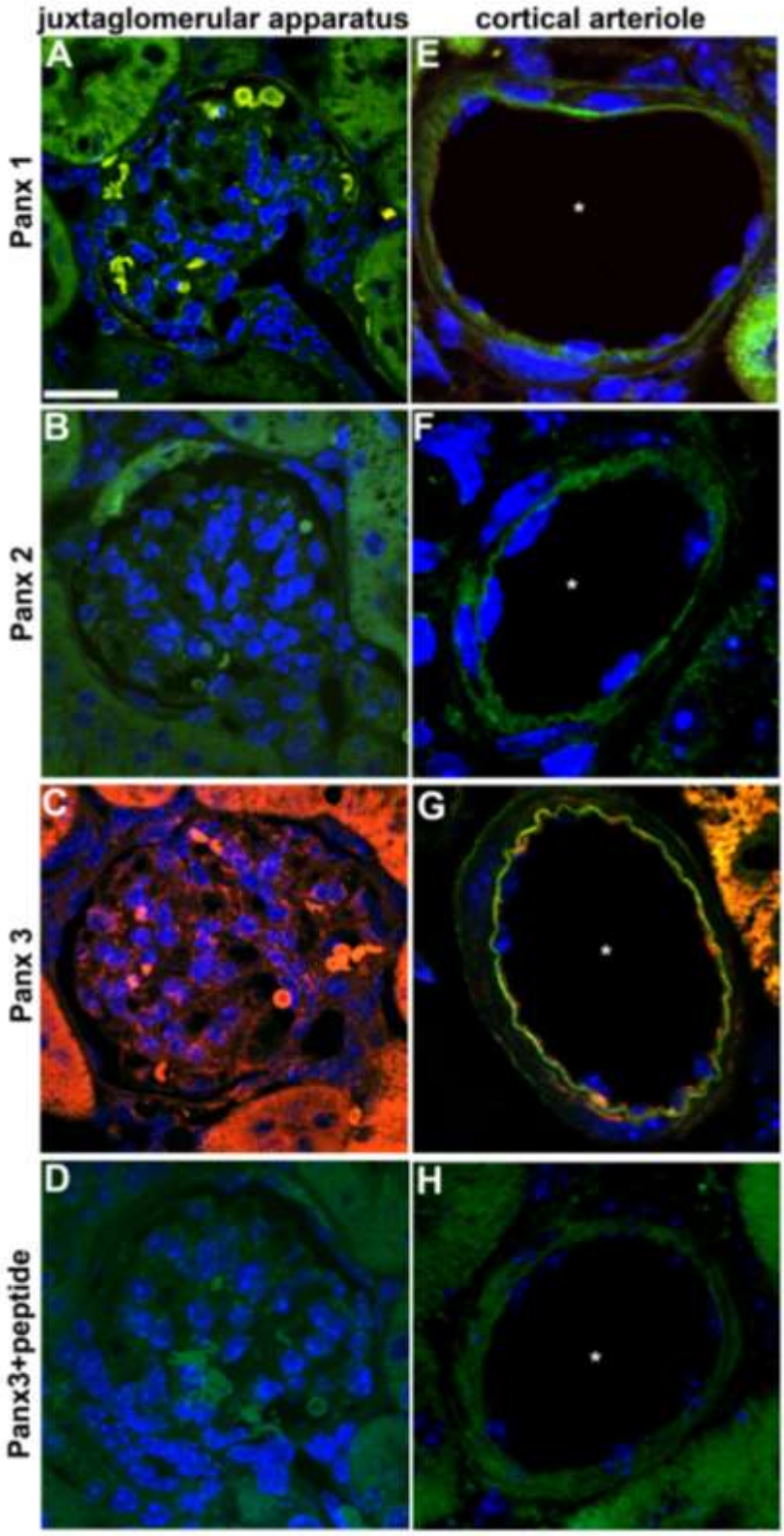

Figure 7. Pannexin expression in the kidney. In **A-D**, the glomerulus of the kidney was stained for Panx1 **(A)**, Panx2 **(B)** and Panx3 **(C)**, with Panx3 peptide competition shown in **D**. In **E-H**, arterioles of the cortical kidney were stained for Panx1 **(E)**, Panx2 **(F)** and Panx3 **(G)**, with Panx3 peptide competition shown in **H**. In all images, pannexins are in red and blue represents DAPI stained nuclei. Scale bar in A is 20 μm and representative for all images.

FIGURE 8. PANNEXIN EXPRESSION IN THE PULMONARY ARTERY AND LUNG ALVEOLI

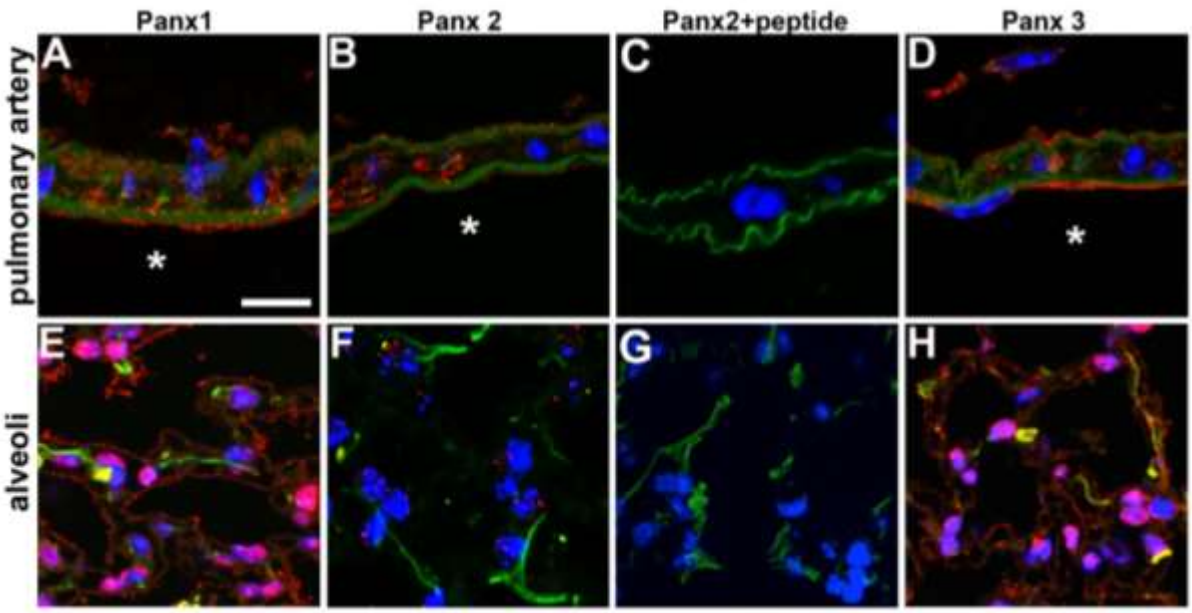

Figure 8. Pannexin expression in the pulmonary artery and lung alveoli. The pulmonary artery (lumen diameter approximately 400 μm) was stained for each of the pannexin isoforms in **A-D**. In **C**, Panx2 peptide competition is shown. The asterisks indicate the luminal side of the arterial wall. In **E-H**, the distal lung alveoli were also stained with each of the pannexin isoforms, with **G** showing Panx2 peptide competition. In all images, green is autofluorescence of matrix proteins and red blood cells **(E-H)**, red is the pannexin isoform of interest, and blue represents DAPI stained nuclei. Scale bars in **A** and **E** are both 20 μm.

3.4. Discussion

Pannexins form plasma membrane channels capable of releasing ATP that provide key physiological functions including regulation of apoptosis and has a potential role in regulation of vasoconstriction by ATP release mechanisms [11,78]. For this reason, we sought to identify whether the various Panx isoforms were expressed across the arterial vasculature and to describe their expression pattern in several vascular beds. We began by extensively testing each of the Panx antibodies on HEK cells which do not endogenously express Panx proteins, although there was some residual Panx mRNA detectable. After transfection of each Panx isoform in HEK cells, we found reactivity for each Panx isoform with corresponding Panx antibodies by Western blotting and immunocytochemistry.

In the blood vessel wall, endothelial cells and vascular smooth muscle cells are in constant communication both within and often between cell types through multiple signaling mechanisms. One such signaling molecule is ATP, which has been implicated in the control of vasoconstriction and vasodilation [31,32,48,51]. Subsequently, ATP sensitive purinergic receptors in vascular endothelial and smooth muscle cells have been extensively characterized [32,34,35,44,235]. In endothelial cells, ATP binding to P2Y purinergic receptors has been shown to mediate vasodilation, whereas in vascular smooth muscle cells, ATP-sensitive P2X purinergic receptors have been shown to control vasoconstriction [32,34,44,235]. While much work has been done characterizing the functional role of extracellular ATP in the vasculature, the conduit for its release from the cells in the arterial wall is a current topic of investigation. As early studies on Panx channels identified the potential for these membrane proteins to release ATP into the

extracellular space, their expression in vascular cells may provide critical insight into the regulation of purinergic signaling at the level of purine release from these cells.

Our observation that Panx1 is expressed in endothelial cells across the arterial tree may suggest that this protein is intimately involved in the release of ATP into the blood vessel lumen and thus may be involved in modulating the extent of vasodilation in response to stimuli such as increases in blood flow and hypoxia, which are known to cause ATP release from endothelial cells [9,19,21,236]. At the smooth muscle cell axis, we have previously suggested a pivotal role for ATP release from Panx1 channels in regulating vasoconstriction in response to stimulation of α_{1D}-adrenergic receptors [11]. The observation that Panx isoforms are highly expressed in smooth muscle cells of small arteries and arterioles, versus selected larger conduit arteries, may suggest a role for these channels in the regulation of peripheral resistance by ATP release mechanisms, as small arterioles have the highest resistance and contribute greatest to total peripheral resistance [237]. While we detected very little Panx expression in smooth muscle cells in large arteries, we did find expression of Panx1 in the endothelial cell layer of these arteries. It is possible that Panx1 channels in endothelial cells across the vasculature may also play a role in mediating the inflammatory response, where ATP released into the vascular lumen has been shown to promote monocyte and macrophage recruitment and migration into the vascular intima [238-241]. With the possibility that Panx channels in the vasculature may play dual roles in regulating blood pressure as well as pathological events such as inflammation, it will be important to identify potential binding partners and regulatory mechanisms that control the gating of these channels and thus ATP release from vascular cells.

Although the main arteries of the systemic arterial vascular tree had only Panx1 expressed, be it in endothelium or smooth muscle, the specialized organs had more variability in terms of the other Panx isoforms expressed. For example, while Panx1 was the main isoform expressed in the coronary circulation, we observed the expression of Panx3 in coronary arteries with an internal diameter less than 100 μm. This is the first evidence for Panx3 expression in vascular cells and may suggest that this isoform plays a special role in cardiac physiology. The coronary arteries play an important role in oxygen delivery to cardiac tissue which has an extremely high metabolic demand and changes in coronary blood flow, and thus changes in oxygen delivery to myocardial tissue, can lead to alterations in myocardial metabolism and ultimately cardiac output. Therefore, changes in coronary blood flow could have profound effects on blood pressure and it is possible that these arteries may require more dynamic ATP release mechanisms to facilitate rapid changes in blood flow to cardiac tissue to match oxygen demand. It will be interesting in the future to determine whether coronary blood flow may be influenced by the expression of the different Panx isoforms in these arteries. Along with Panx1, Panx3 has also been shown to release ATP and expression of this isoform along with Panx1 in critically small arterioles in the coronary circulation may provide additional channels for ATP release. This observation warrants further investigation into the role of Panx channels in the regulation of coronary blood flow, the dynamics of channels composed of Panx3 and whether heteromeric channels can be formed by Panx1 and Panx3 [153,203].

In the renal vasculature, we observed sole expression of Panx3 in both the juxtaglomerular apparatus as well as in the endothelium of arteries in the cortical kidney,

with no detection of Panx1 and Panx2. This was the sole vascular bed in which we did not observe Panx1 expression, which may suggest that the different Panx isoforms may play specific roles in different vascular beds and that the renal vasculature may utilize purinergic signaling cascades distinct from other arterial beds across the arterial tree. This observation may also suggest, as with the coronary circulation, that the different Panx isoforms in the arteries supplying these tissues may play distinct roles in regulating ATP release in different physiological environments that are subject to unique interstitial and intraluminal pressures.

When we examined the pulmonary circulation, we observed expression of Panx1 in both endothelial and smooth muscle cells of the first order intrapulmonary artery while Panx2 was found primarily in the smooth muscle and Panx3 primarily in the endothelium. In the distal lung, we observed expression of Panx1 and Panx3 in the alveoli with Panx2 expressed in a perinuclear fashion.

Lastly, it should be noted that all of the images described in this manuscript were from paraffin embedded tissue blocks. This presents its own unique set of potential issues, including the well-described lack of good signal-to-noise for immunofluorescence and poor antigenicity (as compared to unfixed frozen sections). Although these may be issues, the embedding allowed us to compare directly all the tissue beds and have a consistent amount of antibody applied to each section and consistent setting on the microscope. While these conditions can also be met with frozen tissue sections, the morphology of the tissue is often compromised by sectioning of frozen sections.

In conclusion, the consistent observation from our data was 1. pannexins are expressed throughout the arterial tree, 2. Panx1 is the predominant isoform in the arterial

network, 3. Panx1 is predominantly expressed in the endothelium throughout the arterial network and 4. smooth muscle cells in large conduit arteries express less Panx as compared to the small arteries, where Panx1 is found to be abundantly expressed. Further studies on the ways in which these channels are utilized should provide fascinating new data on the regulation of ATP release in the vasculature.

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CHAPTER 4. S-NITROSYLATION INHIBITS PANX1 CHANNEL FUNCTION

4.1. Abstract

S-nitrosylation is a post-translational modification on cysteine(s) that can regulate protein function and pannexin 1 (Panx1) channels are present in the vasculature, a tissue rich in nitric oxide (NO) species. Therefore, we investigated whether Panx1 can be S-nitrosylated and if this modification can affect channel activity. Using the biotin switch assay, we found that application of the NO donors S-nitrosoglutathione (GSNO) or DEA NONOate to human embryonic kidney (HEK) 293T cells expressing wild type (WT) Panx1 and mouse aortic endothelial cells (mAECs) induced Panx1 S-nitrosylation. Functionally, GSNO and DEA NONOate attenuated Panx1 currents; consistent with a role for S-nitrosylation, current inhibition was reversed by the reducing agent dithiothreitol and unaffected by 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a blocker of guanylate cyclase activity. In addition, ATP release was significantly inhibited by treatment with both NO donors. To identify which cysteine residue(s) were S-nitrosylated, we made single cysteine-to-alanine substitutions in Panx1 ($Panx1^{C40A}$, $Panx1^{C346A}$, $Panx1^{C426A}$). Mutation of these single cysteines did not prevent Panx1 S-nitrosylation; however, mutation of either C40 or C346 prevented Panx1 current inhibition and ATP release by GSNO. This observation suggested that multiple cysteines may be S-nitrosylated to regulate Panx1 channel function. Indeed, we found that mutation of both C40 and C346 ($Panx1^{C40/346A}$) prevented Panx1 S-nitrosylation by GSNO, as well as the GSNO-mediated inhibition of Panx1 current and ATP release. Taken together, these results indicate that S-nitrosylation of Panx1 at C40 and C346 inhibits Panx1 channel currents and ATP release.

4.2. Introduction

Pannexin 1 (Panx1) is a widely expressed integral membrane protein that is thought to form hexameric plasma membrane channels [148]. Since their identification in 2000, Panx1 channels have been characterized as ATP release channels that may play a pivotal role in supporting purinergic signaling in a multitude of cell types [11,30,78,161-166,205,242-244]. Importantly, Panx1 channels mediate ATP release from vascular smooth muscle and endothelial cells [11,205], circulating erythrocytes [30,53], airway epithelial cells [164], astrocytes [158,159,244] and T-cells [161,162]. As purinergic signaling events are critically involved in a number of physiological and pathological processes [245-249], elucidation of the mechanisms controlling the activity of Panx1 channels may provide important insight into how these processes are regulated.

Panx1 channel activation is now known to occur in response to membrane stretch and high extracellular K^+ [154], to activation of α_{1D}-adrenergic receptors [11], PAR-1 receptors [205], and NMDA receptors [250], and by cleavage of its intracellular C-tail by activated caspases 3 and 7 [78,169]. Since sustained ATP release from cells is detrimental to cell viability, it is crucial that the activity of these channels be tightly regulated to prevent depletion of ATP. Even as novel forms of Panx1 channel activation continue to emerge, mechanisms leading to inhibition of these channels remain poorly understood.

Through the initial characterization of Panx1, evidence has arisen indicating that the channel is post-translationally modified by glycosylation at asparagine 254, an event thought to regulate trafficking of Panx1 channels to the plasma membrane [149,151]. Panx1 channels can also be irreversibly modified during apoptosis by cleavage of the

intracellular C-tail by caspases, allowing release of ATP that serves as a “find-me” signal for monocyte recruitment and phagocytosis [78,169]. Whereas recognition of these post-translational modifications has provided critical insight into the regulation of Panx1 trafficking and the role of Panx1 in apoptosis, there are currently no known reversible post-translational modifications involved in the regulation of Panx1 channel activity at the plasma membrane.

Protein S-nitrosylation is a reversible post-translational modification in which nitric oxide (NO) moieties are covalently bound to reactive cysteine thiols via an S-nitrosothiol bond. S-nitrosylation can have profound effects on protein function with modification of even a single cysteine residue dramatically altering protein activity [215,251]. This modification is known to regulate the activity of several membrane channels including, among others, connexin43 (Cx43) gap junctions and hemichannels [215,252], the cardiac slowly activating delayed rectifier potassium channel KCNQ1 [253], the transient receptor potential channel TRPC5 [254] and the ryanodine receptor type 1 [251]. Notably, Cx43, a constituent of gap junction channels with a similar membrane topology and oligomerization state as Panx1, is modified by S-nitrosylation at a single cysteine residue in small arteries leading to an increase in channel permeability to second messengers [215].

Panx1 contains several cysteine residues that may play important roles in the regulation of channel function. As Panx1 is highly expressed in tissues rich in NO, such as the vasculature and nervous system, we sought to determine if Panx1 can be post-translationally modified by S-nitrosylation and whether this modification can affect channel activity.

4.3.Results

Panx1 can be S-nitrosylated.

To determine if Panx1 can be S-nitrosylated, we transfected HEK293T cells with a plasmid encoding murine Panx1 and treated the cells with two independent NO donors: S-nitrosoglutathione (GSNO) or diethylamine NONOate (DEA NONOate). This provides a useful model system for examination of Panx1 modifications since we find that endogenous Panx1 is undetectable in these cells [177]. In addition, Western blot analysis did not reveal expression of any nitric oxide synthase (NOS) isoforms in these cells (data not shown), providing a clean model system with little to no background NO from endogenous sources.

