HYPOXIA-INDUCED LIPID CHANGES AND THEIR EFFECT ON INNATE IMMUNITY

by

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B.S., Kansas State University, 2011

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Ischemia/reperfusion (IR) events result in severe tissue damage and often death. The complex network of molecular and cellular mechanisms that contributes to intestinal IR-induced pathology has hindered a comprehensive understanding of IR-induced injury and limited the success of medical intervention. Although several of the mechanisms contributing to intestinal IR-induced injury have been identified, the initiating event(s) remains unclear. Mouse models have been instrumental in the unraveling of the many components and interactions that ultimately result in tissue damage. It is clear that leukocyte infiltration, complement activation, eicosanoid and pro-inflammatory cytokine production are involved. Toll-like receptors and antibodies also play critical roles. Based on the literature, and especially data demonstrating a significant role for anti-phospholipid antibodies, we hypothesized that ischemia induces phospholipid alterations that result in the exposure of a neoantigen which is recognized by anti-phospholipid antibodies. Furthermore, we hypothesized that endothelial cells are the primary cell type involved in the initial molecular events that result in intestinal IR-induced pathology. A mouse model of intestinal IR as well as an in vitro cell culture system was used to explore these hypotheses. Mass spectrometry-based lipidomics was utilized to assess lipid responses to IR and hypoxia/re-oxygenation (HR). No inherent differences in intestinal phospholipid composition were found between wildtype and several strains of knock-out mice. It was determined that the lack of antibody production by *Rag-1*$^{-/-}$ mice is responsible for protection against intestinal IR-induced injury, as antibody is needed to induce prostaglandin E_2 production, through up-regulation of cyclooxygenase 2 transcription. Unexpectedly, the presence or absence of toll-like receptor 9 was found to be inconsequential for tissue damage caused by intestinal IR. The results of several analyses point to endothelial cells as being directly involved in IR-induced pathology. Importantly, the activation of phospholipid scramblase 1 has been identified as a potential molecular mechanism by which subsequent molecular and cellular responses are elicited as a consequence of IR.

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Abbreviations

4-HNE	4-hydroxynonenal
AA	arachidonic acid
Ab	antibody
APS	Antiphospholipid Syndrome
ATP	adenosine tri-phosphate
β2-GPI	beta2-glycoprotein 1
BCA	bicinchoninic acid
cAMP	cyclic adenosine mono-phosphate
cDNA	complementary deoxyribonucleic acid
Cox	cyclooxygenase
CpG	cytidine-phosphate-guanosine
$cPLA_2$	cytosolic phospholipase A2
CR2	complement receptor 2
CTP	cytidine tri-phosphate
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
ePC	ether linked phosphatidylcholine
ePE	ether linked phosphatidylethanolamine
ESI-MS/MS	electrospray ionization-tandem mass spectrometry
FLAP	5-lipoxygenase activating protein
fMLF	N-formyl methionyl peptide
H & E	Hematoxylin and eosin
HIF	hypoxia inducible factor
HR	hypoxia reoxygenation
HUVEC	human umbilical vein endothelial cells
Ig	immunoglobulin

Iκκ	I kappa kappa B kinase
IL	interleukin
IR	ischemia reperfusion
JNK	Jun-amino-terminal kinase
Lox	lipoxygenase
LPS	lipopolysaccharide
LTB_4	leukotriene B4
MBL	mannose binding lectin
MCP-1	monocyte chemotactic protein 1
MIF	macrophage migration inhibitory factor
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	myeloid differentiation primary response gene 88
NADPH	nicotinamide adenine dinucleotide phosphate
NBD	7-nitrobenzo-2-oxa-1,3-diazole
NEM	N-ethylmaleimide
NfκB	nuclear factor kappa-light-chain-enhancer of activated B cells
PA	phosphatidic acid
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGE_2	prostaglandin E2
$PGF_{2\alpha}$	prostaglandin F2 alpha
PGI_2	prostaglandin I2
PI	phosphatidylinositol
PLSCR	phospholipid scramblase
PS	phosphatidylserine
Rag	recombination activating gene

RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
siRNA	small interfering ribonucleic acid
SLE	Systemic Lupus Erythematous
SM	sphingomyelin
TCPBS	tissue culture phosphate buffered saline
TEER	transepithelial electrical resistance
TIR	Toll/IL-1 receptor homology domain
TLR	toll like receptor
TNFα	tumor necrosis factor alpha
TNFαR1	tumor necrosis factor alpha receptor 1
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAF	tumor necrosis factor receptor associated factor
UPR	unfolded protein response
VEGF	vascular endothelial growth factor

Chapter 1 - Introduction

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. Trauma, shock, routine surgery and organ transplantation are all common scenarios in which IR occurs. The intestinal mucosa is among the organs most sensitive to IR (reviewed in (Guan et al., 2009)) with acute mesenteric arterial or venous thrombosis, embolism, and obstruction being common causes of intestinal IR (reviewed in (Haglund and Bergqvist, 1999; Oldenburg et al., 2004)).

Antonio Beniviene (1443-1502), an Italian physician, was the first to describe intestinal ischemia (reviewed in (Stamatakos et al., 2008)). However, it wasn't until 1895 that a case of acute mesenteric ischemia was successfully treated surgically (reviewed in (Stamatakos et al., 2008)). By the 1960s and 1970s, physicians were becoming better at diagnosing and treating these cases (reviewed in (Stamatakos et al., 2008)). Despite decades of improved imaging devices and medical advances, the mortality rate associated with intestinal IR remains at 50 to 80% (reviewed in (Stamatakos et al., 2008)).

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 (Oldenburg et al., 2004)). Symptoms are non-specific and can be subtle, resulting in a delay of correct diagnosis which further decreases survival probability. Furthermore, the sequelae to many cases of intestinal IR are acute lung injury and multiple organ failure. Bowel resection is indicated in most cases which leaves the surviving patients with bowel issues, such as short bowel syndrome, for the remainder of their lives.

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 (Haglund and Bergqvist, 1999)). The gastrointestinal tract has a very high capillary density, many collateral vessels and receives approximately 25% of total cardiac output at rest (reviewed in (Haglund and Bergqvist, 1999)). Cellular injury in humans is detectable by 20 minutes of

total ischemia and within 60 minutes in the case of partial ischemia (reviewed in (Haglund and Bergqvist, 1999)).

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 (Guan et al., 2009). 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 (Droy-Lefaix et al., 1991; Haglund, 1994; Noda et al., 1998). A recent study with mice illustrates the sensitivity of the intestine to IR damage. Wildtype mice were subjected to 30, 35, 40 or 45 minutes of ischemia. The severity of tissue damage as assessed by histology was positively correlated with the duration of ischemia (Stringa et al., 2012). The authors concluded that ischemia longer than 35 minutes is lethal to mice as 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 (Stringa et al., 2012).

With a loss of epithelial integrity, it has been postulated that intravascular proteins escape into the lumen and that luminal contents, such as commensal bacteria, move into the tissue. While there is evidence that the efficacy of the barrier is decreased following IR, no clear

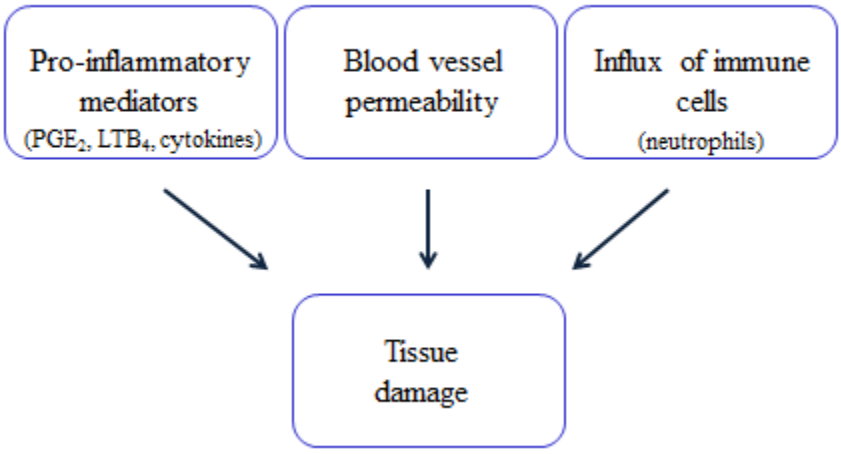


Figure 1.1 Factors contributing to IR-induced tissue damage.

answers exist as to if there is an influx of bacteria into tissues or of its role in pathology. Albumin leakage from blood to lumen was greater in IR versus Sham treated rats and also increased with increasing duration of ischemia (Sun et al., 2000). Other studies using mice have also found increased permeability after IR (Kannan et al., 2011; Williams et al., 1999a). Supporting the premise that reperfusion prevents the translocation of bacteria, no increase in lipopolysaccharide (LPS) was detected between arterial and venous blood samples by Limulus Amebocyte Lysate assay at any point over the two hour reperfusion period in a human jejunum study (Matthijsen et al., 2009).

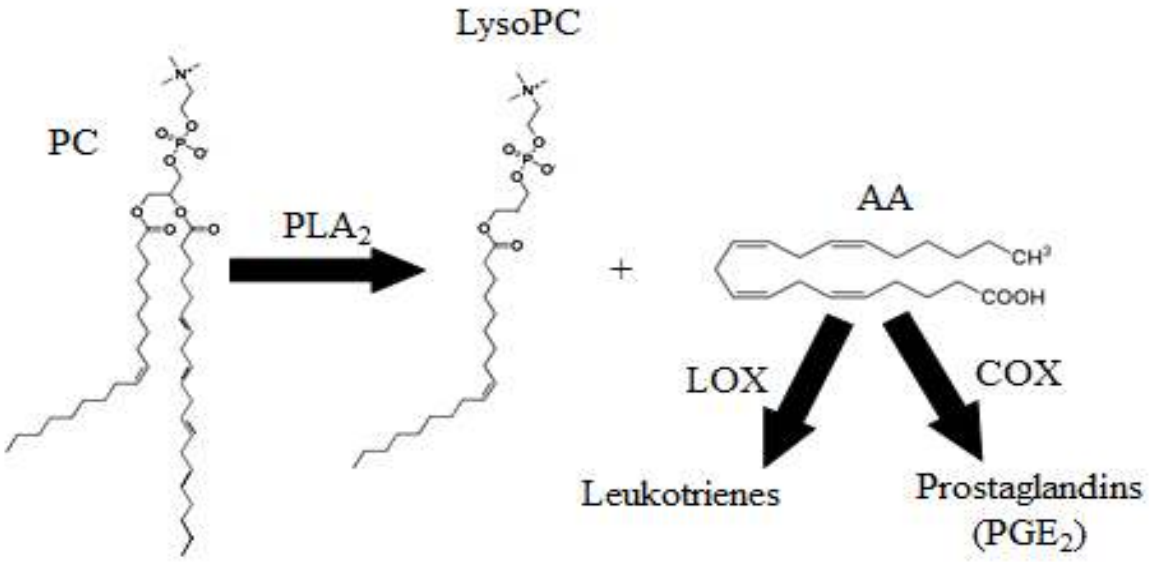


Figure 1.2 Representative diagram of lipid metabolism.

Eicosanoids, derivatives of arachidonic acid, are rapidly produced during the reperfusion period. Prostaglandin E_2 (PGE_2) was found to be required but not sufficient for tissue damage in a mouse model of intestinal IR (Moses et al., 2009). The conversion of arachidonic acid 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. However, the role of Cox 1, the constitutive isoform, in the post-ischemia production of prostaglandins is debated. Increased transcription of Cox 2 was observed in mice two hours after a 30 minute ischemic period (Moses et al., 2009). Similarly, a one hour ischemic period followed by either 30 minutes of reperfusion or two hours of reperfusion resulted in significant up-regulation of Cox 2 transcription in the small intestine of rats (Sato et al., 2005). Interestingly, protein levels were increased after two hours of reperfusion (Sato et al., 2005).

Transcription of Cox 2 was also elevated in a horse model in which mesenteric blood flow was reduced to 20% baseline levels for 75 minutes followed by an equal reperfusion period, however no change in Cox 1 transcription was detected (Hilton et al., 2011). Complete ischemia in horses and pigs also results in higher levels of Cox 2 protein in the intestine (Blikslager et al., 2002; Marshall and Blikslager, 2011) as does partial ischemia in mice (Watanabe et al., 2012). The use of several Cox inhibitors has further demonstrated the importance of this pathway in reperfusion injury. The Cox 2 inhibitor NS-398 reduced villus injury in a dose-dependent manner when administered to rats (Sato et al., 2005). In contrast, the Cox 2 inhibitor FK3311 did not affect villus injury in a dog model of intestinal IR (Kawata et al., 2003). Cox 1 and Cox 2 appear to have some overlapping and some independent effects in IR pathology. Cox 1 and Cox 2 activity contribute to the tissue edema following IR as the Cox 1 selective inhibitor flunixin and the Cox 2 specific inhibitor Celebrex both decreased intestinal edema in rats as measured by wet to dry weight ratio (Arumugam et al., 2003). Both isoforms also contribute to the epithelial permeability that increases following IR as transepithelial resistance in porcine ileum returned to baseline more quickly if the pigs had been dosed with either the Cox 1 selective inhibitor, SC-560, or the Cox 2 selective inhibitor NS-398 (Blikslager et al., 2002). In contrast, only the Cox 2 selective inhibitor Celebrex reduced villus damage in a rat model (Arumugam et al., 2003). Hence, activity of the Cox enzymes contributes substantially to IR injury.

