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ATTENUATION OF ENDOTHELIAL PHOSPHATIDYLSERINE EXPOSURE DECREASES ISCHEMIA-REPERFUSION INDUCED CHANGES IN MICROVASCULAR PERMEABILITY

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Abstract

Introduction—Translocation of phosphatidylserine from the inner leaflet to the outer leaflet of the endothelial membrane via phospholipid scramblase-1 (PLSCR1) is an apoptotic signal responsible for the loss of endothelial barrier integrity after ischemia-reperfusion injury. We hypothesized that inhibiting phosphatidylserine expression on endothelial cells would attenuate ischemia-reperfusion (IRI) induced increases in hydraulic permeability (L_p).

Methods—Mesenteric hydraulic permeability (L_p) was measured in rat post-capillary mesenteric venules subjected to IRI via superior mesenteric artery (SMA) occlusion (45 minutes) and release (300 minutes) in conjunction with several inhibitors of phosphatidylserine exposure as follows: 1) inhibition of PLSCR1 translocation (DTE - dithioerythritol, n=3), 2) inhibition of PLSCR1 membrane trafficking (2-BP - 2-bromopalmitate, n=3), and 3) inhibition of ion exchange necessary for PLSCR1 function (DIDS - 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid, n=3). Under the same IRI conditions, rats were also administered targeted inhibitors of phosphatidylserine exposure including knockdown of phospholipid scramblase-1 (n=3) using RNA interference (RNAi), and as a potential therapeutic tool Diannexin, a selective phosphatidylserine blocker (n=3).

Results—During IRI net hydraulic permeability (L_p) increased by 80% ($p<0.01$). Net reductions of L_p were accomplished by 2-BP (46% reduction, p=0.005), combined DET+2-BP+DIDS (32%

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reduction, p=0.04), RNAi (55% reduction, p=0.002), Diannexin administered pre-SMA artery occlusion (73% reduction, p=0.001) and post-SMA occlusion (70% reduction, p=0.002).

Conclusion—Phosphatidylserine exposure is a key event in the pathogenesis of microvascular dysfunction during ischemia-reperfusion injury. Clinically, inhibition of phosphatidylserine exposure is a promising strategy that may one day be used to mitigate the effects of ischemia-reperfusion injury.

Study Type and Level of Evidence—Therapeutic, Level III

Keywords

Scramblase; Ischemia-Reperfusion; Endothelium; Hydraulic Conductivity; Microvascular Permeability

BACKGROUND

Post-injury endothelial dysfunction is linked to molecular changes that occur at the lipid membrane. Phosphatidylserine (PS) is a normally-appearing lipid substrate that is relegated to the inner cytosolic leaflet of lipid bilayer. This lipid asymmetry in the cell wall bilayer is maintained by an ATP-dependent aminophospholipid translocator that returns exteriorized PS against its concentration gradient to the inner cytosolic leaflet in healthy cells. It is postulated that PS is exteriorized to the outer leaflet during pro-inflammatory states [1] and that PS exposure is thought to act as an apoptotic signal that prompts phagocytes to engulf senescent cells. Phosphatidylserine exteriorization occurs partially via membrane "scramblases," most notably phospholipid scramblase isoform-1 (PLSCR1) and under ATP-depleted states, increased PLSCR1 activity is observed [2]. Additionally, when ATP is depleted, the ATP-dependent aminophospholipid translocator activity becomes less efficient with the end result of increased levels of PS exposed on the outer leaflet of the cell membrane. A proposed mechanism of increased trans-endothelial flux is exteriorization of PS by PLSCR1, leading to scavenging of damaged endothelial cells, loss of the endothelial barrier and increased efflux of extracellular fluid. Endothelial dysfunction promoting extravascular fluid losses can predispose severely injured patients to multiple organ system failure and sepsis.

Phosphatidylserine exposure has been linked to multiple pathologies, such as HIV infection [3], sepsis [4, 5], immunologic function and axon degeneration in the CNS [6, 7], mast cell degranulation [8], autoimmune disease [9], coagulopathy [10] and malignancy [11]. Phosphatidylserine has been studied extensively at the red blood cell (RBC) level, specifically in models of sickle cell anemia [12, 13]. In endothelial cells, PS exposure occurs during the systemic capillary leak syndrome [14] and (LPS)-induced myocardial damage in cardiac endothelium [15]. In models of hypoxia-reoxygenation, PS exposure is linked to IgM binding and promulgation of the inflammatory response [16]. In intestinal ischemia-reperfusion injury, PS exposure promotes intestinal hyperpermeability [17]. Transmembrane exposure of PS causes complement activation *in vitro* after hypoxia-reoxygenation, producing further inflammation and vascular injury [18].

Because apoptosis of endothelial cells is thought to be one mechanism of microvascular dysfunction after ischemia reperfusion injury [19, 20], our hypothesis was that inhibiting phosphatidylserine expression on endothelial cells would attenuate IRI induced increases in hydraulic permeability (L_p). Hydraulic permeability (L_p) is a measure that describes the ease by which water moves across the endothelial barrier, which can be dramatically increased during times of physiologic stress. It is derived from the Starling equation which takes into account factors that impact on trans-endothelial water movement such as hydrostatic pressure and oncotic pressure. Our specific aims were to describe changes in hydraulic permeability after IRI using 1) inhibitors of PS exposure on endothelial cells *in vivo* (dithioerythritol (DTE), 2-bromopalmitate (2-BP), and 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS)), and 2) targeted inhibition of PS exposure (RNAi and Diannexin).

METHODS

All studies were approved by our Institutional Committee for the use of animals in research and complied with animal research protocols.

