

This is the author's final, peer-reviewed manuscript as accepted for publication. The publisher-formatted version may be available through the publisher's web site or your institution's library.

## **Membrane lipid interactions in intestinal ischemia/reperfusion-induced injury**

Emily Archer Slone and Sherry D. Fleming

### **How to cite this manuscript**

If you make reference to this version of the manuscript, use the following information:

Sloane, E. A., & Fleming, S. D. (2014). Membrane lipid interactions in intestinal ischemia/reperfusion-induced injury. Retrieved from <http://krex.ksu.edu>

### **Published Version Information**

**Citation:** Sloane, E. A., & Fleming, S. D. (2014). Membrane lipid interactions in intestinal ischemia/reperfusion-induced injury. *Clinical Immunology*, 153(1), 228-240.

**Copyright:** © 2014 Elsevier Inc.

**Digital Object Identifier (DOI):** doi:10.1016/j.clim.2014.04.018

**Publisher's Link:** <http://www.sciencedirect.com/science/article/pii/S1521661614001260>

This item was retrieved from the K-State Research Exchange (K-REx), the institutional repository of Kansas State University. K-REx is available at <http://krex.ksu.edu>

**Title: Membrane Lipid Interactions in Intestinal Ischemia/reperfusion-induced Injury**

Emily Archer Slone<sup>1</sup> and Sherry D. Fleming<sup>2</sup>

<sup>1</sup>College of Veterinary Medicine and <sup>2</sup> Division of Biology, Kansas State University, Manhattan,  
KS, 66506, USA

eslone@ksu.edu

sdflemin@ksu.edu

Running title: Eicosanoids and lipids in IR-induced injury

Corresponding author:

Sherry D. Fleming, Ph.D.

18 Ackert Hall

Kansas State University

Manhattan, KS 66506

785-532-6130 (voice)

785-532-6653 (fax)

sdflemin@ksu.edu

## **Abstract**

Ischemia, lack of blood flow, and reperfusion, return of blood flow, is a common phenomenon affecting millions of Americans each year. Roughly 30,000 Americans per year experience intestinal ischemia-reperfusion (IR), which is associated with a high mortality rate. Previous studies of the intestine established a role for neutrophils, eicosanoids, the complement system and naturally occurring antibodies in IR-induced pathology. Furthermore, data indicate involvement of a lipid or lipid-like moiety in mediating IR-induced damage. It has been proposed that exposure of neo-antigens are recognized by antibodies, triggering action of the complement cascade. While it is evident that the pathophysiology of IR-induced injury is complex and multi-factorial, we focus this review on the involvement of eicosanoids, phospholipids and neo-antigens in the early pathogenesis. Lipid changes occurring in response to IR, neo-antigens exposed and the role of a phospholipid transporter, phospholipid scramblase 1 will be discussed.

**Key words:** ischemia, reperfusion, lipids, neo-antigen

## Abbreviations

AA	arachidonic acid
ATP	adenosine tri-phosphate
$\beta_2$ -GPI	beta2-glycoprotein 1
cAMP	cyclic adenosine mono-phosphate
Cox	cyclooxygenase
cPLA <sub>2</sub>	cytosolic phospholipase A2
CR2	complement receptor 2
ESI-MS/MS	electrospray ionization-tandem mass spectrometry
HR	hypoxia re-oxygenation
HUVEC	human umbilical vein endothelial cells
Ig	immunoglobulin
IR	ischemia reperfusion
Lox	lipoxygenase
LTB <sub>4</sub>	leukotriene B4
MBL	mannose binding lectin
miRNA	micro ribonucleic acid
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PGE <sub>2</sub>	prostaglandin E2
PGF <sub>2α</sub>	prostaglandin F2 alpha
PGI <sub>2</sub>	prostaglandin I2
PI	phosphatidylinositol
PLA <sub>2</sub>	phospholipase A2
PLSCR	phospholipid scramblase
PS	phosphatidylserine
Rag	recombination activating gene
sCR1	soluble complement receptor type 1
TxB <sub>2</sub>	thromboxane B <sub>2</sub>

## **Ischemia/Reperfusion**

Ischemia, the lack of sufficient blood supply to tissues, results in cellular dysfunction and eventually necrosis. However, the return of blood flow, termed reperfusion, exacerbates the damage begun during the ischemic period. Ischemia and reperfusion (IR) events occur in numerous organs as a result of various insults, such as trauma, shock, routine surgery and organ transplantation. The intestinal mucosa is among the organs most sensitive to IR (reviewed in [1]) with acute mesenteric arterial or venous thrombosis, embolism, and obstruction being common causes of intestinal IR (reviewed in [2, 3]).

Cases of acute mesenteric ischemia are classified by cause. In order of incidence, they are arterial embolism, arterial thrombus, non-occlusive and venous thrombosis (reviewed in [2]). Symptoms are non-specific and can be subtle, resulting in a delay of correct diagnosis which decreases survival probability. Furthermore, common sequelae include acute lung injury and multiple organ failure. Bowel resection is indicated in most cases which leaves surviving patients with bowel problems, such as short bowel syndrome, for the remainder of their lives. Despite decades of improved imaging devices and medical advances, the mortality rate associated with intestinal IR remains at 50 to 80% (reviewed in [4]).

The mesenteric arteries can maintain adequate perfusion of the intestine over a broad range of blood pressures, however below 40 to 45 mm Hg, perfusion is compromised (reviewed in [3]). The gastrointestinal tract has a very high capillary density, many collateral vessels and receives approximately 25% of total cardiac output at rest (reviewed in [3]). Cellular injury in humans is detectable by 20 minutes of total ischemia and within 60 minutes in the case of partial ischemia (reviewed in [3]).

Rodents are commonly used as research models for intestinal IR and have provided a large knowledge base regarding associated physiology and pathogenesis. Studies in mice have shown changes in the internal pH of enterocytes, abruptly increasing from 6.8 to 7.1 at the initiation of ischemia, and rapidly stabilizing at a pH of 6.3 by three minutes into ischemia [1]. Upon reperfusion, cascades of cellular events lead to eicosanoid production, signaling molecules derived from 20 carbon fatty acids via oxidation, formation of reactive oxygen species, secretion of cytokines and activation of the innate immune response. The barrier between luminal contents and intestinal mucosa is compromised as enterocytes are shed, by necrosis and apoptosis, and capillary permeability increases [5-7] ([Fig. 1](#)). The duration of ischemia influences the severity of damage and mortality. A recent study with mice demonstrated increasing severity of tissue damage, as assessed by histology, with increasing duration of ischemia [8]. Furthermore, mice subjected to 30 and 35 minutes of ischemia survived a minimum of 18 hours while mice subjected to 40 and 45 minutes of ischemia all succumbed within six hours post ischemia [8]. In contrast, experimental models of intestinal IR in horses frequently utilize two hours of ischemia and horses survive for 18 hours of reperfusion [9-11]. In fact, 10 of 11 horses subjected to two hours of jejunal ischemia survived 10 days, to the study's end-point [12]. Thus, the duration of ischemia tolerated varies between species.

Several factors contributing to IR-induced pathology will be discussed briefly; however, the majority of this review will focus on lipids, neo-antigens and phospholipid scramblase 1 as they relate to IR-induced pathology. Eicosanoids, derivatives of arachidonic acid, are rapidly produced during the reperfusion period. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) was found to be required but not sufficient for tissue damage in a mouse model of intestinal IR [13]. The conversion of arachidonic acid (AA) to prostaglandins requires the cyclooxygenase (Cox) enzymes. It is well

established that the expression of Cox 2, the inducible isoform, is elevated in the intestinal tissue following ischemia. Several animal models have demonstrated an increase in Cox 2 transcription and translation in the intestine following varying lengths of ischemia and reperfusion [13-18]. The use of Cox inhibitors, NS-398 and FK3311, has further demonstrated the importance of this pathway in reperfusion injury in some but not all animal models [14, 19]. It is possible that structural differences in the inhibitors tested or species differences account for the discrepancy in results.

The role of Cox 1, the constitutive isoform, in the post-ischemia production of prostaglandins is debated in the literature. The expression of Cox 1 has primarily been reported in equine studies. In the equine jejunum, Cox 1 protein is constitutively expressed and is increased, as is Cox 2, at 18 hours reperfusion following a two hour ischemic period [10, 20]. Transcription of Cox 1 also increased, although not significantly, in response to 75 minutes of reduced blood flow to the jejunum [15]. Cox 1 is constitutively expressed in the lamina propria of equine colon and IR significantly increases protein levels in the epithelial cells [9, 11]. Our in vitro model using murine endothelial cells results in significant increases of both Cox 1 and Cox 2, with a greater percent increase over control levels for Cox 1 (unpublished data). The role of Cox 1 in IR-induced pathology may be underappreciated.

The Cox enzymes catalyze the formation of prostaglandins, which, as stated above, are necessary but not sufficient for intestinal IR damage, at least in mice [13]. Prostaglandins are potent signaling molecules primarily derived from AA (reviewed in [21]) that are synthesized upon stimulation rather than being stored by the cell (reviewed in [22]). PGE<sub>2</sub> is often associated with inflammation due to its vasodilatory effect and enhancement of vascular permeability ([23], reviewed in [24]). Several in vivo studies have documented increased PGE<sub>2</sub>



production in response to intestinal IR [13, 25-29]. While increased transcription of the Cox enzymes begins during the ischemic period, reperfusion is necessary for PGE<sub>2</sub> production [27]. Endothelial cells produce prostaglandins (reviewed in [22]); however, not all endothelial cells generate the same prostaglandin profile. PGI<sub>2</sub> is the primary prostaglandin produced from endothelial cells of the large vessels, but microvessel endothelial cells, like those found in the mesenteric vasculature, synthesize more PGE<sub>2</sub> and PGF<sub>2α</sub> [30]. It has long been postulated that PGE<sub>2</sub> may paradoxically contribute to tissue healing by promoting angiogenesis and epithelial cell migration (reviewed in [31]). Higher doses of Cox inhibitors which more profoundly suppress PGE<sub>2</sub> production are inhibitory for mucosal repair following ischemia in a porcine model [17]. An ex vivo study of porcine ileum showed that PGE<sub>2</sub> application could increase intracellular cyclic adenosine monophosphate (cAMP) levels and contribute to closure of leaky tight junctions [23]. It has been proposed that PGE<sub>2</sub> may act to downregulate nuclear factor kappa light chain enhancer of activated B cells (NFκB) activity in a negative feedback fashion (reviewed in [32]). While the precise mechanisms by which PGE<sub>2</sub> exerts opposing effects are not well understood, concentration, timing and ligation of differing receptors are likely possibilities (reviewed in [24]).

Another eicosanoid derived from AA in response to intestinal IR is leukotriene B<sub>4</sub> (LTB<sub>4</sub>). LTB<sub>4</sub>, produced by endothelial cells, is chemotactic for neutrophils and facilitates adherence and degranulation of neutrophils (reviewed in [33]). The chemotactic property of LTB<sub>4</sub> has been well documented, both in vitro and in vivo. Reperfusion greatly increases the number of neutrophils in the intestinal tissue ([34], reviewed in [35]). Increased neutrophil adherence to endothelial cells was observed via intravital microscopy and in ex vivo assays on tissues subjected to intestinal IR [36, 37]. Activated neutrophils produce reactive oxygen

compounds which contribute to the reperfusion damage (reviewed in [38]). Accordingly, IR induces release of myeloperoxidase, an enzyme of neutrophils [39]. LTB<sub>4</sub> receptor antagonists, LTB<sub>4</sub>-DMA and LY-255283, have been shown to attenuate the effects of hypoxia, increased survival and decrease intestinal myeloperoxidase activity, while application of LTB<sub>4</sub> enhanced leukocyte adhesion in a dose-dependent manner [36, 40, 41]. Similarly, LTB<sub>4</sub> production and intestinal myeloperoxidase activity was reduced in dogs subjected to three hours of ischemia and one hour of reperfusion when treated with the 5-lipoxygenase inhibitor A-64077 [42]. Furthermore, *5-Lox*<sup>-/-</sup> mice sustained less intestinal damage, had decreased intestinal myeloperoxidase activity and improved survival compared to wildtype mice following intestinal IR [43].

