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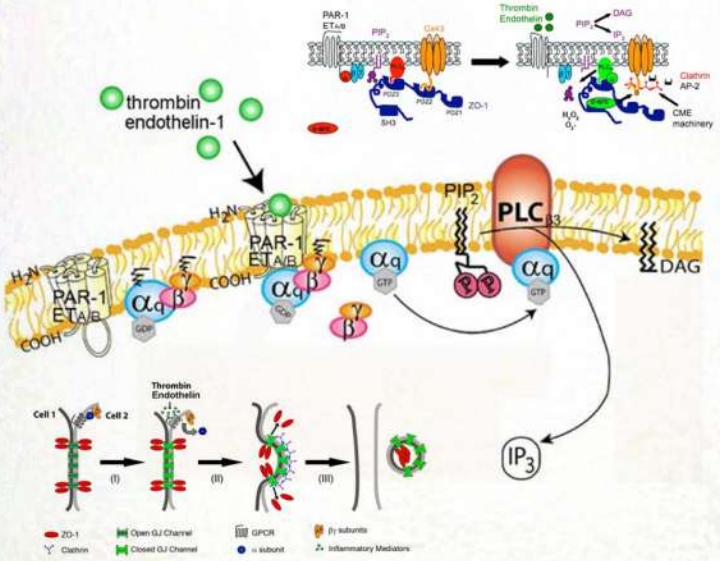
Natural Inflammatory Mediators Thrombin and Endothelin Modulate Gap Junction Intercellular Communication and Cell-Cell Adhesion



Susan M. Baker • Matthias M. Falk

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PREFACE

The vascular endothelium provides a barrier that regulates the flow of proteins and other blood-borne solutes between the vasculature and surrounding tissues. During inflammation, this barrier can become compromised by circulating inflammatory mediators, such as thrombin and endothelin. In vascular endothelial cells, regulated surface expression of gap junction (GJ) channels and proper modulation of gap junctional intercellular communication (GJIC) are required for blood vessels to maintain normal function. Inflammatory mediators such as thrombin and endothelin can elicit inhibition of GJIC and increased permeability of vascular endothelial cells, causing blood vessels to become "leaky." In severe instances, patients may develop life-threatening conditions such as pulmonary edema and acute respiratory distress syndrome (ARDS) in response to these inflammatory mediators. The molecular mechanisms for this down-regulation of GJIC and loss of cellular adhesion are poorly understood, and, to date, there are no effective drug therapies for such conditions. Combining high-resolution fluorescence microscopy and functional assays, we investigated the mechanism of GJIC inhibition and the loss of cell-to-cell adhesion in primary vascular endothelial cells in response to thrombin and endothelin. Activation of endothelial receptors PAR-1 and $ET_{A/B}$ by their natural inflammatory mediator agonists, thrombin and endothelin-1, respectively, or by mastoparan, a constitutive agonist of G-protein coupled receptors (GPCRs), resulted in a rapid and acute internalization of GJs that coincided with the inhibition of GJIC followed by increased vascular endothelial cell permeability. The process is mediated by the endocytic coat protein, clathrin, and the scaffold protein, ZO-1, and

specifically requires binding of connexin 43 (Cx43) to the PDZ2 domain of ZO-1 apparently only on one side of the internalizing GJ plaque. Disruption of Cx43/ZO-1 interaction effectively abolishes GJ internalization in response to thrombin and endothelin, suggesting a novel role for ZO-1 in the internalization process. Moreover, inhibition of GJIC, internalization of GJs and increased vascular endothelial cell permeability were all prevented with the G-protein antagonist, suramin. These findings demonstrate that GJ channel internalization is an efficient mechanism for modulating GJIC and facilitating physical cell-cell uncoupling in vascular endothelium in response to thrombin and endothelin. Additionally, the results presented here identify the PDZ2 domain of ZO-1 as a potential therapeutic target to prevent GJ internalization in vascular endothelial cells during inflammatory response. Overall, we have identified a novel role for the natural inflammatory mediators, thrombin and endothelin, in the regulation of GJIC and physical cell-to-cell adhesion.