Animal Experiments

Animal and Solution Preparations—Red blood cells that are used as flow markers were harvested from female Sprague-Dawley rats (*250-310 g; Hilltop Lab Animals, Scottsdale, PA*). The blood was centrifuged to remove the plasma and buffy coat, and then washed three times in 15 ml of Ringer's solution. The Ringer's solution was prepared daily in distilled de-ionized water and contained 135 mM NaCl; 4.6 mM KCl; 2.0 mM CaCl; 2.46 mM $MgS0_4$; 5.0 mM $NaHCO_3$; 5.5 mM Dextrose; 9.03 mM Hepes Salt (*Research Organics; Cleveland, OH*); 11.04 mM Hepes Acid (*Research Organics*). A 1% bovine serum albumin (*fraction V, fatty acid free - Pierce Biotechnology, Rockford IL*) Ringer's solution was prepared before each experiment by adding the appropriate amount of BSA to the Ringer's. This was used as the perfusate.

Adult female Sprague-Dawley rats were anesthetized with subcutaneous sodium pentobarbital (60 mg/kg body weight). A midline laparotomy incision was performed. The bowel mesentery was gently exposed and positioned on an inverted microscope stage (*Diaphot, Nikon; Melville, NY*). The superior mesenteric artery was identified at the base of the mesentery and a prolene monofilament suture was placed about it circumferentially for occlusion. Throughout this process the animal's body temperature was maintained at 37° C and the mesentery was continuously bathed in Ringer's solution.

In vivo measurement of hydraulic permeability (L_p)

Postcapillary venules, 20 to 30μm in diameter and at least 400μm in length, were identified based on flow patterns. Vessels with no evidence of leukocyte adherence or side branches were chosen. The vessels were cannulated with micropipettes attached to a water manometer to control hydrostatic perfusion pressure.

Single vessel hydraulic permeability (L_p) was determined using the modified Landis micro-occlusion technique [21]. The assumptions and limitations of this technique have been

previously described [22]. Briefly, initial cell velocity (d*l*/d*t*) was obtained by recording marker cell position as a function of time. Transmural water flux per unit area (Jv/S) was calculated by the equation: $J_v/S = (dl/dt) \times (r/2l)$, where *r* is the capillary radius and *l* is the initial distance between the marker cell and the occluded site. Hydraulic permeability (L_p) was determined by using a modified version of Starling's equation of fluid filtration: $L_p = J_v/S[(P_c - P_i) - \sigma\Delta\pi]$, where P_c is the capillary hydrostatic pressure, P_i is the interstitial hydrostatic pressure, σ is the osmotic reflection coefficient, and $\Delta\pi$ is the osmotic pressure difference. Assuming P_i was near zero and $\sigma\Delta\pi$ (units=cm-H_2O) remained constant at 3.78 for 1% BSA Ringer's solution, L_p was calculated from the slope of the regression of J_v/S on P, where $P = (P_c - 3.78)$. This was derived from several occlusions at three different perfusate pressures. Each n signifies that a single vessel was cannulated and the L_p serially measured in a single rat, such that an n=3 means that 3 different vessels were studied in 3 different rats. Control studies that document the stability of this model over time and after multiple recannulations of the vessels have been previously reported [23].

Ischemia-Reperfusion Protocol

The protocol was adapted from previous work [23, 24]. In brief, study microvessels were perfused with 1% Ringer/BSA solution for 10 min before baseline L_p was assessed. After this assessment, a prolene suture snare was placed around the superior mesenteric artery (SMA) and tightened for 45 min, constituting the gut ischemia phase of the protocol. Cessation of blood flow through the study vessel was confirmed by intra-vital microscopy. During this time, the bowel was covered with gauze and bathed with a warm Ringer solution. After 45 min of ischemia, the prolene suture snare was released and reperfusion of the venule was confirmed and continued for an additional 270 minutes, constituting the reperfusion phase of the protocol. Subsequently, L_p was measured at the following time intervals post SMA occlusion – 105, 165, 195, 225, 255, 285, and 315 minutes. Data points were obtained in triplicate at each time interval. For the hydraulic conductivity studies, the reperfusion component of the study lasted 5h. For control animals (sham animals), all steps were carried out in the same manner as the appropriate comparison experimental group, except the prolene suture snare around the SMA was not tightened, thereby eliminating the ischemic and reperfusion components of the study. Measures of L_p was obtained in the same manner as the other study group animals and serve as time controls.

RNA interference studies

Cloning of rat PLSCR1 cDNA—PLSCR1-specific primers were designed from the nucleotide sequences deposited in the *Entrez* data bank. Blast search confirmed that the region of PLSCR1 to be amplified did not share any appreciable identity with any known genes in the non-redundant or EST database. Total RNA (1μg) was reverse transcribed using random hexamers in a 20μl reaction volume and 5μl of RT mix, used in a 50μl PCR reaction using PLSCR1-specific primers. The resulting product was analyzed by agarose gel electrophoresis and cloned into a dual-promoter plasmid (*pTopoII, Invitrogen Corp., Carlsbad, CA*). The cloned product was sequenced to confirm its identity and used to generate dsRNA for the RNAi studies.

Synthesis of dsRNA—Sense and antisense RNAs was synthesized *in vitro* using the mega-riboprobe kit (*Ambion, TX*) from PLSCR1 and control cDNAs cloned in plasmid vectors as described [25]. Equimolar concentrations of sense and antisense RNA was mixed, phenol-extracted and precipitated. The RNA pellet was dissolved in annealing buffer, boiled for one minute in a boiling water bath and allowed to cool to 22°C over 16 hours to form double-stranded RNA. For *in vivo* studies, dsRNA (20μg) was mixed with 1.5μl of lipofectAMINE (*Invitrogen*) and injected directly into the mesentery.