The Cox enzymes also mediate the production of thromboxanes which promote coagulation and are increased by intestinal IR, similar to prostaglandins and leukotrienes. In both rats and dogs, thromboxane B<sub>2</sub> (TxB<sub>2</sub>) increased significantly over baseline during the 60 minute reperfusion period [29, 44]. An equine study also revealed increased TxB<sub>2</sub>, but only during the first of three hours of reperfusion [45]. Thromboxanes contribute to IR-associated systemic effects, such as pulmonary compromise. The release of TxB<sub>2</sub> from the lungs and pulmonary permeability of rats subjected to intestinal IR was significantly greater when compared to Sham treated rats [46]. Furthermore, the pulmonary permeability was attenuated with thromboxane inhibitors [46]. It is clear that the synthesis of eicosanoids from AA potentiate IR-induced injury.

### **Antibodies and Neo-antigens**

The complement system, comprised of over 30 proteins, plays a significant role in the pathology resulting from IR. Traditionally, the complement system is described as having three

different methods of activation, converging at a common endpoint. Several approaches were taken to delineate the contribution of each activation pathway due to the significant overlap and crosstalk between them. The involvement of both the classical and alternative pathways in IR injury was demonstrated in the early 1990s. Administration of soluble complement receptor type 1 attenuated intestinal damage and neutrophil infiltration in a rat model [47]. Later studies using this same complement inhibitor confirmed that both the classical and alternative pathways of complement activation contributed to IR injury [48]. The generation of *factor D*<sup>-/-</sup> mice allowed for investigation of the alternative pathway. Mice deficient in factor D experienced attenuated intestinal injury and neutrophil infiltration following IR [49]. The lectin pathway was subsequently shown to contribute to IR pathology (reviewed in [50]). A supporting study demonstrated the requirement for MBL in IR injury as *MBL*<sup>-/-</sup> mice were protected from IR damage [51, 52] but susceptible after reconstitution with MBL [51]. Two native inhibitors of the complement pathway, complement C1 inhibitor (classical and lectin) and Crry (classical and alternative), attenuate tissue injury and reduce neutrophil infiltration [53, 54].

In accordance with the involvement of the classical complement pathway, antibodies are essential for IR injury. *Rag-1*<sup>-/-</sup> mice do not produce antibodies and do not sustain IR damage; however, administration of pooled wildtype antibodies as well as the IgM fraction alone results in intestinal damage similar to that seen in wildtype animals [27, 55]. It was later shown that human IgM can also elicit injury and complement deposition in *Rag-1*<sup>-/-</sup> and *Rag-2*<sup>-/-</sup> mice [56, 57]. Further support for the involvement of antibodies came out of studies investigating complement receptor 2 (CR2), a B cell membrane protein. *CR2*<sup>-/-</sup> mice produce antibodies but have defects in the generation of the normal antibody repertoire [58, 59]. *CR2*<sup>-/-</sup> mice are protected from IR injury; and, like *Rag-1*<sup>-/-</sup> mice, administration of wildtype IgM results in

intestinal injury and complement deposition [59, 60]. Additional studies with *CR2*<sup>-/-</sup> mice identified specific antibodies capable of inducing IR damage. An anti-phospholipid antibody and anti- $\beta_2$ -glycoprotein I ( $\beta_2$ -GPI) antibody were each able to induce IR injury and complement deposition in *CR2*<sup>-/-</sup> mice; however both antibodies were required in *Rag-1*<sup>-/-</sup> mice [61].

### **IR-Induced Neo-Antigens**

$\beta_2$ -GPI is one of three recently identified proteinaceous neo-antigens involved in IR injury. This 54 kDa protein, originally named apolipoprotein H, is one of the most abundant human plasma proteins with an average concentration of 200  $\mu$ g per ml (reviewed in [62]). Five short consensus repeats comprise the 326 amino acid protein categorizing it as a member of the complement control superfamily (reviewed in [62]).  $\beta_2$ -GPI primarily circulates unaccompanied but can be found bound to circulating lipid (reviewed in [62]). A stretch of lysine residues (amino acids 282-287) in Domain V allows for binding to anionic phospholipids of cellular membranes which can activate the cells and promote apoptosis of the bound cell ([63], reviewed in [62]). The results of the aforementioned study strongly suggested the involvement of  $\beta_2$ -GPI [61] and further studies have demonstrated the efficacy of peptides derived from Domain V, the lipid binding domain, in protecting wildtype mice from IR damage [64]. Additionally, treatment with the  $\beta_2$ -GPI -derived peptides minimized the intestinal injury, complement deposition and eicosanoid production resulting from administration of wildtype antibodies to *Rag-1*<sup>-/-</sup> mice [64]. Interestingly, administration of purified human  $\beta_2$ -GPI to wildtype mice prior to intestinal IR attenuated injury, complement deposition, and PGE<sub>2</sub> production [65]. The authors speculate that the anti- $\beta_2$ -GPI antibodies produced by the mice bind the human  $\beta_2$ -GPI, thus reducing the titer of anti- $\beta_2$ -GPI antibodies available for binding the mouse protein [65]. This hypothesis is supported by further data demonstrating attenuation of IR injury and sequelae when *Rag-1*<sup>-/-</sup>

mice were administered pooled wildtype antibodies that had specifically been depleted of anti- $\beta_2$ -GPI [64].

The other two identified neo-antigens induced by IR are the intracellular proteins non-muscle myosin heavy chain II and annexin IV. Screening experiments probing ischemic tissue with several monoclonal antibodies identified monoclonal antibodies against non-muscle myosin heavy chain II isoforms A and C or annexin IV that rendered *Rag-1*<sup>-/-</sup> mice susceptible to IR injury complement deposition, neutrophil infiltration or eicosanoid production [66-68]. Importantly, the anti-annexin IV monoclonal antibody does not recognize non-muscle myosin or phospholipids [69]. A peptide based on non-muscle myosin heavy chain II provides additional evidence that this protein serves as a neo-antigen following IR [70]. Non-muscle myosin heavy chain II may be involved in other models of IR injury as this same peptide has conferred protection in hind-limb and myocardial IR models [71, 72]. In wildtype mice, recombinant annexin IV reduces tissue injury as well as neutrophil infiltration and eicosanoid production [67, 69]. The authors propose that the recombinant annexin IV binds the circulating anti-annexin IV antibodies, greatly reducing the likelihood of antibody binding annexin IV expressed on damaged tissue [67, 69]. Although multiple neo-antigens have been identified, it is unknown how these proteinaceous neo-antigens interact.

### **The Contribution of Lipids to Cellular Signaling and Processes**

The cellular membrane consists of a bilayer of phospholipids, which under normal conditions, exists in an asymmetric distribution with neutral phospholipids such as phosphatidylcholine (PC) residing in the outer leaflet of the bilayer and anionic aminophospholipids including phosphatidylserine (PS) remaining in the inner leaflet. Despite

advances in “lipidomics”, or mass spectrometry-based lipid analysis, only a few studies have applied this technology to investigate intestinal lipid composition.

PC, PS, phosphatidylinositol (PI), phosphatidylethanolamine (PE) and sphingomyelin were the main lipid classes present in both wildtype and *Rag-1*<sup>-/-</sup> mice after Sham treatment [27]. Similar results were obtained by ESI-MS/MS analysis of untreated mouse and rat intestines [73, 74]. The prominent PC peaks in the rat intestine were composed of 16:0-18:2 PC species [73]. These data correlate with that of Sparkes et al., in which the most prominent PC species was 34:2 [27]. A study by Braun et al. also showed that normal mouse jejunum expressed a 496 Da and 524 Da lysoPC in the highest concentrations [75]. These masses correlate with the predominant 16:0 and 18:0 lysoPC species found in Sham-treated animals [27]. This data is consistent with formation of lysophospholipids by a lipase acting at the sn-2 position since mammalian lipids typically are enriched in saturated fatty acids at the 1-position and polyunsaturated fatty acids at the 2-position.

Phospholipases catalyze phospholipid hydrolysis and are classified by their cleavage sites. Phospholipase A cleaves fatty acyl chains from the glycerol backbone of a phospholipid. Three classes of PLA<sub>2</sub>, secretory, calcium-independent, and cytosolic, cleave the fatty acyl chain from the second carbon of the glycerol backbone (*sn*-2) (reviewed in [76]). The ubiquitous cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) has an affinity for phospholipids containing polyunsaturated fatty acids, particularly AA, at the *sn*-2 position (reviewed in [76-78]). While no preference for a phospholipid head group has been identified [79], AA is most often associated with phospholipids containing choline, ethanolamine or inositol as the head group [79]. The activity of cPLA<sub>2</sub> is calcium-dependent and is synergistically increased by mitogen activated protein kinase mediated phosphorylation [80, 81]. Calcium is required for the translocation of cPLA<sub>2</sub>

from the cytosol to the plasma and intracellular membranes and for binding to a phospholipid substrate (reviewed in [76, 78]). Although the majority of AA is released from PC by the action of PLA<sub>2</sub> [82], the activity of phospholipase C and D can indirectly contribute to the AA pool (reviewed in [83]).

PLA<sub>2</sub> activity not only increases the level of free AA, but also lysophospholipids ([Fig. 2](#)). Several lysophospholipids are biologically active and circulate through the vasculature (reviewed in [84]). The term “lyso” was applied to these lipids because they lyse red blood cells (reviewed in [84]); structurally lysophospholipids are those with a single acyl chain. Just as PC is the most abundant phospholipid of cellular membranes, lysoPC is the most abundant lysophospholipid in serum (reviewed in [84]). Beyond their contribution to cellular structure, phospholipids are biologically active and participate in intracellular signaling.

PA and lysoPA influence several aspects of immune function. Many cells of the immune system express one or more of the nine known G protein coupled receptors for lysoPA (reviewed in [85]). GPR92, the lysoPA receptor mentioned above, is expressed by the resident lymphocytes of the intestine, including those in the epithelial layer, lamina propria, Peyer’s patches and mesenteric lymph nodes [86]. Stimulation of the immune system can increase the concentration of lysoPA, which is chemotactic for leukocytes, elevates intracellular calcium concentrations and compromises the endothelial barrier ([87], reviewed in [85, 88]).

High concentrations (75 μmol/L) of lysoPC are toxic to cells. The addition of exogenous lysoPC (16:0) to cultures of human aortic endothelial cells reduced mitochondrial respiration, as assessed by MTT assay, promoted detachment of cells from the matrix and increased the number of apoptotic cells in a time- and dose- dependent manner [89]. Uptake of extracellular calcium is

promoted by lysoPC, increasing the intracellular calcium concentration, which appears necessary for lysoPC-mediated apoptosis [89, 90].

A hallmark of apoptosis is externalization of PS (reviewed in [91]). As the process of apoptosis begins, cardiolipin, the major constituent of mitochondrial membranes, is oxidized [92]. Cytochrome c is then released into the cytoplasm where it can oxidize PS residing in the inner leaflet of the plasma membrane [92]. Subsequently, oxidized PS is flipped to the outer leaflet where it is recognized as an apoptotic marker by the macrophage membrane protein CD36, promoting phagocytosis [92, 93]. Although transient exposure of PS occurs often, such as during cellular activation or fusion of membranes [94], the phospholipid is rapidly re-internalized under physiological conditions. LysoPS can also be flipped to the outer leaflet; however, lysoPS is not re-internalized, thus serving as a marker for phagocytosis (reviewed in [84]). PS is also involved in the clotting of blood when externalized by platelets and acts as a co-factor for maximal activity of protein kinase C and sodium/potassium ATPase [95].