Chapter 1

GAP JUNCTIONS (GJS): STRUCTURE AND FUNCTION

Gap junction-mediated intercellular communication (GJIC) is a highly regulated cellular activity that plays important roles in numerous cellular functions. Regulation of GJ channels is required for cells to properly modulate GJIC and physical cell-to-cell adhesion. GJ channels form continuous, aqueous pores approximately 1.5 nm in diameter that allow for the free diffusion of small hydrophilic molecules (less than 1 kDa) between cytoplasms of adjacent cells. GJs assemble from four-pass transmembrane proteins, called connexins (Cxs). Connexins consist of four highly conserved transmembrane domains as well as one intracellular and two extracellular loops; both the N- and C-termini are cytoplasmically located. To date, 21 different Cx isoforms have been identified in humans, and the C-terminus of most Cx isoforms contain numerous motifs that are the sites of a variety of protein interactions.

Following biosynthesis of connexins in the endoplasmic reticulum, connexin subunits oligomerize into hexamers to form a connexon, one-half of a gap junction channel, that then are delivered, packaged in vesicles, to the plasma membrane. Two connexons, one from each adjacent cell, dock in the extracellular space between cells to form a double-membrane spanning continuous channel that directly connects the cytoplasms of apposed cells. Once formed, gap junction channels aggregate into plaques that contain potentially thousands of channels and that can range in size from less than a hundred square nanometers to many square micrometers.

Perhaps the most ubiquitously expressed of all Cx isotypes is connexin 43 (Cx43). The Cx43 C-terminal region has been extensively characterized. Numerous studies have revealed that, within the Cx43 C-terminus are binding sites for a wide range of proteins, including: cytoskeletal proteins such as tubulin (Giepmans *et al.*, 2001); adherens- and tight junction-associated proteins such as cadherin (Xu *et al.*, 2001) and ZO-1 (Giepmans and Moolenaar, 1998; Toyofuku *et al.*, 1998); and multiple kinases such as MAPK, c-Src, protein kinase C (PKC) and cdc2 (Loo *et al.*, 1995; Warn-Cramer *et al.*, 1996; Lampe *et al.*, 1998; Duffy *et al.*, 2001). These revelations strongly suggest that, in addition to their role in intercellular communication, Cxs also play essential roles in more common signaling and cellular adhesion events.

Chapter 2

GJS AT THE VASCULAR ENDOTHELIUM

The vascular endothelium provides a barrier that regulates the flow of proteins and other blood-borne solutes between the circulation and surrounding tissues. Vascular endothelial cells maintain tight associations through ubiquitous expression of numerous Cx43-based gap junctional complexes. During inflammation, this barrier can be compromised by circulating inflammatory mediators, such as thrombin and endothelin-1 (Lum and Malik, 1994; Baldwin and Thurston, 2001). In vascular endothelial cells, activation of the thrombin receptor PAR-1 and the endothelin-1 receptors ET_A and ET_B can lead to decreased GJIC, loss of cellular adhesion and increased vascular endothelial cell permeability, causing blood vessels to become "leaky" (Goligorsky *et al.*, 1999; Baldwin and Thurston, 2001; Bogatcheva *et al.*, 2002; Kawamura *et al.*, 2002; Spinella *et al.*, 2003; Ahmmed and Malik, 2005; D'Hondt *et al.*, 2007; van Zeijl *et al.*, 2007). In severe instances, patients may develop life-threatening conditions such as pulmonary edema and acute respiratory distress syndrome (ARDS) in response to these inflammatory mediators. In fact, in cases of acute lung injury (ALI) or acute respiratory distress syndrome, elevated levels of endothelin-1 reportedly are associated with increased vascular permeability, leading to pulmonary edema and sometimes even death (Nakano *et al.*, 2007). It has been suggested that vascular permeability proceeds through a mechanism by which cellular junctions are removed and vascular endothelial cells retract from one another through either passive recoil or active contraction (Baldwin and Thurston, 2001). In either case, in order for

cells to physically separate from one another, all cell-cell junctions, including GJs, must be removed.

Chapter 3

REGULATION OF GJIC AND VASCULAR PERMEABILITY

The molecular mechanisms underlying the regulation of GJIC and removal of GJs from the plasma membrane in response to thrombin and endothelin-1 has been the focus of our recent work. Understanding how the vascular endothelium responds to these inflammatory mediators may not only identify potential targets for drug therapies, but will also provide important insights into the function of GJ channels and the dynamic regulation of GJIC and cell-to-cell adhesion.