The Cox enzymes catalyze the formation of prostaglandins, which, as stated above, are necessary but not sufficient for intestinal IR damage (Moses et al., 2009). Prostaglandins are potent signaling molecules primarily derived from arachidonic acid (reviewed in (Stuart, 1983)). Prostaglandins, having half-lives ranging from seconds to minutes (reviewed in (Egan and FitzGerald, 2006)), are not stored by the cell but newly synthesized upon stimulation (reviewed in (Schror, 1985)). Diffusion allows for autocrine or paracrine action via ligation of G protein coupled receptors (reviewed in (Egan and FitzGerald, 2006; Schuster et al., 2002)). PGE_2 is often associated with inflammation due to its vasodilatory effect and enhancement of vascular permeability ((Blikslager et al., 1997), reviewed in (Egan and FitzGerald, 2006)). Several in vivo studies have documented increased PGE_2 production in response to intestinal IR (Mangino et al., 1989; Moses et al., 2009; Pope et al., 2010; Slone et al., 2012; Sparkes et al., 2010; Turnage et al., 1995). While increased transcription of the Cox enzymes begins during the ischemic period, reperfusion is necessary for PGE_2 production (Sparkes et al., 2010).

Endothelial cells produce prostaglandins (reviewed in (Schror, 1985)); however, not all endothelial cells generate the same prostaglandin profile. PGI_2 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_2 and $PGF_{2\alpha}$ (Charo et al., 1984). It has long been postulated that PGE_2 may paradoxically contribute to tissue healing by promoting angiogenesis and epithelial cell migration (reviewed in (Hawkey and Rampton, 1985)). An ex vivo study of porcine ileum showed that PGE_2 application could increase intracellular cyclic adenosine monophosphate (cAMP) levels and contribute to closure of leaky tight junctions (Blikslager et al., 1997). It has been proposed that PGE_2 may act to downregulate nuclear factor kappa light chain enhancer of activated B cells (NFκB) activity in a negative feedback fashion (reviewed in (Scher and Pillinger, 2009)). While the precise mechanisms by which PGE_2 exerts opposing effects are not well understood, concentration, timing and ligation of differing receptors are likely possibilities (reviewed in (Egan and FitzGerald, 2006)).

Another eicosanoid derived from arachidonic acid in response to intestinal IR is leukotriene B_4 (LTB_4). LTB_4 is produced by endothelial cells, is chemotactic for neutrophils and facilitates adherence and degranulation of neutrophils (reviewed in (Bray, 1982)). The chemotactic property has been well documented, both in vitro and in vivo. Increased neutrophil adherence to endothelial cells was observed in ex vivo assays on tissues from rats subjected to intestinal IR (Karasawa et al., 1991). Intravital microscopy confirms the increase in leukocyte adherence and emigration from mesenteric venules following intestinal ischemia (Oliver et al., 1991). Studies using a whole body hypoxia model in which the animals inhale a gas mixture of 10% O_2 have shown that this level of hypoxia is sufficient to trigger increased leukocyte adherence to and emigration into intestinal tissue (Casillan et al., 2003; Steiner et al., 2001). Additionally, myeloperoxidase, an enzymeof neutrophils, is increased in response to IR (Schmeling et al., 1994). Application of the LTB_4 receptor antagonist LTB_4-DMA attenuated the effects of hypoxia while application of LTB_4 enhanced leukocyte adhesion in a dose-dependent manner (Casillan et al., 2003; Steiner et al., 2001). Rats treated with a different LTB_4 receptor antagonist, LY-255283, and subjected to intestinal IR had a higher survival rate and decreased intestinal myeloperoxidase activity at two hours of reperfusion compared to rats subjected to IR but not receiving the antagonist (Karasawa et al., 1991). Similarly, LTB_4 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 (Mangino et al., 1994). Further evidence for the involvement of the lipoxygenase (Lox) enzymes and LTB_4 in the recruitment of neutrophils to affected tissue was provided by a study with *5-Lox*$^{-/-}$ mice. The *5-Lox*$^{-/-}$ mice sustained less intestinal damage, had decreased intestinal myeloperoxidase activity and improved survival compared to wildtype mice following intestinal IR (Cuzzocrea et al., 2003).

Several pro-inflammatory cytokines are elevated in response to IR, notably tumor necrosis factor α (TNFα), interleukin-6 (IL-6), IL-12p40 and KC (Moses et al., 2009; Pope et al., 2010; Slone et al., 2012; Wu, 2003). The transcription factor NFκB contributes to the regulation of cytokine expression. NFκB translocates from the cytoplasm to the nucleus during reperfusion (Souza and Teixeira, 2005). The use of MOL294, an inhibitor of redox proteins, to inhibit this translocation, thus blocking NFκB-dependent transcription, in a mouse model of IR resulted in lower levels of TNFα, MCP-1 and KC in intestinal supernatants as well as TNFα in the sera (Souza et al., 2005). Furthermore, vascular permeability and neutrophil infiltration of the affected tissue were decreased in comparison to IR treated mice not receiving the NFκB inhibitor (Souza et al., 2005). In addition to decreased serum levels of TNFα and IL-1β, the intestines of *IL-6*$^{-/-}$ mice had lower myeloperoxidase activity and malondialdehyde levels after one hour of reperfusion compared to wiltype mice, indicating attenuated neutrophil infiltration and generation of reactive oxygen species (Cuzzocrea et al., 1999). Similarly, myeloperoxidase and malondialdehyde were reduced from wildtype levels in the intestines of *TNFαR1*$^{-/-}$ mice following IR (Esposito et al., 2007). Unsurprisingly, macrophage migration inhibitory factor (MIF) also contributes to pathology, likely by inducing TNFα release. *MIF*$^{-/-}$ mice have attenuated intestinal damage, reduced vascular permeability and increased survival compared to wildtype mice following IR (Amaral et al., 2007).

As alluded to above, neutrophils and macrophages play a significant role in IR-induced pathology. Circulating neutrophils respond to chemotactic factors, such as LTB_4, and infiltrate the affected tissue (reviewed in (Kong et al., 1998)) . Reperfusion greatly increases the number of neutrophils in the intestinal tissue ((Grosche et al., 2011), reviewed in (Gayle et al., 2000)). Increased adherence of neutrophils to endothelium has been demonstrated in vitro. Primary porcine neutrophils exhibited an increase in adherence to primary porcine endothelial cells after the endothelial cells had been maintained in a hypoxic environment of two percent O_2 for two

hours (Milhoan et al., 1992). Similarly, complete anoxia of primary human endothelial cells for one hour enhanced the adherence of primary human neutrophils (Arnould et al., 1993). Hypoxic conditions stimulate endothelial cells to upregulate expression of several adhesion proteins (reviewed in (Gayle et al., 2000)) and blockade of these proteins with monoclonal antibodies precludes any increase in neutrophil adhesion (Arnould et al., 1993). Activated neutrophils produce reactive oxygen compounds which contribute to the reperfusion damage (reviewed in (Grisham and Granger, 1988)). Evidence suggests that circulating neutrophils, in addition to emigrated neutrophils, are primed in response to IR (Kim et al., 1995). N-formyl-Met-Leu-Phe (fMLP) stimulation of circulating neutrophils following IR led to superoxide secretion (Koike et al., 1995).

Tissue resident macrophages are very abundant in the intestine. Profiling of F4/80 antigen expression, a macrophage surface marker, indicates that the small intestine is one of the most macrophage dense tissues in the rodent body (Lee et al., 1985). Within the muscularis externa, the small intestine is the most densely populated area of the gastrointestinal tract (Kalff et al., 1998). While the resident macrophages of the lamina propria do phagocytose, they do not secrete many cytokines upon stimulation (reviewed in (Smith et al., 2005)). Following tissue injury, circulating monocytes are recruited as these cells secrete large quantities of cytokines (reviewed in (Smith et al., 2005)). Hypoxia has been shown to increase the migration of monocyte-like cells across an endothelial monolayer in vitro (Kalra et al., 1996). Pro-inflammatory cytokine production was attenuated after administration of dichloromethylene-bisphosphonate (Cl2MBP liposomes or claudronate) to deplete macrophages in a rat model of IR or intestinal grafting, respectively (Chen et al., 2004; Schaefer et al., 2007). Furthermore, macrophage depletion reduced the number of damaged villi following IR (Chen et al., 2004). It is clear that both neutrophils and macrophages play a role in the damage sustained following IR.

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. Activation by the classical pathway is primarily antibody dependent while the alternative pathway is antibody-independent (reviewed in (Ehrnthaller et al., 2011)). The more recently described lectin pathway is initiated when mannose binding lectin (MBL) binds mannose moieties (reviewed in (Ehrnthaller et al., 2011)). Several approaches were taken to delineate the contribution of each pathway due to the

significant overlap and crosstalk between the initiation pathways. 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 (Hill et al., 1992a). Later studies using this same complement inhibitor confirmed that both the classical and alternative pathways of complement activation contributed to IR injury (Eror et al., 1999a). The significance of the alternative pathway in IR injury was revealed upon the generation of *factor D*$^{-/-}$ mice. Deficiency of factor D inhibits the alternative pathway and attenuated intestinal injury and neutrophil infiltration following IR (Stahl et al., 2003). The lectin pathway was subsequently shown to contribute to IR pathology (reviewed in (Arumugam et al., 2006)). A supporting study demonstrated the requirement for MBL in IR injury as *MBL*$^{-/-}$ mice were protected from IR damage (Hart et al., 2005; Zhang et al., 2006c) but susceptible after reconstitution with MBL (Hart et al., 2005). 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 (Karpel-Massler et al., 2003; Rehrig et al., 2001b).

In accordance with the involvement of the classical complement pathway, antibodies are essential for IR injury. *Rag-1*$^{-/-}$ 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 (Sparkes et al., 2010; Williams et al., 1999b). It was later shown that human IgM can also elicit injury and complement deposition in *Rag-1*$^{-/-}$ and *Rag -2*$^{-/-}$ mice (Weiser et al., 1996; Zhang et al., 2008). Further support for the involvement of antibodies came out of studies investigating complement receptor 2 (CR2), a B cell membrane protein. *CR2*$^{-/-}$ mice produce antibodies but have defects in the generation of the normal antibody repertoire (Fleming et al., 2002a; Reid et al., 2002a). *CR2*$^{-/-}$ mice are protected from IR injury; and, like *Rag-1*$^{-/-}$ mice, administration of wildtype IgM results in intestinal injury and complement deposition (Fleming et al., 2002b; Reid et al., 2002b). Further studies with *CR2*$^{-/-}$ mice identified specific antibodies capable of inducing IR damage. An anti-phospholipid antibody and anti-β_2-glycoprotein I (β_2-GPI) antibody were each able to induce IR injury and complement deposition in *CR2*$^{-/-}$ mice; however both antibodies were required in *Rag-1*$^{-/-}$ mice (Fleming et al., 2004b).

β_2-GPI is one of three recently identified neoantigens 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 ug per ml (reviewed in (Miyakis et al., 2004)). Five short consensus repeats comprise the 326 amino acid protein categorizing it as a member of the complement control superfamily (reviewed in (Miyakis et al., 2004)). β_2-GPI primarily circulates unaccompanied but can be found bound to circulating lipid (reviewed in (Miyakis et al., 2004)). 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 ((Meroni et al., 2004), reviewed in (Miyakis et al., 2004)). The results of the aforementioned study strongly suggested the involvement of β_2-GPI (Fleming et al., 2004b) and further studies have demonstrated the efficacy of peptides derived from Domain V, the lipid binding domain, in protecting wildtype mice from IR damage. Additionally, *Rag-1*$^{-/-}$ mice infused with pooled wildtype antibodies and treated with the β_2-GPI -derived peptides minimized the intestinal injury, complement deposition and eicosanoid production resulting from administration of wildtype antibodies to *Rag-1*$^{-/-}$ mice (Fleming et al., 2010). Interestingly, administration of purified human β_2-GPI to wildtype mice prior to intestinal IR attenuated injury, complement deposition, and PGE_2 production (Tomasi et al., 2012). The authors speculate that the anti-β_2-GPI antibodies produced by the mice bind the human β_2-GPI, thus reducing the titer of anti-β_2-GPI antibodies available for binding the mouse protein (Tomasi et al., 2012). This hypothesis is supported by further data demonstrating attenuation of IR injury and sequelae when *Rag-1*$^{-/-}$ were administered pooled wildtype antibodies that had specifically been depleted of anti-β_2-GPI(Fleming et al., 2010). Recent studies have suggested that β_2-GPI signals through Toll-like receptor 4 (TLR4) as anti-β_2-GPI antibodies cross react with TLR4, TLR4 co-immunoprecipitates with β_2-GPI and anti-β_2-GPI antibodies have been shown to activate NFκB via myeloid differentiation primary response gene 88 (MyD88) in endothelial cells ((Colasanti et al., 2012; Xie et al., 2013), reviewed in (Cockrell et al., 2008)).

The other two identified neoantigens induced by IR are the intracellular proteins non-muscle myosin heavy chain II and annexin IV. A screening experiment probing ischemic tissue with several monoclonal antibodies produced by different B1 cell clones was performed in an effort to detect specific monoclonal antibodies involved in IR injury. This experiment resulted in the identification of a monoclonal antibody against non-muscle myosin heavy chain II isoforms

A and C that rendered *Rag-1*$^{-/-}$ mice susceptible to IR injury (Zhang et al., 2006b). A peptide based on non-muscle myosin heavy chain II provides additional evidence that this protein serves as a neoantigen following IR. Wildtype mice experience attenuated intestinal injury and less mucosal permeability as a result of intestinal IR if the non-muscle myosin heavy chain II-derived peptide is administered prior to the procedure (Zhang et al., 2006b). 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 (Chan et al., 2006; Haas et al., 2010).

The identification of annexin IV as a neoantigen also resulted from a screening experiment. In vivo experiments in which *Rag-1*$^{-/-}$ mice were administered anti-annexin IV monoclonal antibodies prior to intestinal IR resulted in tissue injury, complement deposition, neutrophil infiltration and eicosanoid production (Elvington et al., 2012; Kulik et al., 2009a). Importantly, the anti-annexin IV monoclonal antibody does not recognize non-muscle myosin or phospholipids (Kulik et al., 2009a). In wildtype mice, recombinant annexin IV reduces tissue injury as well as neutrophil infiltration and eicosanoid production (Elvington et al., 2012; Kulik et al., 2009a). The authors propose that the recombinant annexin IV binds up the circulating anti-annexin IV antibodies, greatly reducing the likelihood of antibody binding annexin IV expressed on damaged tissue (Elvington et al., 2012; Kulik et al., 2009a). Furthermore, anti-annexin IV antibodies bind endothelial cells in vitro following hypoxia and re-oxygenation (HR) (Elvington et al., 2012).