Application of 100 μM GSNO or 50 μM DEA NONOate for 10 minutes induced S-nitrosylation of Panx1 as detected by the biotin switch assay **(Figure 9)**. Treating cells with a reducing agent (DTT, 1 mM) immediately following GSNO or DEA NONOate treatment to reduce S-nitrosothiols prevented biotinylation in this assay. In addition, S-nitrosylation of Panx1 was not observed in untransfected cells or when the ascorbate step was omitted to prevent unmasking of S-nitrosylated cysteines for subsequent biotinylation. The biotin switch assay can detect proteins with cysteine modifications in addition to S-nitrosylation, and GSNO is capable of also modifying cysteine thiols by S-glutathionylation. Therefore, we treated $Panx1^{WT}$ expressing HEK cells with reduced glutathione (GSH, 100 μM). Importantly, we did not observe Panx1 modification by GSH in these cells **(Figure 9A).** Protein S-glutathionylation occurs more readily under conditions of oxidative stress, but we did not observe any Panx1 cysteine modification when $Panx1^{WT}$ expressing cells were exposed to reduced GSH in the presence of 100 μM

H_2O_2. This further supports a role for Panx1 S-nitrosylation over S-glutathionylation **(Figure 9B).** Taken together, these results indicate that Panx1 can be S-nitrosylated.

FIGURE 9. S-NITROSYLATION OF PANX1 BY GSNO AND DEA NONOATE

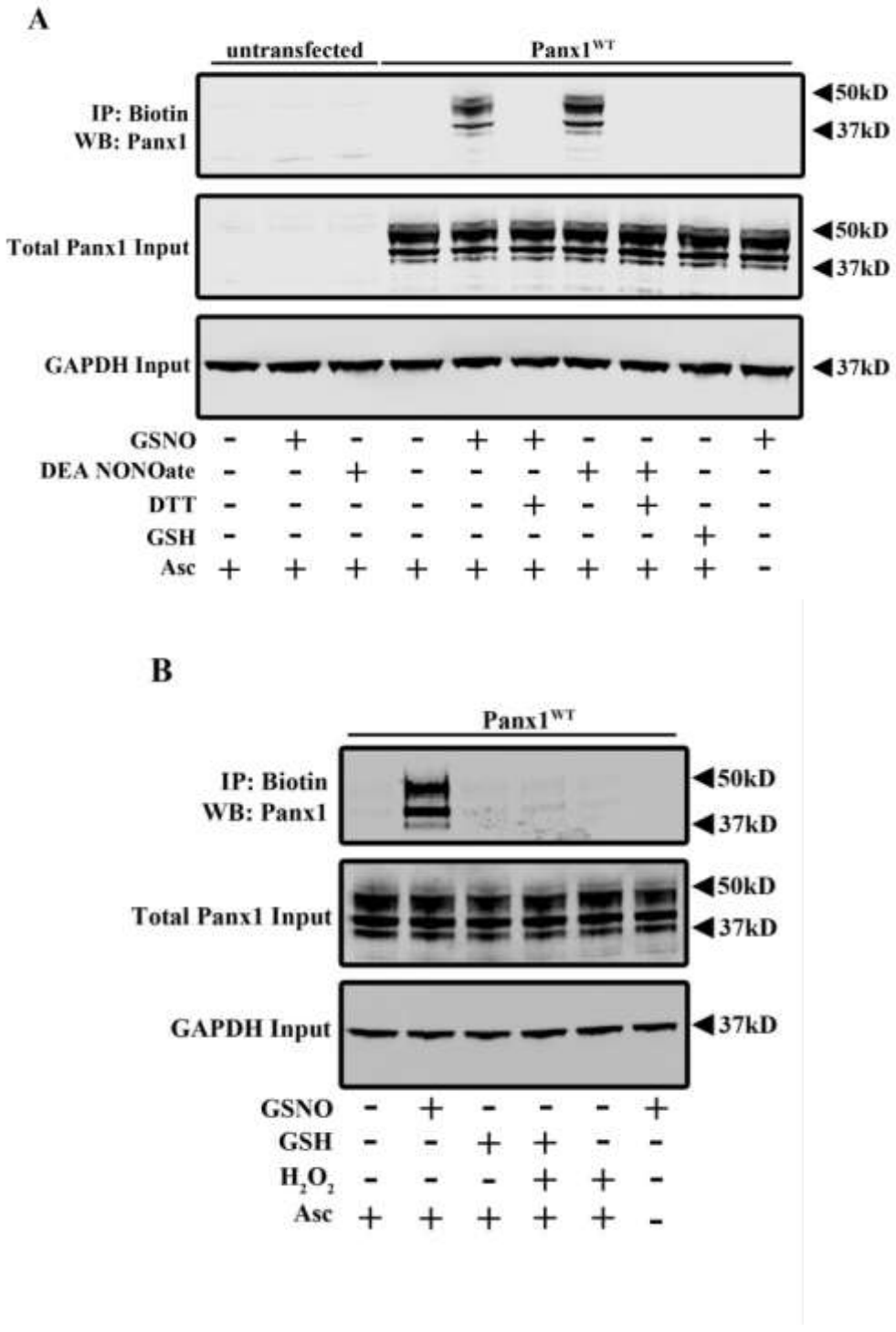

Figure 9. S-nitrosylation of Panx1 by GSNO and DEA NONOate. **A.** Biotin switch assay on untransfected and Panx1WT expressing HEK cells treated with 100 μM GSNO ± 1 mM DTT, 50 μM DEA-NONOate ± 1 mM DTT, or 100 μM GSH. Ascorbate (-Asc) was omitted from the biotin switch assay for the negative control. **B.** Biotin switch assay on Panx1WT expressing HEK cells treated with 100 μM GSNO, 100 μM GSH ± 100 μM H_2O_2, or 100 μM H2O2 alone.

S-nitrosylation inhibits Panx1 channel function.

To determine the functional consequences of S-nitrosylation on Panx1 channel activity, we examined the effects of GSNO and DEA NONOate on whole cell currents from $Panx1^{WT}$ expressing HEK cells. The peak current evoked by voltage ramp protocols (at +80 mV) was strongly inhibited by GSNO (Fig 2*A* and 2*B*). Importantly, and as expected for an effect mediated by S-nitrosylation, GSNO-mediated current inhibition was completely reversed by the reducing agent, DTT **(Figure 10A-B)**. The current restored by DTT was inhibited by the Panx1 channel blocker, carbenoxolone (CBX) and displayed a current-voltage relationship characteristic of Panx1 **(Figure 10B)**; note that GSNO-mediated inhibition was evident over the entire voltage range, including at rest potentials. We quantified the DTT-reversible component of GSNO-inhibited current, relative to the CBX-sensitive current, as a measure of the percentage of Panx1 current that was reduced by S-nitrosylation (~63%, **Figure 10C**). The DTT-reversible inhibition was significantly greater following GSNO treatment, by comparison to that following GSH or GSH with H_2O_2 application, consistent with the biotin switch data which suggested that the channel was modified by S-nitrosylation rather than S-glutathionylation. We also observed DTT-reversible current inhibition following application of DEA NONOate (data not shown). However, the response with DEA NONOate was less consistent than with GSNO (n=2 of 6 cells tested), and effects of the compound were not more evident when applied at higher concentrations (up to 1 mM) or at elevated temperature (30°C). There was no significant activation of current by DTT in Panx1-expressing cells that had not been treated with NO donors, and there were no detectable CBX-sensitive currents in untransfected cells (data not shown).

In addition to S-nitrosylation, increases in NO can activate soluble guanylate cyclase (sGC) and promote downstream cGMP-dependent phosphorylation cascades. Therefore, we examined whether GSNO treatment activated sGC in our cells and if this contributed to GSNO-mediated Panx1 current inhibition. Western blot analysis revealed endogenous expression of sGC in our HEK cells that was not affected by transfection with the murine Panx1 plasmid or by treatment with GSNO **(Figure 10D)**. While GSNO did not affect the expression of sGC, we observed a significant increase in intracellular cGMP concentration following GSNO treatment in $Panx1^{WT}$ expressing HEK cells that could be blocked by pretreatment with the sGC inhibitor ODQ **(Figure 10E)**. To rule out the possibility that GSNO was exerting its functional effects on Panx1 currents by activation of cGMP-dependent signaling cascades, we pretreated $Panx1^{WT}$ expressing HEK cells with 20 μM ODQ and examined the effect of GSNO on Panx1 currents. Importantly, ODQ had no effect on Panx1 current inhibition by GNSO **(Figure 10F)**. Taken together, these data indicate that NO can inhibit Panx1 currents by a mechanism distinct from cGMP signaling, likely by S-nitrosylation of the channel.

While our electrophysiology experiments provide strong evidence that Panx1 channel currents can be inhibited by S-nitrosylation, we also sought to determine if ATP release from $Panx1^{WT}$ expressing HEK cells is functionally affected by S-nitrosylation. Based on previous reports indicating the ability of Panx1 channels to release ATP in response to high extracellular K^+ [154], we treated $Panx1^{WT}$ expressing HEK cells with 100 mM KCl and observed a significant increase in ATP release into the extracellular medium that was strongly inhibited by CBX **(Figure 10G)**. There was no change in ATP release from untransfected HEK cells following KCl stimulation, indicating that Panx1 expression was

required for ATP release in these cells. Importantly, treatment of Panx1^{WT} expressing HEK cells with 100 µM GSNO or 50 µM DEA NONOate attenuated the KCl-evoked ATP release, consistent with our observations on the effects of these NO donor molecules on Panx1 channel currents. Moreover, pretreatment with GSH had no effect on ATP release from these cells **(Figure 10G)**.

To examine the possibility that the reduction in Panx1 current and ATP release following treatment with our NO donors was due to reduced expression of these channels at the plasma membrane, we performed a membrane biotinylation assay on Panx1^{WT} expressing HEK cells treated with GSNO for a time course from 0 to 10 minutes, a time frame consistent with the observed inhibitory effects on Panx1 currents. Treatment of cells with GSNO did not affect Panx1 expression at the plasma membrane **(Figure 10H)**. Moreover, treatment of Panx1^{WT} expressing HEK cells with GSNO for 10 minutes had no effect on Panx1 membrane localization as assessed by immunofluorescence microscopy **(Figure 10I)**. Together, these data indicate that the inhibitory effect of S-nitrosylation on Panx1 channels is unlikely to reflect decreased plasma membrane expression of the channel.

FIGURE 10. GSNO INHIBITS PANX1 CURRENTS AND ATP RELEASE

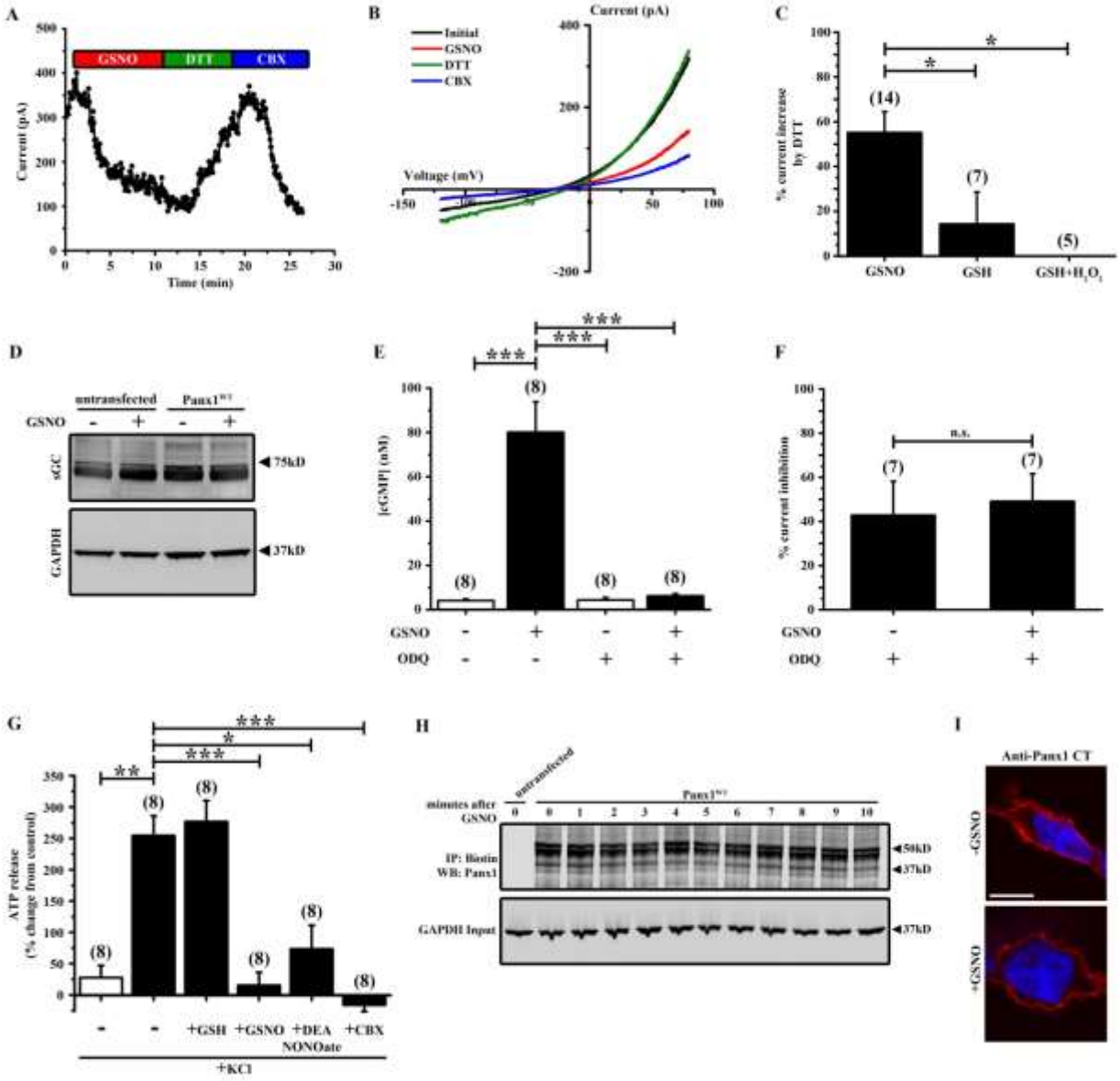

Figure 10. GSNO inhibits Panx1 currents and ATP release. A. Time series showing peak Panx1 whole cell current amplitude during application of 100 μm GSNO, 1 mm DTT, and 50 μm CBX. **B,** current-voltage curves of Panx1 currents from $Panx1^{WT}$-expressing HEK cells under control conditions (*black trace*) and following application of 100 μm GSNO (*red trace*), 1 mm DTT (*green trace*), and 50 μm CBX (*blue trace*). **C,** summary data showing the percentage of Panx1 current inhibition by GSNO, GSH, or GSH with H_2O_2 that was reversible by DTT. **D,** Western blot for sGC expression in untransfected or $Panx1^{WT}$-expressing HEK cells following treatment with 100 μm GSNO or vehicle. **E,** cGMP assay of $Panx1^{WT}$-expressing HEK cells treated with 100 μm GSNO or vehicle and the sGC inhibitor ODQ. **F,** effect of sGC inhibition by ODQ on Panx1 current inhibition by GSNO. **G,** ATP release assay from untransfected (*white bar*) and $Panx1^{WT}$-expressing (*black bars*) HEK cells. Data represent cells treated with 100 mm KCl following pretreatment with 100 μm GSNO, 50 μm DEA NONOate, 100 μm GSH, or 50 μm CBX. all data are presented as percent change in ATP release compared with control (−KCl). **H,** cell surface biotinylation assay of Panx1 from $Panx1^{WT}$-expressing HEK cells following treatment with 100 μm GSNO for 0–10 min. **I,** immunofluorescence micrographs of $Panx1^{WT}$-expressing HEK cells treated with or without 100 μm GSNO for 10 min. *Red* indicates Panx1, and *blue* indicates DAPI-stained nuclei. *Scale bar* in all images, 10 μm. *n* values are indicated in *parentheses*. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$; *n.s.*, not significant. *IP*, immunoprecipitation; *WB*, Western blot; *CT*, C terminus.

Multiple cysteine residues contribute to Panx1 current inhibition by S-nitrosylation.

To determine which cysteines are modified by S-nitrosylation and contribute to Panx1 inhibition, we generated several cysteine-to-alanine mutations in Panx1 by site-directed mutagenesis. A topology map of Panx1 indicating the cysteines in which we made alanine substitutions (red circles) is shown in **Figure 11A.** We identified target cysteines through a combination of experimental and computational evidence. Previous studies have reported increased Panx1 channel currents upon mutation of C40 or C346, suggesting that these residues may be intimately involved in channel regulation (29-31). The C-terminal tail, which contains an additional cysteine (C426), may also be important for channel regulation as removal of the C-tail increases channel activity (19). Additionally, we used the scan-x post-translational modification prediction method [255] to rank the highest probability S-nitrosylation sites, which pointed to C40 as the most likely S-nitrosylation site. Therefore, we constructed single cysteine-to-alanine substitutions at these sites ($Panx1^{C40A}$, $Panx1^{C346A}$ and $Panx1^{C426A}$).

To ensure that mutation of these single cysteines did not affect trafficking to the plasma membrane, we performed immunofluorescence microscopy on mutant expressing cells under non-permeabilizing conditions with an antibody directed against the second extracellular loop of Panx1 (anti-Panx1 EL) and under permeabilizing conditions with an antibody directed against the intracellular C-tail (anti-Panx1 CT). We found that all Panx1 cysteine mutants were able to reach the plasma membrane (see **Figure 11B** and recordings of Cys-substituted channels below). Moreover, we performed cell surface biotinylation assays on HEK cells expressing each of these Panx1 mutants and found that all localize to the membrane and their expression was not affected by treatment with

GSNO **(Figure 11C)**. In addition, there was no significant difference in Panx1 holding current or basal ATP release between WT and the Panx1 cysteine mutant channels **Figure 11D and 11E**, respectively).

To determine which cysteine residue(s) are modified by S-nitrosylation, we treated HEK cells expressing Panx1 single mutant channels with 100 μM GSNO and performed biotin switch assays. Interestingly, S-nitrosylation of Panx1 was preserved in all three constructs with individual cysteine-to-alanine substitutions **(Figure 12A)**. It should be noted that mutation of C40 or C346 resulted in decreased representation of the higher molecular weight glycosylation species of Panx1 in this assay **(Figure 12A)**. Although all three single Cys mutant channels retained the ability to be S-nitrosylated, the inhibitory effects of GSNO on Panx1 currents were abolished specifically in $Panx1^{C40A}$ **(Figure 12B)** and $Panx1^{C346A}$ **(Figure 12C)** expressing cells; GSNO-mediated inhibition was preserved in $Panx1^{C426A}$ expressing cells **(Figure 12D).** Summary data for each Panx1 single cysteine mutant is shown in **Figure 12E** indicating that mutation of C40 or C346, but not C426, significantly prevented Panx1 current inhibition by GSNO. Consistent with a lack of current inhibition by GSNO in the $Panx1^{C40A}$ and $Panx1^{C346A}$ mutants, ATP release from cells expressing these two Panx1 mutants was not affected by GSNO, with the $Panx1^{C426A}$ mutant exhibiting strong inhibition **(Figure 12F)**. The ability of Panx1 to be S-nitrosylated in each Panx1 single cysteine mutant, with a loss of current inhibition and inhibition of ATP release by GSNO only in the $Panx1^{C40A}$ and $Panx1^{C346A}$ mutants, suggested that S-nitrosylation at multiple cysteine residues may be required for channel inhibition.