Connexins have a surprisingly short half-life of only 1.5 - 5 hours, resulting in a rapid GJ and Cx protein turnover. It is well established that, once formed, GJ channels cannot be separated under physiological conditions (Goodenough and Gilula, 1974). Rather, removal of GJ plaques from the plasma membrane requires that GJ channels be endocytosed into one of a pair of neighboring cells. The process results in the formation of unique, double-membrane vesicular structures within the cellular cytoplasm that were termed annular gap junctions (AGJs) or connexosomes (Piehl, *et al.* 2007).

In 2007, Van Zeijl *et al.* reported a rapid (within minutes) inhibition of Cx43-based GJIC in response to thrombin-mediated activation of the $G\alpha_q$/phospholipase $C_{\beta 3}$ ($PLC_{\beta 3}$)/ phosphatidyl-inositol 4,5-bisphosphate (PIP_2) hydrolysis pathway that generates the second messengers diacylglycerol (DAG), and inositol-1,4,5-triphosphate (IP-3) via the hydrolysis of the membrane lipid phosphatidyl-inositol-2-phosphate (PIP_2) (Figure 1). They further showed that PIP_2 hydrolysis was both

necessary and sufficient for GJIC inhibition, with no requirement for the second messengers DAG or IP_3. Since GJIC inhibition was independent of second messengers that were thought to trigger channel closure, we hypothesized that inhibition of GJIC might have been achieved by the internalization of GJ plaques, and we thus sought to characterize the mechanism of thrombin-mediated regulation of GJIC and vascular permeability.

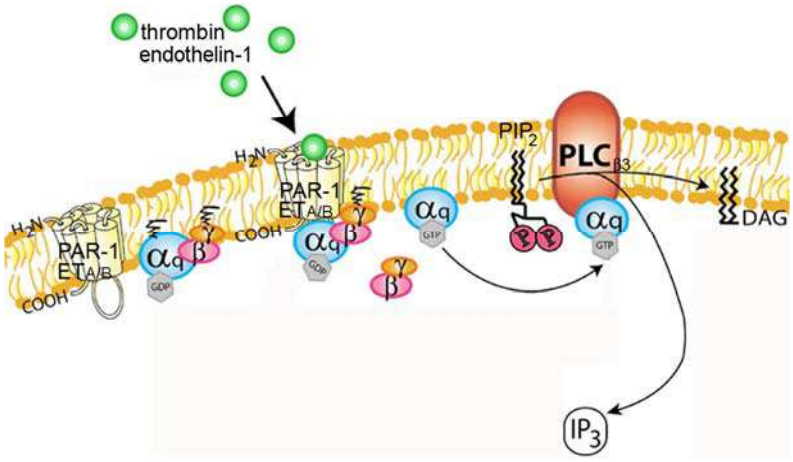

Figure 1. Mechanism of G-protein signaling through thrombin and endothelin-1 mediated activation of PAR-1, ET_A and ET_B G-protein coupled receptors (GPCRs). Activation of $PLC_{\beta 3}$ by $G_{\alpha q}$ subunits leads to cleavage of membrane-bound PIP_2, and generation of the second messengers DAG and IP_3.

In primary porcine pulmonary artery endothelial cells (PAECs), our *in vitro* assays confirmed that thrombin and endothelin-1 stimulated the internalization of GJ plaques through activation of their respective G-protein coupled receptor (GPCR) pathways. Following GPCR activation with thrombin or endothelin-1, primary PAECs began to internalize GJs within minutes that was followed by the loss of physical cell-cell contacts, visible by the separation of cells and an increase in endothelial cell permeability as assessed by a decrease in transendothelial electrical resistance. In addition, functional assays confirmed complete inhibition of GJIC that temporally preceded the internalization of GJs and increased vascular permeability. Control experiments performed with mastoparan,

a constitutive G-protein activator, had similar effects, confirming that G-protein signaling plays a significant role in initiating this process.

Although earlier evidence had been obtained suggesting that Cx43 phosphorylation by c-Src plays a role in regulating GJIC in response to GPCR activation (Postma, *et al*., 1998; Spinella, *et al*., 2003), more recent evidence has shown that PIP_2 hydrolysis alone inhibits GJIC, even when c-Src activity is blocked (van Zeijl, *et al*., 2007). In our studies, we found that GPCR activation by thrombin or endothelin-1 elicited rapid GJ channel closure (within 1-2 minutes), followed by efficient GJ internalization (within 10-30 minutes), followed by physical cell-cell separation and increased vascular permeability (within 30 minutes).