Synthesis of long double-stranded RNA (dsRNA) and RT-PCR—RT-PCR was used to amplify PLSCR1 fragment from rat colon RNA using PLSCR1-specific primers (forward primer: 5′-CGCGTCATCCAGAGTGAG-3′, reverse primer: 5′-TATAATCACCCAG-3′) at an annealing temperature of 58° C. The PLSCR1 PCR product was cloned into pTOPO4 vector (*Invitrogen*) and sequenced to confirm identity. This cloned cDNA was used as template to transcribe sense and antisense RNAs *in vitro* using the Megascript RNA kit (*Ambion, Austin, TX*) according to the manufacturer's specifications and as described previously [26]. A nonspecific 300-bp segment of pBluescript (*Stratagene, La Jolla, CA*) was similarly transcribed for use as control dsRNA. dsRNAs (20μg) for either pBluescript (control RNAi) or PLSCR1 (PLSCR1 RNAi) were mixed with 1.5μl of Lipofectamine 2000 (*Invitrogen*) and brought to a volume of 250μl before being injected into the ileal mesentery [27].

RNAi protocol—After anesthesia was induced using 2% isoflurane and 2% O_2, a midline incision was made, and the small bowel mesentery was gently exposed as described in the animal preparation section. The dsRNA solutions described above were applied onto the thin mesenteric tissue over a mesenteric arcade containing appropriate venule so as not to disrupt the vascular arcades or mesenteric tissue itself. In addition, the dsRNA solution was injected into the mesenteric fat tissue underneath the same arcade by raising a wheal in that area. The ileal wall underneath the injected arcade was marked with a 6-0 Prolene blue monofilament suture to identify the region with knockdown. The laparotomy incision was then sutured closed in two layers, and the rats were allowed to recover from the anesthesia as described previously [23-25]. Three days after dsRNA injections, anesthesia was again induced for ischemia-reperfusion studies below. The midline laparotomy incision was reopened, and the previously marked ileal loop was exposed again. A suitable postcapillary mesenteric venule in the area in which the dsRNA was previously administered was cannulated and measured for hydraulic permeability (L_p) at regular intervals (ischemia-reperfusion protocol described above).

Determination of PS inhibition on L_p after IRI

To determine the effect of PS inhibition on L_p after IRI, we used several inhibitors of PS exposure (DTE, 2-BP, DIDS). Dithioerythritol (DTE) is an inhibitor of the cysteine residue that is essential for PLSCR1 translocation; 2-bromopalmitate (2-BP) is an inhibitor of PLSCR1 trafficking to the plasma membrane, which renders PLSCR-1 nonfunctional; and 4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid (DIDS) is an inhibitor of the anion exchange mechanism necessary for PLSCR1 function. First we measured L_p at baseline (t=0) and during ischemia (t=45 minutes). Serial measurements of L_p were then obtained in

triplicate during reperfusion at the following time intervals: 105, 165, 195, 225, 255, 285, and 315 minutes. The measurements for L_p were then compared at baseline, during IRI, and during IRI with pre-ischemia administration of DTE (0.5nM, n=3), 2-BP (100μM, n=3), DIDS (5mg/kg, n=3), DTE+2-BP+DIDS.

To determine the effect of targeted PS inhibition on L_p after IRI, we used RNAi to specifically knockdown PLSCR-1. Three days after the injection of dsRNA for PLSCR1, rats underwent the IRI protocol as described above (n=3). Next, serial measurements of L_p were obtained in triplicate at baseline (t=0), during ischemia (t=45 minutes), and during reperfusion at the following time intervals: 105, 165, 195, 225, 255, 285, and 315 minutes. The measurements for L_p were then compared at baseline, during IRI and during IRI after PLSCR1 RNAi.

Finally, we wanted to investigate a potential therapeutic tool, Diannexin, that blocks exposed PS from interacting with proteins that are attracted to the negatively charged moieties of PS, and as such, it is a selective phosphatidylserine blocker. Rats underwent the IRI protocol as described above. Diannexin (400μg/kg) was administered prior to SMA occlusion (n=3), and to simulate a more clinically relevant scenario after SMA occlusion was released (n=3). Next, serial measurements of L_p were obtained in triplicate at baseline (t=0), during ischemia (t=45 minutes), and during reperfusion at the following time intervals: 105, 165, 195, 225, 255, 285, and 315 minutes.

Statistical Analysis

Comparisons between control and sample means for *in vivo* modified Landis calculations were made with single-factor analysis-of-variance. L_p test groups differences were compared using the area for the area-under-the-curve (AUC) analysis, which was calculated using the trapezium rule under the Landis curve where the area for each trapezoid = $0.5 \times (b_1 + b_2) \times$ height, where $b_1 = L_p$ at t_1 and $b_2 = L_p$ at t_2 and height = $(t_2) - (t_1)$. Differences between measures of L_p were evaluated with a paired Student's *t*-test or repeated measures two-way ANOVA as appropriate. Tukey's HSD post hoc tests were calculated to verify differences among group comparisons. Percentages are expressed as mean ± SEM based on a normal distribution. P values < 0.05 were considered statistically significant. Values for L_p are expressed as mean ± SEM $\times 10^{-7}$ cm·s $^{-1}$·cmH_2O^{-1}, except when fold increases or percentage change are reported.