Molecular Responses to Hypoxia

Ambient air entering the lungs is approximately 21% O_2. However, in the route from the alveolae of the lungs to the rest of the body, the O_2 content drops to between two and a half and nine percent O_2 depending on the tissue (reviewed in (Imtiyaz and Simon, 2010)). In times of O_2 deprivation, systemic or local, the O_2 content can drop below two percent which is considered the upper limit for hypoxia (reviewed in (Shay and Celeste Simon, 2012)). The microenvironment in areas of tissue damage or infection, is routinely hypoxic, around one percent O_2 (reviewed in (Imtiyaz and Simon, 2010)). The decrease in O_2 availability triggers activity by hypoxia-inducible factors (HIFs).

Three HIF α subunits and three HIF β subunits have been identified in mammals. The oxygen labile α subunits, 1α, 2α and 3α, pair with the constitutively expressed β subunits, 1β, 2β and 3β, to influence gene expression (reviewed in (Lisy and Peet, 2008)). HIF1α is ubiquitously expressed and is the primary HIF mediating the hypoxia response in endothelial cells. While many aspects of HIF biology are applicable to all three HIFs, the remainder of this section will focus on HIF1.

The β subunit is found in the nucleus while the α subunit resides in the cytoplasm. When O_2 is readily available to the cell, the HIF α subunit is targeted for degradation by the proteasome. Prolyl hydroxylases, which require O_2 for activity, hydroxylate the two proline residues in the α subunit. Recognition of the hydroxylated prolines by von Hippel-Lindau E3 ligase leads to polyubiquitinatation by a complex of proteins including von Hippel-Lindau E3 ligase, tagging it for degradation by the 26S proteasome. Thus, although HIF α subunits are continuously translated, the protein has a very short half-life (less than five minutes) when O_2 is readily available (Huang et al., 1996). However, when O_2 becomes scarce, the inactivation of prolyl hydroxylases allows the α subunit to accumulate and move into the nucleus where it binds the β subunit. Once the α subunit binds the β subunit, additional proteins such as p300 and Creb binding protein are recruited to facilitate transcription. Both HIF subunits contact the DNA with p300 and Creb binding protein providing histone acetyltransferase activity to decondense the DNA enabling transcription (reviewed in (Kaluz et al., 2008; Lisy and Peet, 2008; Shay and Celeste Simon, 2012)).

Stabilization of the HIF α subunit can also occur, independently of O_2 content. Pro-inflammatory cytokines, growth factors and other molecules have been shown to influence the stability of the α subunit. For example, the cytokines TNFα and IL-1β, both secreted during HR and IR, contribute to stabilization through NFκB even when O_2 is available to the cell (reviewed in (Imtiyaz and Simon, 2010; Majmundar et al., 2010)). Furthermore, the cellular responses to hypoxia can also be mediated by mammalian target of rapamycin (mTOR) and the unfolded protein response (UPR) (reviewed in (Majmundar et al., 2010)).

The HIF complex ligates hypoxia response elements residing in gene promoters and enhances transcriptional activity. HIFα/β dimers directly interact with a sequence of five nucleotides, (A/G)CGTG, the sequence defining hypoxia response elements (reviewed in

(Loboda et al., 2010)). Additional transcription factors can be recruited to the HIF complex to further enhance transcription of the target gene (reviewed in (Lisy and Peet, 2008)).

The effects of HIF activity are varied and numerous. Activation of HIF1α is estimated to directly affect transcription of well over 100 genes (reviewed in (Loboda et al., 2010)). Many of these genes are involved in cellular metabolism, proliferation, angiogenesis, tumor promotion and inflammation (reviewed in (Majmundar et al., 2010; Shay and Celeste Simon, 2012)). The number of genes influenced by HIF activity has been estimated to be one to five percent of the human genome (reviewed in (Semenza, 2003)). Two percent of endothelial genes are thought to be influenced, either directly or indirectly, by HIF activity (Manalo et al., 2005). Glycolysis becomes the major mode of cellular metabolism, (reviewed in (Shay and Celeste Simon, 2012)) with inhibition of β oxidation and promotion of lipid storage (reviewed in (Majmundar et al., 2010)). Furthermore, pro-inflammatory cytokine production is increased (reviewed in (Shay and Celeste Simon, 2012)).

Studies with HIF1α null mice illustrate the importance of HIF activity. Peritoneal neutrophils and macrophages from HIF1α null mice had reduced levels of adenosine tri-phosphate (ATP) even under normoxic conditions (Cramer et al., 2003). Several abnormalities in the behavior of the HIF1α null peritoneal macrophages were noted, including decreased migration and invasiveness and lack of TNFα production when exposed to hypoxia or LPS stimulation (Cramer et al., 2003). In a model of acute inflammation, TPA (12-O-tetradecanoylphorbol-13-acetate) application to the ears of HIF1α null mice resulted in a reduced inflammatory response compared to wildtype mice (Cramer et al., 2003). The weight of the ears from wildtype mice, an indicator of edema, were heavier and contained more neutrophils, as assessed by myeloperoxicase activity, than the HIF1α null mice (Cramer et al., 2003). An attenuated inflammatory response was also observed in a second skin model as well as a joint model of inflammation (Cramer et al., 2003). Moreover, HIF1α was found to be necessary and sufficient to promote reperfusion injury in a model of skeletal muscle IR (Bosch-Marce et al., 2007). HIF1α heterozygotes also respond differently to IR. Intestinal IR did not stimulate increased transcription of TNFα, IL-1β, IL-6 or Cox 2 in the intestinal tissue of HIF1α heterozygous mice, unlike the response in wildtype mice (Feinman et al., 2010; Kannan et al., 2011). Although intestinal myeloperoxidase activity was not different from wildtype, intestinal damage was attenuated in the heterozygous mice (Feinman et al., 2010; Kannan et al., 2011).

Systemic hypoxia also has a profound effect on the vasculature. Intravital microscopy of mesenteric venules revealed an increase in leukocyte adherence when rats were exposed to 10% O_2 for 10 minutes followed by a 10 minute recovery period with ambient air (Casillan et al., 2003). A four hour exposure to 10% O_2 led to increased vascular permeability to albumin and significant emigration of leukocytes (Casillan et al., 2003).

Cells respond to hypoxic conditions by altering their metabolic activities. With an increase in glycolysis, lactic dehydrogenase and lactic acid begin to accumulate (Gloria et al., 2006; Lum et al., 1992). MTT assays and luciferin/luciferase assays indicate a reduction in ATP (Arnould et al., 1992; Zhang et al., 2011; Zhao et al., 2012). Morphologically, endothelial cells appear swollen (Busija et al., 1996) and viability decreases with increasing lengths of hypoxia (Michiels et al., 1992). Similar to the effect of reperfusion, re-oxygenation of cultured endothelial cells results in further damage, manifested by an additional decrease in viability (Michiels et al., 1992).

Endothelial Responses to Hypoxia

Many of the in vivo responses to IR are replicated in endothelial cultures exposed to HR. Although no one in vitro model of HR is used consistently in the literature, the data are remarkably consistent providing confidence in the legitimacy of the results. The effects of HR on endothelial cells include production of cytokines and eicosanoids and increased permeability and affinity for leukocytes. Furthermore, involvement of complement, antibodies and neo-antigens are apparent.

Many of the same pro-inflammatory cytokines produced in response to IR are made by endothelial cells subjected to HR. Several studies using primary human umbilical vein endothelial cells (HUVEC) have demonstrated an increase in IL-1 production in response to HR. Higher levels of IL-1α and IL-1β were detected in supernatants of cultures exposed to five hours of zero percent O_2 hypoxia followed by 19 hours of re-oxygenation than supernatants of control cultures (Ala et al., 1992). A shorter period of hypoxia, two hours at one percent O_2, followed by 24 hours of re-oxygenation also elicited increased production and secretion of IL-1β into the supernatant than was detected in supernatants of control cultures (Harmon et al., 2004). A time course study revealed that IL-1 production positively correlates with the duration of hypoxia and

also demonstrated up-regulation of IL-1 transcription during the hypoxic period (Shreeniwas et al., 1992). IL-6 production is also increased in HUVECs and microvascular endothelial cells subjected to HR (Ala et al., 1992; Zhang et al., 2011). Re-oxygenation promoted the release of TNFα from microvascular endothelial cells after a six hour exposure to one percent O_2 (Zhang et al., 2011). Just as NFκB has been shown to translocate to the nucleus and regulate cytokine production during reperfusion (Souza and Teixeira, 2005), the protein level of NFκB increases in the nucleus of HUVECs during re-oxygenation (Collard et al., 1999a; Collard et al., 1998).

HR treated endothelial cells upregulate Cox and Lox enzymes and increase production of PGE_2 and LTB_4. Increased transcription of Cox 2 requires HIF activity at the hypoxia response element located in the gene's promoter sequence (Cook-Johnson et al., 2006; Csiki et al., 2006). Transfection of stable HIF1α resulted in a fourfold increase in Cox 2 transcription by HUVECs in response to one percent O_2 hypoxia (Cook-Johnson et al., 2006). Cox 2 transcription can be further enhanced by the ligation of the NFκB consensus elementby the p65 subunit of NFκB (Schmedtje et al., 1997). Several studies have followed the increase in Cox 2 transcription over time. Most agree with the pattern of early detection, as early as 30 minutes of one percent O_2 exposure, peak transcription two to three hours into one percent O_2 exposure and a gradual decline with longer hypoxic periods, remaining significantly elevated with as long as 24 hours of hypoxia (Cook-Johnson et al., 2006; Schmedtje et al., 1997; Zhao et al., 2012). Increased protein expression of Cox 2 was detected after one and three hours of hypoxia in HUVECs (Zhao et al., 2012) as well as following HR of cerebrovascular endothelial cells (Busija et al., 1996) While many prostaglandins are produced by endothelial cells, PGE_2 is the primary prostaglandin produced by both HUVECs and primary coronary microvascular endothelial cells (Gerritsen and Cheli, 1983; Weksler et al., 1977). The literature contains conflicting data as to when PGE_2 is produced in response to HR. In agreement with in vivo data, a study with human umbilical artery endothelial cells found PGE_2 production to increase during the re-oxygenation period (Soler et al., 1997). In contrast, the production of PGE_2 increased during the hypoxic period with no further increase during the re-oxygenation period in a study using HUVECs (Michiels et al., 1993). The discrepancy may be due to the differing endothelial cells used, arterial versus venous, as well as the different HR protocols.

Shortly after the identification of 5-Lox and LTB_4 generation by neutrophils, data suggesting the production and release of LTB_4 by endothelial cells was published. Stimulation

of primary bovine pulmonary vessel endothelial cells with thiourea resulted in migration of neutrophils (O'Brien et al., 1984). Further studies demonstrated that treatment with a Cox inhibitor only slightly attenuated this effect whereas application of a non-specific 5-Lox/Cox inhibitor eliminated the neutrophil migration towards the endothelial cells (O'Brien et al., 1984). In conjunction with additional data, the authors concluded that a neutrophil chemoattractant, likely LTB_4, was secreted by stimulated endothelial cells (O'Brien et al., 1984). More recently, a mechanism for the upregulation of LTB_4 production in response to hypoxia has been described. In addition to 5-Lox, 5-lipoxygenase activating protein (FLAP) is needed for the conversion of arachidonic acid to LTB_4. The promoter region of FLAP contains four hypoxia response elements as well as a binding motif for NFκB (Gonsalves and Kalra, 2010). Hypoxia stabilizes HIF1α, which binds to the FLAP promoter and initiates transcription of FLAP (Gonsalves and Kalra, 2010). Further regulation of FLAP transcription occurs as the NFκB motif is critical and microRNAs (miRNA 135a and 199a-5p) are involved (Gonsalves and Kalra, 2010). Exposure of bovine pulmonary microvascular endothelial cells to four hours of hypoxia and 30 minutes of re-oxygenation resulted in the increased production and secretion of LTB_4 (Wiles et al., 1993). Further increases in LTB_4 were observed with the addition of primary neutrophils to the hypoxic culture (Wiles et al., 1993).

The chemoattractant property of LTB_4 for neutrophils contributes to the neutrophil influx observed in IR. In their migration from the blood to tissue, neutrophils must traverse the endothelial layer. Increased adherence of HL60 cells, a neutrophil-like cell line, to HUVECs was observed following a 16 hour hypoxic and four hour re-oxygenation period (Shreeniwas et al., 1992). Re-oxygenation was critical in this study, as no change in adherence was detected with hypoxia alone (Shreeniwas et al., 1992). Examination of a time course revealed an increased number of primary human neutrophils adhering to and rolling along a monolayer of primary HUVECs when exposed to hypoxia for 30 minutes and re-oxygenation for 10 minutes (Rainger et al., 1995). Lower O_2 levels and increased lengths of hypoxia further increased the adherence of neutrophils to HUVECs (Rainger et al., 1995). Similarly, increased adherence of neutrophils to bovine pulmonary and cerebral microvascular endothelial cells was elicited by HR (Cuzzocrea et al., 1999; Wiles et al., 1993). Furthermore, addition of TNFα or IL-1α resulted in even greater adherence (Cuzzocrea et al., 1999). A more recently published study reports a decrease in adherence of neutrophils to endothelial cells following HR (Schmitz et al., 2011).

The specific conditions of this study, use of dermal microvascular endothelial cells and neutrophils from a single donor, may account for the disparate result.