Chapter 4

PROTEINS INVOLVED IN GJ INTERNALIZATION

In the following we will discuss proteins and molecular mechanisms that are involved in the process of inflammatory mediator-induced GJ internalization as we have, thus far, characterized them. Using high-resolution immunofluorescence microscopy and colocalization studies, we observed that the coat-protein, clathrin, colocalized with endogenous Cx43-based GJ plaques and with internalized AGJ structures. These results suggest that GJ plaque internalization occurs in a clathrin-mediated endocytic process as we confirmed in Cx43-GFP expressing HeLa cells (Piehl et al., 2007; Gumpert et al., 2008). In addition, GJ internalization further appears to be mediated by the scaffolding protein, zonula occludens-1 (ZO-1).

ZO-1 is a complex, 220-kDa protein found in multi-protein associations in various cell types and is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins. These proteins are often found associated with cellular junctions at the plasma membrane and may function to form large protein complexes that couple signaling pathways with the cytoskeleton. Numerous studies, including NMR titration, immunoprecipitation and yeast two-hybrid studies have shown that the C-terminus of Cx43 interacts with the second PDZ domain of ZO-1 (Giepmans and Moolenaar, 1998; Toyofuku, *et al*., 1998; Jin, *et al*., 2000; Sorgen, *et al*. 2004). With the recent body of evidence within the last several years, it has become increasingly clear that, far from playing merely a passive, scaffolding role at cell-cell

junctions, ZO-1 functions in a highly dynamic fashion to regulate cellular activities. In untreated PAECs, we observed ZO-1 ubiquitously colocalized at GJ plaque peripheries in agreement with earlier observations by Hunter, *et al.* (2005). However, when GJs were internalized in response to thrombin or endothelin-1, ZO-1 appeared to be localized in the center of AGJ vesicles, but not on their outer surface. This suggests that ZO-1 was displaced from that surface of the GJ plaque to which internalization occurred. Interestingly, a similar distribution of ZO-1 remaining only on the internalizing surface of GJs was also observed by Gilleron, *et al.* (2008) after treating 42GPA9 Sertoli cells with the non-genomic carcinogen, lindane (γ-hexachlorocyclohexane).

These observations, summarized in Figure 2, then allow us to propose a model of the sequence of events that occur in the acute internalization of GJs in response to natural inflammatory mediators, thrombin and endothelin-1, in vascular endothelial cells (see also Baker, *et al.*, 2008).

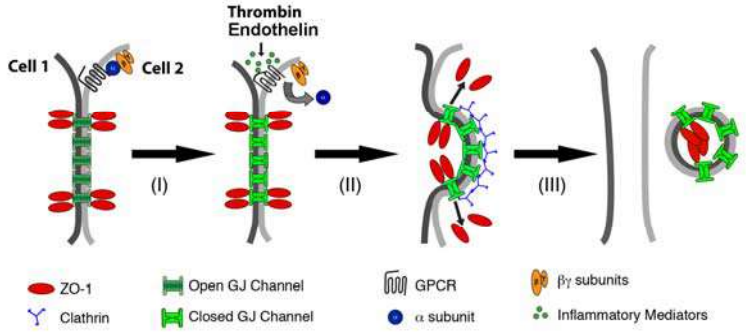

Figure 2. Schematic representation of gap junction internalization in vascular endothelial cells in response to GPCR activation by inflammatory mediators, such as thrombin and endothelin-1. Three events occur in sequence: (I) gap junction channel closure and inhibition of GJIC (fast, within a few minutes); (II) ZO-1 displacement and GJ internalization via clathrin-mediated endocytosis (medium, within about 20 minutes); and (III) cellular retraction and increased endothelial permeability (slow, within 1 hour).