To determine if multiple cysteines can be S-nitrosylated, we generated double cysteine-to-alanine substitutions in Panx1 ($Panx1^{C40/346A}$, $Panx1^{C40/426A}$ and $Panx1^{C346/426A}$) and tested the ability of GSNO to S-nitrosylate and inhibit the double mutant channels. Each of the Panx1 double cysteine mutants trafficked to the plasma membrane and expression was unaffected by GSNO **(Figure 11C)**. The GSNO-induced S-nitrosylation was preserved in Panx1 constructs that retained either C40 or C346, but was lost in Panx1 channels missing both C40 and C346 ($Panx1^{C40/346A}$), indicating that these two cysteines are the sites of modification (**Figure 13A)**. All double mutant constructs generated CBX-sensitive Panx1 currents, but GSNO-mediated current inhibition was not observed in any of the double mutant constructs, each of which had a substitution at either C40 or C346 **(Figure 13B-E)**. Consistent with this, GSNO was unable to inhibit ATP release from cells expressing any of the Panx1 double mutant constructs (**Figure 13F)**. Together, these data support the idea that both C40 and C346 can be S-nitrosylated and modification at both sites is required for GSNO-mediated inhibition of Panx1.

FIGURE 11. TRAFFICKING OF PANX1 CYSTEINE MUTANTS TO THE PLASMA MEMBRANE IS UNAFFECTED BY GSNO

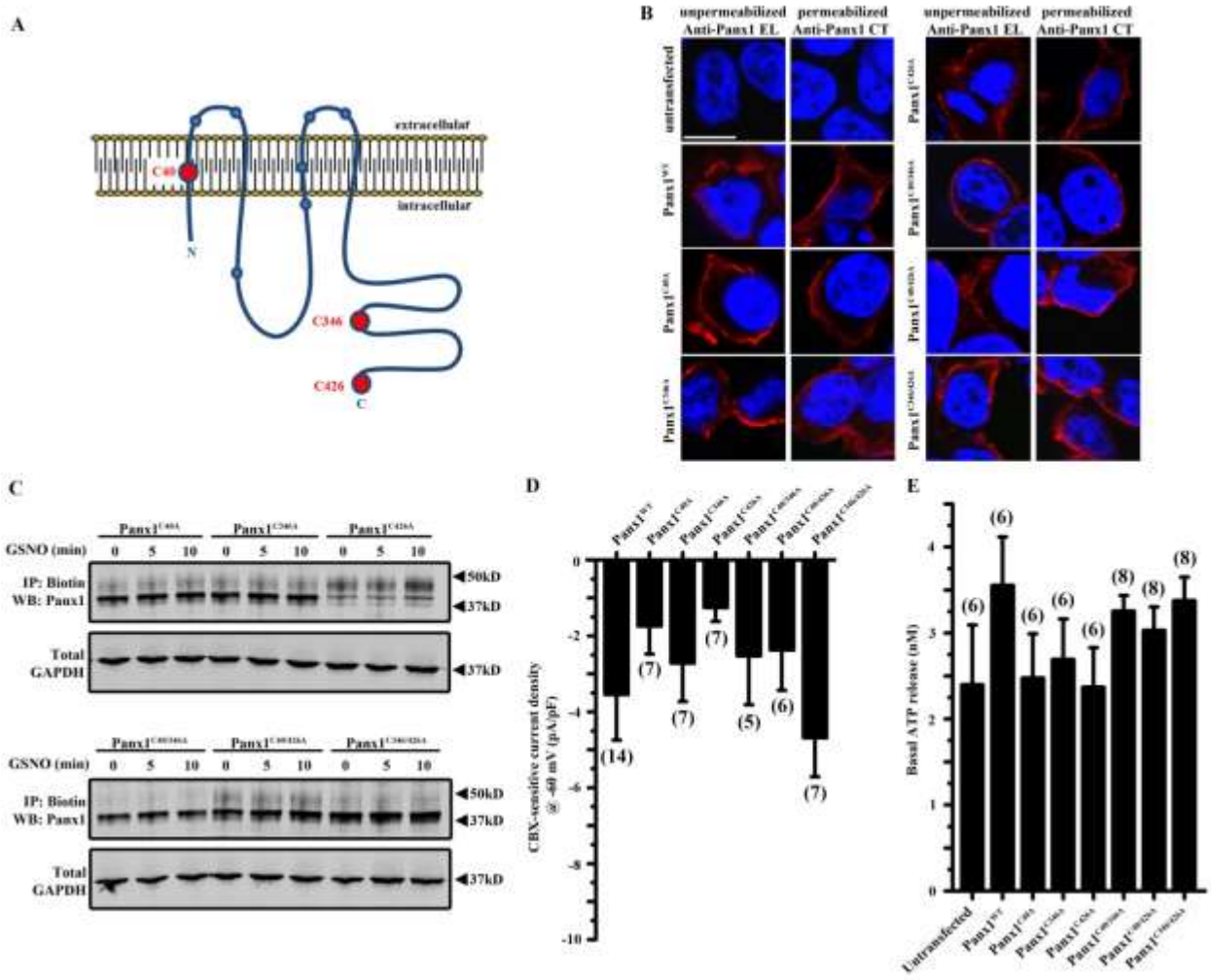

Figure 11. Trafficking of Panx1 cysteine mutants to the plasma membrane is unaffected by GSNO. A. Cell surface biotinylation assay of Panx1 from Panx1WT expressing HEK cells following treatment with 100 μM GSNO for 0-10 minutes. **B.** Immunofluorescence micrographs of Panx1WT expressing HEK cells treated with or without 100μM GSNO for 10 minutes. Red is Panx1 and blue is DAPI stained nuclei. **C.** Topology map of Panx1 showing the predicted location of cysteine residues (circles) within the Panx1 protein. The red circles indicate the three cysteines that were mutated to alanines within the Panx1 polypeptide (C40A, C346A and C426A). **D.** Immunofluorescence micro-graphs of HEK cells depicting Panx1 mutants at the plasma membrane. Samples were processed for immunofluorescence either under non-permeabilizing conditions where they were labeled with an antibody directed against the second extracellular loop of Panx1 (anti-Panx1 EL) or under permeabilizing conditions where they were labeled with an antibody against the C-terminus of Panx1 (anti-Panx1 CT). Red is Panx1 and blue is DAPI stained nuclei. Scale bar is 10 μm in all images.

FIGURE 12. SINGLE MUTATION OF C40 OR C346 PREVENTS PANX1 CURRENT INHIBITION AND ATP RELEASE BY GSNO.

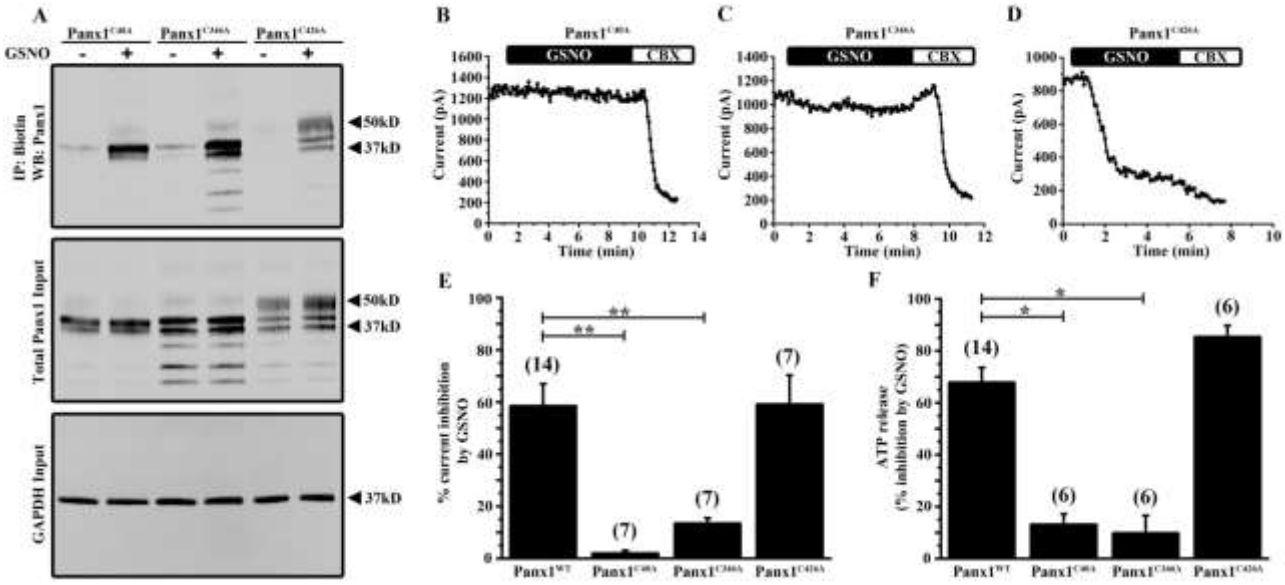

Figure 12. Single mutation of C40 or C346 prevents Panx1 current inhibition and ATP release by GSNO. A. Biotin switch assay on HEK cells expressing each of the Panx1 single cysteine mutants (Panx1C40A, Panx1C346A, Panx1C426A). Panx1 mutant expressing cells were treated with or without 100 μM GSNO. **B-D.** Time series of the peak Panx1 current amplitudes from HEK cells expressing Panx1C40A **(B)**, Panx1C346A **(C)** or Panx1C426A **(D)** mutant constructs and treated with 100 μM GSNO. Transfected cells were treated with 50 μM CBX near the end of each recording protocol to demonstrate that currents could be blocked. **E.** Summary data showing the percent of Panx1 current inhibition by GSNO from HEK cells expressing the Panx1 single cysteine mutant constructs. **F.** ATP release assay from HEK cells expressing Panx1 single cysteine mutants. Data are presented as a % inhibition of ATP release by GSNO. n-values are indicated in brackets. *, $p<0.05$; **, $p<0.001$; ***, $p<0.0001$.

FIGURE 13. S-NITROSYLATION OF BOTH C40 AND C346 IS REQUIRED TO INHIBIT PANX1 CURRENTS AND ATP RELEASE.

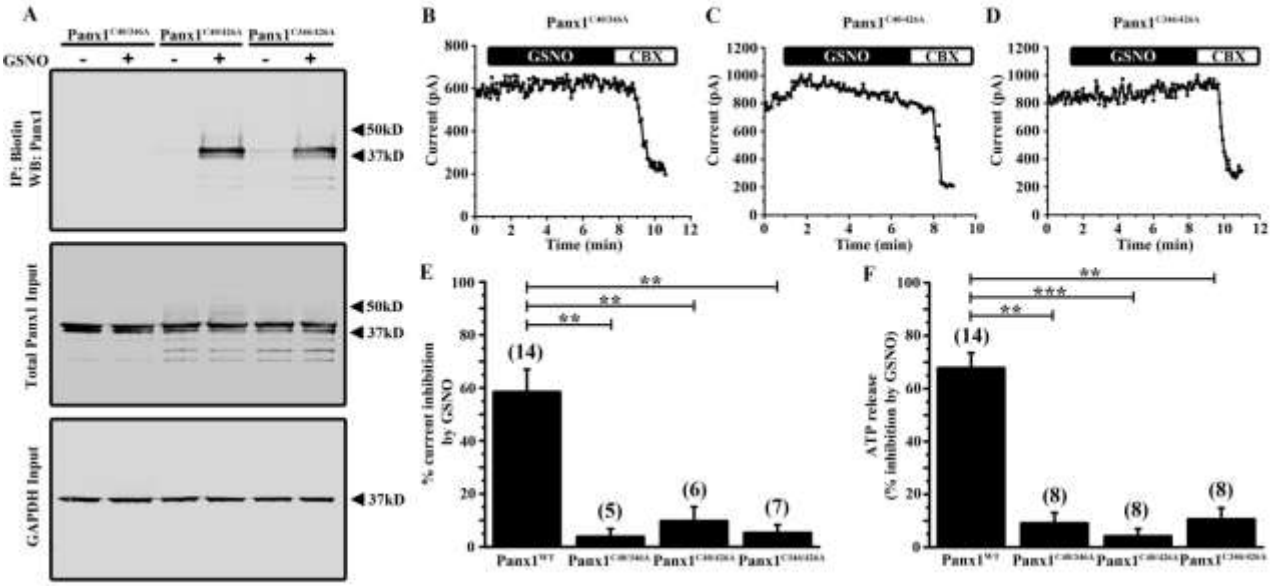

Figure 13. S-nitrosylation of both C40 and C346 is required to inhibit Panx1 currents and ATP release. A. Biotin switch assay on HEK cells transfected with each Panx1 double cysteine mutant construct (Panx1C40/346A, Panx1C40/426A, Panx1C346/426A). Panx1 mutant expressing cells were treated with or without 100 μM GSNO. **B-D.** Time series of the peak Panx1 current amplitudes from HEK cells expressing Panx1C40/346A **(B)**, Panx1C40/426A **(C)** and Panx1C346/426A **(D)** and treated with 100 μM GSNO. Transfected cells were treated with 50 μM CBX near the end of each recording protocol. **E.** Summary data of the percent of Panx1 current inhibition by GSNO from HEK cells expressing the Panx1 double cysteine mutant constructs. **F.** ATP release assay from HEK cells expressing Panx1 single cysteine mutants. Data are presented as a % inhibition of ATP release by GSNO. n-values are indicated in brackets. **, $p<0.001$; *** $p<0.0001$.

S-nitrosylation of Panx1 in endothelial cells inhibits channel function.

Panx1 is highly expressed in endothelial cells across the arterial tree (35). Because these cells express eNOS and are exposed to large amounts of NO, we sought to determine if Panx1 can be modified by S-nitrosylation in a native cell. We utilized primary cultures of mouse aortic endothelial cells (mAEC) which express eNOS and the endothelial cell markers VE-cadherin (Cdh5) and PECAM-1 (data not shown). Treatment of mAECs with GSNO or DEA NONOate for 10 minutes induced S-nitrosylation of endogenous Panx1 **(Figure 14A)** Consistent with our HEK cell data, S-nitrosylation of Panx1 in mAECs by GSNO or DEA NONOate was reversed by treatment with DTT. Moreover, treatment with GSH alone had no effect on Panx1 cysteine modification.

To determine if S-nitrosylation of Panx1 in mAECs inhibits channel function, we conducted whole-cell patch clamp recordings and ATP release assays on mAECs. Single mAECs were patched in the whole cell configuration and Panx1 currents were identified by their sensitivity to CBX and their characteristic I/V relationship (**Figure 14B-C)**. Consistent with our previous observations, peak Panx1 currents at +80 mV were significantly inhibited by GSNO and this inhibition was substantially reversed by treatment with DTT **(Figure 14B-D)**. Treatment with GSH or DTT alone did not affect Panx1 current in these cells indicating that S-nitrosylation of endogenous Panx1 causes potent inhibition of channel currents. We next sought to determine whether ATP release from Panx1 channels in mAECs was affected by S-nitrosylation. Because thrombin is a stimulus for ATP release from Panx1 channels in ECs (14), we stimulated mAECs with 1U/mL of thrombin for 5 minutes. Thrombin stimulation significantly increased ATP

release that was inhibited by both CBX and siRNA knockdown of endogenous Panx1 **(Figure 14E).** Moreover, GSNO and DEA-NONOate significantly attenuated ATP release from mAECs, indicating that S-nitrosylation inhibits endogenous Panx1 channel function, confirming our findings on HEK cells.

FIGURE 14. S-NITROSYLATION INHIBITS PANX1 CURRENTS AND ATP RELEASE FROM MOUSE AORTIC ENDOTHELIAL CELLS.

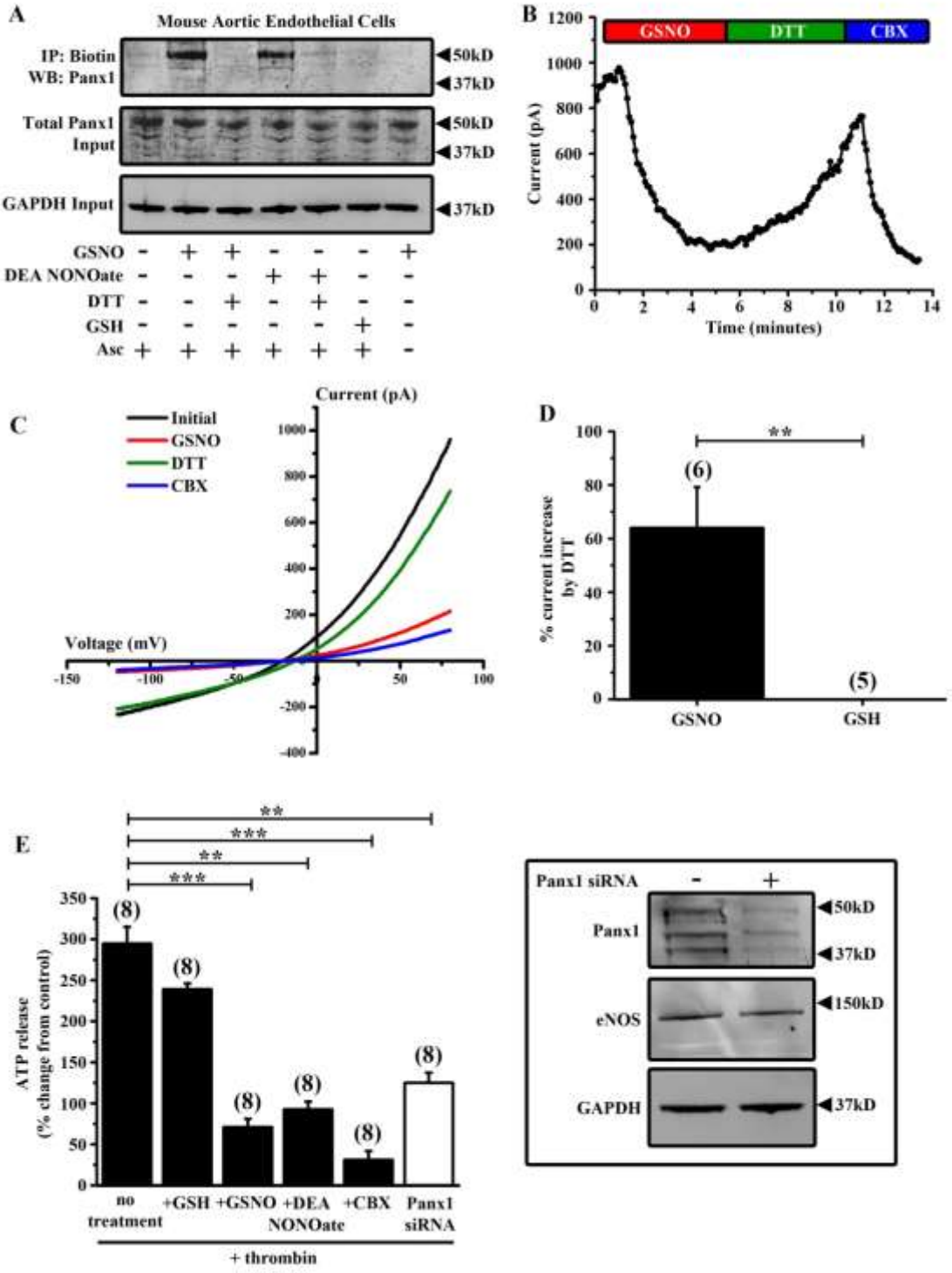

Figure 14. S-nitrosylation inhibits Panx1 currents and ATP release from mouse aortic endothelial cells. A. Biotin switch assay on primary mouse aortic endothelial cells (mAECs) treated with 100 μM GSNO ± 1 mM DTT, 50 μM DEA NONOate ± 1 mM DTT or 100 μM GSH. Ascorbate was omitted from the assay as a negative control. **B.** Time series showing peak Panx1 whole cell current amplitude from mAECs during application of GSNO, DTT and CBX. **C.** I/V curves of Panx1 currents from primary mAECs under control conditions (black trace), following application of 100 μM GSNO (red trace), 1 mM DTT (green trace) and CBX (blue trace). **D.** Summary data showing the percent of Panx1 current inhibition by GSNO or GSH that was reversible by DTT. **E.** ATP release assay from mAECs stimulated with 1U/mL mouse thrombin. Cells were pretreated with 100 μM GSNO, 50 μM DEA NONOate, 100 μM GSH or 50 μM CBX. A subset of cells was transfected with siRNA against mouse Panx1 to knockdown the endogenous protein. All data are presented as a % change in ATP release compared to control (-thrombin). Panel E insert depicts a Panx1 Western blot of untransfected and Panx1 siRNA transfected mAECs. eNOS and GAPDH were used as loading controls. n-values are indicated in brackets. **, $p<0.001$; *** $p<0.0001$.