Hypoxia activated PLA<sub>2</sub> in a study exposing primary human umbilical vein endothelial cells (HUVEC) to zero percent O<sub>2</sub> for two hours [96]. However, in the context of IR, reperfusion seems essential for stimulating PLA<sub>2</sub> activity and elevating lysophospholipid content [97]. A decrease in total phospholipids is observed after intestinal IR and IR studies in rats indicated that administration of quinacrine, a non-specific inhibitor of PLA<sub>2</sub> enzymes, reduced intestinal permeability and lowered the ratio of lysoPC to PC suggesting a role for PLA<sub>2</sub> in IR injury [97, 98]. Quinacrine also attenuated the loss of total phospholipids following IR in a porcine heart model [99]. The total phospholipid content per gram of tissue following ischemia of the heart decreased in an investigation of the metabolic effects due to ischemia in cardiac tissue [99, 100].



An ex vivo study of myocardial cells indicated loss of PC and PE with a concomitant increase of lysoPC and lysoPE in response to four hours of zero percent O<sub>2</sub> exposure [101]. An increase in fatty acids including AA was detected in the culture supernatant [101]. PC and PE were similarly decreased and lysophospholipids increased in a rat model of cerebral IR [102]. Two independent studies found a decrease in total phospholipids, up to 20%, when primary porcine pulmonary artery endothelial cells were exposed to zero percent O<sub>2</sub> for 24 to 48 hours [103, 104]. In accordance with the myocardial cell study, culture supernatants contained elevated levels of AA and other free fatty acids [103, 104]. Primary HUVECs exposed to zero percent O<sub>2</sub> for two hours followed by 45 minutes re-oxygenation released approximately 15% more AA than the controls [96]. Accordingly, eicosanoid production was augmented when HUVECs and bovine aortic endothelial cells were enriched with fatty acids in a hypoxia/re-oxygenation (HR) study [105]. Thus, several in vitro and ex vivo studies suggest a role for phospholipids and lysolipids in IR-induced damage.

The limited investigation into the composition of the healthy lipid profile extends to diseased tissue. A small number of studies have used ESI-MS/MS to study lipids in intestinal disease with variable results depending on the disease model. IR increased lysoPC with a concomitant decrease in PC [27]. Furthermore, phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity and the subsequent lysoPC:PC ratio increased in response to IR [97]. These data correlate with previous studies indicating that lysoPC increases the intestinal permeability and PLA<sub>2</sub> activity as a result of intestinal IR [106]. In addition to increased lysoPC levels following IR treatment, intestinal levels of free AA increased.

The altered phospholipid composition resulting from hypoxia increases the fluidity of the cellular membrane, an effect that is reversible with a sufficient re-oxygenation period [103]. In

vivo studies demonstrated the effect of lysophospholipids on endothelial permeability. Addition of lysoPC to the lumen of the ileum resulted in increased permeability to molecules as large as 70 kDa [107]. LysoPC also enhanced the permeability that ischemia alone causes [108].

Inflamed tissue exhibits some of the same lipid changes found in HR studies. Colon biopsies from inflammatory bowel disease patients and inflamed intestinal mucosal samples contained significantly more AA than biopsies and mucosa from healthy control patients [109, 110]. LysoPE in the inflamed intestinal mucosal samples was also elevated in comparison to healthy control samples [110].

Evidence of lipid oxidation is found in both in vitro HR and in vivo IR studies. An increase in malondialdehyde occurred in primary pulmonary artery endothelial cells after a hypoxic period of eight hours [103]. However, evidence of free radical production and lipid oxidation was detected with a 45 minute hypoxia, 15 minute re-oxygenation treatment of aortic endothelial cells in a separate study [111]. A rat model of intestinal IR showed that reperfusion was necessary for an increase in malondialdehyde [97]. Interestingly, a model of oxidative tissue damage in which mice are exposed to  $\gamma$ -irradiation revealed an increase in oxidation of cardiolipin and PS but no other phospholipids, suggesting an increased susceptibility of cardiolipin and PS to oxidative stress [74]. Together, these studies suggest that the lipid profile of a tissue can change and that lysophospholipids and oxidized lipids are important components of intestinal reperfusion injury.

### **The Response of Phospholipid Scramblase 1 to Hypoxia**

The cellular membrane consists of a bilayer of phospholipids which form a hydrophobic barrier between the cellular constituents and the outside environment. This bilayer is not a passive barrier however; the composition and distribution of membrane phospholipids are highly

regulated. Under normal conditions, the membrane exists in an asymmetric distribution, with neutral phospholipids residing in the outer leaflet of the bilayer and anionic aminophospholipids remaining in the inner leaflet. This asymmetric distribution of phospholipids is not present in all membranes that fuse with the cellular membrane, such as during endocytosis. Thus, transmembrane proteins shuttle phospholipids from one leaflet to the other as a means of maintaining the asymmetric distribution of phospholipids ([Fig. 3](#)).

Phospholipids move between membranes by several mechanisms. Phospholipids can be transferred by vesicles and proteins (reviewed in [112]). Proteins specific for the transport of phospholipids between organelles reside in the cytoplasm (reviewed in [113]). Additionally, contact between membranes allows for lateral diffusion of phospholipids whereby relocation can be achieved (reviewed in [112]).

The first of these lipid transporting proteins to be characterized was aminophospholipid translocase [114]. Use of spin-labeled analogs with bovine serum albumin back-extraction confirmed an affinity for PS and PE and demonstrated more rapid internalization of PS compared to PE [115, 116]. This aminophospholipid translocase was found to be ATP-dependent with a stoichiometry of one ATP per PS or PE transported [114, 117]. There is some evidence that basic fibroblast growth factor signaling regulates activity as incubation with an anti-basic fibroblast growth factor immunoglobulin inhibited aminophospholipid translocase activity in cultured bovine aortic endothelial cells [118].

A counterpart to the aminophospholipid translocase, which transports aminophospholipids from the outer leaflet to the inner leaflet of the bilayer, was proposed for the cell to maintain shape and function. Early evidence for such a counterpart was provided in studies with red blood cells [119]. Subsequently, this activity was also shown to be ATP-

dependent, although independent of the aminophospholipid translocase [116]. This putative transporter was called flippase; for consistency and simplicity, the aminophospholipid translocase became known as floppase.

A third class of transmembrane proteins involved in regulation of membrane asymmetry were discovered nearly a decade later and named scramblases. Four members of this protein class have been identified in human ([120, 121], reviewed in [122]) and in mouse (reviewed in [122]). The properties of the scramblases are in stark contrast to those of the floppases and flippases. While floppases and flippases transport phospholipids in one direction, from inner leaflet to outer leaflet or vice versa, respectively, scramblases are bi-directional transporters, capable of moving phospholipids between leaflets in both directions (reviewed in [122]). Additionally, scramblases show very little specificity for phospholipid head-groups, transporting both neutral and aminophospholipids at a similar rate (reviewed in [123, 124]). Thus, scramblase activity serves to disrupt and reduce the membrane asymmetry ([125, 126], reviewed in [122]). The exposure of aminophospholipids, particularly PS, is at times desired, such as for initiation of the coagulation cascade and clearance of apoptotic cells (reviewed in [127]). Conceptually, scramblase activity should be tightly regulated even in cases of physiologic benefit. Experimentally, activation of scramblase corresponds with very specific intracellular conditions. Scramblase is activated by high concentrations of intracellular calcium [125, 126, 128-130] and acidic pH [130]. In contrast to the constitutive activity of floppases and flippases, scramblase activity is highly regulated and ATP-independent.

The scramblases are a conserved family, with orthologs found in several diverse organisms including the model organisms *Mus musculus*, *Drosophila melanogaster*, *Danio rerio* and *Saccharomyces cerevisiae* (reviewed in [122]). The four human homologs are similar in

sequence with PLSCR2 – 4 exhibiting 46 to 59% protein identity with PLSCR1 [120]. Each of the four known scramblases appears to have distinct localizations and functions. The localization of each murine protein has been investigated and all findings regarding the localization of human homologs are consistent with the murine data. Phospholipid scramblase 1 (PLSCR1), the first to be characterized [128, 129], primarily localizes to the cellular membrane [131-134] while PLSCR3 resides in the outer mitochondrial membrane [135]. Expression of PLSCR2 is only detected in the testes [120] and PLSCR4 appears to distribute both to the cellular membrane and the nucleus [136], though the functions of these two family members remain unidentified.

Activation of PLSCR1 involves the binding of calcium or potentially other divalent cations [128, 137] to a predicted single cation binding site [137] in the cytoplasmic portion. Several studies have demonstrated an increase in phospholipid scrambling in the presence of calcium [128, 129, 138]. Recent work confirms the importance of the C-terminal alpha helix for both calcium binding and scrambling activity [121, 139]. It is hypothesized that calcium binding is followed by a conformational change and perhaps self-aggregation [131, 137, 138]. In the absence of calcium, acidic conditions ( $\text{pH} < 6.0$ ) activate PLSCR1 in erythrocyte-derived inside out vesicles [130]. Data suggest that protein kinase C $\delta$  phosphorylates PLSCR1 at the threonine residue at position 161 following calcium binding [140]. This phosphorylation appears to be required for PLSCR1 activity; as either specific inhibition of protein kinase C $\delta$  or transfection of PLSCR1 alone in cells intrinsically lacking both protein kinase C $\delta$  and PLSCR1 resulted in loss of phospholipid scrambling [140]. Additionally, PLSCR1 and epidermal growth factor receptor are primarily localized to lipid rafts. There is evidence that PLSCR1 is a component of the epidermal growth factor receptor complex as epidermal growth factor stimulation allows for the

co-immunoprecipitation of phosphorylated PLSCR1, epidermal growth factor receptor and the adaptor protein Shc [133]. Furthermore, data suggest that signaling via epidermal growth factor receptor activates synthesis of PLSCR1 [133].

Normally, PLSCR1 is found in the cellular membrane and to a lesser extent membranes of secretory vesicles, often associated with lipid rafts [131, 133, 134, 141]. The protein contains 18 cysteine residues [132], each of which could potentially serve as a site of palmitoylation. Using a mutated PLSCR1 that cannot be palmitoylated or the native structure with inhibition of palmitoylation revealed that palmitoylation is required for the protein's association with the membrane [132]. Site directed mutagenesis studies in which alanine residues were systematically substituted for cysteine residues provided insight into which cysteine residues were important for palmitoylation. Only when all five of the cysteine residues between amino acids 184 and 189 were changed to alanine residues was palmitoylation lost [132]. Additionally, the binding affinity for calcium and activity of PLSCR1 is greatly decreased in the absence of palmitoylation [142].

In the absence of palmitoylation, PLSCR1 is found diffusely in the nucleus [132]. While PLSCR1 does not contain a classical nuclear localization signal [143], the amino acid sequence from residues 257 to 266 is necessary for active import into the nucleus by importin  $\alpha$  and  $\beta$  [143, 144]. However, the full significance of PLSCR1's function in the nucleus is still unclear.

A study by Rami et al. performed immunohistochemical staining for PLSCR1 on the hippocampal region of human brain samples from patients who had experienced and temporarily recovered from an ischemic insult and found an increase in PLSCR1 protein versus control samples in which IR did not occur [145]. Our unpublished data indicate a decrease in endothelial cell protein levels during a period of acute hypoxia but a significant increase in

transcription within 15 minutes of re-oxygenation. While it is important to point out the different cell types under investigation, neurons in the Rami study and endothelial cells in ours, both suggest an increase in PLSCR1 shortly after reperfusion is established. As for the decrease in PLSCR1 protein during the hypoxic period that we observed, the potential for PLSCR1 to be released from the cell membrane must be considered and may be due to shedding of the protein into the culture media as was recently described for PLSCR3 [146].

## Summary

Although the phenomenon of intestinal IR has been recognized for centuries and medical knowledge and technology have progressed immensely, a high mortality rate remains for those afflicted. The pathogenesis is multi-factorial and the numerous molecular and cellular interactions contribute to the difficulty of designing effective therapeutics. While several aspects of intestinal IR-induced pathogenesis have been identified, the initial molecular response remains unknown. Figure 4 represents one possibility for initiation of the cascade.