RESULTS

Ischemia-Reperfusion Studies

The effect of PS inhibition on L_p after IRI is displayed in Figure 1. Inhibition by DTE attenuated the IRI-induced increase in L_p at all time points during reperfusion except for the 45, 165, and 195 minute time points ($p < 0.004$). Inhibition by 2-BP attenuated the IRI-induced increase in L_p at all time points during reperfusion except for the 165 and 195 minute time points ($p < 0.01$). Similarly, inhibition by DIDS attenuated the IRI-induced increase in L_p at all time points during reperfusion except for the 165 and 196 minute time points ($p < 0.02$). Combined inhibition by DTE + DIDS + 2-BP reduced the IRI-induced

increase in L_p at all time points during reperfusion except for the 45, 165, and 196 minute time points ($p < 0.002$).

Targeted inhibition by RNA interference and its effect on L_p after IRI is displayed in Figure 2. RNAi had a very robust effect on reducing the IRI-induced increase in L_p after IRI. RNAi attenuated the IRI-induced increase in L_p at all time points during reperfusion ($p < 0.02$). This effect held even for the 45, 165, and 195 minute time points, that weren't significantly different in the experiments on inhibition with DTE, 2-BP or DIDS.

Targeted inhibition with Diannexin had the most prominent effect on L_p after IRI, and this is displayed in Figure 3. Both pre- and post-ischemia Diannexin attenuated the IRI-induced increase in L_p at all time points during reperfusion ($p < 0.02$). This effect held even for the 45, 165, and 195 minute time points that weren't significantly different in the experiments of inhibition with DTE, 2-BP or DIDS. Diannexin administered pre-ischemia had a slightly more robust effect than Diannexin administered post-ischemia, as pre-ischemia Diannexin returned the IRI-induced increase in L_p to baseline levels at the 45 and 105 minute time periods ($p < 0.05$).

An area under the curve analysis was performed for all inhibitors during IRI. With the exception of DTE and DIDS, all agents demonstrated significant attenuation of the IRI-induced increase in L_p ($p < 0.001$). This is shown in Figure 4.

DISCUSSION

Endothelial dysfunction promoting extravascular fluid losses can predispose severely injured patients to multiple organ system failure and sepsis. Extravascular fluid loss may be due to loss of the endothelial barrier, in part mediated by PS-exposing cells that serve as an extrinsic signal for phagocytosis. Our hypothesis was that inhibiting phosphatidylserine expression on endothelial cells would attenuate IRI induced increases in hydraulic permeability (L_p). In our first specific aim we sought to determine whether inhibitors of PS exposure on endothelial cells *in vivo* (DTE, 2-BP, and DIDS) would attenuate the increase in hydraulic permeability observed during IRI. In our second specific aim, we sought to determine whether the increase in hydraulic permeability could be attenuated pharmacologically during IRI using targeted inhibition of PS exposure (RNAi and Diannexin). We found that DTE, 2-BP, and DIDS were modestly effective at decreasing hydraulic permeability and that targeted inhibition of PS exposure via RNAi and Diannexin were very effective at reducing hydraulic permeability during IRI. These results add further support that phosphatidylserine exposure is a key event in the pathogenesis of microvascular dysfunction during ischemia-reperfusion injury and suggests that inhibition of phosphatidylserine exposure is a promising strategy that may one day be used to mitigate the effects of ischemia-reperfusion injury.

Our *in vivo* studies interrogated the production transport, insertion, modification, flipping, and antagonist properties of PLSCR1 (and resultant PS exposure). The compound 2-BP inhibited the transport and insertion of PLSCR1 into the cell membrane, leading to decreased hydraulic permeability. Using the known enzymatic structure and function of

PLSCR1 we used thiol modification of the cysteine residues by DTE to interrupt the enzymatic conformational change required for PLSCR1 function, leading to decreased hydraulic permeability. The combined inhibitors approach (2-BP+DTE+DIDS) was inferior to 2-BP alone, possibly due to DTE and DIDS interfering with 2-BP efficacy (decreased uptake or diminished function). The biodegradation of 2-BP by DTE and DIDS, both with some reducing properties, is one hypothetical mechanism of diminished function. Another plausible mechanism would be DTE competing with 2-bromopalmitate for cysteine residues, preferentially undergoing reduction instead of palmitoylation inhibition, exhibiting a weaker effect than 2-BP alone [28]. Another potential mechanism involves modulation of downstream inflammatory mediator production that contributes to endothelial permeability. For example, DIDS has been shown to inhibit serine palmitoyltransferase activity and downstream sphingolipid synthesis [29], and nuclear sphingolipids propagate genes that are involved in inflammation [30]. As opposed to 2-BP, DIDS may exhibit a dominant effect on palmitoyltransferase activity and downstream sphingolipid production, rendering less of a protective effect compared to 2-BP alone.

With targeted RNAi we were able to significantly attenuate hydraulic permeability due to the decreased production of the PLSCR1 enzyme. Finally, targeted PS inhibition via the specific blocker, Diannexin, significantly decreased the hydraulic permeability observed after IRI when given either before, or after, the initiation of ischemia. Diannexin was more effective than RNAi, possibly due to the completeness of the PS blockade with Diannexin compared to knockdown of PLSCR1 via RNAi or from cells potentially increasing production of PLSCR1 mRNA during progressive IRI. Moreover, the administration of Diannexin before ischemia was more effective compared to Diannexin administration after ischemia, which makes intuitive sense. In the setting of traumatic injury, Diannexin post-ischemia would more closely resemble real-life therapy during the initial injury phase, however, theoretically Diannexin could be administered prophylactically after the initial resuscitation but before an additional systemic insult occurs (such as in the damage control surgery setting before repeated operations) and potentially prevent the sequelae of the second hit phenomenon.