4.4. Discussion

In this study, we identify a novel inhibitory mechanism for Panx1 channels: we show that Panx1 can be modified by S-nitrosylation and that this post-translation modification leads to inhibition of Panx1-mediated currents and ATP release. In brief, we used a biotin switch assay to show that GSNO and DEA NONOate, two NO donor molecules, induce a DTT-sensitive Panx1 modification that is consistent with S-nitrosylation; also, it was not observed when GSH was substituted for GSNO in control or oxidative conditions, ruling out an alternative glutathionylation mechanism. Likewise, inhibition of whole cell Panx1 currents by GSNO and DEA NONOate was reversed by DTT, an action that was also not mimicked by GSH. In addition, GSNO-mediated current inhibition was not dependent on sGC activity, supporting S-nitrosylation over other mechanisms that can contribute to NO actions. In support of a direct inhibitory effect on the channel, substitution of either C40 or C346 on Panx1 prevented GSNO-mediated channel inhibition, while mutation of both C40 and C346 blocked S-nitrosylation of the channel. In sum, these data demonstrate that modification at these two critical cysteines is required for Panx1 channel inhibition by S-nitrosylation, and they suggest a functional role for NO in regulating the activity of Panx1 channels.

The role of Panx1 channels in ATP release from cells has been a growing area of investigation, with multiple studies identifying mechanisms by which Panx1 channels can be activated. By contrast, there remains little understanding as to how Panx1 channels are inhibited. The results of our current study provide a novel mechanism by which Panx1 channels can be inhibited, supporting a functional role for NO in controlling the

activity of Panx1 channels at the plasma membrane of cells and suggesting a way to negatively regulate ATP release from cells.

Although our results indicate an inhibitory role imparted by NO on Panx1 channels through S-nitrosylation, a recent study has implicated this reactive oxygen species in Panx1 channel activation [256]. In that work, it was suggested that NO generated during oxygen-glucose deprivation can activate Panx1 channels expressed in cultured hippocampal neurons. While it is possible that NO signaling may result in differential Panx1 channel regulation in different physiological contexts, it is important to point out that the aforementioned study used calcein dye leakage as a functional readout for Panx1 channel activity. Since cultured hippocampal neurons express connexin proteins, which are both permeable to calcein dye and activated by S-nitrosylation [215,252], it is possible that the dye leakage observed in this study represented activity of connexins. Moreover, it is possible that cultured hippocampal neurons express other pannexin isoforms, such as Panx2, which is highly expressed in the central nervous system [257,258]. As such, other pannexin isoforms may be regulated differently by NO. In our work, we assessed S-nitrosylation and recorded whole cell currents as well as ATP release from recombinant Panx1 channels, providing direct evidence for inhibition of Panx1 by NO.

We performed cysteine mutagenesis of Panx1 to identify the specific residues required for S-nitrosylation and channel inhibition. Interestingly, the two sites that we identified as critical for this inhibitory modification -- C40 and C346 -- were previously reported to enhance activity of Panx1 channels in mutagenesis studies [259,260]. In that other work, serine substitution at either C40 [260] or C346 [259] produced “leaky” or

constitutively active channels. Together with our results, these observations suggest that C40 and C346 may be localized to regions that are important for dynamic up- and down-regulation of Panx1 channel activity.

We noted that mutation of either C40 or C346 affected the banding pattern of Panx1 on a Western blot. It is known that Panx1 exists in three forms: a core unglycosylated species (Gly0), a high-mannose species (Gly1) and a complex glycosylated form (Gly2), each contributing to the characteristic triple banding pattern on a Western blot. Mutation of C40 or C346 resulted in a marked reduction in the Gly2 species, with the protein detected mainly as Gly0 and Gly1 forms. Since glycosylation has been implicated in trafficking of Panx1 channels to the plasma membrane, we examined the cellular localization of the cysteine mutants and found that each mutant appeared at the plasma membrane. Even though these cysteine substitutions may have affected Panx1 glycosylation status, it is important to point out that Panx1 currents and ATP release were detected for all mutant constructs, indicating that they could form functional channels at the plasma membrane. This is consistent with previous work indicating that all Panx1 Gly species can reach the plasma membrane [151]. Also, a previous study identified a loss in the Gly2 species in functional, plasma membrane-localized Panx1C346S [259].

S-nitrosylation of Panx1 channels may function physiologically as a negative feedback mechanism, mediating inhibition of channel activity following their activation to prevent chronic release of ATP. In the vasculature, endothelial cells that line the blood vessel lumen express functional Panx1 channels at the plasma membrane and multiple studies have emerged implicating these channels in ATP release [11,30,53,177,205]. Recently, it was suggested that thrombin, by activation of

endothelial cell PAR-1 receptors, promotes ATP release from Panx1 channels into the extracellular compartment [205]. Our data utilizing mouse aortic endothelial cells indicates that thrombin-induced ATP release from these cells can be significantly inhibited by S-nitrosylation of the channel. This novel regulatory mechanism may play an important role in controlling the extent of ATP release from the vascular endothelium and therefore modulate purinergic signaling events in the vasculature. In addition, circulating erythrocytes release ATP via Panx1 into the blood vessel lumen during conditions of low oxygen tension and membrane deformation, conditions that arise in small arterioles and capillaries [30,53]. Released ATP activates endothelial cell P2Y receptors causing NO generation which diffuses to and relaxes adjacent smooth muscle cells [261]. It is possible that the endothelial-derived NO could also diffuse into the blood vessel lumen and S-nitrosylate erythrocyte Panx1 channels, inhibiting further ATP release and preventing excess vasodilation. Regulation of Panx1 channels by S-nitrosylation in these cells could therefore play an important role in the matching of blood flow to tissue oxygen demand.

While the inhibitory effect of S-nitrosylation on Panx1 channel function was demonstrated, the mechanism by which this modification elicits its effects on the channel is not as clear. In this respect, our data indicate that S-nitrosylation has no effect on Panx1 cell membrane localization suggesting the possibility that channel activity is directly inhibited. Recent evidence has indicated that the C terminal tail may form a portion of the Panx1 channel pore [262] and that proteolytic cleavage of the C terminal tail increases channel currents by its removal from the Panx1 pore [169]. It is possible that S-nitrosylation of C346 and C40 promotes a conformation that stabilizes the C-

terminal:pore interaction, and thus the channel closed state. Future studies utilizing structural and biophysical techniques may reveal the precise mechanism by which Panx1 S-nitrosylation inhibits the channel. Nonetheless, our data has provided novel evidence for a post-translational mechanism inhibiting Panx1 channels at the plasma membrane.

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CHAPTER 5. PANX1-DEPENDENT ATP RELEASE FROM VENOUS ENDOTHELIAL CELLS POTENTIATES ACUTE VASCULAR INFLAMMATION

5.1. Abstract

Acute vascular inflammation is the first response defense system that targets circulating inflammatory cells to local sites of tissue injury and infection. This physiological process has been widely characterized in the scope of cell-to-cell interactions between the vascular endothelium (primarily at the level of post-capillary venules) and circulating leukocytes, involving complex signaling networks that influence cellular expression and localization of adhesion molecules with paralleled synthesis and secretion of pro-inflammatory cytokines [62]. A prominent signaling pathway integrated in these cellular changes is mediated by the ATP sensitive purinergic receptors [263]. Most notably, endothelial cell localized P_2Y purinergic receptors regulate a number of inflammatory cytokine signaling processes, including those initiated by tumor necrosis factor alpha (TNFα) [16,17]. However, while it has become increasingly clear that extracellular signaling by ATP plays an implicit role in vascular inflammation, the mechanism regulating cellular ATP release in this response from vascular cells has remained elusive. Here we show that the ATP release channel Pannexin 1 (Panx1) expressed in venous endothelial cells opens to release ATP downstream of TNF receptor type 1 (TNFR1) activation. Dissection of the molecular signaling pathway initiating this response revealed that Panx1 is activated by Src Family Kinase (SFK)-dependent phosphorylation at tyrosine residue 198 located on the intracellular loop of the channel. Utilizing an inducible, endothelial cell specific Panx1 knockout mouse we found that TNFα induced vascular inflammation in mice expressing physiological levels of the ATP release channel, but mice with selective EC deletion of Panx1 displayed significant reductions in leukocyte rolling, adhesion and emigration to the inflammatory stimuli.

Taken together, these findings identify a novel mediator in the vascular inflammatory response poising Panx1 channels at the center of cytokine cross-talk with purinergic signaling in the venous endothelium.

5.2. Introduction/Results:

The pro-inflammatory cytokine TNFα is synthesized and released from macrophages, lymphocytes and endothelial cells during systemic vascular inflammation [264]. Through activation of its complement cell surface receptors (TNFR1 and TNFR2), endothelial cell exposure to TNFα induces the activation of NFκB and induction of pro-inflammatory gene transcription promoting an inflammatory endothelial cell phenotype that signals recruitment of circulating neutrophils to aid in resolution of local tissue injury and infection. Chronic exposure to the cytokine induces processing of intracellular pro-caspase enzymes to their active form leading to apoptosis. While purinergic receptor activation has been implicated in TNFα-mediated inflammatory responses, it is unknown whether extracellular ATP is released from the venous endothelium in response to TNFα stimulation. To this end, we utilized *in vitro* static ATP release assays on two primary venous endothelial cell types, human umbilical vein (HUVEC) and human saphenous vein (HSaVEC). In response to stimulation with TNFα, we observed a significant increase in ATP efflux from both HUVEC and HSaVEC **(Figure 15a-b)**. This effect increased in a dose- and time-dependent manner with maximal accumulation of extracellular ATP achieved following activation of ECs with 10 ng/mL TNFα for 10 minutes. Interestingly, two arterial endothelial cell types, human aortic (HAoEC) and human coronary artery (HCoAEC) failed to release ATP in response to TNFα stimulation, suggesting differential roles for purinergic signaling in the arterial and

venous circulation during inflammatory stress. To assess the potential for venous ECs to release ATP to TNFα in a more physiological setting, we perfused TNFα into the lumen of isolated mouse mesenteric venules and quantified ATP accumulation in the perfusate. Similar to cultured venous ECs, TNFα exerted a significant increase in luminal ATP accumulation in *ex vivo* cannulated mesenteric venules as compared to vehicle perfused controls **(Figure 15c)**. Consistent with observations seen *in vitro*, TNFα did not promote ATP release from isolated mesenteric arterioles under the conditions tested **(Figure 15d)**. To determine if ATP release was a direct effect of TNFR1 activation in venous endothelial cells, we blocked TNFR1 activation utilizing a peptide antagonist for the receptor, WP9QY. Treatment of HUVEC and HSaVEC with WP9QY prevented ATP release in response to increasing doses of TNFα, indicating that ATP release is dependent on TNFR1 activation **(Figure 15e-f)**.

FIGURE 15. VENOUS ENDOTHELIAL CELLS (ECs) RELEASE ATP WHEN ACTIVATED BY TNFα.

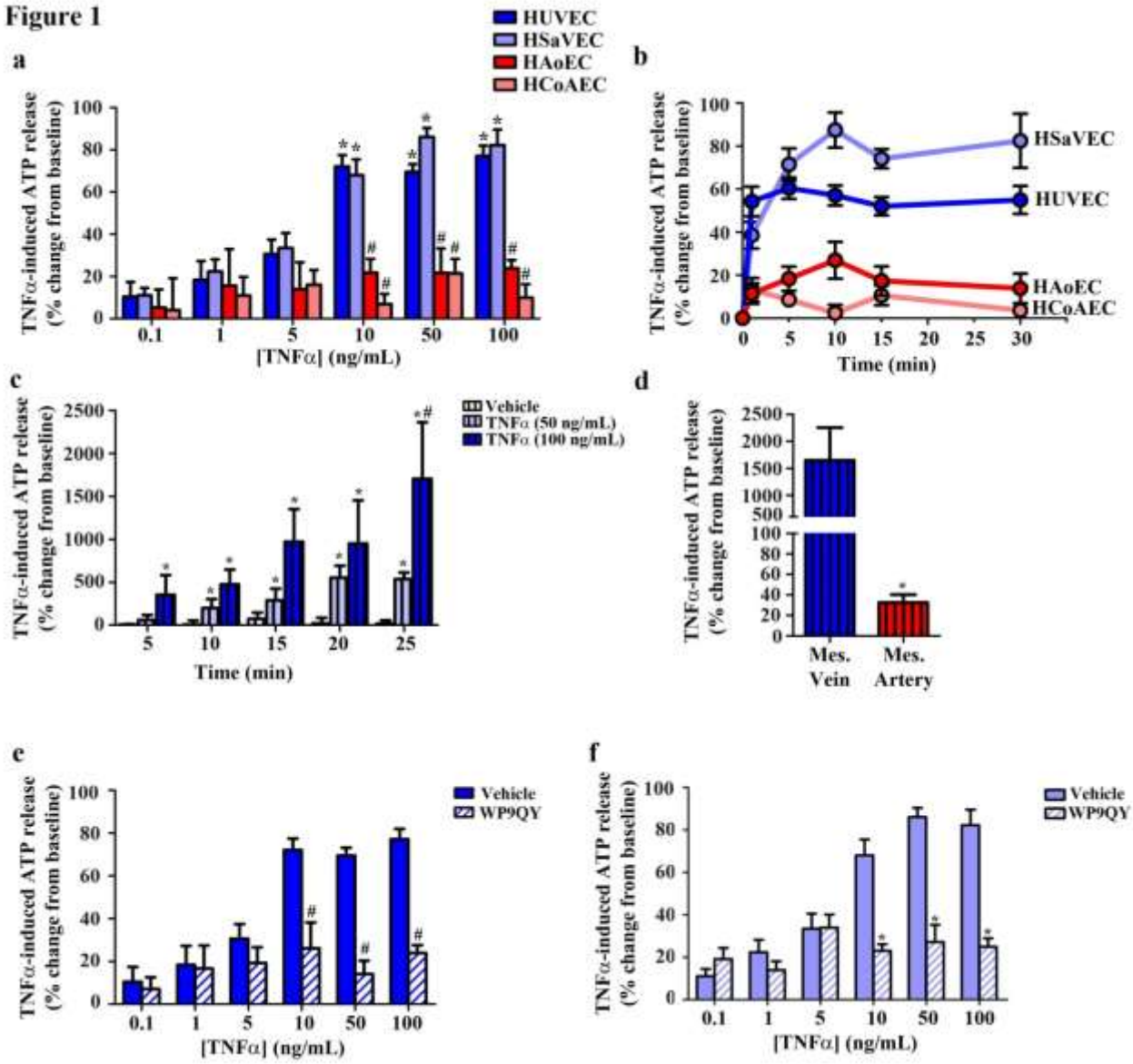

Figure 15: Venous endothelial cells (ECs) release ATP when activated by TNFα.

a. Dose response of cultured human arterial (HAoEC and HCoAEC) and venous (HUVEC and HSaVEC) endothelial cells to TNFα. HUVEC: human umbilical vein endothelial cell, HSaVEC: human saphenous vein endothelial cell, HAoEC: human aortic endothelial cell, HCoAEC: human coronary artery endothelial cell. * indicates a significant increase from unstimulated cells ($p<0.01$) and # indicates significant difference as compared to venous cells ($p<0.005$). **b.** Time course of TNFα-induced ATP release from cultured arterial and venous endothelial cells. **c.** ATP release from isolated murine mesenteric veins perfused with TNFα. * indicates significant difference as compared to vehicle ($p<0.001$) and # indicates a significant difference as compared to the 50 ng/mL dose ($P<0.01$). **d.** ATP release from isolated mesenteric veins and paired arteries in response to TNFα (50 ng/mL) perfusion. * indicates $p<0.001$). **e-f.** Dose response of HUVEC **(e)** and HSaVEC **(f)** to TNFα following inhibition of TNFR1 with the peptide antagonist WP9QY. * indicate $p<0.01$ as compared to vehicle.