Recent studies have identified neo-antigens as potential triggers, but their roles remain undefined [64, 66, 68]. The involvement of a lipid moiety is suggested by data in the literature. Antibodies are known to be one of the required components for IR-induced tissue damage as antibody-deficient *Rag-I*<sup>-/-</sup> mice are protected and reconstitution of *Rag-I*<sup>-/-</sup> mice with antibodies from wildtype mice prior to intestinal IR renders the *Rag-I*<sup>-/-</sup> mice susceptible to IR-induced injury [27]. Likewise, reconstitution with a monoclonal anti-phospholipid antibody and anti-phospholipid binding protein,  $\beta_2$ -GPI, (both found in wildtype sera) produces IR-induced pathology in the otherwise protected *Rag-I*<sup>-/-</sup> mice [147].

Mass spectrometry has been used to compare the intestinal lipid profiles of polar lipids and free fatty acids between different strains of mice, C57Bl6, *Rag-I*<sup>-/-</sup>, *TLR9*<sup>-/-</sup>, *TLR2*<sup>-/-</sup> and

*CR2*<sup>-/-</sup> ([26, 27] and unpublished data). IR treatment results in considerable changes in the lipid composition; however these alterations are largely independent of genetic background.

Importantly, the levels of lysolipids increased, especially lysoPC and lysoPE, as did the levels of AA. Release of AA through phospholipase activity is essential for production of eicosanoids. These data show that cellular lipids are altered in response to intestinal IR and provide support for the hypothesis that a lipid moiety participates in the pathogenesis of IR-induced injury.

As the lipid profiles from various strains of mice prior to and following IR are quite similar, this may be the inciting stimulus for the pathogenic cascade leading to tissue damage. Ischemia activates PLSCR1 resulting in disruption of the phospholipid bilayer ([Fig. 4](#)). Following exposure of negatively charged phospholipids, such as PS, circulating  $\beta_2$ -GPI is able to deposit on the cellular membranes of endothelial cells. The conformational change that occurs in  $\beta_2$ -GPI upon binding allows for antibody recognition of the now exposed neo-antigens. The complement cascade can then be activated and an inflammatory response initiated.

### **Future Directions**

In addition to their role in activating the complement cascade, antibodies are required for the up-regulation of Cox 2 transcription. In the absence of antibodies, Cox 2 transcription is not up-regulated and the production of PGE<sub>2</sub>, another required component for IR-induced damage, is not increased [27]. The mechanism by which antibodies influence Cox 2 transcription remains unidentified. Future studies addressing the relationship between antibodies and Cox 2 transcription will be important not only for the development of therapeutics for IR-induced injury but also for other conditions in which a Cox 2-mediated inflammatory response is detrimental. The contribution of the Cox 3 isoform to IR-induced injury is another area of study that may be of therapeutic value.



The identification of IR-induced neo-antigens is an important step in further dissecting the early molecular events involved in the pathogenesis. The mechanism by which these neo-antigens, being large intracellular or serum proteins, are exposed to the extracellular milieu remains a mystery. It seems likely that facilitated transport of some sort, either direct or indirect, must occur to allow for the intracellular neo-antigens to traverse the membrane. Likewise, it is probable that some change at the membrane triggers the deposition of the serum neo-antigen,  $\beta_2$ -GPI.

PLSCR1 is sensitive to changes in  $O_2$  tension. Transcription and activity of PLSCR1 are promoted by hypoxia. The activity of PLSCR1 involves disruption of the cellular membrane, with phospholipids flipping between the inner and outer leaflets of the membrane. To successfully disrupt the membrane, this action is fairly non-specific and affects a relatively large area of the membrane. Accordingly, PLSCR1 activity could provide a means for externalization of intracellular neo-antigens. Going forward, with the knowledge that PLSCR1 is activated under hypoxic conditions (unpublished data), it will be important to assess the relationship between PLSCR1 activation and neo-antigen externalization. In addition to determining if the intracellular neo-antigens are exposed to the extracellular environment via PLSCR1 activity, studies examining the binding of  $\beta_2$ -GPI to endothelial cells following hypoxia in the absence of PLSCR1 will further clarify the potential value in pursuing PLSCR1 as a therapeutic target. Hypothesized to be one of the first cellular responses to hypoxia, PLSCR1 may become an important target for the development of IR-related therapeutics.

## **Acknowledgements**

This work was supported by grants from National Institutes of Health [R01 AI061691 to S.D.F, P20 GM103418, RR016475]; the American Heart Association [to S.D.F.]; the Kansas State University National Science Foundation GK-12 program [to E.A.S] and Kansas State University. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Institute of Health or National Science Foundation or Kansas State University.

## References

- [1] Y. Guan, R.T. Worrell, T.A. Pritts, M.H. Montrose, Intestinal ischemia-reperfusion injury: reversible and irreversible damage imaged in vivo, *Am. J. Physiol. Gastrointest. Liver Physiol.*, 297 (2009) G187-196.
- [2] W.A. Oldenburg, L.L. Lau, T.J. Rodenberg, H.J. Edmonds, C.D. Burger, Acute mesenteric ischemia: a clinical review, *Archives of Internal Medicine*, 164 (2004) 1054-1062.
- [3] U. Haglund, D. Bergqvist, Intestinal ischemia -- the basics, *Lang. Arch. Surg.*, 384 (1999) 233-238.
- [4] M. Stamatakos, C. Stefanaki, D. Mastrokalos, H. Arampatzi, P. Safioleas, C. Chatziconstantinou, C. Xiromeritis, M. Safioleas, Mesenteric ischemia: still a deadly puzzle for the medical community, *Tohoku J Exp Med*, 216 (2008) 197-204.
- [5] T. Noda, R. Iwakiri, K. Fujimoto, S. Matsuo, T.Y. Aw, Programmed cell death induced by ischemia-reperfusion in rat intestinal mucosa, *Am J Physiol*, 274 (1998) G270-276.
- [6] U. Haglund, Gut ischaemia, *Gut*, 35 (1994) S73-S76.
- [7] M.T. Droy-Lefaix, Y. Drouet, G. Geraud, D. Hosford, P. Braquet, Superoxide dismutase (SOD) and the PAF-antagonist (BN 52021) reduce small intestinal damage induced by ischemia-reperfusion, *Free radical research communications*, 12-13 Pt 2 (1991) 725-735.
- [8] P. Stringa, N. Lausada, D. Romanin, M. Machuca, A. Cabanne, M. Rumbo, G. Gondolesi, Defining the nonreturn time for intestinal ischemia reperfusion injury in mice, *Transplant Proc*, 44 (2012) 1214-1217.
- [9] S.A. Matyjaszek, A.J. Morton, D.E. Freeman, A. Grosche, M.M. Polyak, H. Kuck, Effects of flunixin meglumine on recovery of colonic mucosa from ischemia in horses, *American journal of veterinary research*, 70 (2009) 236-246.
- [10] V.L. Cook, J. Jones Shults, M.R. McDowell, N.B. Campbell, J.L. Davis, J.F. Marshall, A.T. Blikslager, Anti-inflammatory effects of intravenously administered lidocaine hydrochloride on ischemia-injured jejunum in horses, *American journal of veterinary research*, 70 (2009) 1259-1268.
- [11] A.J. Morton, A. Grosche, A.K. Rotting, S.A. Matyjaszek, A.T. Blikslager, D.E. Freeman, Expression of cyclooxygenase-1 and -2 in the left dorsal colon after different durations of ischemia and reperfusion in horses, *American journal of veterinary research*, 70 (2009) 1536-1544.
- [12] R.M. Dabareiner, N.A. White, 2nd, L. Donaldson, Evaluation of Carolina Rinse solution as a treatment for ischaemia reperfusion of the equine jejunum, *Equine veterinary journal*, 35 (2003) 642-646.
- [13] T. Moses, L. Wagner, S.D. Fleming, TLR4-mediated Cox-2 expression increases intestinal ischemia/reperfusion-induced damage, *J Leukoc Biol*, 86 (2009) 971-980.
- [14] N. Sato, R.A. Kozar, L. Zou, J.M. Weatherall, B. Attuwaybi, S.D. Moore-Olufemi, N.W. Weisbrodt, F.A. Moore, Peroxisome proliferator-activated receptor gamma mediates protection against cyclooxygenase-2-induced gut dysfunction in a rodent model of mesenteric ischemia/reperfusion, *Shock*, 24 (2005) 462-469.
- [15] H. Hilton, J.E. Nieto, P.F. Moore, F.A. Harmon, D.K. Naydan, J.R. Snyder, Expression of cyclooxygenase genes in the jejunum of horses during low-flow ischemia and reperfusion, *American journal of veterinary research*, 72 (2011) 681-686.

- [16] J.F. Marshall, A.T. Blikslager, The effect of nonsteroidal anti-inflammatory drugs on the equine intestine, *Equine Vet J*, 43 Suppl 39 (2011) 140-144.
- [17] A.T. Blikslager, D.N. Zimmel, K.M. Young, N.B. Campbell, D. Little, R.A. Argenzio, Recovery of ischaemic injured porcine ileum: evidence for a contributory role of COX-1 and COX-2, *Gut*, 50 (2002) 615-623.
- [18] T. Watanabe, A. Kobata, T. Tanigawa, Y. Nadatani, H. Yamagami, K. Watanabe, K. Tominaga, Y. Fujiwara, K. Takeuchi, T. Arakawa, Activation of the MyD88 signaling pathway inhibits ischemia-reperfusion injury in the small intestine, *Am. J. Physiol.*, 303 (2012) G324-334.
- [19] K. Kawata, I. Takeyoshi, K. Iwanami, Y. Sunose, H. Tsutsumi, S. Ohwada, K. Matsumoto, Y. Morishita, The effects of a selective cyclooxygenase-2 inhibitor on small bowel ischemia-reperfusion injury, *Hepato-gastroenterology*, 50 (2003) 1970-1974.
- [20] V.L. Cook, C.T. Meyer, N.B. Campbell, A.T. Blikslager, Effect of firocoxib or flunixin meglumine on recovery of ischemic-injured equine jejunum, *American journal of veterinary research*, 70 (2009) 992-1000.
- [21] M.J. Stuart, Prostaglandins and hemostasis: an overview, *Advances in pediatrics*, 30 (1983) 321-364.
- [22] K. Schror, Prostaglandins, other eicosanoids and endothelial cells, *Basic Res Cardiol*, 80 (1985) 502-514.
- [23] A.T. Blikslager, M.C. Roberts, J.M. Rhoads, R.A. Argenzio, Prostaglandins I2 and E2 have a synergistic role in rescuing epithelial barrier function in porcine ileum, *J. Clin. Invest.*, 100 (1997) 1928-1933.
- [24] K. Egan, G.A. FitzGerald, Eicosanoids and the vascular endothelium, *Handbook of experimental pharmacology*, (2006) 189-211.
- [25] M.R. Pope, S.M. Hoffman, S. Tomlinson, S.D. Fleming, Complement regulates TLR4-mediated inflammatory responses during intestinal ischemia reperfusion, *Mol. Immunol.*, 48 (2010) 356-364.
- [26] E.A. Slone, M.R. Pope, M. Roth, R. Welti, S.D. Fleming, TLR9 is dispensable for intestinal ischemia/reperfusion-induced tissue damage, *Am J Clin Exp Immunol*, 1 (2012) 124-135.
- [27] B.L. Sparkes, E.E. Slone, M. Roth, R. Welti, S.D. Fleming, Intestinal lipid alterations occur prior to antibody-induced prostaglandin E2 production in a mouse model of ischemia/reperfusion, *Biochim Biophys Acta*, 1801 (2010) 517-525.
- [28] R.H. Turnage, K.M. Kadesky, L. Bartula, K.S. Guice, K.T. Oldham, S.I. Myers, Splanchnic PGI2 release and "no reflow" following intestinal reperfusion, *J Surg Res*, 58 (1995) 558-564.
- [29] M.J. Mangino, C.B. Anderson, M.K. Murphy, E. Brunt, J. Turk, Mucosal arachidonate metabolism and intestinal ischemia-reperfusion injury, *Am J Physiol*, 257 (1989) G299-307.
- [30] I.F. Charo, S. Shak, M.A. Karasek, P.M. Davison, I.M. Goldstein, Prostaglandin I2 is not a major metabolite of arachidonic acid in cultured endothelial cells from human foreskin microvessels, *J Clin Invest*, 74 (1984) 914-919.
- [31] C.J. Hawkey, D.S. Rampton, Prostaglandins and the gastrointestinal mucosa: are they important in its function, disease, or treatment?, *Gastroenterology*, 89 (1985) 1162-1188.
- [32] J.U. Scher, M.H. Pillinger, The anti-inflammatory effects of prostaglandins, *Journal of investigative medicine : the official publication of the American Federation for Clinical Research*, 57 (2009) 703-708.
- [33] M.A. Bray, Leukotriene B4: an inflammatory mediator with vascular actions in vivo, *Agents and actions. Supplements*, 11 (1982) 51-61.