Phosphatidylserine exposure during hypoxia-reoxygenation is associated with microvascular dysfunction. *In vitro*, hypoxia (2h) and reoxygenation (1h) in murine endothelial cells is associated with increased extracellular beta-2 glycoprotein binding to endothelium (beta-2 glycoprotein has high affinity for anionic phospholipids such as PS) as demonstrated by increased Annexin-V immunofluorescence [16]. In that study, PLSCR1 mRNA was upregulated in hypoxia-treated cells, and hypoxia with subsequent reoxygenation increased protein expression of PLSCR1. In HUVECs, hypoxia and reoxygenation in cells exposing PS by Annexin-V demonstrated increased C3/C5 production and activation. Complement activation increases microvascular dysfunction during reperfusion injury through increased binding and activation of neutrophils [18]. In systemic capillary leak syndrome, endothelial cells exposed to patient sera demonstrated increased PS exposure, DNA fragmentation and increased bax/bcl-2 ratios [14], suggesting that PS exposure and resultant microvascular leak is associated with endothelial loss via apoptosis. During sepsis, lipopolysaccharide (LPS)-induced microparticles (small 1μm vesicles that bud from damaged endothelial cells) exposing PS can cause microvascular dysregulation via endothelial apoptosis, possibly by a nitric oxide intermediate [31]. Microvascular dysfunction and increased endothelial

permeability in vivo is associated with PS-exposure [32]. In a murine model of pulmonary sepsis, endothelial barrier dysfunction and increased permeability to albumin was associated with increased PS exposure [33]. In terms of quantifying the deleterious nature of PS production and translocation, this study demonstrated a 10-fold increase in exposure of PS, which corresponded to a 6-fold increase in apoptosis, a 10-fold increase in pulmonary endothelial cell death and a 3-fold increase in microvascular fluid leak.

In our current study, hydraulic permeability during IRI was reduced significantly by inhibiting PLSCR1 and PS exposure, suggesting that PS may act through an apoptotic mechanism to produce vascular dysfunction. A limitation of our *in vivo* studies was an inability to quantify endothelial apoptosis and cite this as the cause for increased hydraulic permeability during IRI in our experiments.

Phosphatidylserine exposure has deleterious effects beyond endothelial apoptosis. Exteriorization of PS is associated with prothrombinase complex formation and recruitment of cells that participate in further inflammation [34]. As thrombosis and permeability are intrinsically linked, shielding PS-rich surfaces decreases catalytic efficiency of tenase and prothrombinase complexes by 200- and 1000-fold [35] and pharmacologic compounds that act on PS production, trafficking, exteriorization or that modulate PLSCR1 activity may be beneficial. In preclinical models, PS antagonism has been shown to be effective. In a mouse model of bilateral renal IRI, Diannexin pretreatment decreased proximal tubule damage (diminished tubular cast formation), decreased leukocyte influx (decreased granulocyte staining on immunohistochemistry), decreased neutrophil eliciting factors (reduced mRNA production of KIM-1 and NGAL). Specifically, PS inhibition reduced mRNA markers of acute kidney injury by 62-67% and protein expression by 71-74%, correlating to mildly damage renal tubules (approximately 1% scattered casts in tubules) in contrast to untreated controls (25% casts in tubules), which improved renal recovery (normalized urine flow and urinary glucose levels) [36]. In a model of hepatic ischemia-reperfusion, PS inhibition reduced levels of liver transaminases (80% reduction), and decreased necrosis of liver tissue on H&E staining [37]. The authors of both studies concluded that PS inhibition via Diannexin blocked leukocyte activation and recruitment, limiting inflammation in the renal and hepatic microcirculation, preserving parenchymal function.

In conclusion, microvascular dysfunction after ischemia-reperfusion injury is in part linked to endothelial cells with increased levels of PS exposed on their cell surfaces which promotes inflammation and injury. Plausible therapeutic targets exist for targeting both intracellular and extracellular mechanisms of inhibiting PS exposure. Further study on the intracellular mechanistic aspects of PS exposure in endothelial cells and the impact on microvascular dysfunction are warranted.