Prolonged ATP release from cells can be detrimental to cell viability, eventually leading to apoptosis and necrosis. In fact, the chronic accumulation of extracellular ATP is now known to act as a potent "find-me" signal for phagocyte recruitment[78,265]. However, loss of endothelial integrity is detrimental to cardiovascular health and the resolution of inflammation; therefore, we hypothesized that TNFα-mediated ATP release from venous endothelial cells is mediated via a non-lytic mechanism. Multiple regulated ATP release pathways have been reported in the vasculature, including prominent roles for vesiclular and channel-dependent ATP release primarily involving connexins (Cx) hemichannels and the recently identified pannexin (Panx) channels[245]. Cx hemichannels constitute hexameric single membrane "half-channels" that comprise the building blocks for intercellular gap junction formation. The Panx channels, composed of three members: Panx1, Panx2 and Panx3, are predicted to be structurally similar to Cx hemichannels; however, Panxs exist solely as single membrane channels and do not form gap junctions *in vivo*[150]. Rather, the primary function ascribed to channels formed by the Panx1 isoform is cellular ATP release. Initially, to discriminate between the involvement of Cx hemichannels and Panx1 channels in TNFα-mediated ATP we selectively depleted Cx hemichannels from the EC plasma membrane without affecting the localization of Panx1. Taking into account the relative half-life of each channel at the plasma membrane (Cx hemichannels: 1-5 hours[266], Panx1 channels: >6 hours[152]) we treated HUVEC and HSaVEC with the vesicular exocytosis inhibitor brefeldin A (BFA) to prevent trafficking of newly synthesized channels to the cell surface while preserving internalization of older channels. Inhibition of vesicular trafficking decreased the expression of Cx43 hemichannels at the EC surface without significantly altering the

expression of Panx1 in both HUVEC **(Figure 16a)** and HSaVEC **(Figure 17a).** Under these experimental conditions, the ability of TNFα to induce ATP release was unaffected, suggesting a role for Panx1 channels, but not Cx hemichannels, as possible conduits for ATP liberation **(Figure 16b, Figure 17b)**. To more directly interrogate the contribution of Panx1 channels to ATP release from venous ECs, we used two independent Panx1 pharmacological antagonists to block channel function, carbenoxolone (CBX) and an inhibitory peptide called ^{10}panx1. In HUVEC, Panx1 channel blockade with CBX (50μM) and ^{10}panx1 (200μM) significantly reduced TNFα-mediated ATP release by 86.4% and 70.9%, respectively **(Figure 16c-d)**. Similar inhibition was observed in HSaVECs (CBX: 69.7%,^{10}panx1: 75.2%) **(Figure 17c)**. Additionally, pharmacological blockade of vesicular ATP release with BFA and a newly identified member of ATP release channels CALHM1 with ruthenium red (RuR) did not affect TNFα-induced ATP release **(Figure 16e)**. Employing RNA interference, we genetically knocked down the expression of Panx1 in HUVEC and HSaVEC with siRNA duplexes targeting the 3rd and 4th exons of the *PANX1* gene. Knockdown efficiency was ~65% and ~75% in HUVEC **(Figure 16f)** and HSaVEC **(Figure 17d)**, respectively. Concurrent with pharmacological inhibition of EC Panx1 channels, siRNA knockdown of Panx1 significantly reduced TNFα-induced ATP release in both EC types **(Figure 16g, Figure 17e)**. Finally, to directly investigate the contribution of venous EC Panx1 channels to TNFα-mediated ATP release in the intact venous circulation, we developed an inducible, endothelial cell-specific Panx1 knockout mouse which harbors loxP sites flanking exon 3 of the *Panx1* gene (Panx1$^{fl/fl}$) and a tamoxifen-inducible Cre recombinase whose expression is driven by the vascular endothelial cell cadherin (VECadER^{T2}) promoter. Following 10 days of

tamoxifen injections, VECadER^{T2}/Panx1$^{fl/fl}$ mice displayed a substantial reduction in Panx1 expression in the endothelium of mesenteric venules **(Figure 16h)**. Luminal perfusion of TNFα in isolated mesenteric venules from mice lacking Panx1 in the endothelium (i.e. tamoxifen injected) showed a marked inhibition of ATP release as compared to littermate controls expressing normal levels of the channel (i.e. peanut oil (vehicle) injected) **(Figure 16i)**. Taken together, these studies indicate a direct role for Panx1 channel-dependent ATP release in response to endothelial cell activation by the inflammatory cytokine TNFα.

FIGURE 16. PANNEXIN 1 CHANNELS MEDIATE TNFα-INDUCED ATP RELEASE FROM VENOUS ECs.

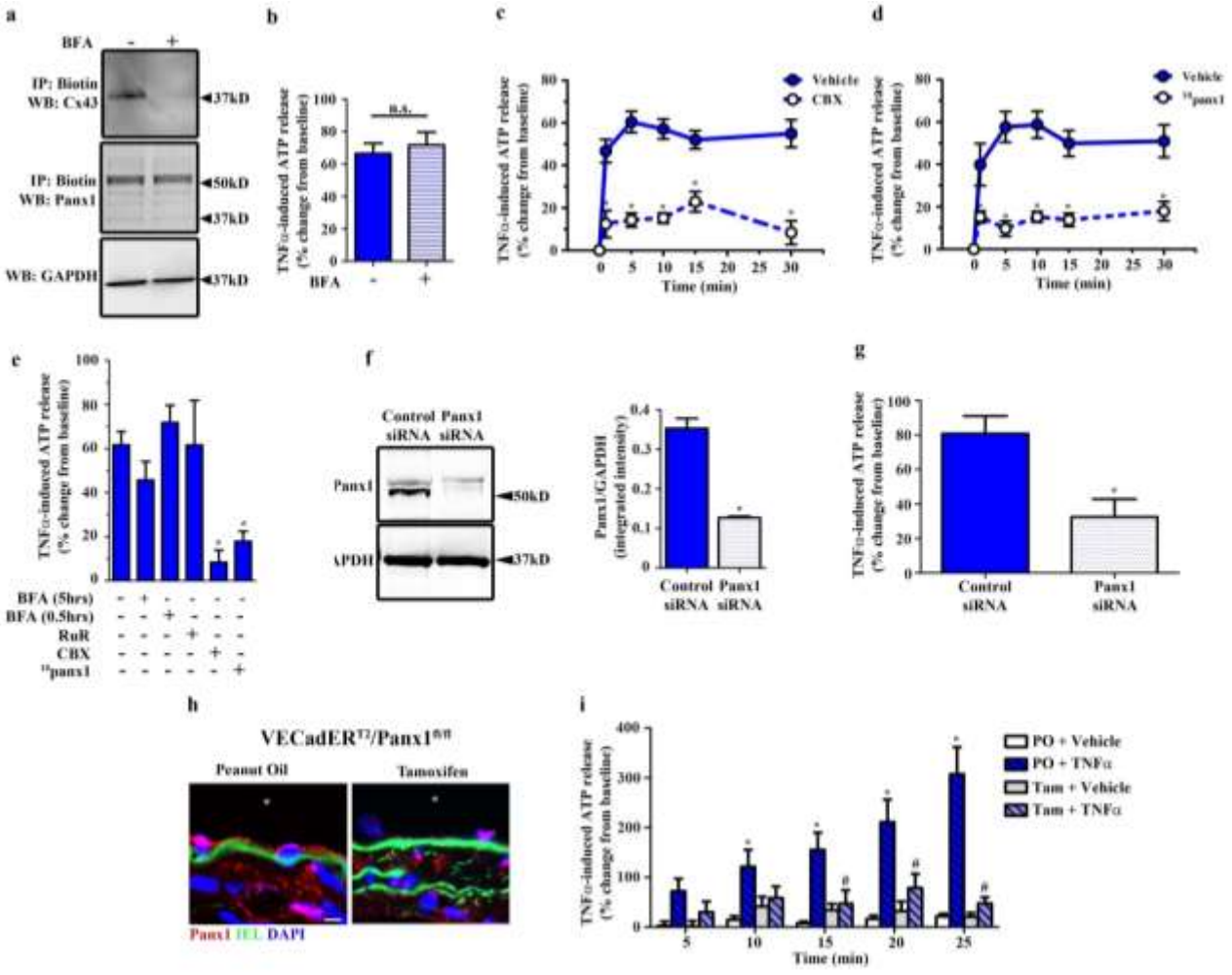

Figure 16: Pannexin 1 channels mediate TNFα-induced ATP release from venous ECs.

a. Representative Western blot of HUVEC subjected to treatment with brefeldin A (BFA) for 5 hours and subsequent cell surface biotinylation of membrane proteins. Panx1 and Cx43 expression were assessed. **b.** ATP release from BFA treated HUVEC in response to TNFα (10 ng/mL) treatment for 30 minutes. **c-d.** Time course of TNFα-induced ATP release from HUVEC following inhibition of Panx1 channels with carbenoxolone (CBX: 50μM) **(c)** and the Panx1 blocking peptide ^{10}panx1 (200μM) **(d).** **e.** Summary data of pharmacological inhibitors assessed for inhibition of TNFα-induced ATP release from HUVEC. BFA (30min): inhibition of vesicular release, Ruthenium Red (RuR): antagonist of CALHM1 channels. **f.** siRNA knockdown of Panx1 from HUVEC. **g.** ATP release from siRNA treated HUVEC from **f** in response to TNFα (10 ng/mL). **h.** Immunofluorescence of Panx1 expression in the endothelium of mesenteric veins isolated from the inducible, endothelial cell specific Panx1 knockout mice (VECadER^{T2}/Panx1$^{fl/fl}$) injected with tamoxifen (Panx1$^{-/-}$) or its vehicle peanut oil (WT) for 10 days. * indicates the blood vessel lumen nuclei are stained with DAPI and the autofluorescence of the internal elastic lamina (IEL) is in green. **i.** ATP release from isolated mesenteric veins from VECadER^{T2}/Panx1$^{fl/fl}$ mice injected with tamoxifen (Tam) or peanut oil (PO) for 10 days and subjected to perfusion with TNFα.

FIGURE 17. HSaVEC RELEASE ATP VIA PANX1 CHANNELS IN RESPONSE TO TNFα STIMULATION.

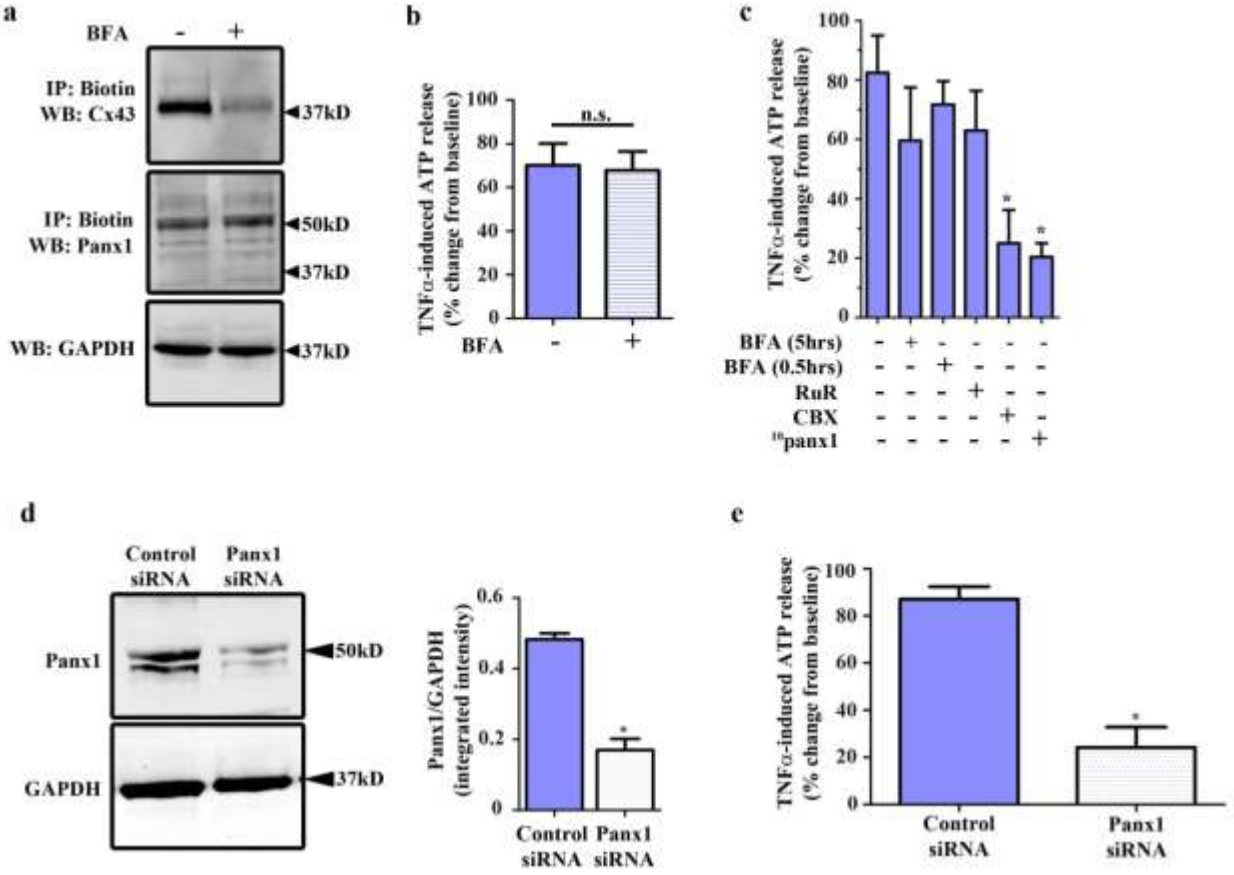

Figure 17: HSaVEC release ATP via Panx1 channels in response to TNFα stimulation.

a. Representative Western blot of HSaVEC subjected to treatment with brefeldin A (BFA) for 5 hours and subsequent cell surface biotinylation of membrane proteins. Panx1 and Cx43 expression were assessed. **b.** ATP release from BFA treated HSaVEC in response to TNFα (10 ng/mL) treatment for 30 minutes. **c.** Summary data of pharmacological inhibitors assessed for inhibition of TNFα-induced ATP release from HSaVEC. BFA (30min): inhibition of vesicular release, Ruthenium Red (RuR): antagonist of CALHM1 channels. **d.** siRNA knockdown of Panx1 from HSaVEC. **e.** ATP release from siRNA treated HSaVEC from **d** in response to TNFα (10 ng/mL).

Next, we aimed to elucidate the molecular mechanism by which activation of TNFR1 in venous ECs promotes Panx1 channel activation. TNFR1 activation has been reported to induce the activity of a number of intracellular kinases, including the Src Family Tyrosine Kinases (SFK) [267,268]. In addition, endothelial cell and inflammatory cell SFKs are intimately involved in promoting the breakdown of endothelial barrier function and increased recruitment, adhesions and transmigration of circulating neutrophils, monocytes and macrophages to inflamed tissues [269]. As such, we sought to determine the potential role for SFKs in TNFα-induced ATP release from venous ECs. SFK activation can be assessed by autophosphorylation of a conserved Y416 residue which stabilizes a substrate-permissive active site conformation in the kinases [270]. Using a phospho-specific Y416 SFK antibody, we detected an increase in Y416 phosphorylation in HUVEC **(Figure 18a)** and HSaVEC **(Figure 19a)** following 5 minutes of exposure to recombinant human TNFα. This effect was specific to SFK activation as treatment of both cell types with the selective SFK inhibitor PP2 reduced Y416 phosphorylation to baseline levels, while PP3, the inactive analog of PP2, had no effect. Pharmacological inhibition of SFKs with PP2 significantly blunted TNFα-induced ATP release from HUVEC **(Figure 18b)** and HSaVEC **(Figure 19b)**, with PP3 showing no significant effect. In brief, these observations provide novel insight into the molecular signaling between TNFα and Panx1 channels in the venous endothelium, implicating SFK activation and downstream phosphorylation of an important regulatory tyrosine residue in the Panx1 intracellular loop in the induction of ATP release to facilitate autocrine/paracrine purinergic signaling cascades during vascular inflammation.

FIGURE 18. TNFα INDUCES SRC FAMILY KINASE (SFK) ACTIVATION IN VENOUS ECs INDUCING PANX1 CHANNEL ACTIVATION

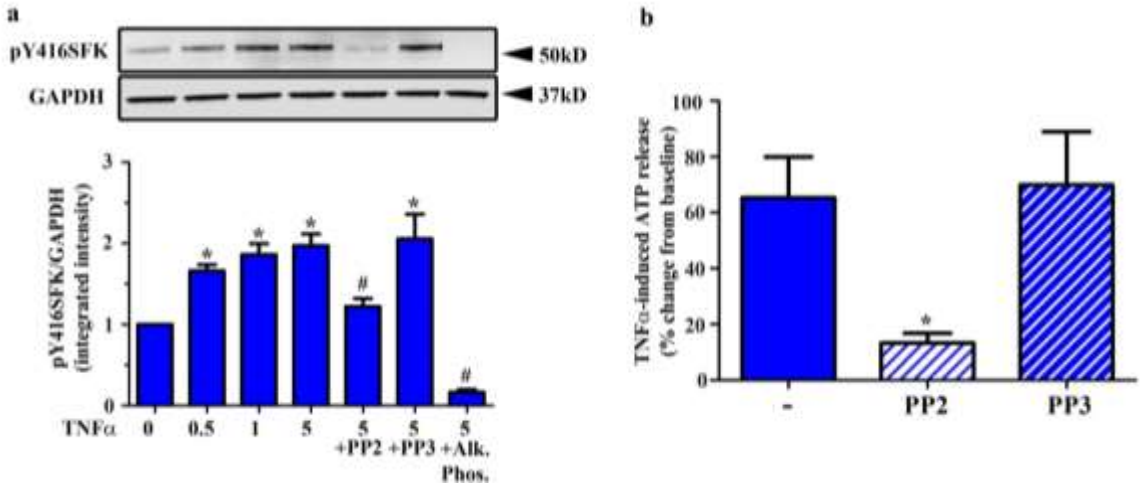

Figure 18: TNFα induces Src Family Kinase (SFK) activation in venous ECs inducing Panx1 channels activation.

a. Western blot analysis of SFK activation in HUVEC in response to TNFα stimulation. A phospho-specific antibody against Y416 in SFKs (pY416SFK) was used as an indicator of SFK activation. SFK activation was blocked with the pharmacological antagonist PP2 but not by its inactive analog PP3. Antibody specificity for the phosphorylated form of the protein was confirmed by dephosphorylating proteins in cell lysates with alkaline phosphatase. **b.** TNFα-induced ATP release from HUVEC following SFK inhibition with PP2.

FIGURE 19. TNFα ACTIVATES SRC-FAMILY KINASES IN HSaVECs INDUCING PANX1 CHANNEL ACTIVATION

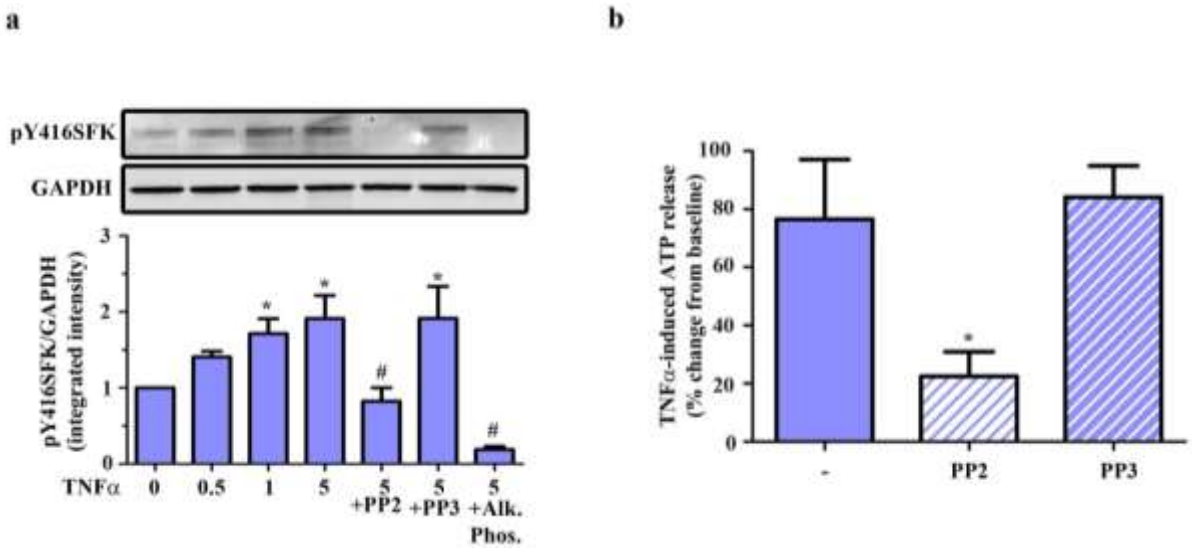

Figure 19: TNFα activates SFKs in HSaVECs inducing Panx1 channel activation.

a. Western blot analysis of SFK activation in HSaVEC in response to TNFα stimulation. A phospho-specific antibody against Y416 in SFKs (pY416SFK) was used as an indicator of SFK activation. SFK activation was blocked with the pharmacological antagonist PP2 but not by its inactive analog PP3. Antibody specificity for the phosphorylated form of the protein was confirmed by dephosphorylating proteins in cell lysates with alkaline phosphatase. **b.** TNFα-induced ATP release from HSaVEC following SFK inhibition with PP2.