- [34] A. Grosche, A.J. Morton, A.S. Graham, J.F. Valentine, J.R. Abbott, M.M. Polyak, D.E. Freeman, Mucosal injury and inflammatory cells in response to brief ischaemia and reperfusion in the equine large colon, *Equine veterinary journal. Supplement*, (2011) 16-25.
- [35] J.M. Gayle, A.T. Blikslager, S.L. Jones, Role of neutrophils in intestinal mucosal injury, *Journal of the American Veterinary Medical Association*, 217 (2000) 498-500.
- [36] A. Karasawa, J.P. Guo, X.L. Ma, P.S. Tsao, A.M. Lefer, Protective actions of a leukotriene B4 antagonist in splanchnic ischemia and reperfusion in rats, *Am J Physiol*, 261 (1991) G191-198.
- [37] M.G. Oliver, R.D. Specian, M.A. Perry, D.N. Granger, Morphologic assessment of leukocyte-endothelial cell interactions in mesenteric venules subjected to ischemia and reperfusion, *Inflammation*, 15 (1991) 331-346.
- [38] M.B. Grisham, D.N. Granger, Neutrophil-mediated mucosal injury. Role of reactive oxygen metabolites, *Dig Dis Sci*, 33 (1988) 6S-15S.
- [39] D.J. Schmeling, M.G. Caty, K.T. Oldham, K.S. Guice, Cytoprotection by diclofenac sodium after intestinal ischemia/reperfusion injury, *Journal of pediatric surgery*, 29 (1994) 1044-1048.
- [40] D.R. Steiner, N.C. Gonzalez, J.G. Wood, Leukotriene B(4) promotes reactive oxidant generation and leukocyte adherence during acute hypoxia, *Journal of applied physiology*, 91 (2001) 1160-1167.
- [41] A.J. Casillan, N.C. Gonzalez, J.S. Johnson, D.R. Steiner, J.G. Wood, Mesenteric microvascular inflammatory responses to systemic hypoxia are mediated by PAF and LTB4, *J Appl Physiol*, 94 (2003) 2313-2322.
- [42] M.J. Mangino, M.K. Murphy, C.B. Anderson, Effects of the arachidonate 5-lipoxygenase synthesis inhibitor A-64077 in intestinal ischemia-reperfusion injury, *J. Pharmacol. Exp. Therap.*, 269 (1994) 75-81.
- [43] S. Cuzzocrea, A. Rossi, I. Serraino, R. Di Paola, L. Dugo, T. Genovese, A.P. Caputi, L. Sautebin, 5-lipoxygenase knockout mice exhibit a resistance to splanchnic artery occlusion shock, *Shock*, 20 (2003) 230-236.
- [44] S. Murthy, Q. Hui-Qi, T. Sakai, D.E. Depace, J.D. Fondacaro, Ischemia/reperfusion injury in the rat colon, *Inflammation*, 21 (1997) 173-190.
- [45] R.M. Moore, W.W. Muir, A.L. Bertone, J.L. Oliver, Effect of platelet-activating factor antagonist L-691,880 on low-flow ischemia-reperfusion injury of the large colon in horses, *Veterinary surgery : VS*, 27 (1998) 37-48.
- [46] R.H. Turnage, J.L. LaNoue, K.M. Kadesky, Y. Meng, S.I. Myers, Thromboxane A2 mediates increased pulmonary microvascular permeability after intestinal reperfusion, *Journal of applied physiology*, 82 (1997) 592-598.
- [47] J. Hill, T.F. Lindsay, F. Ortiz, C.G. Yeh, H.B. Hechtman, F.D. Moore, Soluble complement receptor type 1 ameliorates the local and remote organ injury after intestinal ischemia-reperfusion in the rat, *J. Immunol.*, 149 (1992) 1723-1728.
- [48] A.T. Eror, A. Stojadinovic, B.W. Starnes, S.C. Makrides, G.C. Tsokos, T. Shea-Donohue, Anti-inflammatory effects of soluble complement receptor type 1 promote rapid recovery of ischemia/reperfusion injury in rat small intestine, *Clin. Immunol.*, 90 (1999) 266-275.
- [49] G.L. Stahl, Y. Xu, L. Hao, M. Miller, J.A. Buras, M. Fung, H. Zhao, Role for the alternate complement pathway in ischemia/reperfusion injury, *Am. J. Pathol.*, 162 (2003) 449-455.
- [50] T.V. Arumugam, T. Magnus, T.M. Woodruff, L.M. Proctor, I.A. Shiels, S.M. Taylor, Complement mediators in ischemia-reperfusion injury, *Clinica Chimica Acta*, (2006).

- [51] M.L. Hart, K.A. Ceonzo, L.A. Shaffer, K. Takahashi, R.P. Rother, W.R. Reenstra, J.A. Buras, G.L. Stahl, Gastrointestinal ischemia-reperfusion injury is lectin complement pathway dependent without involving C1q, *J. Immunol.*, 174 (2005) 6373-6380.
- [52] M. Zhang, K. Takahashi, E.M. Alicot, T. Vorup-Jensen, B. Kessler, S. Thiel, J.C. Jensenius, R.A. Ezekowitz, F.D. Moore, M.C. Carroll, Activation of the lectin pathway by natural IgM in a model of ischemia/reperfusion injury, *J. Immunol.*, 177 (2006) 4727-4734.
- [53] G. Karpel-Massler, S.D. Fleming, M. Kirschfink, G.C. Tsokos, Human C1 esterase inhibitor attenuates murine mesenteric ischemia/reperfusion induced local organ injury, *J Surg Res*, 115 (2003) 247-256.
- [54] S. Rehrig, S.D. Fleming, J. Anderson, J.M. Guthridge, J. Rakstang, C.E. McQueen, V.M. Holers, G.C. Tsokos, T. Shea-Donohue, Complement inhibitor, complement receptor 1-related gene/protein  $\gamma$ -Ig attenuates intestinal damage after the onset of mesenteric ischemia/reperfusion injury in mice, *J. Immunol.*, 167 (2001) 5921-5927.
- [55] J.P. Williams, T.T.V. Pechet, M.R. Weiser, R. Reid, L. Kobzik, F.D. Moore, M.C. Carroll, H.B. Hechtman, Intestinal reperfusion injury is mediated by IgM and complement, *J. Appl. Physiol.*, 86 (1999) 938-942.
- [56] M. Zhang, E.M. Alicot, M.C. Carroll, Human natural IgM can induce ischemia/reperfusion injury in a murine intestinal model, *Mol. Immunol.*, 45 (2008) 4036-4039.
- [57] M.R. Weiser, J.P. Williams, F.D. Moore, L. Kobzik, M. Ma, H.B. Hechtman, M.C. Carroll, Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement, *J. Exp. Med.*, 183 (1996) 2343-2348.
- [58] S.D. Fleming, T. Shea-Donohue, J.M. Guthridge, L. Kulik, T.J. Waldschmidt, M.G. Gipson, G.C. Tsokos, V.M. Holers, Mice deficient in complement receptors 1 and 2 lack a tissue injury-inducing subset of the natural antibody repertoire, *J Immunol*, 169 (2002) 2126-2133.
- [59] R.R. Reid, S. Woodcock, A. Shimabukuro-Vornhagen, W.G. Austen, L. Kobzik, M. Zhang, H.B. Hechtman, F.D. Moore, M.C. Carroll, Functional activity of natural antibody is altered in Cr2-deficient mice, *J. Immunol.*, 169 (2002) 5433-5400.
- [60] S.D. Fleming, T. Shea-Donohue, J.M. Guthridge, L. Kulik, T.J. Waldschmidt, M.G. Gipson, G.C. Tsokos, V.M. Holers, Mice deficient in complement receptors 1 and 2 lack a tissue injury-inducing subset of the natural antibody repertoire, *J. Immunol.*, 169 (2002) 2126-2133.
- [61] S.D. Fleming, R.P. Egan, C. Chai, G. Girardi, V.M. Holers, J. Salmon, M. Monestier, G.C. Tsokos, Anti-phospholipid antibodies restore mesenteric ischemia/reperfusion-induced injury in complement receptor 2/complement receptor 1-deficient mice, *J. Immunol.*, 173 (2004) 7055-7061.
- [62] S. Miyakis, B. Giannakopoulos, S.A. Krilis, Beta 2 glycoprotein I--function in health and disease, *Thromosis Research*, 114 (2004) 335-346.
- [63] P.L. Meroni, E. Raschi, C. Testoni, A. Parisio, M.O. Borghi, Innate immunity in the antiphospholipid syndrome: role of toll-like receptors in endothelial cell activation by antiphospholipid antibodies, *Autoimmun. Rev.*, 3 (2004) 510-515.
- [64] S.D. Fleming, M.R. Pope, S.M. Hoffman, T. Moses, U. Bukovnik, J.M. Tomich, L.M. Wagner, K.M. Woods, Domain V peptides inhibit beta2-glycoprotein I-mediated mesenteric ischemia/reperfusion-induced tissue damage and inflammation, *J Immunol*, 185 (2010) 6168-6178.
- [65] M. Tomasi, Y. Hiromasa, M.R. Pope, S. Gudlar, J.M. Tomich, S.D. Fleming, Human B2-glycoprotein I attenuates mouse intestinal ischemia/reperfusion induced injury and inflammation, *Mol. Immunol.*, 52 (2012) 207-216.