Chapter 5

GJ INTERNALIZATION, GJIC INHIBITION AND INCREASED VASCULAR PERMEABILITY; CLINICAL RELEVANCE

The finding that ZO-1 displacement occurs on only one side of an internalizing GJ plaque provokes the question of how ZO-1 is functioning in this pathway and why ZO-1 is displaced from the side to which internalization occurs. Since ZO-1 is also tethered to elements of the cellular cytoskeleton, remaining ZO-1 residues may serve to provide tensile forces that are required for the invagination and internalization of such a large, double-membrane domain. On the other hand, if ZO-1 binding to connexons regulates accretion of connexons to GJ plaques, as reported recently by Rhett *et al.* (2011) removal or prevention of ZO-1 binding could trigger the process of internalization. To address this question, we performed a series of experiments in which ZO-1/Cx43 binding was disrupted by tagging the C-terminus of Cx43 with a GFP-protein tag that hindered Cx43 from binding to the PDZ2 domain of ZO-1. Since the pulmonary artery endothelial cells we used in these experiments also expressed endogenous Cx43 that can continue to bind to ZO-1, we expected to merely see a reduction in GJ internalization in response to thrombin or endothelin-1. Surprisingly, however, even disruption of only a portion of the Cx43/ZO-1 interaction was sufficient to *completely prevent* GJ internalization and vascular permeability under these experimental conditions (S.M. Baker and M.M. Falk, unpublished results). These findings suggest that the PDZ2 domain of ZO-1 may

qualify as a valid target for potential therapies that aim at the prevention of GJ internalization and endothelial cell permeability that occurs during acute inflammatory responses. In fact, recent evidence has shown that a PDZ-binding peptide that targets the PDZ2 domain of ZO-1 has proven to be effective in wound healing in a cardiac arrhythmia model (Gourdie *et al*., 2006), leading to the exciting notion that disrupting specific PDZ/target protein interactions may be of widespread therapeutic interest (Houslay, 2009).

Finally, we tested the efficacy of blocking the GPCR pathways using suramin, a polysulfonated naphthylurea, which has been used as a GPCR inhibitor that both disrupts association of the Gα subunit and prevents guanine nucleotide exchange. By using suramin in relatively low concentrations (240 nmol/L), we were able to prevent GJ internalization and increased vascular endothelial cell permeability in cultured PAE cells, even in the presence of thrombin or endothelin-1. These results suggest that the administration of suramin or similar synthetic GPCR antagonists might reduce or even prevent increased vascular permeability during an acute inflammatory response with only minor, or at least manageable side effects.

Taken together, the preceding body of work suggests that ZO-1 assembles a multi-protein complex on the Cx43 C-terminal domain that can be activated by natural inflammatory mediators. Figure 3 is a hypothetical illustration of how we suggest the complex may mediate GJ internalization and loss of cellular adhesion. In particular, it is understood that thrombin is a generator of reactive oxygen species such as superoxide (O_2^-) and H_2O_2. Redox-mediated loss of cellular adhesion involves tyrosine-phosphorylation-dependent disruption of cell adhesion complexes with ZO-1 through c-src activation (Chan, *et al.* 2010). Since asymmetric ZO-1 displacement is required for efficient GJ internalization, it is possible that internalization is mediated through generation of reactive oxygen species (ROS) in the cell into which the GJ is internalized, activating c-Src and disrupting ZO-1 complexes. In addition, $G_{\beta\gamma}$ subunits of GPCRs are also known to mediate downstream activation of kinases like c-Src. We propose that, during acute inflammatory response, tyrosine phosphorylation of Cx43 by active c-Src may also mediate displacement of Cx43 from the PDZ2 domain of ZO-1, ultimately resulting in inhibition of GJIC, clathrin-mediated internalization of GJs and increased vascular cell permeability.

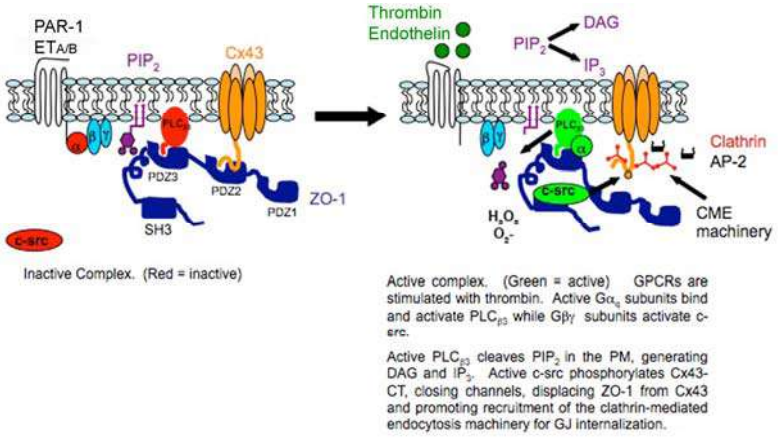

Figure 3. Proposed model of how ZO-1 may assemble a multi-protein complex on the Cx43 C-terminal domain. The complex mediates GJ internalization and loss of cellular adhesion, either through generation of reactive oxygen species (ROS), GPCR activation or a combination of both.

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