We next sought to investigate the physiological contribution of Panx1 channel activation and ATP release in the venous circulation to acute vascular inflammation. Initially, using an *in vitro* leukocyte adhesion assay, we found that pharmacological inhibition of EC Panx1 channels with CBX and ^{10}panx1 significantly reduced adhesion of THP-1 monocytes to TNFα primed HUVEC **(Figure 20a-b)**. In agreement with a role for Panx1-dependent ATP release in this process, degradation of extracellular ATP with the ATP metabolizing enzyme Apyrase significantly reduced monocyte adhesion following TNFα stimulation. Finally, we utilized intravital microscopy of the exteriorized mouse cremaster muscle preparation to quantitatively assess the role of Panx1 channels in vascular inflammation *in vivo.* This technique allows real-time quantitation of endogenous leukocyte rolling flux and velocities, cell adhesion and emigration in post-capillary venules in response to inflammatory stress. Topical application of TNFα (50 ng/mL) to the cremaster muscle in WT C57Bl/6 mice significantly increased leukocyte interactions with the venous endothelium **(Figure 20c-d)**. Specifically, leukocyte adhesion increased ~4-fold after 90 minutes of TNFα exposure and leukocyte emigration into the inflamed muscle increased by ~3.5 fold after 120 minutes of TNFα stimulation. Genetic deletion of Panx1 specifically in the endothelium (VECadER^{T2}/Panx1$^{fl/fl}$ mice, tamoxifen injected) potently blunted leukocyte adhesion and emigration in response to TNFα as compared to peanut oil injected littermates, indicating a direct functional role for endothelial cell Panx1 channels in promoting leukocyte homing inflamed tissues.

In conclusion, we have dissected a novel signaling pathway linking TNFα signaling to the regulated release of ATP via Panx1 channels in the venous endothelium which

promotes leukocyte-EC interactions important for the acute inflammatory response **(Figure 21)**. We provide novel evidence for the involvement of SFKs in the activation of Panx1 channels downstream of inflammatory stimuli, suggesting a potential role for channel phosphorylation. Physiologically, Panx1 channels represent a previously unidentified target for regulating the purinergic input to inflammatory signaling through TNFα in the venous circulation and in the future may prove efficacious as a therapeutic target to dampen chronic inflammatory conditions.

Acknowledgements: Karen Stokes, Igor Leskov and Tara Stokes performed intravital microscopy experiments and subsequent data analysis. Artwork in Figure 21 was created by Anita Impagliazzo.

FIGURE 20. VENOUS EC PANX! CHANNELS PROMOTE VASCULAR INFLAMMATION.

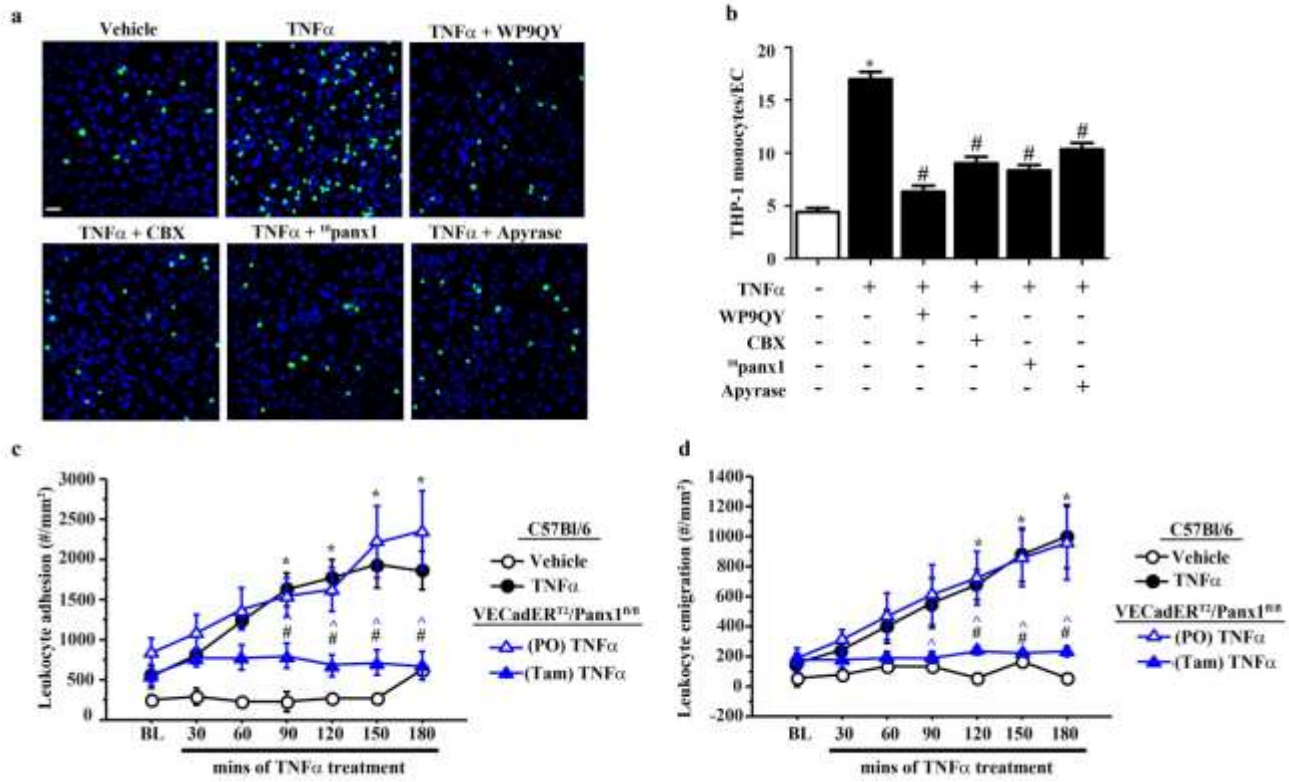

Figure 20: Venous EC Panx1 channels promote vascular inflammation. a. THP-1 monocyte adhesion assay on TNFα-stimulated HUVEC. HUVEC nuclei are stained with DAPI in blue and adherent calcein-AM loaded THP-1 monocytes are in green. **b.** Quantification of TNFα-induced monocyte adhesion from **a**. * indicates a significant increase from control conditions ($p<0.01$) and # indicates a significant difference as compared to TNFα treated cells in the absence of inhibitors. **c-d.** Quantification of endogenous leukocyte adhesion and emigration in WT mice and mice lacking Panx1 specifically in the endothelium ($VECadER^{T2}/Panx1^{fl/fl}$) during acute inflammation. Acute inflammation was induced by topically applying recombinant murine TNFα to exteriorized cremaster muscles in anesthetized mice. Tamoxifen (Tam)-injected mice display significantly decreased levels of Panx1 in the endothelium as compared to C57Bl/6 and peanut oil (PO)-injected controls. * indicates a significant increase from baseline ($p<0.01$), # indicates a significant difference as compared to C57Bl/6 mice treated with TNFα ($p<0.005$) and ^ indicates a significant difference as compared to PO injected $VECadER^{T2}/Panx1^{fl/fl}$ mice treated with TNFα.

FIGURE 21. MECHANISM OF TNFα-INDUCED ATP RELEASE FROM VENOUS ENDOTHELIAL CELLS DURING ACUTE VASCULAR INFLAMMATION.

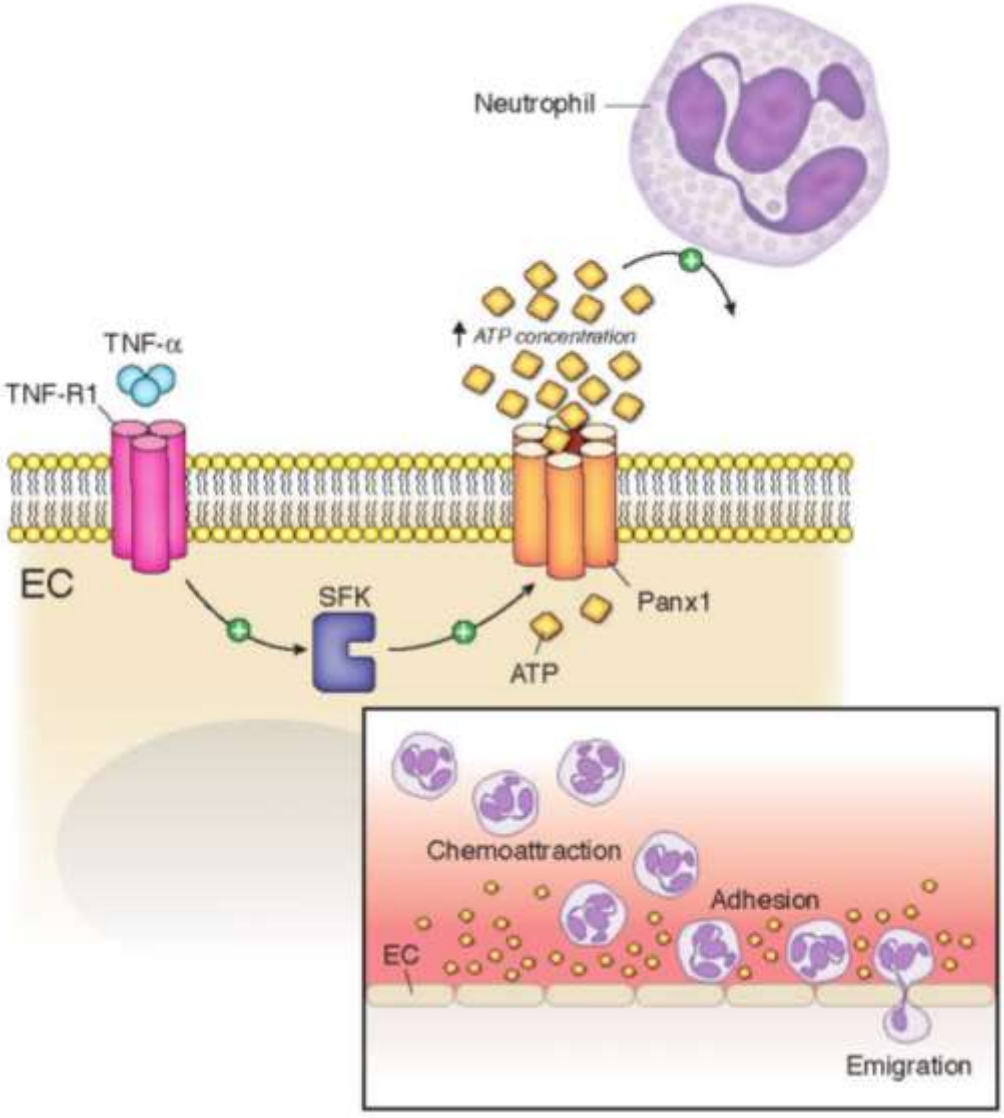

Figure 21. Mechanism of TNFα-induced ATP release from venous endothelial cells during acute vascular inflammation. In the acute inflammatory response, TNFα binds to the TNFR1 on venous endothelial cells (EC). This induces activation of Src Family Kinases (SFK) and downstream activation of Panx1 channels. Opening of Panx1 channels propels ATP release which promotes leukocyte interactions with the endothelium.

CHAPTER 6. GENERAL DISCUSSION AND FUTURE DIRECTIONS

General Discussion and Future Directions

In the vasculature, extracellular nucleotides signal in a variety of physiological and pathophysiological process, ranging from the maintenance of proper vascular tone to aberrant signaling in pathologies including atherosclerosis and inflammation. The mechanistic intricacies of purinergic signaling cascades in these states have been closely examined; however, until recently little attention has been focused on the mechanisms controlling purine nucleotide release from vascular cells. While several candidate pathways have been suggested to date, including vesicular release, transport via ABC transporters or channel-dependent release by connexin hemichannels and pannexin channels, the latter have received the most attention. These two protein families form hexameric membrane channels and harbor a similar membrane topology, but lack sequence homology. Importantly, connexin-based membrane channels readily dock with opposed hemichannels on neighboring cells, forming an intercellular gap junction channel. Pannexin channels lack this intrinsic ability and the reason for this difference has been debated [150]. Current evidence suggests that the high state of glycosylation on the extracellular domains of the Panx channels may hinder docking between opposed Panx channels. These observations have placed the primary function of Panx channels in regulating the exchange of intracellular and extracellular constituents, namely ATP. As the focus on Panx biology has increased over the past decade, we are beginning to uncover novel aspects of channel regulation and the translational importance of Panx signaling in both health and disease.

The work presented in this thesis has centered on the function and regulation of Panx1 channels in the vascular endothelium and the central role these channels play in

the acute vascular inflammatory response. Initially, we assessed the specific expression and localization profiles of the Panx isoforms (Panx1, Panx2 and Panx3) across the systemic arterial network [177]. Our analysis revealed a robust expression of the Panx1 isoform in all vessels studied, ranging from large conduit arteries (Aorta, carotid) to the small arterioles found in the spinotrapezius and cremaster muscles. This extensive characterization revealed homogenous expression of Panx1 in the endothelium regardless of blood vessel source or size, whereas Panx1 expression in the smooth muscle layer was only evident in the small arteries and arterioles. Our examination of more specialized vascular networks, including the lung, kidney and coronary circulations, indicated more heterogeneous expression patterns including differences in the isoform expressed and cell-type localization.

In order to assess the function of Panx1 channels in the vasculature, we utilized a multifaceted approach, employing both pharmacological and genetic manipulation of the channel. One current limitation in the study of Panx1 channel function lies at the level of pharmacological blockers. As mentioned previously, Panx channels and Cx hemichannels share a similar intrinsic membrane topology and are both thought to oligomerize into hexamers. Probably due to these similarities, many currently employed antagonists harbor overlapping pharmacology. Notably, the Panx1 blockers CBX and ^{10}panx1 have been reported to inhibit both Cx hemichannel and Panx channel function in a number of model systems [271]. To overcome this confounding issue, we performed a number of biochemical and genetic manipulations of Panx1 including RNAi-mediated knockdown and the generation of a novel, inducible Panx1 knockout mouse. In an initial attempt to examine the functional effect of EC Panx1 deletion, we created an EC-specific

Panx1 knockout mouse by crossing our $Panx1^{fl/fl}$ mice to mice harboring a Cre recombinase under the control of the Tie-2 promoter. Tie-2 has been used as an endothelial cell specific marker extensively [272-275]. Characterization of EC Panx1 expression by immunofluorescence microscopy revealed a minimal change in expression in knockout mice as compared to $Cre^{-}/Panx1^{WT}$ littermate controls **(Figure 22).** It is interesting to note that in a separate global Panx1 knockout mouse line, the germline deletion of Panx1 caused a compensatory increase in Panx3 expression in the vasculature **(Figure 23)**. These puzzling findings may point towards a compensatory mechanism by which Panx3 is up-regulated to fulfill the role of Panx1 in these cells. Comparison of the sequences between the three Panx isoforms has also indicated that Panx1 and Panx3 have higher sequence homology than Panx2 [152]. In addition, Panx3 channels have been reported to release ATP, placing these channels as possible candidates to replace Panx1 function [276]. In this sense, it is also interesting to speculate the involvement of Panx3 channels in purinergic regulation of vascular function in the heart, lung and kidney, tissues where Panx3 is co-expressed with Panx1 in the vascular endothelium (see **Figure 5,7 and 8**, respectively). Due to the incomplete deletion using the Tie-2 mouse model, we turned to an inducible system in which we could spatially and temporally modulate the levels of Panx1 to reduce the effect of compensation. To accomplish this task, we crossed our $Panx1^{fl/fl}$ mice with mice harboring a tamoxifen-inducible Cre recombinase under the control of the vascular endothelial cadherin promoter ($VECadCre^{+}$). Following 10 days of intraperitoneal injection of tamoxifen, mice expressing our inducible Cre showed almost complete loss of Panx1 in the endothelial cell layer (see **Figure 15h).**

Interestingly, endothelial cell deletion of Panx1 dramatically reduced leukocyte-EC interactions as assessed by intravital microscopy.

Based on our finding that Panx1 channels integrate inflammatory and purinergic signaling in vascular inflammation in a venous endothelial cell-specific manner, the robust expression of Panx1 in the endothelium across the systemic arterial vasculature may suggest alternative functions for this channel in the arterial circulation. For example, Panx1 channels are mechanosensitive and shear-stress induced by changes in blood flow has been shown to induce ATP release from endothelial cells [9,19,154]. Panx1 channels may therefore constitute a mechanism for flow-induced vasodilation, with mechanical activation inducing ATP release and activation of EC P2Y receptors for subsequent NO production.

Alternatively, Panx1 channels may remain in a preferentially closed state in the arterial endothelium and become activated under more chronic inflammatory conditions. In support, an active role for vessel wall purinergic receptors has been ascribed during atherosclerosis, a chronic inflammatory disease involving endothelial dysfunction [277-280]. Future efforts will be aimed towards evaluating the role for EC Panx1 channels in the arterial endothelium in promoting monocyte and T-cell infiltration and atherosclerotic plaque development. To this end we are currently crossing our inducible, endothelial cell specific Panx1 knockout mice to an $ApoE^{-/-}$ background to examine the effect of EC Panx1 deletion in a murine model of atherosclerotic plaque development. These mice will be valuable tools for assessing the *in vivo* role of Panx1 channels on the development and progression of atherosclerosis. We hypothesize that deletion of Panx1 from the

arterial endothelium will decrease atherosclerotic plaque burden by reducing the inflammatory component of the disease.

FIGURE 22. PANX1 EXPRESSION IN Tie2Cre^{+}/Panx1$^{fl/fl}$ MICE

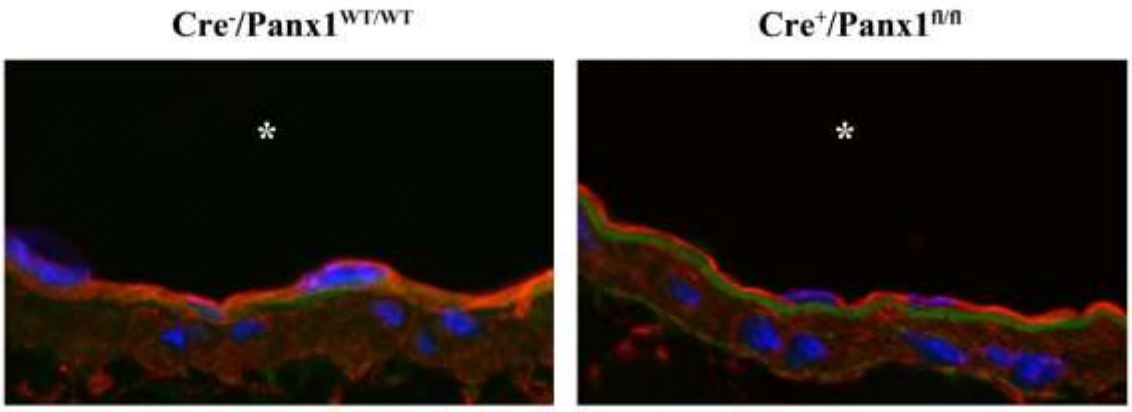

Figure 22. Panx1 expression in Tie2Cre^{+}/Panx1$^{fl/fl}$ mice. Immunofluorescence micrographs of isolated thoracodorsal arteries from Tie2Cre^{+}/Panx1$^{fl/fl}$ mice stained with and antibody against the murine Panx1 C-tail (red). Autofluorescence of the IEL is shown in green and the nuclei are stained with DAPI. * indicate the blood vessel lumen.