In association with the secretion of LTB_4 and adhesion of neutrophils prior to extravasation, the endothelial layer becomes more permeable with HR. Permeability to albumin increased when bovine pulmonary artery endothelial cells were subjected to 90 minutes of zero percent O_2 followed by an equal length of re-oxygenation (Inauen et al., 1990). A second study using albumin translocation as an indicator of permeability found that neither two nor four hours of zero percent O_2 were sufficient, but that 12 and 24 hours of hypoxia did result in greater permeability (Lum et al., 1992). The difference in the length of hypoxia required for passage of albumin may be due to the use of different cells. The first study used macrovascular endothelial cells from the pulmonary artery while the second study utilized endothelial cells from the pulmonary microvasculature which may be more resistant to hypoxia. Work with HUVECs illustrated an analogous trend. Determination of permeability via trans-endothelial electrical resistance (TEER) demonstrated correlation between severity of hypoxia and TEER values, where decreasing TEER values coincide with increasing severity of hypoxia (Ali et al., 1998). Nine hours of exposure to one percent O_2 was required for TEER values to begin dropping and by 18 hours of hypoxia TEER values were only 60% of the values from control HUVECs (Ali et al., 1998). To further investigate the cellular changes contributing to increased permeability, studies examining the actin cytoskeleton were performed. A 24 hour time course of zero percent O_2 revealed the formation of actin stress fibers and intercellular gaps (Partridge, 1995). Dextran of 71.2 kDa traversed the monolayer during the hypoxic period (Partridge, 1995). However, the effects of hypoxia are reversible as permeability to 71.2 kDa dextran returned to control levels within four hours of re-oxygenation (Partridge, 1995). A single hour of hypoxia was sufficient to alter the actin cytoskeleton in a similar manner in a second study (Wojciak-Stothard et al., 2005). As with the previous study, permeability increased during the hypoxic period, as evaluated with 42 kDa dextran, and was largely reversible with re-oxygenation (Wojciak-Stothard et al., 2005). Furthermore, the surface area of the individual cells in a monolayer of bovine pulmonary microvascular endothelial cells decreased in response to HR (Wiles et al., 1993). These physical changes correlate with the increased permeability of endothelial cells observed following HR.

Just as complement and antibodies are deposited in response to IR, deposition also occurs when using an in vitro HR model. C3 deposition occurred in HUVEC cultures if normal human sera was provided during re-oxygenation but not if sera depleted of C1q and Factor B was used (Mold and Morris, 2001). Addition of C1q to the depleted sera resulted in C3 deposition, suggesting activation of the classical complement pathway (Mold and Morris, 2001). Additionally, C1q, C3d and IgM were primarily bound to annexin V positive cells as determined by flow cytometry (Mold and Morris, 2001). Complement deposition (iC3b) appears to be enhanced with longer periods of hypoxia followed by longer periods of re-oxygenation (Collard et al., 1998). In addition to C3 and its degradation products, HR also elicits deposition of C5b-9 (Collard et al., 1999b). An in vitro model with endothelial cells has revealed increased deposition of β_2-GPI, a neoantigen, when exposed to HR (Fleming et al., 2010). Thus, the interaction between neoantigens, antibodies and complement are supported by in vitro studies.

The Contribution of Lipids to Cellular Signaling and Processes

The majority of lipid biosynthesis occurs at the endoplasmic reticulum (reviewed in (Bishop and Bell, 1988)). Phospholipids, and all other acylglycerol lipids, are derived from phosphatidic acid (PA) (Athenstaedt and Daum, 1999). Acylation of sn-glycerol-3-phosphate yields 1-acyl-sn-glycerol-3-phosphate, lysoPA (reviewed in (Bishop and Bell, 1988)). A second acylation, by microsomal acetyltransferase, generates PA (reviewed in (Bishop and Bell, 1988)). PA can then be hydrolyzed by phosphatidic acid phosphatase to diacylglycerol from which phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and triacylglycerol are derived (reviewed in (Bishop and Bell, 1988)). Alternatively, PA can react with the nucleotide CTP (cytidine tri-phosphate), forming CDP-diacylglycerol through the action of CDP-diacylglycerol synthase. PI, PG and the mitochondrial lipid cardiolipin are produced from CDP-diacylglycerol (reviewed in (Bishop and Bell, 1988)).

Transfers of the choline and ethanolamine moieties of CDP-choline and CDP-ethanolamine to diacylglycerol yield PC and PE, respectively. PG results from the reaction of CDP-diacylglycerol with glycerol-3-phosphate. The production of PI and PS occurs by the addition of inositol or serine, respectively, to CDP-diacylglycerol. Alternatively, PS can result from a base-exchange reaction in which serine is exchanged for ethanolamine on PE ((Yamaji-

Hasegawa and Tsujimoto, 2006), reviewed in (Baranska, 1982)). This base-exchange reaction occurs primarily at the inner mitochondrial membrane ((Yamaji-Hasegawa and Tsujimoto, 2006), reviewed in (Baranska, 1982)).

Phospholipids move between membranes by several mechanisms. Phospholipids can be transferred by vesicles and proteins (reviewed in (Bishop and Bell, 1988)). Proteins specific for the transport of phospholipids between organelles reside in the cytoplasm (reviewed in (Baranska, 1982)). Additionally, contact between membranes allows for lateral diffusion of phospholipids whereby relocation can be achieved (reviewed in (Bishop and Bell, 1988)).

Phospholipases are lipid hydrolases that catalyze phospholipid hydrolysis and enzymes are classified by their cleavage sites. Phospholipase C separates the phosphate and alcohol from the glycerol backbone of a phospholipid, while phospholipase D removes the alcohol only. Phospholipase A cleaves fatty acyl chains from the glycerol backbone of a phospholipid. Three classes of phospholipase A_2, secretory, calcium-independent, and cytosolic, cleave the fatty acyl chain from the second carbon of the glycerol backbone (*sn-2*) (reviewed in (Leslie, 1997)). The ubiquitous cytosolic phospholipase A_2 ($cPLA_2$) has an affinity for phospholipids containing polyunsaturated fatty acids, particularly arachidonate, at the *sn-2* position (reviewed in (Anderson et al., 1994; Clark et al., 1995; Leslie, 1997)). While no preference for a phospholipid head group has been identified (Lister et al., 1988), arachidonate is most often associated with phospholipids containing choline, ethanolamine or inositol as the head group (Lister et al., 1988). The activity of $cPLA_2$ is calcium-dependent and is synergistically increased by mitogen activated protein kinase mediated phosphorylation (Lin et al., 1992; Lin et al., 1993). Calcium is required for the translocation of $cPLA_2$ from the cytosol to the plasma and intracellular membranes and for binding to a phospholipid substrate (reviewed in (Clark et al., 1995; Leslie, 1997)). Phosphorylation of $cPLA_2$ is important for activation of enzymatic activity (reviewed in (Leslie, 1997)). The majority of arachidonic acid is released from phosphatidylcholine (PC) by the action of PLA_2 (Schoonderwoerd and Stam, 1992). The activity of phospholipase C and D can indirectly contribute to the arachidonate pool (reviewed in (Rink and Khanna, 2011)).

PLA_2 activity not only increases the level of free arachidonic acid, but also lysophospholipids (Fig 1.2). Several lysophospholipids are biologically active and circulate through the vasculature (reviewed in (Frasch and Bratton, 2012)). The term “lyso” was applied to these lipids because they lyse red blood cells (reviewed in (Frasch and Bratton, 2012));

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 (Frasch and Bratton, 2012)). The concentration of lysoPA in serum is typically greater than 1 μM and between 3 and 300 nM at the tissue level ((Goetzl and Lynch, 2000), reviewed in (Goetzl et al., 2004)). In contrast to lysoPC and lysoPA, lysophosphatidylserine (PS) is barely detectable in serum (reviewed in (Frasch and Bratton, 2012)). In circulation, many of the smaller and more water soluble acyl lipids, including lysophospholipids, are bound to albumin or other plasma proteins (reviewed in (Lum, 2001; Rosen and Goetzl, 2005)).

The generation of reactive oxygen species, a feature of reperfusion or re-oxygenation, promotes lipid oxidation. Polyunsaturated fatty acids are the preferred target of reactive oxygen species (Tyurin et al., 2008). In fact, the greater the number of double bonds in the fatty acid, the more likely it is to be oxidized (reviewed in (Sparvero et al., 2010)). Due to the unstable nature of reactive oxygen species and oxidized molecules, a proxy for the extent of lipid oxidation is often assessed (reviewed in (Rink and Khanna, 2011)). The reactive aldehydes malondialdehyde and 4-hydroxynonenal (4-HNE) covalently bind proteins, enhancing their stability and allowing for detection (reviewed in (Rink and Khanna, 2011)).

Beyond their contribution to cellular structure, phospholipids are biologically active and participate in cellular signaling. For example, the interaction of PA with mTOR fosters cell survival and proliferation (Fang et al., 2001) (reviewed in (Wang et al., 2006)). Signaling through one of its receptors, GPR92, lysoPA affects numerous signaling cascades by increasing phosphoinositide hydrolysis and cAMP production (Kotarsky et al., 2006). The increase in cAMP resulting from lysoPA signaling can activate protein kinase C, as can arachidonic acid (Hwang et al., 1996; McPhail et al., 1984).

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 (Goetzl et al., 2004)). 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 (Kotarsky et al., 2006). 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 ((Hines et al., 2000), reviewed in (Goetzl et al., 2004; Lum, 2001)). Furthermore, PA can enhance the

respiratory burst of neutrophils, through interaction with the p47phox subunit of NADPH oxidase (reviewed in (Wang et al., 2006)). Macrophage adherence to endothelial cells is promoted by12-hydroxyeicosatetraenoic acid (12-HETE), an arachidonic acid metabolite (Navab et al., 2012). Activation of macrophages elevates the levels PA and lysoPC as phospholipases act on the increased number of PC-containing microvesicles that are released (reviewed in (Rosen and Goetzl, 2005)). An in vitro study found that the addition of exogenous arachidonic acid led to translocation of NFκB from the cytoplasm to the nucleus of cells within 30 minutes (Hughes-Fulford et al., 2006).

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 (Matsubara and Hasegawa, 2005). Uptake of extracellular calcium is promoted by lysoPC, increasing the intracellular calcium concentration, which appears necessary for lysoPC-mediated apoptosis (Chaudhuri et al., 2003; Matsubara and Hasegawa, 2005).

A hallmark of apoptosis is externalization of PS (reviewed in (Leventis and Grinstein, 2010)). As the process of apoptosis begins, cardiolipin, the major constituent of mitochondrial membranes, is oxidized (Tyurin et al., 2008). Cytochrome c is then released into the cytoplasm where it can oxidize PS residing in the inner leaflet of the plasma membrane (Tyurin et al., 2008). 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 (Fadeel and Xue, 2009; Tyurin et al., 2008). Although transient exposure of PS occurs often, such as during cellular activation or fusion of membranes (Smrz et al., 2008), 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 (Frasch and Bratton, 2012)). Macrophage clearance of aged neutrophils is facilitated by the exposure of lysoPS on the neutrophil membrane at sites of inflammation (reviewed in (Frasch and Bratton, 2012)). 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 (Yamaji-Hasegawa and Tsujimoto, 2006).

Hypoxia was demonstrated to activate PLA_2 in a study exposing primary HUVECs to zero percent O_2 for two hours (Michiels et al., 1993). However, in the context of IR, reperfusion seems essential for stimulating PLA_2 activity and elevating lysophospholipid content (Otamiri et al., 1987). 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_2 enzymes, reduced intestinal permeability and lowered the ratio of lysoPC to PC suggesting a role for PLA_2 in IR injury (Otamiri et al., 1987; Otamiri and Tagesson, 1989). Quinacrine also attenuated the loss of total phospholipids following IR in a porcine heart model (Das et al., 1986). 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 (Das et al., 1986; Shaikh and Downar, 1981).

An ex vivo study of myocardial cells indicated loss of PC and phosphatidylethanolamine (PE) with a concomitant increase of lysoPC and lysoPE in response to four hours of zero percent O_2 exposure (Kawaguchi et al., 1991). An increase in fatty acids including arachidonic acid was detected in the culture supernatant (Kawaguchi et al., 1991). PC and PE were similarly decreased and lysophospholipids increased in a rat model of cerebral IR (Drgova et al., 2004). 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_2 for 24 to 48 hours (Bhat and Block, 1992; Block et al., 1989). In accordance with the myocardial cell study, culture supernatants contained elevated levels of arachidonic acid and other free fatty acids (Bhat and Block, 1992; Block et al., 1989). Primary HUVECs exposed to zero percent O_2 for two hours followed by 45 minutes re-oxygenation released approximately 15% more arachidonic acid than the controls (Michiels et al., 1993). Accordingly, eicosanoid production was augmented when HUVECs and bovine aortic endothelial cells were enriched with fatty acids in a HR study (Oudot et al., 1998).

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 (Block et al., 1989). 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 (Tagesson et al., 1985). LysoPC also enhanced the permeability that ischemia alone causes (Otamiri et al., 1986).

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 arachidonic acid than biopsies and mucosa from healthy control patients (Morita et al., 1999; Pacheco et al., 1987). LysoPE in the inflamed intestinal mucosal samples was also elevated in comparison to healthy control samples (Morita et al., 1999).

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 (Block et al., 1989). However, evidence of free radical production and lipid oxidation was detected with a 45 minute hypoxia, 15 minute reoxygenation treatment of aortic endothelial cells in a separate study (Kramer et al., 1995). A rat model of intestinal IR showed that reperfusion was necessary for an increase in malondialdehyde (Otamiri et al., 1987). Interestingly, a model of oxidative tissue damage in which mice are exposed to γ-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 (Tyurina et al., 2008).

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 are shuttling phospholipids from one leaflet to the other as a means of maintaining the asymmetric distribution of phospholipids.