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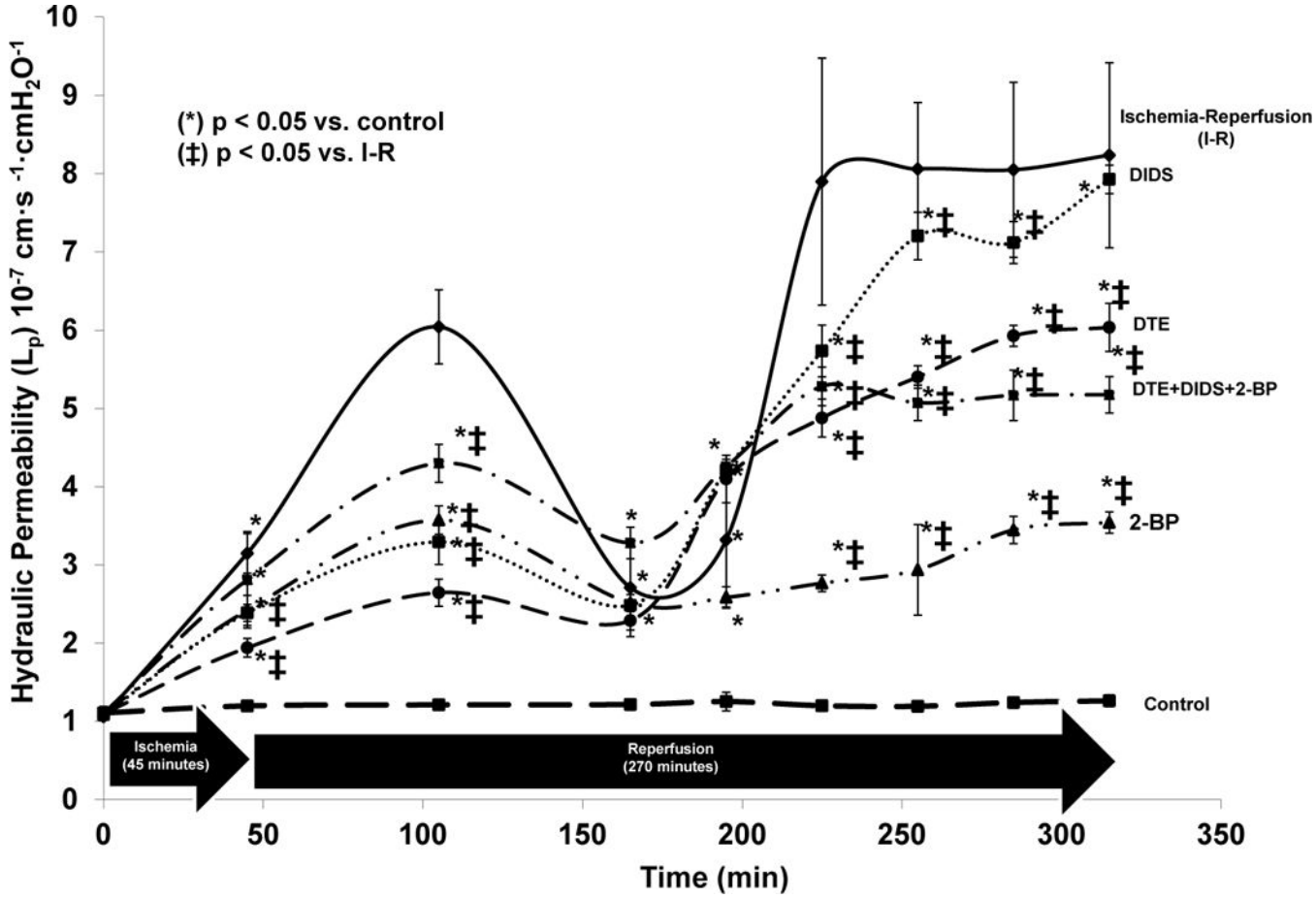

Figure 1. Impact of Phosphatidylserine Inhibition on Hydraulic Permeability in Rat Mesenteric Venules During Ischemia/Reperfusion

The modified Landis technique with SMA occlusion (45 minutes) and reperfusion (270 minutes) was used to assess for changes in hydraulic permeability after IRI. Potential therapy targeting the trafficking mechanism of PLSCR1 to the endothelial membrane (2-BP) was particularly effective in attenuating the increased hydraulic permeability associated with IRI. Abbreviations = Dithioerythritol (DTE), 2-Bromopalmitate (2-BP), 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic (DIDS), Phospholipid Scramblase-1 (PLSCR1). Units for L_p are expressed as mean ± SEM × 10^{-7} cm·s $^{-1}$·cmH_2O^{-1}. (*) denotes $p < 0.05$ vs. control, (‡) denotes $p < 0.05$ vs. ischemia-reperfusion (I-R).