FIGURE 23. PANX3 IS UPREGULATED IN THE ARTERIAL WALL OF A GLOBAL PANX1 KNOCKOUT MOUSE.

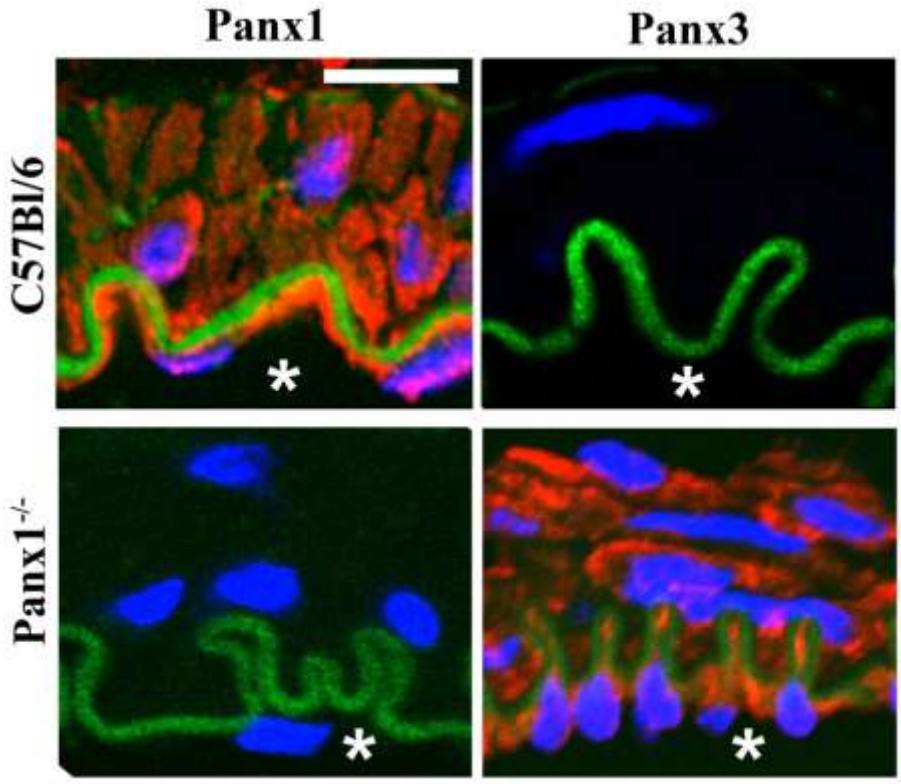

Figure 23. Panx3 is upregulated in the arterial wall of a global Panx1 knockout mouse. Immunocytochemical analysis of Panx1 and Panx3 expression in isolated mouse thoracodorsal arteries (TDA). In wild type C57Bl/6 animals (top panels) Panx1 is abundantly expressed in the endothelial and smooth muscle cells, with minimal Panx3 expression detected. Parallel analysis of Panx3 expression in isolated TDA from a global Panx1 knockout mouse ($Panx1^{-/-}$) revealed a dramatic upregulation of Panx3 when Panx1 was genetically ablated (lower panels). * indicate the blood vessel lumen. Nuclei are stained with DAPI (blue) and autofluorescence of the internal elastic lamina is in green. Scale bar = 20 μm. Immunolabeling was performed by Brant Isakson.

Diving into the molecular aspects of Panx1 function in vascular inflammation, we identified a central role for channel activation and ATP release specifically from venous endothelial cells. In these studies, cellular release of ATP was assayed by measuring the accumulation of extracellular ATP in the media surrounding cultured ECs or the perfusate from cannulated veins using conventional luciferin:luciferase bioluminescence. These approaches harbor a few limitations. First, assessing ATP concentration in this manner results in dilution of ATP as it exits and diffuses from the cell. In this regard, little information is gained on the local concentration of ATP that accumulates at the extracellular side of the cell membrane. The pro-inflammatory effects of ATP in our *in vitro* and *in vivo* analyses point towards a probable role for the purine in activating endothelial cell purinergic receptors or leukocyte purinergic receptors following the induction of rolling. This is evident by the lack of an effect of EC Panx1 deletion on leukocyte rolling dynamics, but a prominent reduction in adhesion and emigration downstream of rolling. While our assessment of ATP release revealed the activation of Panx1 channels downstream of TNFα-induced activation, future studies will be aimed at identifying the downstream target of released ATP both at the cell type and purinergic receptor subtype level. Second, we utilized 2nd order mesenteric veins to measure ATP release. These vessels mimicked the results obtained in our vascular EC cultures; however, much of the interactions between leukocytes and ECs occur in the proximal post-capillary venules. Due to the relatively small size of these venules and the sensitivity of conventional luciferin:luciferase bioluminescence, it is not feasible to measure ATP release. Nonetheless, the functional impact of Panx1 deletion was evident in the small post-capillary venules in the cremaster preparation, supporting our

observations made in the mesenteric circulation. To overcome some of these issues, future efforts to increase both temporal and spatial resolution of ATP release, particularly at microdomains within and outside of the cell, will aid in our understanding of purinergic dynamics in inflammation. Currently, small microelectrode ATP biosensors are beginning to be employed to measure local ATP concentrations in restricted spaces [281]. Additionally, it remains unclear as to whether other purine nucleotides are released via Panx1 channels during this process. Luciferin:luciferase-based bioluminescence is selective for ATP quantification and is insensitive to other purine nucleotides including ADP, AMP, and the uridine nucleotides UTP and UDP. A role for uridine nucleotides in phagocytosis and platelet aggregation has been observed, suggesting their potential involvement in the vascular inflammatory response [78].

Our observation that venous, and not arterial, ECs release ATP downstream of activation by TNFα is in agreement with previous reports identifying the venous circulation as the primary location for leukocyte extravasation during inflammatory stress. However, upon examination of Panx1 expression between ECs of venous and arterial origin, there were no apparent differences in channel expression or localization to the plasma membrane **(Figure 24b)** and the intracellular ATP content did not differ between cell types **(Figure 24a)**. In addition, the expression of TNFR1 was not different between cell types, indicating that the heterogeneity observed in ATP release was not due to differential expression of the receptor or channel **(Figure 24b)**.

FIGURE 24. COMPARISON OF VENOUS AND ARTERIAL ENDOTHELIAL CELL PANX1 AND TNFR1 EXPRESSION, AND ATP CONTENT.

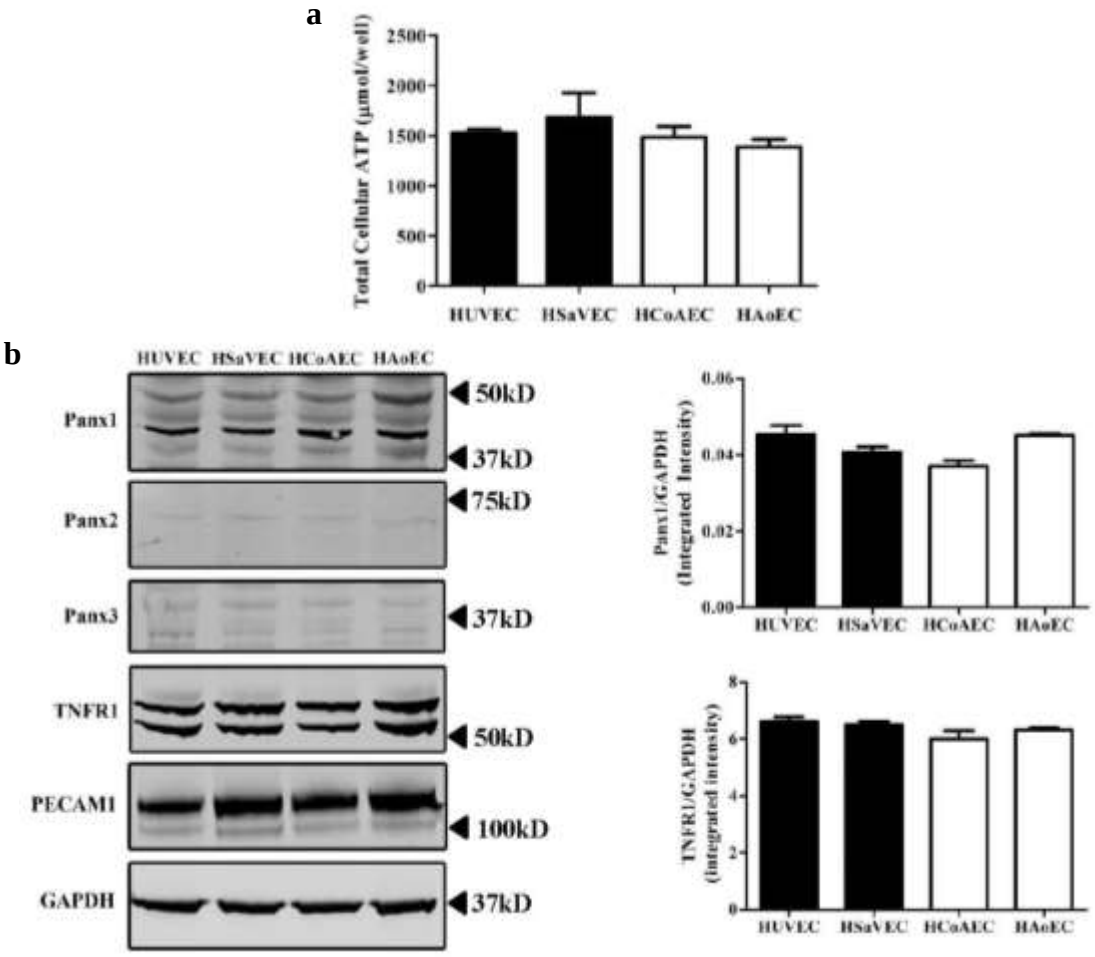

Figure 24. Comparison of venous and arterial endothelial cell Panx1 and TNFR1 expression, and ATP content. a. Total cellular ATP content was analyzed across both venous (HUVEC, HSaVEC) and arterial (HAoEC, HCoAEC) cell types by luciferin:luciferase bioluminescence. **b.** Western blot anlaysis of total cellular expression of Panx isoforms and TNFR1. Protein expression was normalized to GAPDH.

In an attempt to identify the mechanistic difference between arterial and venous endothelial cells in this signaling pathway, we performed gene microarray analyses on each of our four EC types (in triplicate from independent donors). **Table 2** shows the most differentially expressed genes between our arterial and venous endothelial cell types. The majority of differentially expressed genes were more highly expressed in the venous ECs (19 of 25 genes). One of the most differentially expressed genes was *Cldn11*. This gene encodes claudin-11, a tight-junction protein implicated in determining permeability between myelin sheaths in the central nervous system. Further biochemical analysis revealed this protein to be a specific marker for venous endothelium, which may provide a useful tool for distinguishing between the two **(Figure 25)**. A pathway analysis was performed and one of the most perturbed pathways between cell types based on differential gene expression is the ECM-receptor and focal adhesion pathway **(Figure 26)**. In this flow diagram, red highlighted genes are more highly expressed in venous as compared to arterial ECs, or the pathway is perturbed in the arterial ECs comparatively. Of interesting note, there is a significant difference in the expression of genes regulating extracellular matrix-receptor interactions and cytokine signaling. Both of these pathways converge to regulate a number of downstream targets, one being Src. As our analysis of SFK activation between arterial and venous endothelial cells revealed the activation of the kinases in both cell types downstream of TNFα stimulation, it is unclear whether different SFK members are activated in each cell type. Our analysis took advantage of an antibody that detects phosphorylation of an activating residue which is conserved between all SFK members. This approach limited our ability to identify the specific SFK member regulating Panx1 channel function. Since these different family members can

selectively target downstream proteins, the difference in Panx1 activation between arterial and venous ECs may lie in the specific SFK member activated in each respective cell type. Currently, there are few selective inhibitors for the individual SFK members so future differentiation between these enzymes may rely on genetically manipulating each gene. In addition, many of these perturbed pathways converge at the level of actin cytoskeleton dynamics. Previous reports have indicated reciprocal interactions between the actin cytoskeleton and Panx1 channels, affecting the localization and stabilization of the channel at the plasma membrane, and the overall function of the channel. Future studies will be aimed at targeting these differential genes in arterial endothelial cells to determine their effect on inflammatory phenotypic switching. It will be interesting to assess our microarray datasets alongside gene expression data from arterial ECs in diseased arteries to determine whether these cells adopt a "venous-like" phenotype.

TABLE 2: DIFFERENTIALLY EXPRESSED GENES BETWEEN ARTERIAL AND VENOUS ENDOTHELIAL CELLS

Gene	Gene Name	adj. P-value
family with sequence similarity 174, member B	FAM174B	2.0E-06
claudin 11	CLDN11	0.000270
laminin, alpha 2	LAMA2	0.000853
forkhead box P2	FOXP2	0.006649
DnaJ (Hsp40) homolog, subfamily C, member 12	DNAJC12	0.006649
hydroxysteroid (17-beta) dehydrogenase 2	HSD17B2	0.006649
vav 3 guanine nucleotide exchange factor	VAV3	0.006649
kelch-like family member 4	KLHL4	0.006649
placenta-specific 8	PLAC8	0.007310
POU class 4 homeobox 1	POU4F1	0.008930
aldehyde dehydrogenase 1 family, member A2	ALDH1A2	0.008930
alkylglycerol monooxygenase	AGMO	0.014918
glycophorin C (Gerbich blood group)	GYPC	0.019754
neuronatin	NNAT	0.021968
G protein-coupled receptor 65	GPR65	0.021968
bone morphogenetic protein receptor, type IB	BMPR1B	0.023971
solute carrier organic anion transporter family, member 3A1	SLCO3A1	0.023971
endothelin receptor type B	EDNRB	0.023971
blood vessel epicardial substance	BVES	0.023971
growth regulation by estrogen in breast cancer-like	GREB1L	0.027615
neuregulin 3	NRG3	0.029613
mannose receptor, C type 2	MRC2	0.029613
sema domain, immunoglobulin domain (Ig), (semaphorin) 3A	SEMA3A	0.037274
tumor-associated calcium signal transducer 2	TACSTD2	0.037274
SH3 domain containing ring finger 2	SH3RF2	0.047863

Table 2. Differentially expressed genes between arterial and venous endothelial cells. Gene microarray analysis of arterial (HCoAEC, HAoEC) and venous (HUVEC, HSaVEC) endothelial cells. The results above show the most significant differentially expressed genes between cell types. Genes highlighted in blue are more highly expressed in venous as compared to arterial ECs and genes highlighted in red are more highly

expressed in arterial as compared to venous ECs. Significance is indicated by an adjusted p-value <0.05. Yongde Bao and the UVA microarray core facility performed Affymetrix microarray experiments and Stephen Turner performed data analysis.

FIGURE 25. CLAUDIN-11 IS A VENOUS ENDOTHELIAL CELL SPECIFIC PROTEIN

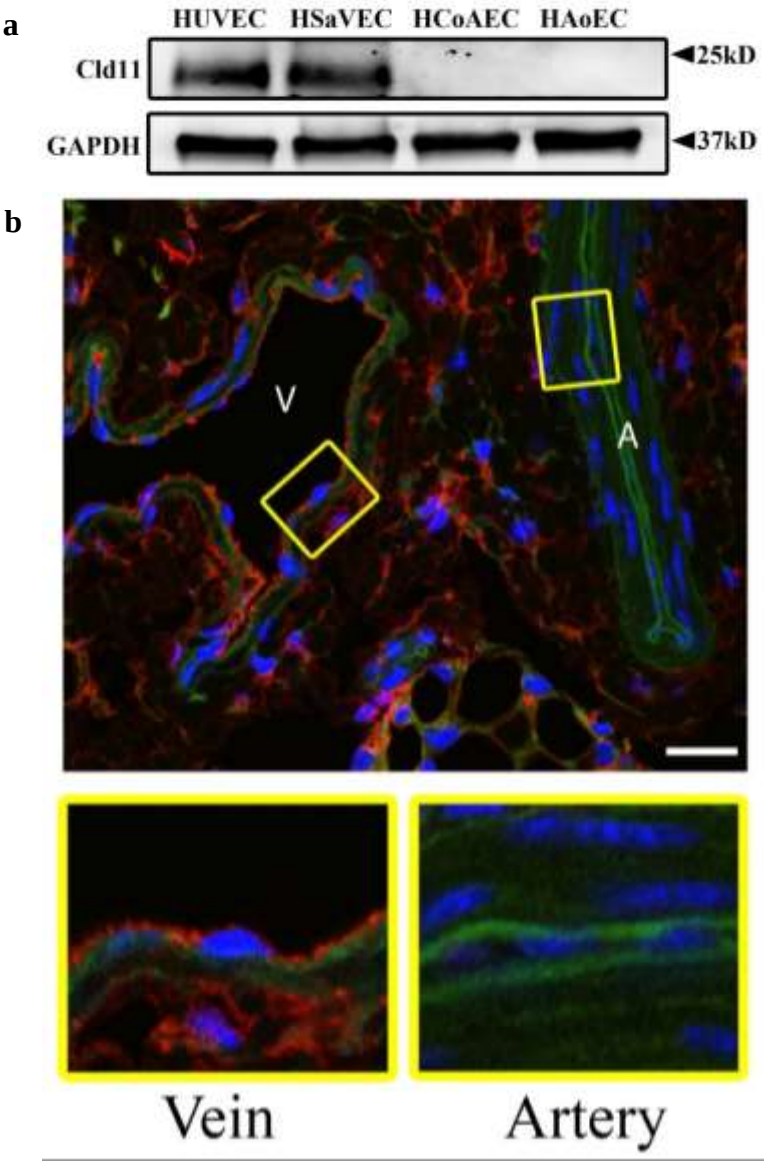

Figure 25. Claudin-11 is a venous endothelial cell specific protein. a. Western blot of human venous and arterial endothelial cell types for Claudin-11 (Cld11). **b.** Immunofluorescence micrographs showing Cld11 staining (red) in venous (V) but not arterial (A) ECs. Zoomed images of V and A are shown at bottom.