- [66] L. Kulik, S.D. Fleming, C. Moratz, J.W. Reuter, A. Novikov, K. Chen, K.A. Andrews, A. Markaryan, R.J. Quigg, G.J. Silverman, G.C. Tsokos, V.M. Holers, Pathogenic natural antibodies recognizing annexin IV are required to develop intestinal ischemia-reperfusion injury, *J Immunol*, 182 (2009) 5363-5373.
- [67] A. Elvington, C. Atkinson, L. Kulik, H. Zhu, J. Yu, M.S. Kindy, V.M. Holers, S. Tomlinson, Pathogenic natural antibodies propagate cerebral injury following ischemic stroke in mice, *J. Immunol.*, 188 (2012) 1460-1468.
- [68] M. Zhang, E.M. Alicot, I. Chiu, J. Li, N. Verna, T. Vorup-Jensen, B. Kessler, M. Shimaoka, R. Chan, D. Friend, U. Mahmood, R. Weissleder, F.D. Moore, M.C. Carroll, Identification of the target self-antigens in reperfusion injury, *J Exp Med*, 203 (2006) 141-152.
- [69] L. Kulik, S.D. Fleming, C. Moratz, J.W. Reuter, A. Novikov, K. Chen, K.A. Andrews, A. Markaryan, R.J. Quigg, G.J. Silverman, G.C. Tsokos, V.M. Holers, Pathogenic natural antibodies recognizing annexin IV are required to develop intestinal ischemia-reperfusion injury, *J. Immunol.*, 182 (2009) 5363-5373.
- [70] M. Zhang, E.M. Alicot, I. Chiu, J. Li, N. Verna, T. Vorup-Jensen, B. Kessler, M. Shimaoka, R. Chan, D. Friend, U. Mahmood, R. Weissleder, F.D. Moore, M.C. Carroll, Identification of the target self-antigens in reperfusion injury, *J. Exp. Med.*, 203 (2006) 141-152.
- [71] R.K. Chan, N. Verna, J. Afnan, M. Zhang, S. Ibrahim, M.C. Carroll, F.D. Moore, Jr., Attenuation of skeletal muscle reperfusion injury with intravenous 12 amino acid peptides that bind to pathogenic IgM, *Surgery*, 139 (2006) 236-243.
- [72] M.S. Haas, E.M. Alicot, F. Schuerpf, I. Chiu, J. Li, F.D. Moore, M.C. Carroll, Blockade of self-reactive IgM significantly reduces injury in a murine model of acute myocardial infarction, *Cardiovasc. Res.*, 87 (2010) 618-627.
- [73] A.M. Hicks, C.J. DeLong, M.J. Thomas, M. Samuel, Z. Cui, Unique molecular signatures of glycerophospholipid species in different rat tissues analyzed by tandem mass spectrometry, *Biochim Biophys Acta*, 1761 (2006) 1022-1029.
- [74] Y.Y. Tyurina, V.A. Tyurin, M.W. Epperly, J.S. Greenberger, V.E. Kagan, Oxidative lipidomics of gamma-irradiation-induced intestinal injury, *Free Radic Biol Med*, 44 (2008) 299-314.
- [75] A. Braun, I. Treede, D. Gotthardt, A. Tietje, A. Zahn, R. Ruhwald, U. Schoenfeld, T. Welsch, P. Kienle, G. Erben, W.D. Lehmann, J. Fuellekrug, W. Stremmel, R. Ehehalt, Alterations of phospholipid concentration and species composition of the intestinal mucus barrier in ulcerative colitis: a clue to pathogenesis, *Inflamm Bowel Dis*, 15 (2009) 1705-1720.
- [76] C.C. Leslie, Properties and regulation of cytosolic phospholipase A2, *J Biol Chem*, 272 (1997) 16709-16712.
- [77] B.O. Anderson, E.E. Moore, A. Banerjee, Phospholipase A2 regulates critical inflammatory mediators of multiple organ failure, *J Surg Res*, 56 (1994) 199-205.
- [78] J.D. Clark, A.R. Schievella, E.A. Nalefski, L.L. Lin, Cytosolic phospholipase A2, *Journal of lipid mediators and cell signalling*, 12 (1995) 83-117.
- [79] M.D. Lister, R.A. Deems, Y. Watanabe, R.J. Ulevitch, E.A. Dennis, Kinetic analysis of the Ca<sup>2+</sup>-dependent, membrane-bound, macrophage phospholipase A2 and the effects of arachidonic acid, *J Biol Chem*, 263 (1988) 7506-7513.
- [80] L.L. Lin, A.Y. Lin, J.L. Knopf, Cytosolic phospholipase A2 is coupled to hormonally regulated release of arachidonic acid, *Proc Natl Acad Sci U S A*, 89 (1992) 6147-6151.
- [81] L.L. Lin, M. Wartmann, A.Y. Lin, J.L. Knopf, A. Seth, R.J. Davis, cPLA2 is phosphorylated and activated by MAP kinase, *Cell*, 72 (1993) 269-278.

- [82] K. Schoonderwoerd, H. Stam, Lipid metabolism of myocardial endothelial cells, *Mol Cell Biochem*, 116 (1992) 171-179.
- [83] C. Rink, S. Khanna, Significance of brain tissue oxygenation and the arachidonic acid cascade in stroke, *Antioxid Redox Signal*, 14 (2011) 1889-1903.
- [84] S.C. Frasch, D.L. Bratton, Emerging roles for lysophosphatidylserine in resolution of inflammation, *Prog Lipid Res*, 51 (2012) 199-207.
- [85] E.J. Goetzl, W. Wang, C. McGiffert, M.C. Huang, M.H. Graler, Sphingosine 1-phosphate and its G protein-coupled receptors constitute a multifunctional immunoregulatory system, *J Cell Biochem*, 92 (2004) 1104-1114.
- [86] K. Kotarsky, A. Boketoft, J. Bristulf, N.E. Nilsson, A. Norberg, S. Hansson, C. Owman, R. Sillard, L.M. Leeb-Lundberg, B. Olde, Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes, *J Pharmacol Exp Ther*, 318 (2006) 619-628.
- [87] O.J. Hines, N. Ryder, J. Chu, D. McFadden, Lysophosphatidic acid stimulates intestinal restitution via cytoskeletal activation and remodeling, *J Surg Res*, 92 (2000) 23-28.
- [88] H. Lum, Lysophospholipids in the regulation of endothelial barrier function, *Am J Physiol Lung Cell Mol Physiol*, 281 (2001) L1335-1336.
- [89] M. Matsubara, K. Hasegawa, Benidipine, a dihydropyridine-calcium channel blocker, prevents lysophosphatidylcholine-induced injury and reactive oxygen species production in human aortic endothelial cells, *Atherosclerosis*, 178 (2005) 57-66.
- [90] P. Chaudhuri, S.M. Colles, D.S. Damron, L.M. Graham, Lysophosphatidylcholine inhibits endothelial cell migration by increasing intracellular calcium and activating calpain, *Arterioscler Thromb Vasc Biol*, 23 (2003) 218-223.
- [91] P.A. Leventis, S. Grinstein, The distribution and function of phosphatidylserine in cellular membranes, *Annu Rev Biophys*, 39 (2010) 407-427.
- [92] V.A. Tyurin, Y.Y. Tyurina, P.M. Kochanek, R. Hamilton, S.T. DeKosky, J.S. Greenberger, H. Bayir, V.E. Kagan, Oxidative lipidomics of programmed cell death, *Methods Enzymol*, 442 (2008) 375-393.
- [93] B. Fadeel, D. Xue, The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease, *Crit Rev Biochem Mol Biol*, 44 (2009) 264-277.
- [94] D. Smrz, P. Lebduska, L. Draberova, J. Korb, P. Draber, Engagement of phospholipid scramblase 1 in activated cells: implication for phosphatidylserine externalization and exocytosis, *J Biol Chem*, 283 (2008) 10904-10918.
- [95] A. Yamaji-Hasegawa, M. Tsujimoto, Asymmetric distribution of phospholipids in biomembranes, *Biol Pharm Bull*, 29 (2006) 1547-1553.
- [96] C. Michiels, T. Arnould, I. Knott, M. Dieu, J. Remacle, Stimulation of prostaglandin synthesis by human endothelial cells exposed to hypoxia, *Am J Physiol*, 264 (1993) C866-874.
- [97] T. Otamiri, L. Franzen, D. Lindmark, C. Tagesson, Increased phospholipase A2 and decreased lysophospholipase activity in the small intestinal mucosa after ischaemia and revascularisation, *Gut*, 28 (1987) 1445-1453.
- [98] T. Otamiri, C. Tagesson, Role of phospholipase A2 and oxygenated free radicals in mucosal damage after small intestinal ischemia and reperfusion, *Am J Surg*, 157 (1989) 562-565; discussion 566.
- [99] D.K. Das, R.M. Engelman, H. Otani, J.A. Rousou, R.H. Breyer, S. Lemeshow, Effect of superoxide dismutase and catalase on myocardial energy metabolism during ischemia and reperfusion, *Clin Physiol Biochem*, 4 (1986) 187-198.