The first of these lipid transporting proteins to be characterized was aminophospholipid translocase (Seigneuret and Devaux, 1984). Incorporation of spin-labeled analogs of PC, PS and PE were analyzed using electron spin resonance spectroscopy (Seigneuret and Devaux, 1984). Studies showed incorporation of all analogs to the membrane of red blood cells; however, less of the PS and PE analogs were reduced with sodium ascorbate with increasing lengths of

incubation, providing evidence that these analogs were internalized to the inner leaflet of the lipid bilayer (Seigneuret and Devaux, 1984). Use of spin-labeled analogs with bovine serum albumin back-extraction provided similar results with regard to specificity, confirming an affinity for PS and PE, and rate of transport, demonstrating more rapid internalization of PS (Connor et al., 1992; Morrot et al., 1989). This aminophospholipid translocase was found to be ATP-dependent with a stoichiometry of one ATP per PS or PE transported (Beleznay et al., 1993; Seigneuret and Devaux, 1984). 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 (Julien et al., 1995).

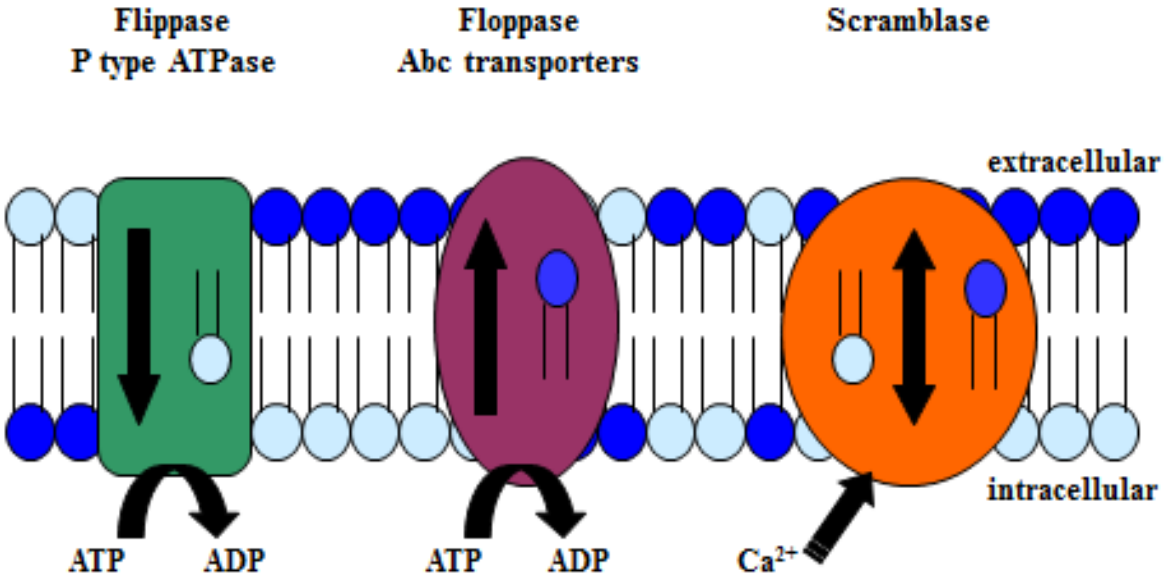


Figure 1.3 Schematic of membrane proteins involved in regulating phospholipid asymmetry.

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 (Andrick et al., 1991). Subsequently, this activity was also shown to be ATP-dependent, although independent of the aminophospholipid translocase (Connor et al.,

1992). This putative transporter was called floppase; for consistency and simplicity, the aminophospholipid translocase became known as flippase.

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 ((Francis et al., 2013; Wiedmer et al., 2000), reviewed in (Sahu et al., 2007)) and in mouse (reviewed in (Sahu et al., 2007)). 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 (Sahu et al., 2007)). Additionally, scramblases show very little specificity for phospholipid head-groups, transporting both neutral and aminophospholipids at a similar rate (reviewed in (Bevers, 1996; Sims and Wiedmer, 2001)). Thus, scramblase activity serves to disrupt and reduce the membrane asymmetry ((Williamson et al., 1995; Williamson et al., 1992), reviewed in (Sahu et al., 2007)). 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 (Bevers and Williamson, 2010)). 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 (Basse et al., 1996; Stout et al., 1997; Williamson et al., 1995; Williamson et al., 1992; Zhou et al., 1997) and acidic pH (Stout et al., 1997). 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 cerevisae* (reviewed in (Sahu et al., 2007)). The four human homologs are similar in sequence with PLSCR2 – 4 exhibiting 46 to 59% protein identity with PLSCR1 (Wiedmer et al., 2000). 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 (Basse et al., 1996; Zhou et al., 1997), primarily localizes to the cellular membrane (Frasch et al., 2004; Ory et al., 2013; Sun

et al., 2002; Wiedmer et al., 2003) while PLSCR3 resides in the outer mitochondrial membrane (Liu et al., 2003). Expression of PLSCR2 is only detected in the testes (Wiedmer et al., 2000) and PLSCR4 appears to distribute both to the cellular membrane and the nucleus (Francis et al., 2012), though the functions of these two family members remain unidentified.

The first cloning of the human PLSCR1 gene suggested a type 2 transmembrane protein of 318 amino acids with a single transmembrane helix but no signal sequence (Zhou et al., 1997). A second cloning provided additional information about the human PLSCR1 gene. The gene is located on chromosome three and consists of nine exons the first of which is untranslated, and eight introns with the open reading frame beginning at exon two (Wiedmer et al., 2000). There is evidence that the murine gene for PLSCR1 may contain an alternative splicing site resulting in transcription of mTRA1a, a truncated form of PLSCR1 which may be the closest murine ortholog to human PLSCR1 (Wiedmer et al., 2000). Although a crystal structure of the protein, human or mouse, has not been solved, a three-dimensional structural model based on homology modeling exists (Bateman et al., 2009).

Activation of PLSCR1 involves the binding of calcium or potentially other divalent cations (Basse et al., 1996; Sahu et al., 2009) to a predicted single cation binding site (Sahu et al., 2009) in the cytoplasmic portion. Several studies have demonstrated an increase in phospholipid scrambling in the presence of calcium (Basse et al., 1996; Stout et al., 1998; Zhou et al., 1997). It is hypothesized that calcium binding is followed by a conformational change and perhaps self-aggregation (Frasch et al., 2004; Sahu et al., 2009; Stout et al., 1998). In the absence of calcium, acidic conditions ($pH < 6.0$) activate PLSCR1 in erythrocyte-derived inside out vesicles (Stout et al., 1997). Data suggest that protein kinase Cδ phosphorylates PLSCR1 at the threonine residue at position 161 following calcium binding (Frasch et al., 2000). This phosphorylation appears to be required for PLSCR1 activity; as either specific inhibition of protein kinase Cδ or transfection of PLSCR1 alone in cells intrinsically lacking both protein kinase Cδ and PLSCR1 resulted in loss of phospholipid scrambling (Frasch et al., 2000). 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 (Sun et al., 2002).

Furthermore, data suggest that signaling via epidermal growth factor receptor activates synthesis of PLSCR1 (Sun et al., 2002).

Normally, PLSCR1 is found in the cellular membrane and to a lesser extent membranes of secretory vesicles, often associated with lipid rafts (Frasch et al., 2004; Merregaert et al., 2010; Ory et al., 2013; Sun et al., 2002). The protein contains 18 cysteine residues (Wiedmer et al., 2003), 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 (Wiedmer et al., 2003). 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 (Wiedmer et al., 2003). Additionally, the binding affinity for calcium and activity of PLSCR1 is greatly decreased in the absence of palmitoylation (Zhao et al., 1998).

In the absence of palmitoylation, PLSCR1 is found diffusely in the nucleus (Wiedmer et al., 2003). While PLSCR1 does not contain a classical nuclear localization signal (Ben-Efraim et al., 2004), the amino acid sequence from residues 257 to 266 is necessary for active import into the nucleus by importin α and β (Ben-Efraim et al., 2004; Chen et al., 2005). Recent studies suggest a role for PLSCR1 in the interferon response as an interferon regulatory factor is found upstream of the gene's promoter (Wiedmer et al., 2000) and the untranslated exon one contains an interferon stimulated response element (Zhou et al., 2000). Following translation, PLSCR1 decreases the synthesis of viral proteins (Yang et al., 2012). However, the full significance of PLSCR1's function in the nucleus is still unclear.

Toll-Like Receptors and Ischemia/Reperfusion

TLRs are a highly conserved (Roach et al., 2005) group of pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs). TLRs are transmembrane receptors belonging to the IL-1 superfamily as their intracellular domains are homologous to that of the IL-1 receptor. TLRs are further characterized by leucine-rich repeats in the extracellular domains. As pattern recognition receptors, TLRs function as components of the innate immune response;

recognizing and responding to various bacterial and viral components. Ten functional TLRs have been identified in humans and twelve in mice. Importantly, TLR 1-9 are conserved between the two species (reviewed in (Bryant and Monie, 2012)).

A critical TLR for IR injury in multiple organs is TLR4. Several studies have demonstrated the participation of TLR4 in intestinal, myocardial, cerebral and renal IR pathology (Gao et al., 2009b; Li and Cherayil, 2004; Moses et al., 2009; Takeishi and Kubota, 2009; Victoni et al., 2010; Wu et al., 2007; Yang et al., 2008a). IR damage to the liver requires TLR9 (Bamboat et al., 2010; Huang et al., 2011). Like most TLRs, TLR4 and TLR9 signal through MyD88, which is necessary for intestinal IR damage (Moses et al., 2009). These data prompt the question as to the involvement of TLR9 in intestinal IR injury.

TLR9 is an intracellular pattern recognition receptor. TLR9 is trafficked to the membrane of endosomes where it interacts with ligands. One of the first identified ligands of TLR9 is unmethylated CpG (cytidine-phosphate-guanosine) dinucleotides (Hemmi et al., 2000) which are abundant in bacterial genomes but limited in mammalian genomes. Viral RNA (Krug et al., 2004a; Krug et al., 2004b; Lund et al., 2003) and synthetic single stranded oligodeoxynucleotides (reviewed in (Jurk and Vollmer, 2007)) also ligate TLR9 resulting in signaling. More recently, self-nucleotides (mammalian as opposed to bacterial) and chromatin, when presented in immunoglobulin complexes, have been identified as ligands for TLR9 (Boule et al., 2004; Means et al., 2005).

Early studies indicated that the majority of translated TLR9 remains in the endoplasmic reticulum prior to phagocytosis or endocytosis by the cell (Ahmad Nejad et al., 2002; Kim et al., 2008; Latz et al., 2004; Leifer et al., 2006; Leifer et al., 2004), after which it is rapidly shuttled to endosomes (Kim et al., 2008; Latz et al., 2004; Leifer et al., 2006). The Golgi apparatus appeared to be bypassed as endosomal TLR9 remains sensitive to endoglycosidase H digestion (Leifer et al., 2006; Leifer et al., 2004). More recent work has provided compelling evidence that TLR9 does in fact pass through the Golgi apparatus prior to localization in endosomes (Chockalingam et al., 2009; Ewald et al., 2008). However, the Golgi-processed protein remains sensitive to digestion by endoglycosidase H (Chockalingam et al., 2009). Gp96, an endoplasmic reticulum paralog of heat shock protein 90 (Yang and Li, 2005), serves as a chaperone, assisting in the proper folding of TLR9 (Yang et al., 2007). Both the cytoplasmic and ectodomain, but not the transmembrane domain, are important for correct localization of TLR9 to endosomes (Leifer

et al., 2006) and two endoplasmic reticular proteins, UNC9B1 and protein associated with TLR4 A (PRAT4A), have been identified as critical for the proper shuttling of TLR9 to endosomes (Kim et al., 2008; Takahashi et al., 2007). Endosomal maturation and acidification (Ahmad-Nejad et al., 2002) as well as proteolytic cleavage of the TLR9 ectodomain are required prior to signaling (Ewald et al., 2008; Park et al., 2008). Binding of stimulatory ligands induces a conformational change in the ectodomains of the homodimers allowing for recruitment of the Toll/IL-1R homology domain (TIR) domain adaptor protein MyD88 (Latz et al., 2007). TNF receptor associated factor (TRAF) 6 is required to mediate signaling via the Jun-amino-terminal kinase (JNK; one of the mitogen activated protein kinases) and I kappa kappa B kinase (IKK) pathways (Hacker et al., 2000). Nuclear translocation of NFκB results in the production of several cytokines. TLR9 stimulation by CpG oligodinucleotides elicits production of IL-6 (Butt et al., 2012; Suwarti et al., 2013), KC, the murine homolog of IL-8 (Agrawal and Gupta, 2011; He et al., 2012; Johnson et al., 2005), IL-12 (Cowdery et al., 1999; Mason et al., 2002; Sasai et al., 2010; Yi et al., 2002) and TNFα (Agrawal and Gupta, 2011; Butt et al., 2012; Chockalingam et al., 2012).

Summary

The pathogenesis of intestinal IR-induced injury involves several cell types and signaling cascades. The lack of oxygen during ischemia initiates an altered physiological state within affected cells, such as transcription of HIF-regulated genes and a switch to anaerobic metabolism (reviewed in (Shay and Celeste Simon, 2012)). While reperfusion is required for salvage of the organ or tissue (provided viable cells remain), the collateral damage is significant. As the interface between the tissue and blood, endothelial cells are critically important in the pathogenesis of intestinal IR.

Apoptosis can be induced during the ischemic phase, particularly in the cells furthest from the vasculature (Droy-Lefaix et al., 1991; Haglund, 1994). Reperfusion initiates the formation of reactive oxygen species, activation of the complement cascade and production of pro-inflammatory cytokines and eicosanoids. The endothelial barrier is compromised as permeability increases and recruitment of neutrophils, and later macrophages, occurs. Yet, the initial event(s) that sets the aforementioned processes in motion remains elusive.