FIGURE 26. THE FOCAL ADHESION PATHWAY

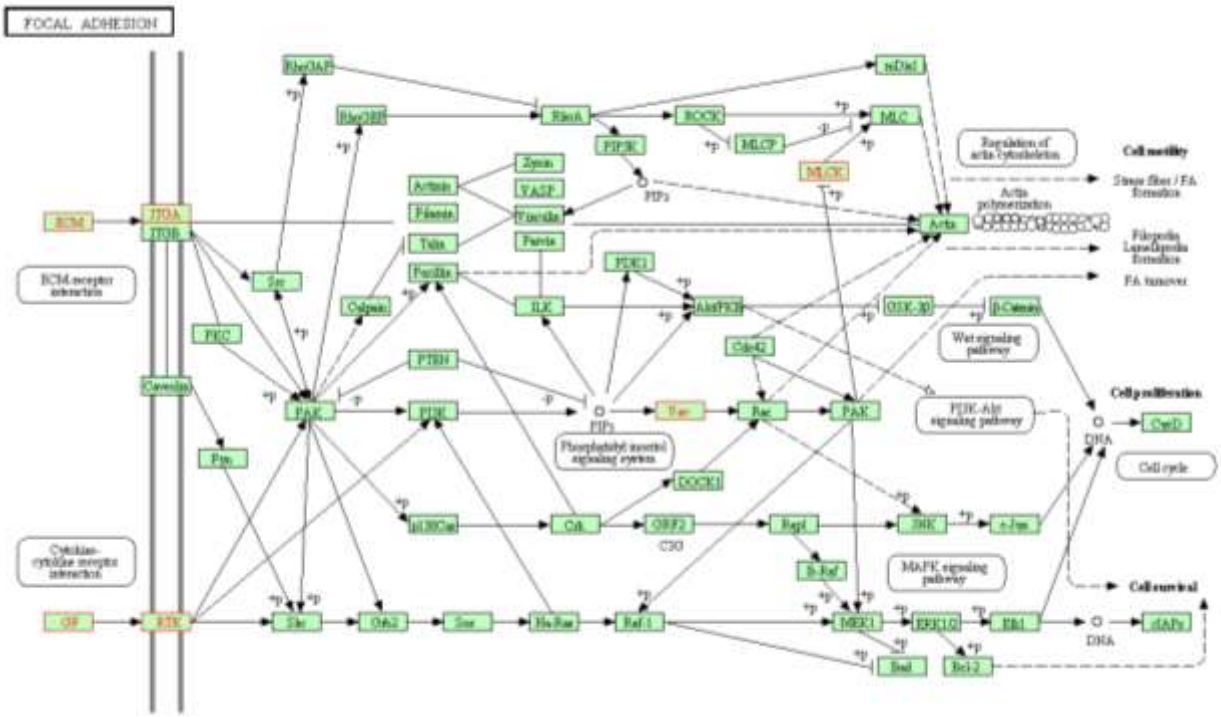

Figure 26. The Focal Adhesion Pathway. Pathway analysis from human endothelial cell microarray datasets showing perturbed genes in the focal adhesion cascade in arterial as compared to venous ECs. Stephen Turner performed pathway analysis.

Following activation of TNFR1, SFKs were activated in both venous **(Figure 18/19)** and arterial (data not shown) endothelial cells as assessed by the phosphorylation of Y416 in SFKs. In venous ECs SFK participation in the activation of Panx1 channels was evident by the potent inhibition in ATP release by the SFK inhibitor PP2. These data led us to examine potential tyrosine residues in the Panx1 channel that may be targeted for phosphorylation. Several post-translational modifications have been reported for Panx1, including N-linked glycosylation [152], S-nitrosylation [217], caspase cleavage of its C-terminal tail [78,282] and potentially phosphorylation. In regard to channel phosphorylation, several studies have implicated kinases belonging to the Src and Rho families in Panx1 channel activation, but to date it is unclear whether Panx1 is a direct target for these kinases [164,250]. Based on these observations we utilized a phosphorylation prediction program (PhosphoSitePlus) to identify potential tyrosine residues in Panx1 for SFK-mediated phosphorylation. Our analysis identified Y198 in the intracellular loop region of Panx1 as a prominent candidate for tyrosine phosphorylation and we generated a custom phospho-specific antibody against Y198 of Panx1 to determine if this site is a target of SFKs in TNFα-stimulated venous ECs. Preliminary results in **Figure 27a-c** suggest that Panx1 may be ultimately phosphorylated downstream of TNFR1 and SFK activation in the venous endothelium. This effect was reduced in the presence of PP2 and the specificity of our antibody for phosphorylation was confirmed by treating protein samples with alkaline phosphatase. Currently, this antibody is being validated against mutant Panx1 constructs that cannot be phosphorylated at Y198. Future studies will aid in discriminating the ability of Panx1 to become phosphorylated in venous and arterial endothelial cells.

FIGURE 27. ANALYSIS OF EC PANX1 PHOSPHORYLATION AT Y198 IN RESPONSE TO TNFα STIMULATION

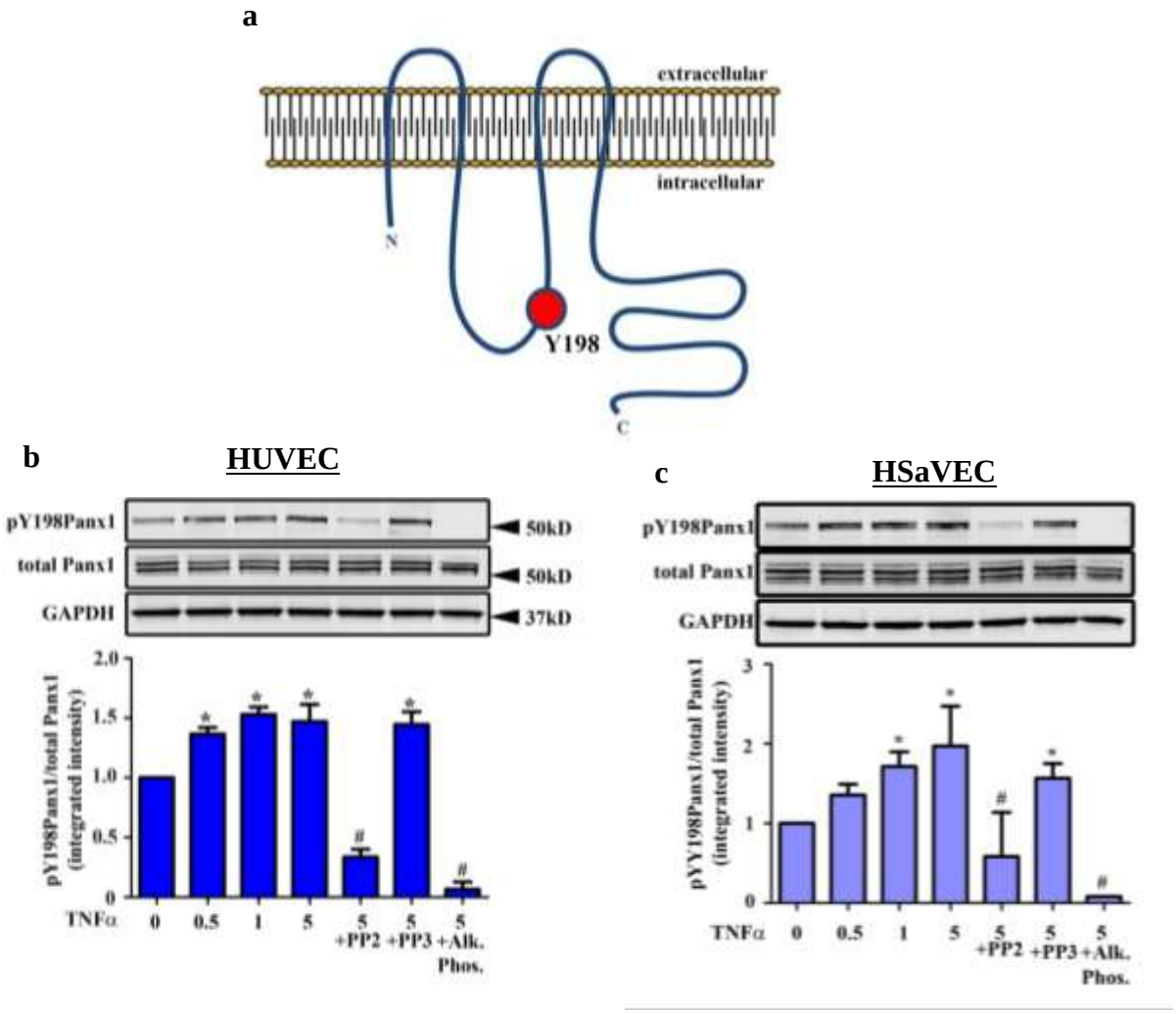

Figure 27. Analysis of EC Panx1 phosphorylation at Y198 in response to TNFα stimulation. a. Topological schematic of Panx1 indicating a predicted tyrosine (Y) residue for phosphorylation by SFKs. Y198 resides in the intracellular loop region of the Panx1 protein. **b.** Western blot analysis of Panx1 phosphorylation at Y198 in HUVEC in response to TNFα stimulation. A custom phospho-specific antibody directed against

Y198 in Panx1 (pY198Panx1) was generated to assess the targeted phosphorylation of this residue. **c.** Western blot analysis of Panx1 phosphorylation at Y198 in HSaVEC in response to TNFα stimulation.

Another potential discrepancy between arterial and venous endothelial responses to TNFα lies in the bioavailability of the nitric oxide (NO). Our recent data has revealed the potential for Panx1 channels to become S-nitrosylated by NO [204]. Functionally, S-nitrosylation of intracellular cysteines 40 and 346 imparts negative regulation on the channel, preventing ATP release and channel currents. Across the vascular endothelium, the basal expression and activity of endothelial nitric oxide synthase (eNOS) varies with the steady-state activity of eNOS and basal NO bioavailability being higher in the arterial endothelium as compared to the venous circulation. This raises the possibility that under normal homeostatic conditions, Panx1 is more highly S-nitrosylated in arterial ECs as compared to venous ECs, increasing the threshold for channel activation and subsequent ATP release. In support of this notion, multiple lines of evidence have defined NO as an important anti-inflammatory mediator, inhibiting neutrophil interactions with the endothelium and maintaining proper barrier function [283-287]. Moreover, in our experiments, the addition of the NO donor GSNO to cultured venous ECs reduces TNFα-mediated ATP release indicating the ability of NO to negatively regulate channel activation by the cytokine **(Figure 28a)**. While inhibiting eNOS activity in these cells with L-NAME did not significant increase ATP release in response to TNFα, we observed a trend in that direction. Finally, in our static adhesion model, application of GSNO reduced leukocyte-EC interactions, while L-NAME increased adhesion **(Figure 28b)**. Taken together, our results suggest that NO can modulate the extent of Panx1 channel activity during the acute inflammatory response, potentially providing a mechanism for arterio-venous heterogeneity in the response.

The ability of NO to post-translationally modify pannexins is not limited to the Panx1 isoform. Recently, we reported the ability of GSNO to induce S-nitrosylation of the Panx3 isoform **(Figure 29)** [288]. Future studies will be instrumental in determining the specific functional impact of S-nitrosylation of Panx3 and the physiological context in which this modification may be relevant. Panx3 has been implicated in a number of cellular processes including its involvement in the regulation of keratinocyte and chondrocyte proliferation and differentiation, participation in Ca^{2+} leak from the endoplasmic reticulum (ER) and the promotion of bone development [167,223,276,289]. The participation of NO signaling events in a number of these processes has also been well documented. For example, NO promotes bone and cartilage development by influencing osteoblast and chondrocyte differentiation, processes that are influenced by activation of Panx3 channels at the plasma membrane and ER [290,291]. In this context, targeted S-nitrosylation of Panx3 in these cells may serve as an important regulatory element promoting cell growth and differentiation. Nitric oxide also promotes both the proliferation, at low concentrations, and differentiation, at high concentrations, of keratinocytes [292,293]. In these cells, Panx1 and Panx3 act to suppress proliferation and augment differentiation [167,294]. Concurrently, the ability of Panx1 and Panx3 to be modified by S-nitrosylation may serve a role in regulating the activity or localization of these pannexins during skin development and wound repair. These provocative observations may place the pannexin family at the heart of a number of NO-mediated responses, providing novel targets for clinical intervention.

In conclusion, the pannexin family of proteins plays important roles in vascular homeostasis and analysis of these channels in the vasculature has revealed novel information on their post-translational regulation. The ability of Panx1 channels to promote inflammatory signaling in the vascular endothelium may provide a novel target for clinical intervention, particularly in the context of chronic inflammatory diseases where these channels may become upregulated or overactive.

FIGURE 28. GSNO INHIBITS AND L-NAME PROMOTES TNFα-INDUCED ATP RELEASE AND LEUKOCYTE-EC INTERACTIONS *IN VITRO*.

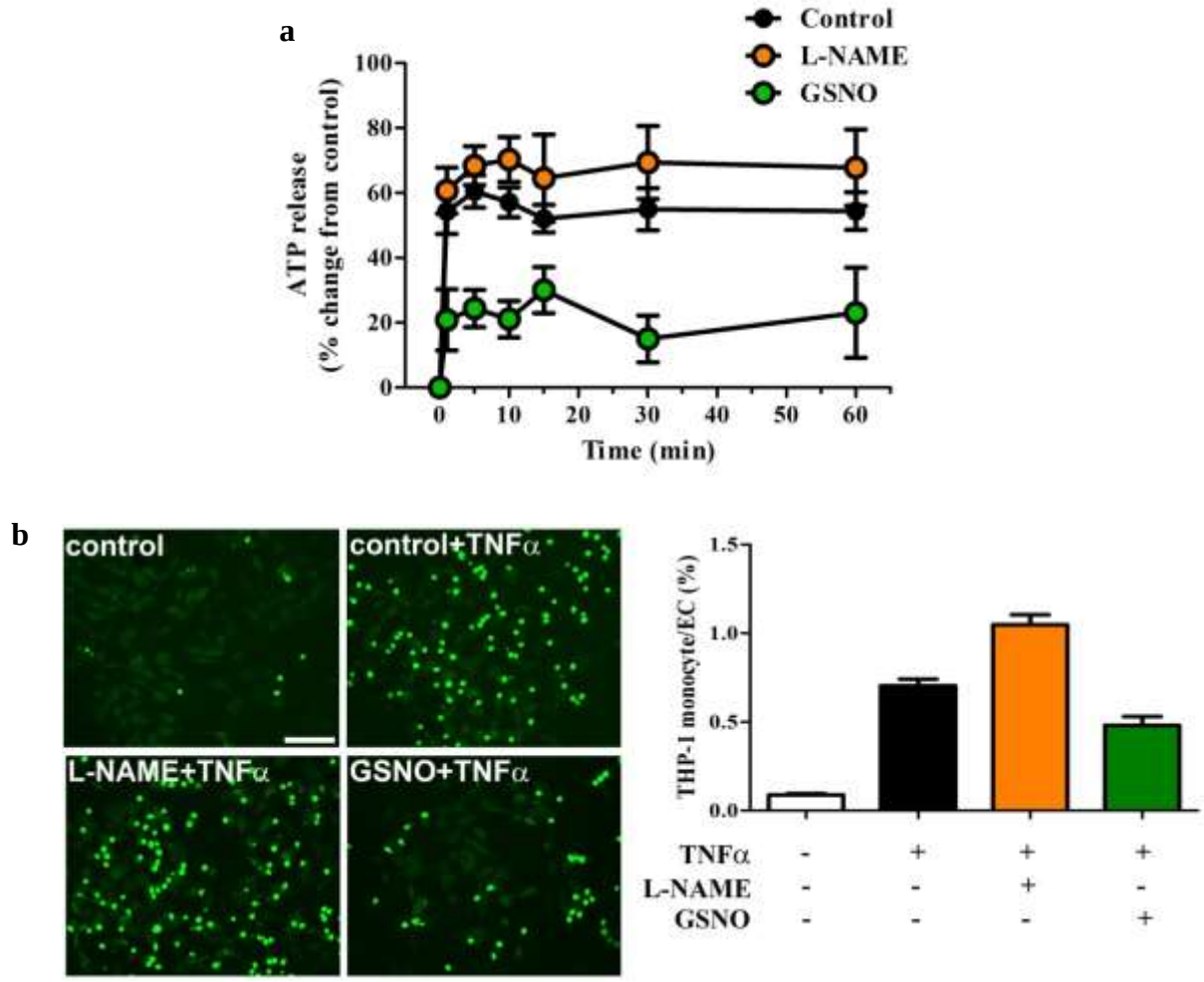

Figure 28. GSNO inhibits and L-NAME promotes TNFα-induced ATP release and leukocyte-EC interactions. a. Effect of S-nitrosoglutathione (GSNO; NO donor) and L-NAME (NOS inhibitor) on TNFα-induced ATP release from cultured HUVEC. **b.** *in vitro* static adhesion of calcein-AM loaded THP-1 monocytes to TNFα-stimulated HUVEC monolayers. Cells were treated with GSNO or L-NAME prior to TNFα-stimulation. Adam Straub performed adhesion assays.

FIGURE 29. PANX1 AND PANX3 CAN BE POST-TRANSLATIONALLY MODIFIED BY S-NITROSYLATION.

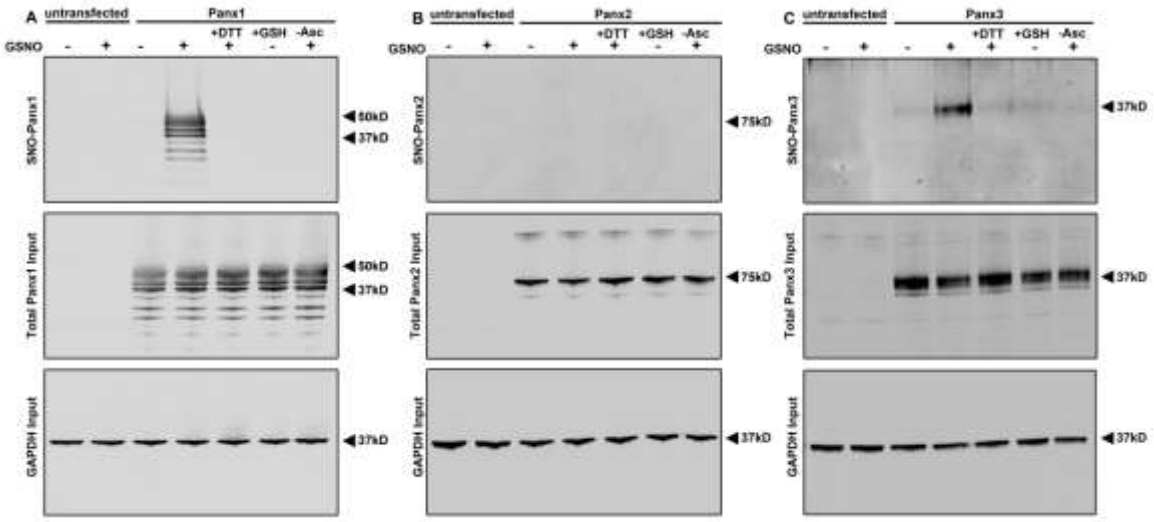

Figure 29. Panx1 and Panx3 can be post-translationally modified by S-nitrosylation. Biotin switch assay on Panx1 **(A)**, Panx2 **(B)** and Panx3 **(C)** expressing HEK293T cells treated with the nitric oxide donor S-nitrosoglutathione (GSNO). Treatment with GSNO induced S-nitrosylation of Panx1 and Panx2, but not Panx2, which was reversible by reduction of S-nitrosothiols with the reducing agent DTT. There was no detectable modification of any Panx isoforms by treatment with the reduced form of GSNO, L-glutathione (GSH), indicating that the effects of GSNO were due to S-nitrosylation and not S-glutathionation. Ascorbate (Asc) was omitted from the biotin switch assay as a negative control.

CHAPTER 7. REFERENCES

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