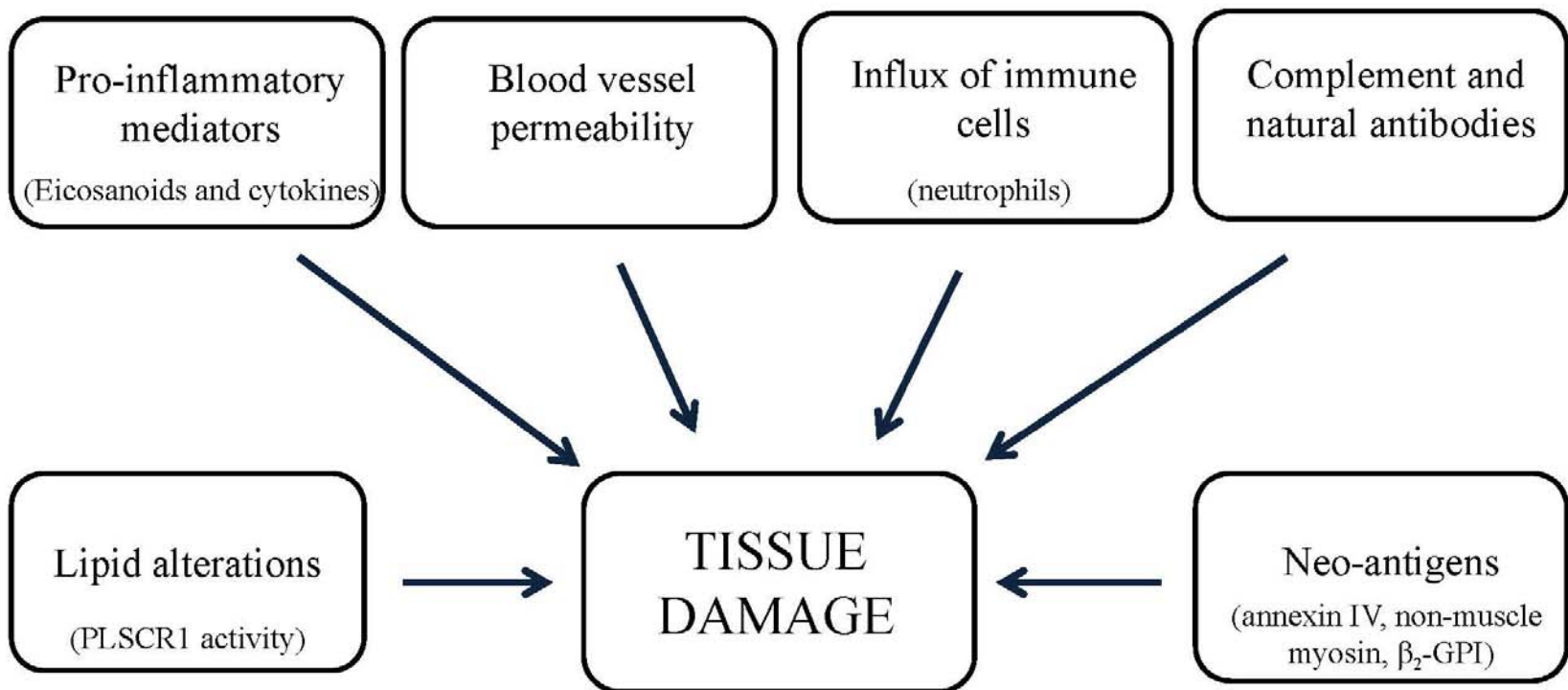


- [100] N.A. Shaikh, E. Downar, Time course of changes in porcine myocardial phospholipid levels during ischemia. A reassessment of the lysolipid hypothesis, *Circ. Res.*, 49 (1981) 316-325.
- [101] H. Kawaguchi, M. Shoki, K. Iizuka, H. Sano, Y. Sakata, H. Yasuda, Phospholipid metabolism and prostacyclin synthesis in hypoxic myocytes, *Biochim Biophys Acta*, 1094 (1991) 161-167.
- [102] A. Drgova, K. Likavcanova, D. Dobrota, Changes of phospholipid composition and superoxide dismutase activity during global brain ischemia and reperfusion in rats, *Gen Physiol Biophys*, 23 (2004) 337-346.
- [103] E.R. Block, J.M. Patel, D. Edwards, Mechanism of hypoxic injury to pulmonary artery endothelial cell plasma membranes, *Am J Physiol*, 257 (1989) C223-231.
- [104] G.B. Bhat, E.R. Block, Effect of hypoxia on phospholipid metabolism in porcine pulmonary artery endothelial cells, *Am J Physiol*, 262 (1992) L606-613.
- [105] F. Oudot, C. Cordelet, J.P. Sergiel, A. Grynberg, Polyunsaturated fatty acids influence prostanoid synthesis in vascular endothelial cells under hypoxia and reoxygenation, *Int J Vitam Nutr Res*, 68 (1998) 263-271.
- [106] T.J. Cunningham, L. Yao, A. Lucena, Product inhibition of secreted phospholipase A2 may explain lysophosphatidylcholines' unexpected therapeutic properties, *J Inflamm (Lond)*, 5 (2008) 17.
- [107] C. Tagesson, L. Franzen, G. Dahl, B. Westrom, Lysophosphatidylcholine increases rat ileal permeability to macromolecules, *Gut*, 26 (1985) 369-377.
- [108] T. Otamiri, R. Sjodahl, C. Tagesson, Lysophosphatidylcholine potentiates the increase in mucosal permeability after small-intestinal ischaemia, *Scand J Gastroenterol*, 21 (1986) 1131-1136.
- [109] S. Pacheco, K. Hillier, C. Smith, Increased arachidonic acid levels in phospholipids of human colonic mucosa in inflammatory bowel disease, *Clin Sci (Lond)*, 73 (1987) 361-364.
- [110] H. Morita, K. Nakanishi, T. Dohi, E. Yasugi, M. Oshima, Phospholipid turnover in the inflamed intestinal mucosa: arachidonic acid-rich phosphatidyl/plasmenyl-ethanolamine in the mucosa in inflammatory bowel disease, *J Gastroenterol*, 34 (1999) 46-53.
- [111] J.H. Kramer, B.F. Dickens, V. Misik, W.B. Weglicki, Phospholipid hydroperoxides are precursors of lipid alkoxy radicals produced from anoxia/reoxygenated endothelial cells, *J Mol Cell Cardiol*, 27 (1995) 371-381.
- [112] W.R. Bishop, R.M. Bell, Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movement and intracellular translocation, *Annual review of cell biology*, 4 (1988) 579-610.
- [113] J. Baranska, Biosynthesis and transport of phosphatidylserine in the cell, *Advances in lipid research*, 19 (1982) 163-184.
- [114] M. Seigneuret, P.F. Devaux, ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes, *Proc Natl Acad Sci U S A*, 81 (1984) 3751-3755.
- [115] G. Morrot, P. Herve, A. Zachowski, P. Fellmann, P.F. Devaux, Aminophospholipid translocase of human erythrocytes: phospholipid substrate specificity and effect of cholesterol, *Biochemistry*, 28 (1989) 3456-3462.
- [116] J. Connor, C.H. Pak, R.F. Zwaal, A.J. Schroit, Bidirectional transbilayer movement of phospholipid analogs in human red blood cells. Evidence for an ATP-dependent and protein-mediated process, *J Biol Chem*, 267 (1992) 19412-19417.

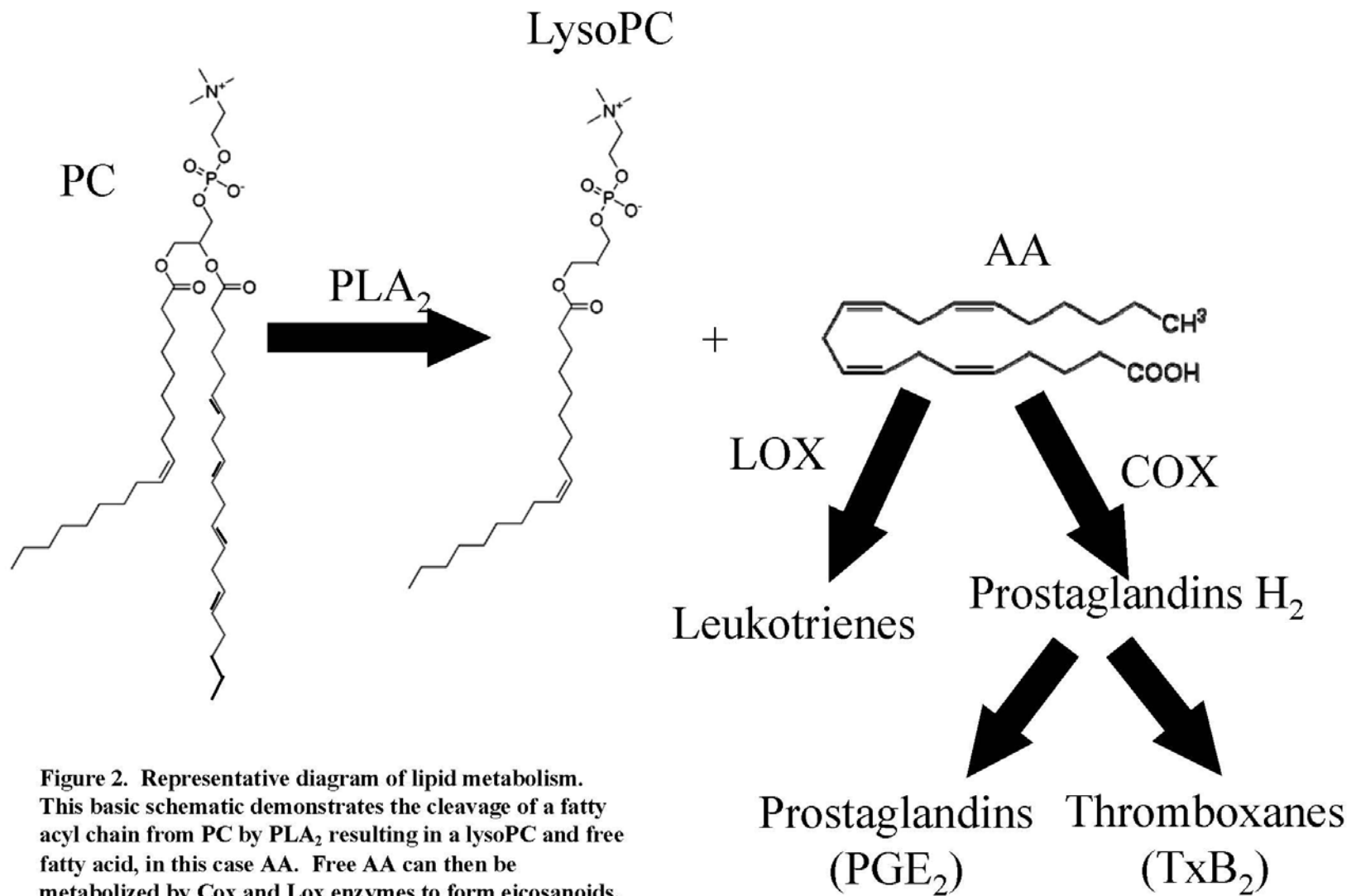
- [117] Z. Beleznyay, A. Zachowski, P.F. Devaux, M.P. Navazo, P. Ott, ATP-dependent aminophospholipid translocation in erythrocyte vesicles: stoichiometry of transport, *Biochemistry*, 32 (1993) 3146-3152.
- [118] M. Julien, J.F. Tournier, J.F. Tocanne, Basic fibroblast growth factor modulates the aminophospholipid translocase activity present in the plasma membrane of bovine aortic endothelial cells, *Eur J Biochem*, 230 (1995) 287-297.
- [119] C. Andrick, K. Broring, B. Deuticke, C.W. Haest, Fast translocation of phosphatidylcholine to the outer membrane leaflet after its synthesis at the inner membrane surface in human erythrocytes, *Biochim Biophys Acta*, 1064 (1991) 235-241.
- [120] T. Wiedmer, Q. Zhou, D.Y. Kwok, P.J. Sims, Identification of three new members of the phospholipid scramblase gene family, *Biochim Biophys Acta*, 1467 (2000) 244-253.
- [121] V.G. Francis, A.M. Mohammed, G.K. Aradhyam, S.N. Gummadi, The single C-terminal helix of human phospholipid scramblase 1 is required for membrane insertion and scrambling activity, *FEBS J*, 280 (2013) 2855-2869.
- [122] S.K. Sahu, S.N. Gummadi, N. Manoj, G.K. Aradhyam, Phospholipid scramblases: an overview, *Arch Biochem Biophys*, 462 (2007) 103-114.
- [123] P.J. Sims, T. Wiedmer, Unraveling the mysteries of phospholipid scrambling, *Thromb Haemost*, 86 (2001) 266-275.
- [124] E. Bevers, Comfurius, P, Zwaal, RFA, Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiologic implications, *Lupus*, 5 (1996) 480-487.
- [125] P. Williamson, A. Kulick, A. Zachowski, R.A. Schlegel, P.F. Devaux, Ca<sup>2+</sup> induces transbilayer redistribution of all major phospholipids in human erythrocytes, *Biochemistry*, 31 (1992) 6355-6360.
- [126] P. Williamson, E.M. Bevers, E.F. Smeets, P. Comfurius, R.A. Schlegel, R.F. Zwaal, Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets, *Biochemistry*, 34 (1995) 10448-10455.
- [127] E.M. Bevers, P.L. Williamson, Phospholipid scramblase: an update, *FEBS Lett*, 584 (2010) 2724-2730.
- [128] F. Basse, J.G. Stout, P.J. Sims, T. Wiedmer, Isolation of an erythrocyte membrane protein that mediates Ca<sup>2+</sup>-dependent transbilayer movement of phospholipid, *J Biol Chem*, 271 (1996) 17205-17210.
- [129] Q. Zhou, J. Zhao, J.G. Stout, R.A. Luhm, T. Wiedmer, P.J. Sims, Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids, *J Biol Chem*, 272 (1997) 18240-18244.
- [130] J.G. Stout, F. Basse, R.A. Luhm, H.J. Weiss, T. Wiedmer, P.J. Sims, Scott syndrome erythrocytes contain a membrane protein capable of mediating Ca<sup>2+</sup>-dependent transbilayer migration of membrane phospholipids, *J Clin Invest*, 99 (1997) 2232-2238.
- [131] S.C. Frasch, P.M. Henson, K. Nagaosa, M.B. Fessler, N. Borregaard, D.L. Bratton, Phospholipid flip-flop and phospholipid scramblase 1 (PLSCR1) co-localize to uropod rafts in formylated Met-Leu-Phe-stimulated neutrophils, *J Biol Chem*, 279 (2004) 17625-17633.
- [132] T. Wiedmer, J. Zhao, M. Nanjundan, P.J. Sims, Palmitoylation of phospholipid scramblase 1 controls its distribution between nucleus and plasma membrane, *Biochemistry*, 42 (2003) 1227-1233.