Recent studies have identified neo-antigens as a potential trigger, but their roles remain undefined (Fleming et al., 2010; Kulik et al., 2009b; Zhang et al., 2006a). 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-1*$^{-/-}$ mice are protected. Reconstitution of *Rag-1*$^{-/-}$ mice with antibodies from wildtype mice prior to intestinal IR renders the *Rag-1*$^{-/-}$ mice susceptible to IR-induced injury (Sparkes et al., 2010). Likewise, reconstitution with a monoclonal anti-phospholipid antibody and anti-phospholipid binding protein, β_2-GPI, (both found in wildtype sera) produces IR-induced pathology in the otherwise protected *Rag-1*$^{-/-}$ mice (Fleming et al., 2004a). Thus, the hypothesis that phospholipids of the cellular membrane were modified as a result of IR was examined. Furthermore, endothelial cells were hypothesized to be the primary cell type involved in intestinal IR-induced damage.

Several TLRs, pattern-associated molecular pattern receptors, have been shown to be involved in IR-induced pathogenesis as well. *TLR2* and TLR4 signaling promotes IR-induced injury in the kidney, heart, brain and intestine (Gao et al., 2009b; Li and Cherayil, 2004; Li et al., 2013; Moses et al., 2009; Takeishi and Kubota, 2009; Victoni et al., 2010; Wu et al., 2007; Yang et al., 2008a). TLR9 is a key component in hepatic IR-induced damage (Bamboat et al., 2010; Huang et al., 2011). The role of TLR9 in intestinal IR-induced injury, however, had not been investigated. Given that TLR9 recognizes self-DNA, TLR9 was hypothesized to contribute to intestinal IR-induced injury. The following studies were performed to address these hypotheses.

Chapter 2 - Intestinal lipid alterations occur prior to antibody-induced PGE_2 production in a mouse model of ischemia/reperfusion

Summary

Ischemia/reperfusion (IR) induced injury results in significant tissue damage in wildtype but not antibody deficient *Rag-1*$^{-/-}$ mice. However, *Rag-1*$^{-/-}$ mice sustain intestinal damage after administration of wildtype antibodies or naturally occurring, specific anti-phospholipid related monoclonal antibodies, suggesting involvement of a lipid antigen. We hypothesized that IR initiates metabolism of cellular lipids, resulting in production of an antigen recognized by anti-phospholipid antibodies. At multiple time points after Sham or IR treatment, lipids extracted from mouse jejunal sections were analyzed by electrospray ionization triple quadrupole mass spectrometry. Within 15 min of reperfusion, IR induces significantly more lysophosphatidylcholine (lysoPC), lysophosphatidylglycerol (lysoPG) and free arachidonic acid (AA) production than Sham treatment. While lysoPC, lysoPG and free AA levels were similar in C57Bl/6 (wildtype) and *Rag-1*$^{-/-}$ mice, IR activated Cox 2 and prostaglandin E_2 (PGE_2) production in wildtype, but not in the antibody deficient *Rag-1*$^{-/-}$ mice. Administration of wildtype antibodies to *Rag-1*$^{-/-}$ mice restored PGE_2 production and intestinal damage. These data indicate that IR-induced intestinal damage requires antibodies for Cox 2 stimulated PGE_2 production but not for production of lysoPC and free AA.

Introduction

Ischemia, a condition in which a lack of oxygen and nutrients results in severe inflammation and cellular damage, is a common medical pathology (Mallick et al., 2004; Zimmerman and Granger, 1994). Ischemic cells undergo biological and chemical changes including activation of numerous proteases and lipases which induce tissue damage (Zhang and Carroll, 2007). Subsequent reperfusion to the ischemic region results in far greater injury than observed as a result of ischemia alone (Fleming et al., 2002a; Mallick et al., 2004; Zimmerman and Granger, 1994). The intestines are thought to be the organ most sensitive to reperfusion induced damage (Clark and Coopersmith, 2007; Mallick et al., 2004). Mesenteric reperfusion damage is

associated with multiple organ failure, resulting in a mortality rate ranging from 60 to 80% in humans (Clark and Coopersmith, 2007; Deitch, 2001; Leaphart and Tepas, 2007). Very little is understood about prevention and diagnosis of this condition, and therapeutic treatments are limited. Thus, there is strong incentive to understand the mechanisms leading to IR injury in the intestine.

The pathology of intestinal IR involves neutrophil infiltration and complement activation, as either neutrophil depletion (Crawford et al., 1988; Hernandez et al., 1987; Simpson et al., 1993) or complement blockade (Eror et al., 1999b; Hill et al., 1992b; Rehrig et al., 2001a) attenuates injury. Although naturally resistant to mesenteric IR-induced injury, antibody-deficient *Rag-1*$^{-/-}$ mice sustain significant inflammation and damage following administration of antibodies (Ab) from wildtype mice. Indeed, Ab play a critical role in both neutrophil recruitment and complement activation (Fleming et al., 2004a; Williams et al., 1999a). After administration of monoclonal Ab against phospholipids and a phospholipid binding protein, these damage resistant mice sustained inflammation and intestinal injury at levels seen in wildtype mice (Fleming et al., 2004a; Williams et al., 1999a). These data suggest that a newly expressed lipid antigen (neo-antigen) may be important in reperfusion-induced damage. Identification of lipid alterations during IR might suggest therapeutic targets for reperfusion-induced damage.

Despite advances in "lipidomics", or mass spectrometry-based lipid analysis, only a few studies have applied this technology to investigate intestinal lipid composition. One study utilized electrospray ionization-tandem mass spectrometry (ESI-MS/MS) to determine the relative prevalence of 10 glycerophospholipid classes in normal rat intestine (Hicks et al., 2006). Another study showed that phosphatidylcholine (PC) and lysophosphatidylcholine (lysoPC) were decreased in the intestinal mucus of ulcerative colitis patients (Braun et al., 2009; Ehehalt et al., 2004). Using lipidomics, a recent study found the mouse duodenum and jejunum contained the highest concentrations of PC and lysoPC in normal intestines (Braun et al., 2009). These data indicate that lipidomics is a useful tool for identification of intestinal lipid changes associated with disease. Since intestinal lipid changes in the IR model were not investigated in previous studies, ESI-MS/MS may be useful in determining the IR-induced lipid antigen. In the current study, we test the hypothesis that mesenteric IR alters total intestinal lipid composition and examine the role of Ab in IR-induced lipid changes and subsequent tissue injury.

Methods

Mice

C57Bl/6 wild-type mice and *Rag-1*$^{-/-}$ mice (Jackson Laboratories) were bred and maintained at Kansas State University. Male mice were between 10 and 16 weeks of age when used in experiments. Mice were maintained in a 12 h light/dark cycle with constant access to standard rodent chow and water and were not fasted prior to experimental use. All procedures were approved by the Institutional Animal Care and Use Committee and were in compliance with the Animal Welfare Act.

Intestinal Ischemia/Reperfusion

Mice were subjected to IR as described previously (Fleming et al., 2004a). Briefly, mice were anaesthetized by an intraperitoneal injection of 8 mg per kg xylazine and 16 mg per kg ketamine. All subsequent manipulations were performed on a heat pad to maintain body temperature. After performing a midline laparotomy, blood flow to the superior mesenteric artery was occluded for 30 min using a small vascular clamp. Following removal of the clamp and suturing the body wall, the intestines were reperfused for 15, 30 or 120 min. Mice were then euthanized and one to two cm long jejunal sections (approximately 10 cm distal of the gastroduodenal junction) were removed and fixed in formalin or frozen in liquid nitrogen. Sham treated animals underwent the same surgical procedure without occlusion of the superior mesenteric artery. Additional *Rag-1*$^{-/-}$ mice received an intravenous injection of 100 μg of purified Ab 15 to 20 min prior to occlusion of the superior mesenteric artery. All tissues collected were assayed in a blinded manner.

Injury Scoring of Intestinal Villi

A formalin fixed, hematoxylin and eosin stained transverse jejunum section from each mouse was scored for intestinal damage based on a six tiered scale adapted from Chui et al. (Chiu et al., 1970). Each villus was assigned a score according to the following criteria: 0: intact villus with no damage, 1: bulging of the epithelium, 2: Guggenheim's space, 3: visible breakage of the

epithelium, 4: exposure of the intact lamina propria, 5: exuding of the lamina propria, and 6: blood loss and denuding of the lamina propria. Scores from 75 to 150 villi in a two cm section were averaged to determine the injury score for that mouse.

Lipid Extraction

Frozen two cm jejunal samples were homogenized into a fine powder in liquid nitrogen using a steel mortar and pestle and extracted as described previously with adaptations (Bligh and Dyer, 1959; Folch et al., 1957). A portion of the homogenized tissue was retained for bicinchoninic acid (BCA, Pierce) protein analysis. The remaining tissue (80 to 150 mg) was resuspended in one ml cold water. One ml chloroform and three ml methanol were added and the sample was shaken vigorously. An additional one ml chloroform and one ml water were added and the sample was centrifuged at 4000 rpm for five min at 4ºC. The lower organic layer was removed and one ml chloroform added to the aqueous layer. The sample was again centrifuged and the process repeated for a total of three chloroform extractions. The lower organic layers from each extraction were pooled and washed with one ml water. The final organic layer was submitted for mass spectrometry analysis.

Mass Spectrometry Analysis

An automated electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) approach was used and data acquisition and analysis carried out at the Kansas Lipidomics Research Center as described previously (Bartz et al., 2007; Devaiah et al., 2006) with modifications. Solvent was evaporated from the pooled organic layers of the extracts and each sample was dissolved in one ml of chloroform. An aliquot of 20 µl of extract in chloroform was analyzed. Precise amounts of internal standards, obtained and quantified as previously described (Welti et al., 2002), were added in the following quantities (with some small variation in amounts in different batches of internal standards): 0.66 nmol di14:0-PC, 0.66 nmol di24:1-PC, 0.66 nmol 13:0-lysoPC, 0.66 nmol 19:0-lysoPC, 0.36 nmol di14:0-PE, 0.36 nmol di24:1-PE, 0.36 nmol 14:0-lysoPE, 0.36 nmol 18:0-lysoPE, 0.36 nmol 14:0-lysoPG, 0.36 nmol 18:0-lysoPG, 0.36 nmol di14:0-PA, 0.36 nmol di20:0(phytanoyl)-PA, 0.24 nmol di14:0-PS, 0.24 nmol di20:0(phytanoyl)-PS, 0.20 nmol 16:0-18:0-PI, 0.16 nmol di18:0-PI and 1 nmol 15:0 fatty acid. The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform/methanol/300 mM

ammonium acetate in water was 300/665/35, and the final volume was 1.2 ml. These unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 µl per min.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: PC, SM and lysoPC, [M + H]+ ions in positive ion mode with Precursor of 184.1 (Pre 184.1); PE and lysoPE, [M + H]+ ions in positive ion mode with Neutral Loss of 141.0 (NL 141.0); PI, [M + NH4]+ in positive ion mode with NL 277.0; PS, [M + H]+ in positive ion mode with NL 185.0; PA, [M + NH4]+ in positive ion mode with NL 115.0; and free fatty acids (i.e., free arachidonic acid, (AA)), [M - H]- in negative mode with single stage MS analysis. SM was determined from the same mass spectrum as PC (precursors of m/z 184 in positive mode) (Brügger et al., 1997; Liebisch et al., 2004) and by comparison with PC internal standards using a molar response factor for SM (in comparison with PC) determined experimentally to be 0.39. Acyl,alk(en)yl ("ether-linked") ePCs and ePEs were determined in relation to the same standards as diacyl PC and PE species, and no response factors were applied. The scan speed was 50 or 100 u per sec. The collision gas pressure was set at two (arbitrary units). The collision energies, with nitrogen in the collision cell, were +28 V for PE, +40 V for PC (and SM), +25 V for PI, PS and PA. Declustering potentials were +100 V. Entrance potentials were +15 V for PE, +14 V for PC (and SM), PI, PA and PS. Exit potentials were +11 V for PE, +14 V for PC (and SM), PI, PA and PS. The mass analyzers were adjusted to a resolution of 0.7 u full width at half height. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode. The source temperature (heated nebulizer) was 100°C, the interface heater was on, +5.5 kV or -4.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units) and the two ion source gases were set at 45 (arbitrary units).

The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software. The lipids in each class were quantified in comparison to the two internal standards of that class. The first and

typically every 11th set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the "internal standards only" spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each "internal standards only" set of spectra were used to correct the data from the following 10 samples. Finally, the data were expressed as mole percent of total lipid analyzed. Each class of lipid was also normalized to intestinal proteins and expressed as nmol lipid class per mg protein.

Acyl ions (product ions) of PE, PI and PS species were identified after collision induced dissociation of the [M - H]- ions, and acyl ions of PC species were identified following collision induced dissociation of the [M + OAc]- ions.

Prostaglandin E_2 Concentrations

A freshly excised mid-jejunal section of intestine was used for analysis of prostaglandin E_2 (PGE_2) as described previously (Fleming et al., 2002a; Miner et al., 1999b; Rehrig et al., 2001a). Briefly, the intestinal section was minced in oxygenated Tyrode's buffer (Sigma), washed three times to remove feces and incubated for 20 min at 37ºC in freshly oxygenated Tyrode's buffer. The supernatant was collected and stored at -80ºC until assayed for PGE_2 by EIA (Cayman Chemicals). The PGE_2 concentration was normalized to total intestinal protein which was determined by BCA analysis and expressed as pg PGE_2 per mg protein.