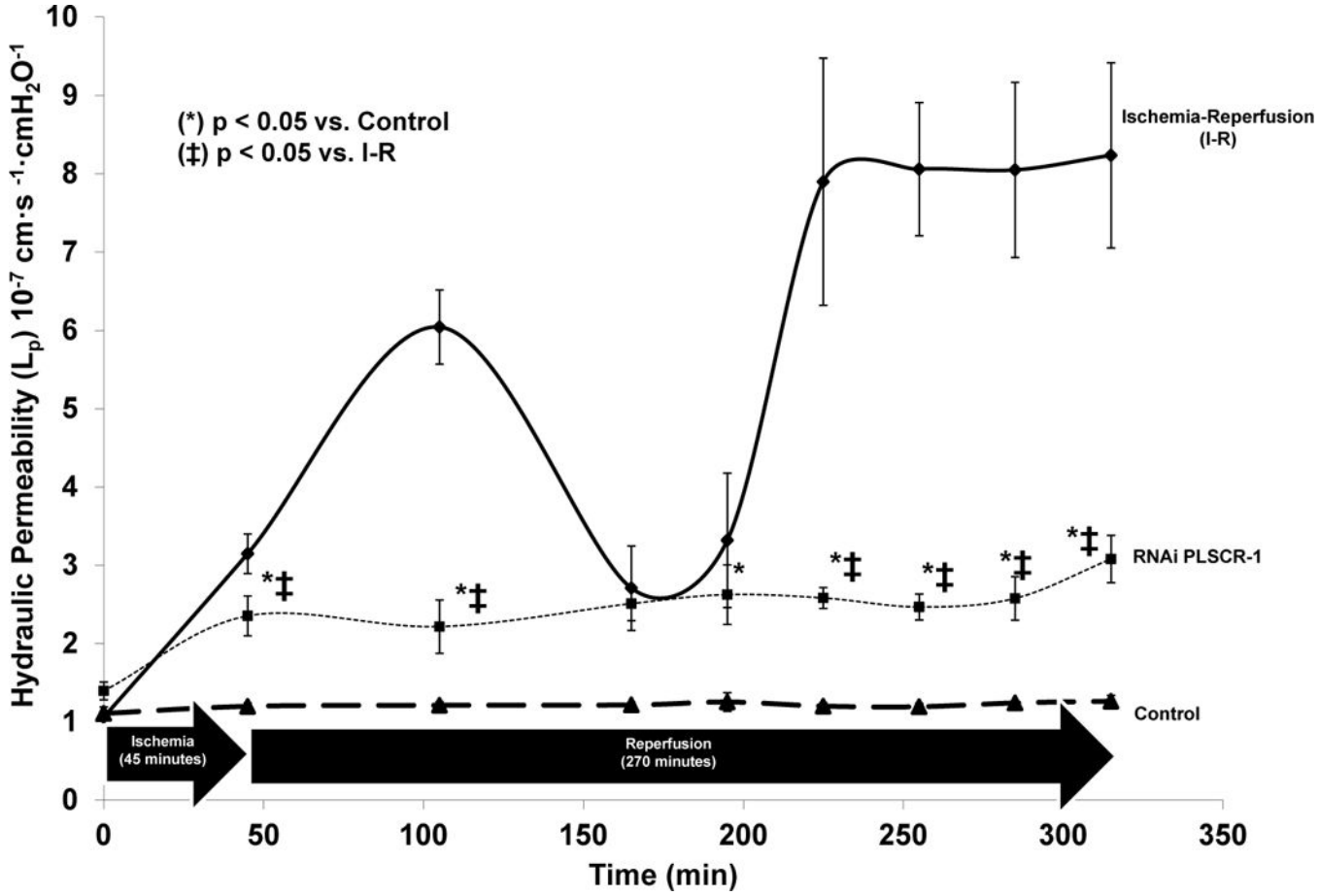

Figure 2. Impact of Targeted Phosphatidylserine Inhibition in Rat Mesenteric Venules via RNA Interference

RNAi had a very robust effect on reducing the IRI-induced increase in L_p after IRI. RNAi attenuated the IRI-induced increase in L_p at all time points during reperfusion. Abbreviations = RNA interference (RNAi), Units for L_p are L_p are expressed as mean ± SEM × 10^{-7} cm·s $^{-1}$·cmH_2O^{-1}. (*) denotes $p < 0.05$ vs. control, (‡) denotes $p < 0.05$ vs. ischemia-reperfusion (I-R).

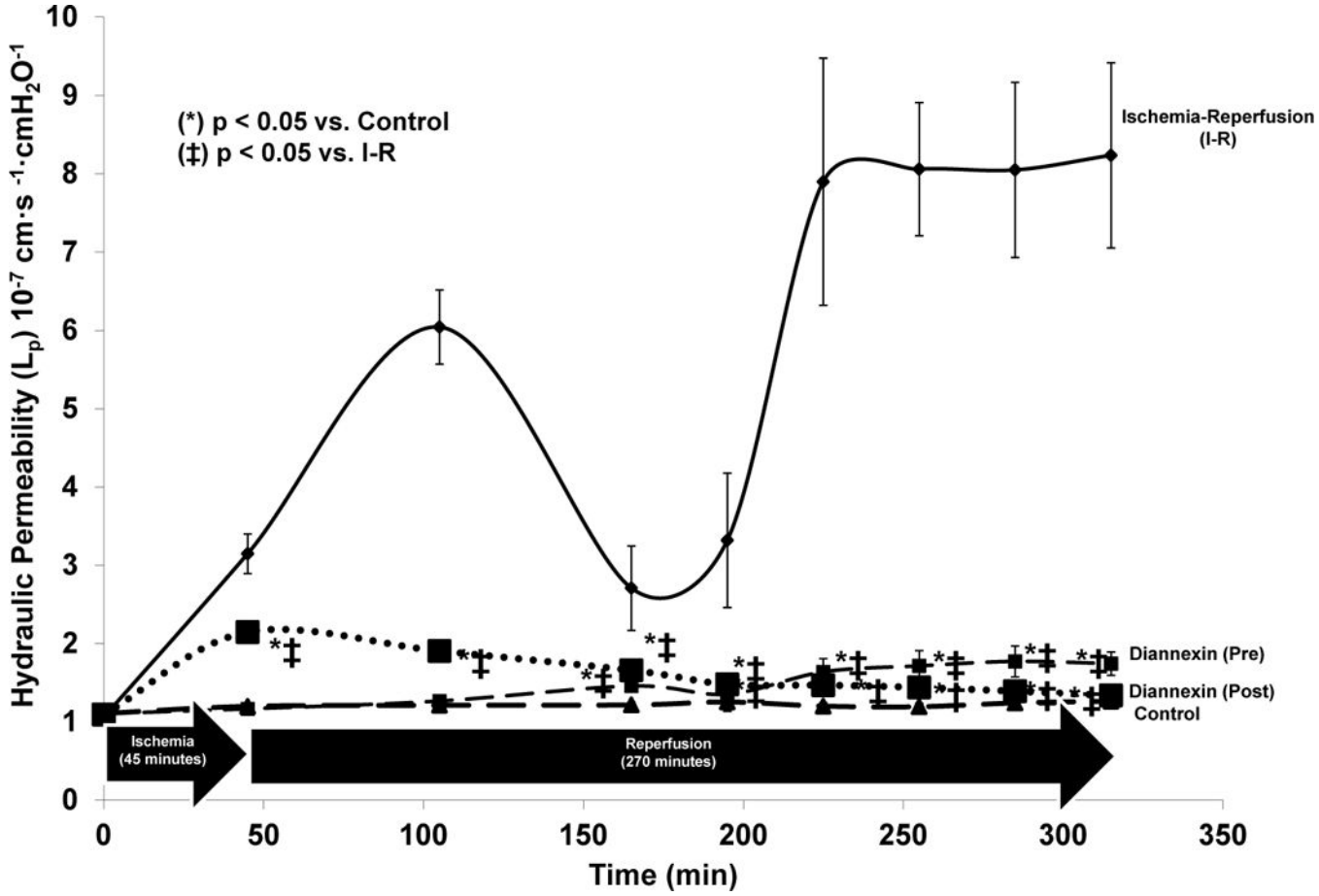

Figure 3. Impact of Targeted Phosphatidylserine Inhibition in Rat Mesenteric Venules During Ischemia/Reperfusion

Both pre- and post-ischemia administered Diannexin attenuated the IRI-induced increase in L_p at all time points during reperfusion. Diannexin administered pre-ischemia had a slightly more robust effect than Diannexin administered post-ischemia, as pre-ischemia Diannexin returned the IRI-induced increase in L_p to baseline levels at the 45 and 105 minute time periods. Abbreviations = Phospholipid Scramblase-1 (PLSCR1). Units for L_p are L_p are expressed as mean ± SEM × 10^{-7} cm·s $^{-1}$·cmH_2O^{-1}. (*) denotes $p < 0.05$ vs. control, (‡) denotes $p < 0.05$ vs. ischemia-reperfusion (I-R).