- [133] J. Sun, M. Nanjundan, L.J. Pike, T. Wiedmer, P.J. Sims, Plasma membrane phospholipid scramblase 1 is enriched in lipid rafts and interacts with the epidermal growth factor receptor, *Biochemistry*, 41 (2002) 6338-6345.
- [134] S. Ory, M. Ceridono, F. Momboisse, S. Houy, S. Chasserot-Golaz, D. Heintz, V. Calco, A.M. Haerberle, F.A. Espinoza, P.J. Sims, Y. Bailly, M.F. Bader, S. Gasman, Phospholipid scramblase-1-induced lipid reorganization regulates compensatory endocytosis in neuroendocrine cells, *J Neurosci*, 33 (2013) 3545-3556.
- [135] J. Liu, Q. Dai, J. Chen, D. Durrant, A. Freeman, T. Liu, D. Grossman, R.M. Lee, Phospholipid scramblase 3 controls mitochondrial structure, function, and apoptotic response, *Mol Cancer Res*, 1 (2003) 892-902.
- [136] V.G. Francis, M.A. Majeed, S.N. Gummadi, Recovery of functionally active recombinant human phospholipid scramblase 1 from inclusion bodies using N-lauroyl sarcosine, *J Ind Microbiol Biotechnol*, 39 (2012) 1041-1048.
- [137] S.K. Sahu, G.K. Aradhyam, S.N. Gummadi, Calcium binding studies of peptides of human phospholipid scramblases 1 to 4 suggest that scramblases are new class of calcium binding proteins in the cell, *Biochim Biophys Acta*, 1790 (2009) 1274-1281.
- [138] J.G. Stout, Q. Zhou, T. Wiedmer, P.J. Sims, Change in conformation of plasma membrane phospholipid scramblase induced by occupancy of its Ca<sup>2+</sup> binding site, *Biochemistry*, 37 (1998) 14860-14866.
- [139] L. Sanchez-Magraner, I.M. Posada, N. Andraka, F.X. Contreras, A.R. Viguera, D.M. Guerin, J.L. Arrondo, H.L. Monaco, F.M. Goni, The C-terminal transmembrane domain of human phospholipid scramblase 1 is essential for the protein flip-flop activity and Ca(2+)-binding, *J Membr Biol*, 247 (2014) 155-165.
- [140] S.C. Frasch, P.M. Henson, J.M. Kailey, D.A. Richter, M.S. Janes, V.A. Fadok, D.L. Bratton, Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta, *J Biol Chem*, 275 (2000) 23065-23073.
- [141] J. Merregaert, J. Van Langen, U. Hansen, P. Ponsaerts, A. El Ghalbzouri, E. Steenackers, X. Van Ostade, S. Sercu, Phospholipid scramblase 1 is secreted by a lipid raft-dependent pathway and interacts with the extracellular matrix protein 1 in the dermal epidermal junction zone of human skin, *J Biol Chem*, 285 (2010) 37823-37837.
- [142] J. Zhao, Q. Zhou, T. Wiedmer, P.J. Sims, Palmitoylation of phospholipid scramblase is required for normal function in promoting Ca<sup>2+</sup>-activated transbilayer movement of membrane phospholipids, *Biochemistry*, 37 (1998) 6361-6366.
- [143] I. Ben-Efraim, Q. Zhou, T. Wiedmer, L. Gerace, P.J. Sims, Phospholipid scramblase 1 is imported into the nucleus by a receptor-mediated pathway and interacts with DNA, *Biochemistry*, 43 (2004) 3518-3526.
- [144] M.H. Chen, I. Ben-Efraim, G. Mitrousis, N. Walker-Kopp, P.J. Sims, G. Cingolani, Phospholipid scramblase 1 contains a nonclassical nuclear localization signal with unique binding site in importin alpha, *J Biol Chem*, 280 (2005) 10599-10606.
- [145] A. Rami, J. Sims, G. Botez, J. Winckler, Spatial resolution of phospholipid scramblase 1 (PLSCR1), caspase-3 activation and DNA-fragmentation in the human hippocampus after cerebral ischemia, *Neurochem Int*, 43 (2003) 79-87.
- [146] T. Inuzuka, A. Inokawa, C. Chen, K. Kizu, H. Narita, H. Shibata, M. Maki, ALG-2-interacting Tubby-like protein superfamily member PLSCR3 is secreted by exosomal pathway and taken up by recipient cultured cells, *Biosci. Rep.*, (2013).

[147] S.D. Fleming, R.P. Egan, C. Chai, G. Girardi, V.M. Holers, J. Salmon, M. Monestier, G.C. Tsokos, Anti-phospholipid antibodies restore mesenteric ischemia/reperfusion-induced injury in complement receptor 2/complement receptor 1-deficient mice, *J Immunol*, 173 (2004) 7055-7061.

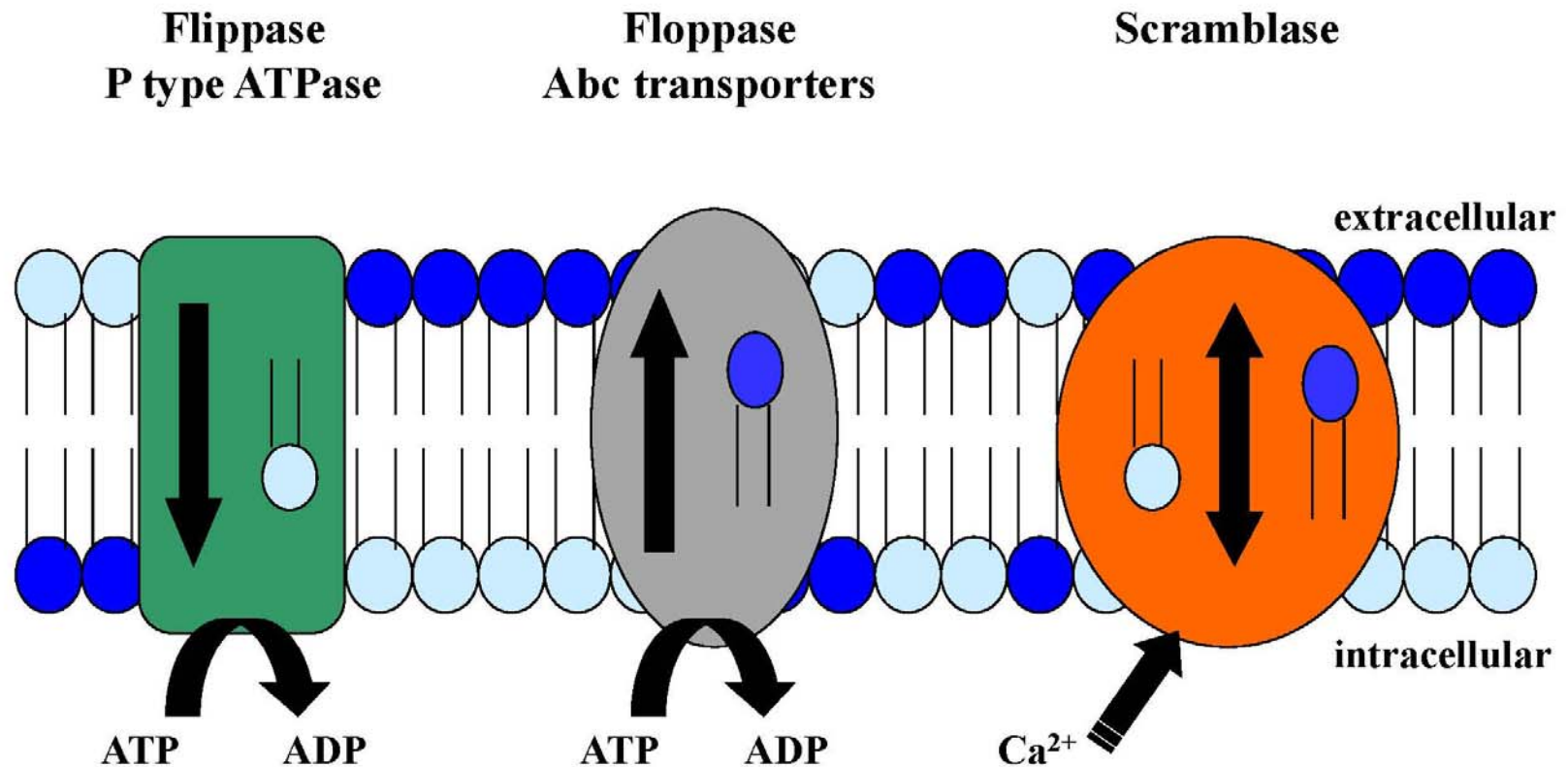


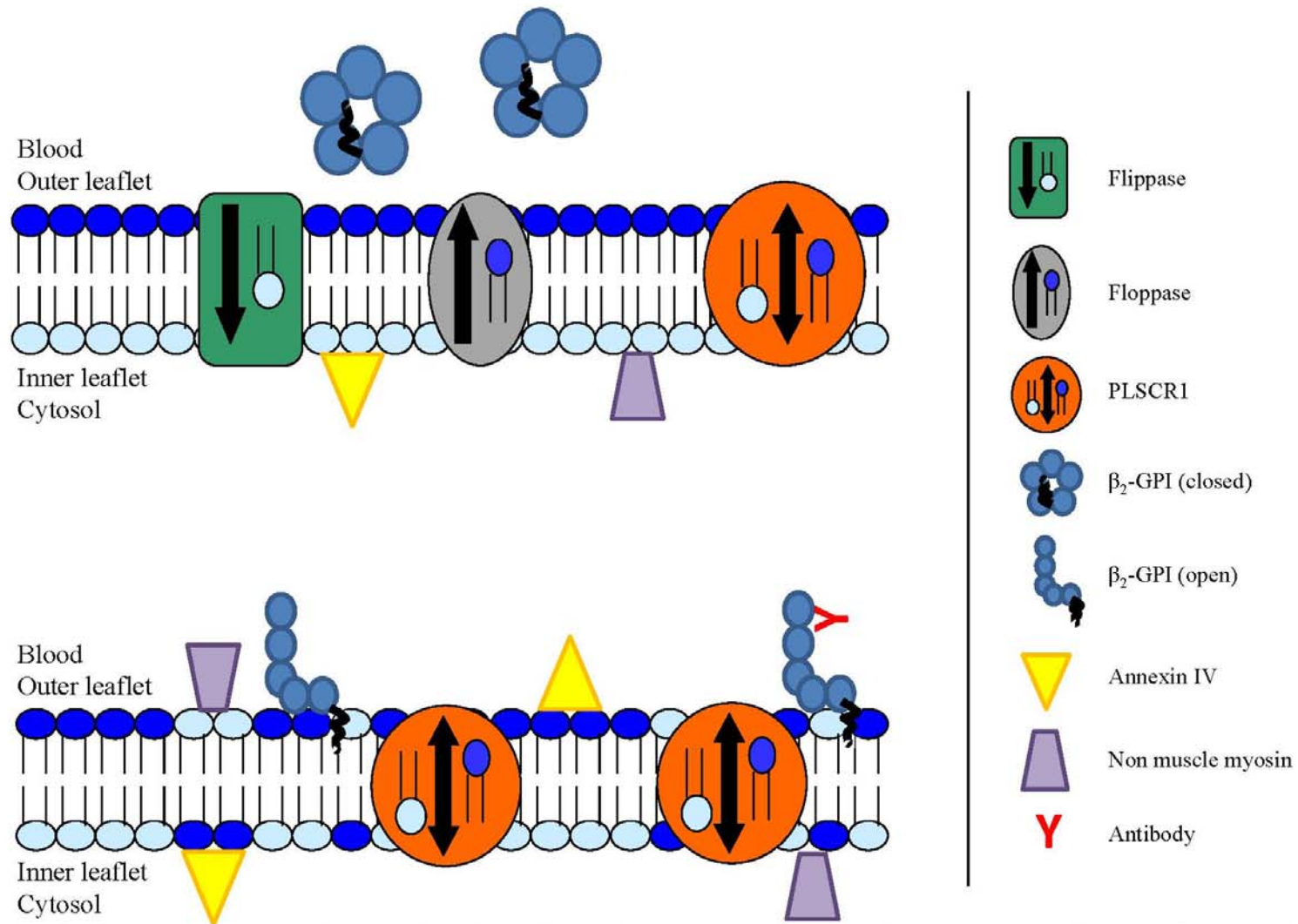
**Figure 1. Factors contributing to IR-induced tissue damage. The pathogenesis of IR-induced injury is complex with many contributing factors. Shown are a few of the factors involved.**



**Figure 2. Representative diagram of lipid metabolism.** This basic schematic demonstrates the cleavage of a fatty acyl chain from PC by  $PLA_2$  resulting in a lysoPC and free fatty acid, in this case AA. Free AA can then be metabolized by Cox and Lox enzymes to form eicosanoids.

**Figure 3. Schematic of membrane proteins involved in regulating phospholipid asymmetry. The three main classes of proteins involved in regulating the phospholipid bilayer are illustrated. Flippases and floppases are ATP-dependent and move specific phospholipids in one direction. Scramblases are activated by calcium and transport many different phospholipids in both directions.**





**Figure 4. Proposed model of early events in the pathogenesis of IR-induced injury.** During normoxia, the asymmetry of the bilayer is maintained by flippases, floppases and scramblases. The neo-antigen  $\beta_2$ -GPI circulates in a closed conformation and annexin IV and non-muscle myosin, additional neo-antigens, remain intracellular. Upon oxygen deprivation (ischemia or hypoxia) PLSCR1 is activated and the bilayer asymmetry disrupted with translocation of anionic phospholipids such as PS to the outer leaflet. Upon binding anionic phospholipids,  $\beta_2$ -GPI adopts an open conformation exposing an epitope for antibody binding which can serve as a trigger for initiation of the complement cascade and inflammatory responses. The intracellular neo-antigens may also be exposed to the extracellular environment as a result of PLSCR1 activation.