Cox 2 mRNA Expression

Mid-jejunal intestinal sections were snap-frozen in liquid nitrogen and stored at -80ºC until homogenized in Trizol (Invitrogen) and RNA extracted using the manufacturer's protocol. RNA concentration was determined by spectrophotometry (Nanodrop 1000 spectrophotometer). RNA (1 µg) was reverse transcribed with a first strand cDNA synthesis kit (MBI Fermentas, Hanover, MD) with random hexamer primers. The cDNA was subjected to real-time PCR on a mini-Opticon thermocycler (Bio-Rad). PCR was performed using SYBR green in 25 µl volumes with the following amplification conditions: 95°C for 3 min followed by 50 cycles of 95°C for 10 sec,

58°C for 30 sec and 72°C for 10 sec. All reactions were performed in duplicate. After normalization to 18s, relative gene expression between treatment groups was determined using the comparative ΔCt method. The fold change in mRNA expression for Cox 2 expression was determined relative to the C57Bl/6 (wildtype) Sham treated control group after normalization to 18s rRNA. 18s rRNA was selected as the internal standard based on preliminary studies indicating no significant differences in the 18s rRNA quantities between treatment groups. Cox 2 primers were: sense, 5`-ATCCTGCCAGCTCCACCG- 3'; anti-sense, 5`-TGGTCAAATCCTGTGCTC ATACAT -3' and 18s primers were: sense, 5`-GGTTGATCCTGCCAGTAGC- 3'; anti-sense, 5`-GCGACCAAAGGAACCATAAC -3'. Primer sequences were designed by Beacon Designer 5.0 (Premier Software, Palo Alto, CA) and synthesized by Integrated DNA Technologies (Coralville, IA).

Antibody Purification

Immunoglobulins (Ig) from C57Bl/6 sera were purified using protein L bead columns (Pierce) to allow purification of all antibody isotypes. Subsequent multiplex analysis indicated that IgM, IgG_1, IgG_{2b} and IgG_3 were present in similar proportions to that found in the sera (data not shown). Beads were packed into a 0.5 ml bead bed and 0.5 ml sera applied to each column. Multiple Ig fractions were collected and each analyzed by spectrophotometer. Those fractions containing the highest absorbance were pooled and dialyzed at 4°C overnight in phosphate buffered saline (PBS). The PBS was changed twice during dialyzation. Dialyzed fractions were concentrated to one mg per ml using Centriplus concentrators (Millipore).

Statistical Analysis

Statistical analysis was performed using an unpaired t-test or one-way ANOVA with Newman-Keuls post hoc analysis (Graphpad Prism 4). Data were expressed as the mean ± SEM analyses. Data were deemed to be statistically different when $p < 0.05$.

Results

Lipidomic analysis of the molecular composition of intestinal phospholipids, sphingomyelin, and free arachidonic acid in Sham treated wildtype mice

Using ESI-MS/MS, characterization of the intestinal polar glycerophospholipids and sphingomyelin species of Sham treated wildtype (C57Bl/6) mice revealed that phosphatidylcholines, including diacyl (PC), acyl,alk(en)yl (ePC), and monoacyl (lysoPC) species, and phosphatidylethanolamines, including diacyl (PE), acyl,alk(en)yl (ePE), and monoacyl (lysoPE) species, are the major phospholipids of the intestine making up 61 mol% and 13 mol% respectively (Fig. 1A). Phosphatidylinositol (PI), sphingomyelin (SM) and phosphatidylserine (PS) represented six, seven and five mol% respectively of the measured phospholipids in C57Bl/6 wildtype mice, while phosphatidic acid (PA), phosphatidylglycerol (PG), monoacyl PG (lysoPG) and free arachidonic acid (AA) were present in lesser amounts (Fig. 1A).

The fatty acyl compositions of the various phospholipid classes from intestines were different (Fig. 1B). In the PC class, species containing a total of 34 and 36 fatty acyl carbons were most abundant, while in PE and PS, species containing 36, 38 and 40 carbons were most abundant. The 38:4 species of PI represented nearly half (45%) of the total PI. Mass spectral product ion analysis indicated that each of the major diacylphospholipid classes included molecular species containing arachidonic acid (AA). For example, 36:4 PC and 38:4 PC were primarily 16:0-20:4 and 18:0-20:4 PC, 38:4 PE was primarily 18:0-20:4 PE, 38:4 PS was primarily 18:0-20:4 PS and 38:4 PI was primarily 18:0-20:4 PI (Fig. 1B).

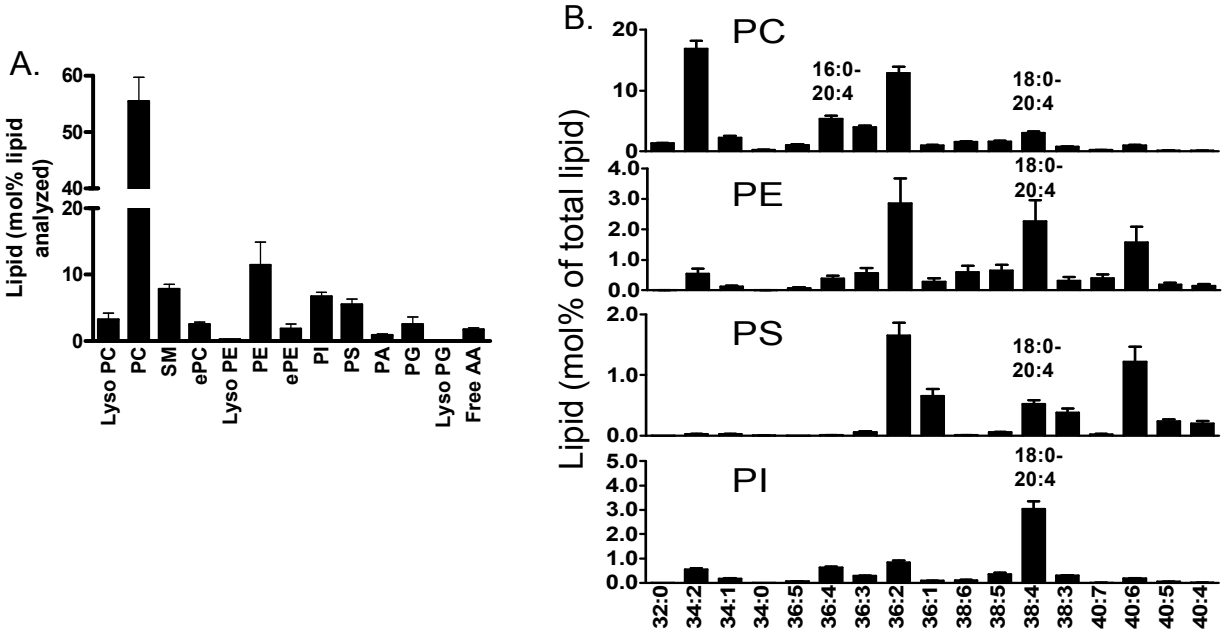


Figure 2.1 ESI-MS/MS identifies mid-jejunal lipids in Sham treated C57Bl/6 mice.
Lipids were extracted from mid-jejunal sections from Sham treated C57Bl/6 mice and analyzed by ESI-MS/MS to determine mol% of (A) polar intestinal glycerophospholipids, sphingomyelin and free arachidonic acid and (B) fatty acyl compositions of the major phospholipid classes. Each bar represents the mean ± SEM of 7 to 8 mice.

Following IR, Rag-1$^{-/-}$ mice sustain significantly decreased damage compared to wildtype mice

Rag-1$^{-/-}$ mice sustain significantly less IR-induced mesenteric injury than C57Bl/6 wildtype mice at two h post ischemia (Fleming et al., 2004a). To verify this finding and examine the extent of injury at earlier time points of reperfusion, *Rag-1*$^{-/-}$ mice and wildtype mice were subjected to 30 min ischemia followed by 15, 30 or 120 min reperfusion. As indicated in Figure 2A, intestinal damage in *Rag-1*$^{-/-}$ mice was significantly attenuated compared to wildtype mice at all observed time points. Sham treatment of either mouse strain resulted in no damage as indicated by the low injury scores (Fig. 2A). Wildtype mice sustained significant damage compared to the Sham treated mice by 15 min reperfusion (Fig. 2A, B). The damage increased slightly by two h post ischemia (Fig. 2A, C). In contrast, *Rag-1*$^{-/-}$ mice sustained minimal damage at all time points measured (Fig. 2A, D, E). Administration of wildtype Ab to *Rag-1*$^{-/-}$ mice results in significantly increased intestinal damage by 15 min post ischemia (Fig. 2A). The data indicate that Ab-

mediated intestinal damage occurs within 15 min after reperfusion begins in wildtype mice. The damage was further increased by 2 h post ischemia (Fig. 2A, F).

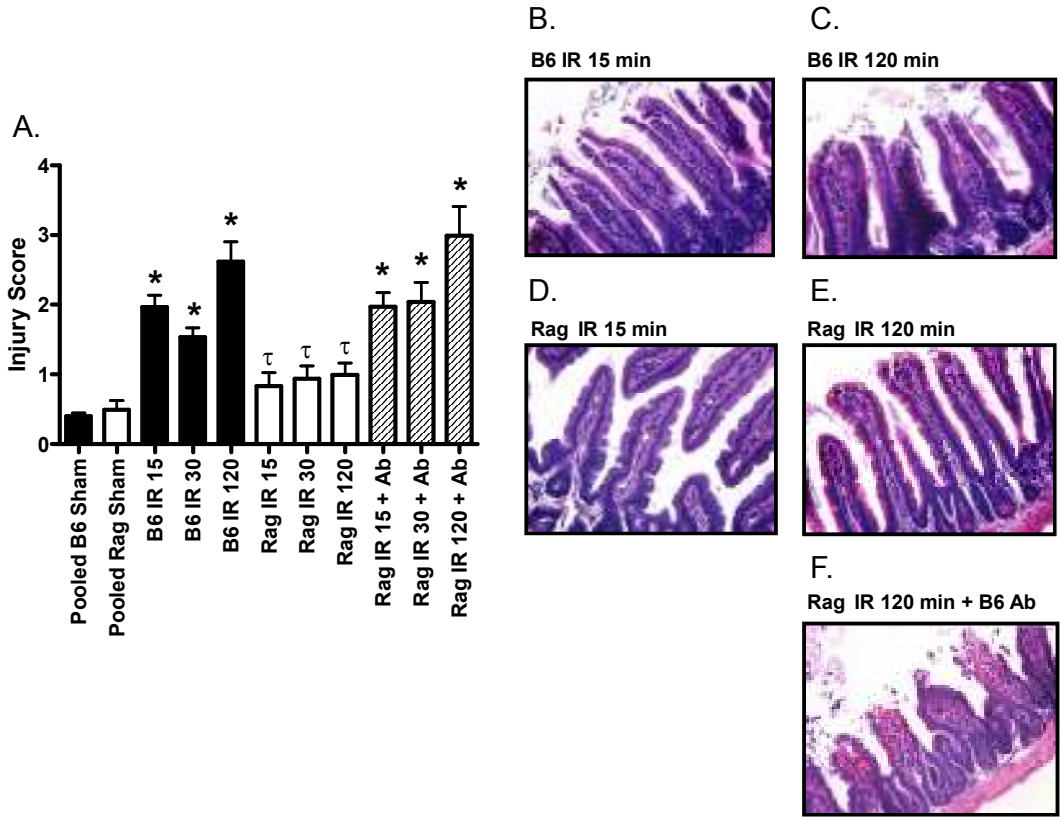


Figure 2.2 *Rag-1*$^{-/-}$ mice sustain significantly less IR-induced intestinal damage than wildtype mice.

Rag-1$^{-/-}$ (open bars) and C57Bl/6 (solid bars) mice were subjected to mesenteric IR with 15, 30 or 120 min reperfusion or Sham treatment. Additional *Rag-1*$^{-/-}$ mice received 100 µg purified C57Bl/6 antibody prior to IR with 120 min reperfusion (striped bar). Formalin fixed, H & E stained intestinal sections were scored for mucosal injury as described in *Methods*. Average injury scores ± SEM for each treatment group of 4 to 10 mice are shown (A). Representative sections of intestinal tissue after 15 min reperfusion (B, D) or 120 min reperfusion (C, E) from *Rag-1*$^{-/-}$ mice (D, E) or C57Bl/6 mice (B, C) are shown. Also shown is a representative section of intestinal tissue at 120 min reperfusion from *Rag-1*$^{-/-}$ mice receiving wildtype Ab prior to IR treatment (F). A one-way ANOVA with Newman-Keuls post-hoc test was used to determine significance; * indicates significant difference ($p < 0.05$) from Sham treatment and τ indicates significant difference from wildtype.

In response to IR, palmitoyl and stearoyl lysoPCs and free arachidonic acid levels are significantly increased in intestines

Mass spectrometry analysis allows identification of individual lipid molecular species. The data suggest that lysoPC and lysoPE rise quickly after IR, with lysoPC becoming significantly higher than the Sham group at 30 min after ischemia (Fig. 3A). LysoPG also rose over time with significant increases from Sham group at 30 and 120 min post ischemia (Fig. 3A). PC levels decreased upon reperfusion, with significant differences from Sham group at 30 and 120 min reperfusion (Fig. 3B). Neither PE nor lysoPE were significantly altered during reperfusion (Fig. 3A, B). The levels of lysoPC were maximal at 30 min after IR with 6.0 ± 1.1 (SD) mol% compared to 3.5 ± 2.6 (SD) mol% following Sham treatment. Figure 4 shows that five major lysoPC molecular species, 16:0, 18:2, 18:1, 18:0 and 20:4, increased following IR with palmitoyl (16:0) and stearoyl (18:0) lysoPCs the most prominent. Free arachidonic acid (AA) levels were increased by IR treatment at 15 min post ischemia (Fig. 3C). By 30 min reperfusion, free AA levels in Sham treated wildtype mice were 1.7 mol%, while free AA levels of IR treated wildtype mice were 4.5 mol%. The elevated free AA was maintained at 120 min post ischemia. Taken together, these data suggest that activation of acyl hydrolytic activity during or following IR results in hydrolysis of phospholipids to lysolipids and free fatty acids, in particular free AA.