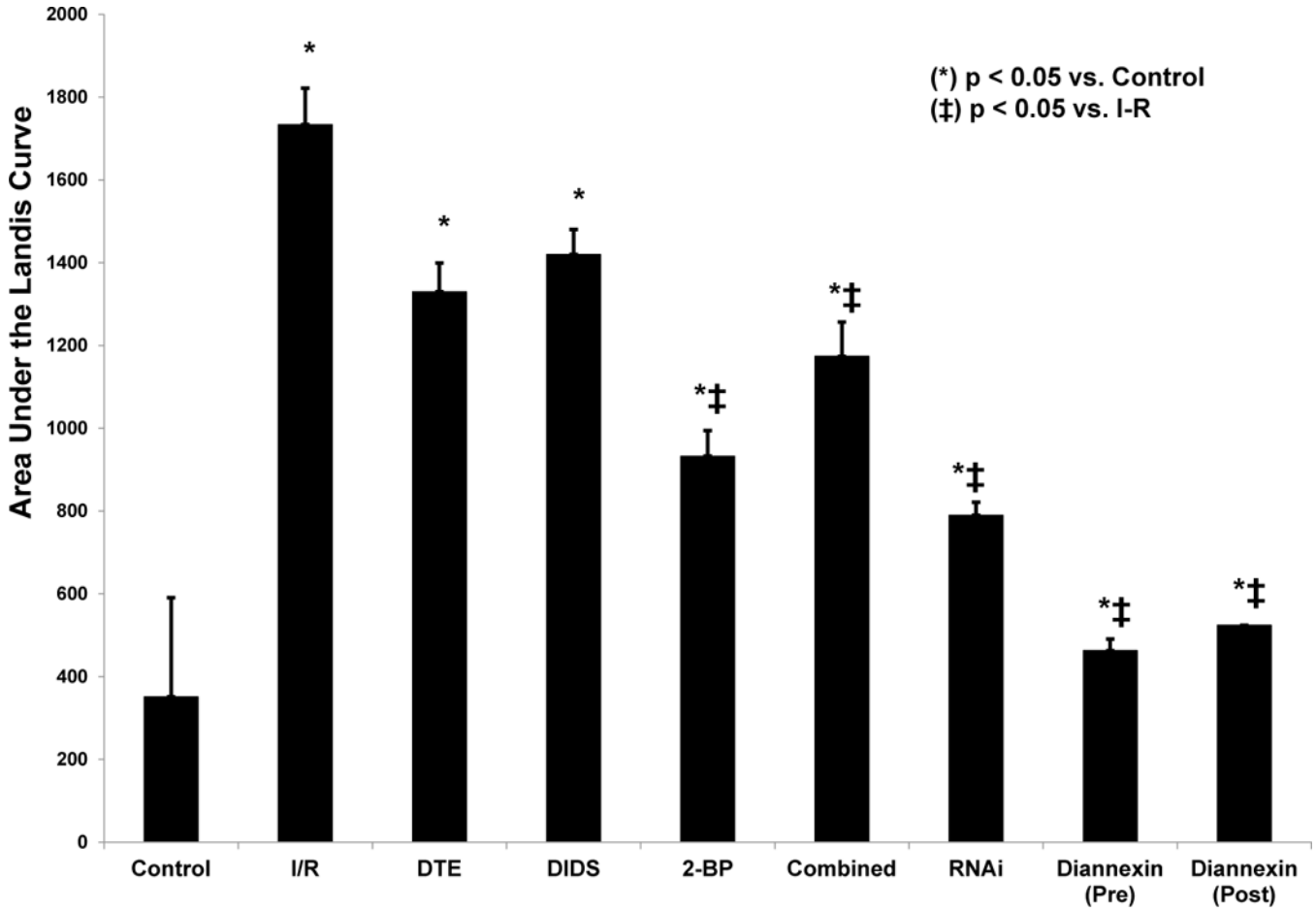

Figure 4. Area Under the Curve (AUC) Analysis: Phosphatidylserine Inhibition During Ischemia/Reperfusion

The cumulative area under the Landis curve was calculated for each inhibitor of PS. During IRI net hydraulic permeability (L_p) increased by 80% ($p<0.01$). Net reductions of L_p were accomplished by 2-BP (46% reduction, p=0.005), combined DTE+2-BP+DIDS (32% reduction, p=0.04), RNAi (55% reduction, p=0.002), Diannexin pre-SMA occlusion (73% reduction, p=0.001) and post-SMA occlusion (70% reduction, p=0.002). As shown, the most effective inhibition (as represented by reduction of the cumulative area) was accomplished by targeted knockdown of PLSCR1 with RNAi and by targeted blockade of PS with Diannexin ($p < 0.01$ for both). (*) denotes $p < 0.05$ vs. control, (‡) denotes $p < 0.05$ vs. ischemia-reperfusion (I-R).