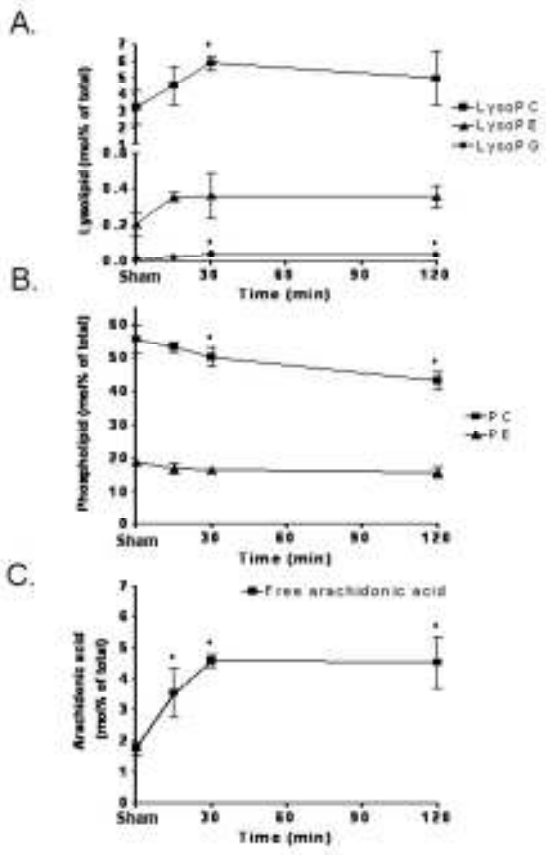


Figure 2.3 IR induces a rapid increase in intestinal lysolipids and free arachidonic acid with a concurrent decrease in phospholipid.

Mid-jejunal lipid extracts from IR treated C57Bl/6 mice were analyzed by ESI-MS/MS. Mol% lysoPC, lysoPE and lysoPG (A), PC and PE (B) and free arachidonic acid (C) were examined at 0 (Sham treatment), 15, 30 and 120 min post ischemia. Mean ± SEM of 3 to 7 mice per group are shown. One-way ANOVA with Newman-Keuls post-hoc test was used to determine significance; * indicates significance from Sham treatment where $p < 0.05$.

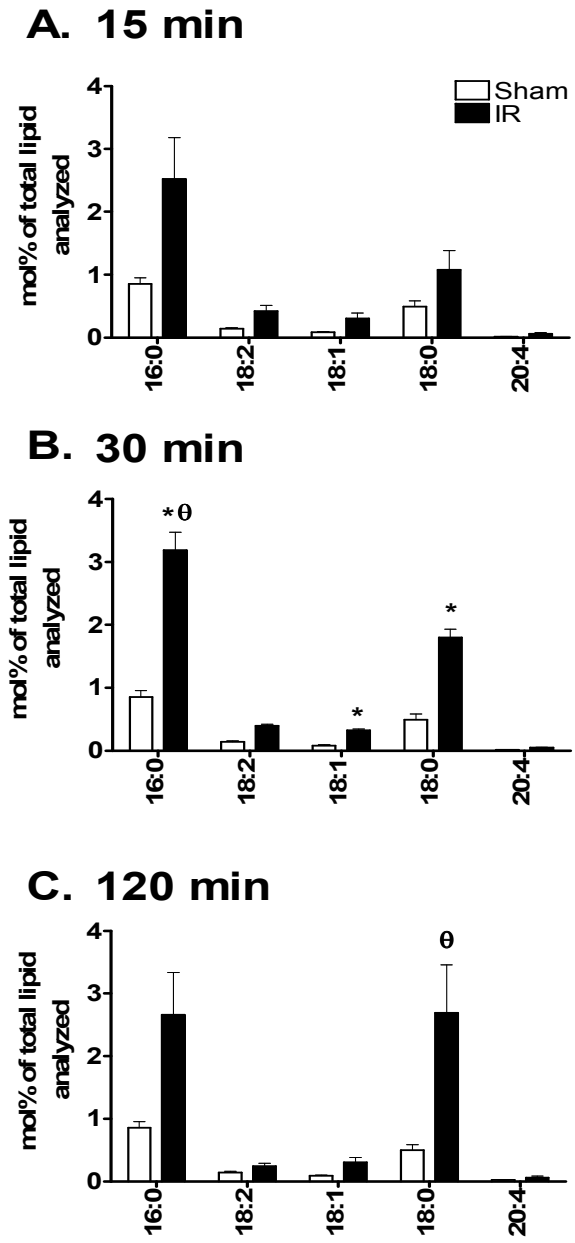


Figure 2.4 Specific lysoPC molecular species increase significantly within the intestine in response to IR.

C57Bl/6 mice were subjected to Sham (open bars) or IR (solid bars) with 15 (A) 30 (B) or 120 (C) min reperfusion prior to ESI-MS/MS determination of mol% of lipid analyzed within the intestine. The major lysoPC species which increased in response to IR, lysoPC 16:0, 18:2, 18:1, 18:0 and 20:4 are illustrated. Each bar represents the mean ± SEM with 4 to 5 mice per group. An unpaired t-test determined significant difference ($p < 0.05$) between Sham and IR treatment within a molecular species (*) and one way ANOVA with Newman-Keuls post hoc analysis was used to determine significance of a species over time (θ) where $p < 0.05$.

Total jejunal lipid composition is similar between Rag-1$^{-/-}$ mice and wildtype mice during mesenteric reperfusion

Rag-1$^{-/-}$ mice are naturally resistant to IR-induced damage. Examination of total jejunal lipids between mouse strains revealed no significant differences (Table 1). Additionally, comparison of intestinal lipid composition of Sham-treated mice (Table 2) showed no significant

Total Lipid nmol/mg protein mean ± SEM[1]	Sham	IR 15	IR 30	IR 120
B6	253 ± 85	114 ± 21	104 ± 23	152 ± 36
Rag	110 ± 13	81 ± 17	72 ± 26	129 ± 3

Table 2.1 Total jejunal lipids analyzed in Sham and IR treated C57Bl/6 and *Rag-1*$^{-/-}$ mice.

[1] Each value is the nmol total lipids per mg tissue analyzed and is a mean of 4 to 8 mice per time point.

Lipid Class[1]	B6 (mol% ± SEM) [2]	Rag (mol% ± SEM)
Lyso PC	2.298 ± 0.443	2.196 ± 0353
PC	58.920 ± 2.885	51.900 ± 0.804
SM	8.194 ± 0.721	7.977 ± 0.225
ePC	2.758 ± 0.234	2.478 ± 0.092
Lyso PE	0.200 ± 0.069	0.252 ± 0.039
PE	18.580 ± 0.924	17.690 ± 0.294
ePE	3.186 ± 0.621	3.650 ± 0.272
PI	6.191 ± 0.465	6.003 ± 0.302
PS	4.888 ± 0.696	4.223 ± 0.395
PA	0.874 ± 0.205	0.604 ± 0.104
Lyso PG	1.433 ± 0.164	1.398 ± 0.155
PG	0.013 ± 0.004	0.016 ± 0.004
Free AA	1.743 ± 0.261	1.464 ± 0.221

Table 2.2 C57Bl/6 and *Rag-1*$^{-/-}$ mice maintain similar intestinal lipid classes.

[1] Lipids were extracted from mid-jejunal intestinal sections from C57Bl/6 (B6) and *Rag-1*$^{-/-}$ (Rag) Sham treated mice. ESI-MS/MS analysis determined mol% of polar intestinal glycerophospholipids, sphingomyelin and free arachidonic acid of major lipid classes.

[2] The mean ± SEM of 5 to 9 mice.

differences in the lipid class compositions between wildtype and *Rag-1*$^{-/-}$ mice. Acyl composition within classes was also similar between wildtype and *Rag-1*$^{-/-}$ mice (Table 3). Because *Rag-1*$^{-/-}$ mice sustain less IR-induced intestinal damage as compared to wildtype mice, we tested the hypothesis that changes in lipid composition in response to IR would differ between strains. Results indicated that total lipids from mid-jejunal samples collected at 15, 30 and 120 min post ischemia did not display differences in composition between the strains of mice at any time of reperfusion examined (Fig. 5). The major change occurring in response to IR in the measured phospholipids of the *Rag-1*$^{-/-}$ mice, as in the wildtype mice, was the production of lysoPC species (Fig. 5). Free AA levels also increased in response to IR in the *Rag-1*$^{-/-}$ mice, similar to the increase observed in wildtype (C57Bl/6) mice (Fig. 5), with maximal *Rag-1*$^{-/-}$ free AA levels at 120 min after reperfusion at 1.4 mol% in Sham-treated and 4.9 mol% in the IR group.

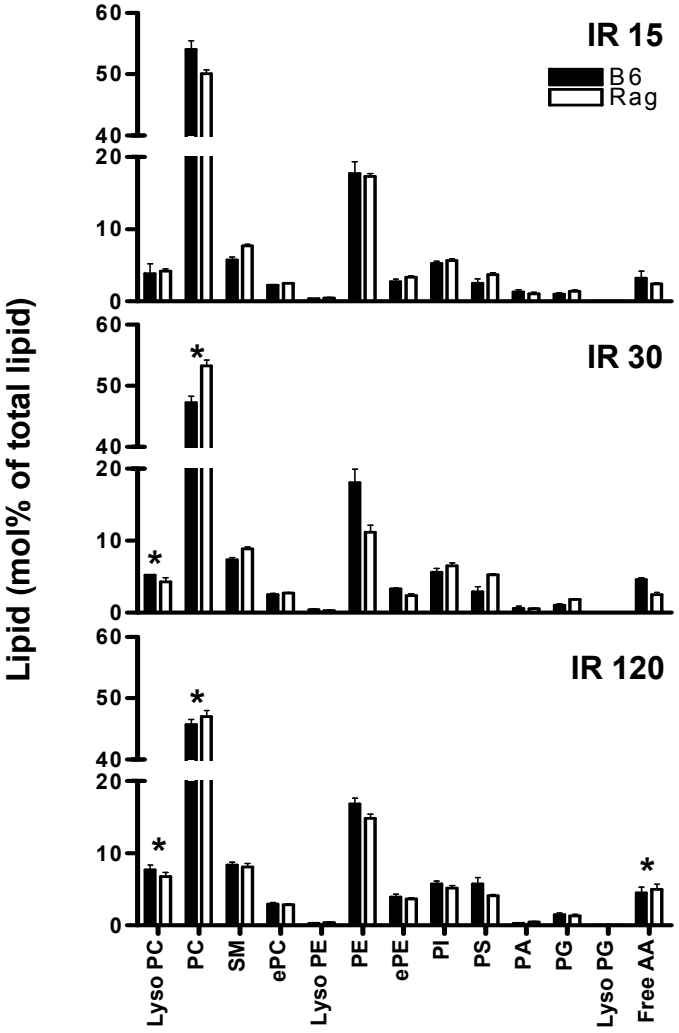


Figure 2.5 Intestinal lipid composition is conserved between C57Bl/6 and *Rag-1*$^{-/-}$ mice. C57Bl/6 (solid bars) and *Rag-1*$^{-/-}$ (open bars) mice were subjected to IR treatment with 15, 30 or 120 min reperfusion prior to collection of the mid-jejunum. After intestinal lipid extraction, ESI-MS/MS analysis determined mol% lipid analyzed from the mid-jejunum of each animal. Each bar represents the mean ± SEM with 3 to 4 mice per group. A one way ANOVA with Newman-Keuls post hoc analysis was used to determine significance of a species over time (*) where $p < 0.05$ (both B6 and Rag, from 15 min).

	PC		PE		PI		PS	
Acyl chains	**B6**	**Rag**	**B6**	**Rag**	**B6**	**Rag**	**B6**	**Rag**
32:0	0.977 ± 0.173	1.199 ± 0.026	0.005 ± 0.002	0.008 ± 0.001				
34:2	13.790 ± 2.709	13.950 ± 0.783	1.031 ± 0.305	0.757 ± 0.056	0.653 ± 0.112	0.486 ± 0.036	0.036 ± 0.006	0.022 ± 0.004
34:1	2.075 ± 0.372	2.174 ± 0.150	0.168 ± 0.042	0.164 ± 0.011	0.243 ± 0.068	0.168 ± 0.016	0.039 ± 0.015	0.024 ± 0.002
34:0	0.182 ± 0.066	0.251 ± 0.018	0.005 ± 0.002	0.011 ± 0.002			0.004 ± 0.001	0.006 ± 0.001
36:5	0.772 ± 0.228	1.002 ± 0.066	0.139 ± 0.039	0.133 ± 0.011	0.077 ± 0.015	0.063 ± 0.006	0.001 ± 0.001	0.001 ± 0.001
36:4	4.047 ± 0.773	5.253 ± 0.109	0.770 ± 0.214	0.597 ± 0.013	0.793 ± 0.099	0.621 ± 0.043	0.008 ± 0.002	0.008 ± 0.002
36:3	2.527 ± 0.644	3.499 ± 0.082	1.168 ± 0.329	0.719 ± 0.030	0.348 ± 0.073	0.258 ± 0.022	0.067 ± 0.015	0.050 ± 0.007
36:2	9.034 ± 2.151	12.840 ± 0.497	5.449 ± 1.558	4.163 ± 0.183	1.050 ± 0.215	0.702 ± 0.041	1.896 ± 0.443	1.290 ± 0.095
36:1	0.635 ± 0.115	0.938 ± 0.081	0.413 ± 0.103	0.443 ± 0.013	0.110 ± 0.031	0.072 ± 0.005	0.819 ± 0.285	0.508 ± 0.077
38:6	1.077 ± 0.225	1.474 ± 0.030	1.048 ± 0.221	0.873 ± 0.020	0.153 ± 0.039	0.095 ± 0.008	0.009 ± 0.003	0.008 ± 0.001
38:5	1.082 ± 0.264	1.683 ± 0.032	1.322 ± 0.381	1.086 ± 0.035	0.431 ± 0.076	0.324 ± 0.023	0.071 ± 0.013	0.060 ± 0.004
38:4	1.900 ± 0.503	3.265 ± 0.094	4.539 ± 1.243	3.944 ± 0.105	4.047 ± 0.792	2.683 ± 0.119	0.730 ± 0.239	0.467 ± 0.044
38:3	0.458 ± 0.125	0.772 ± 0.038	0.433 ± 0.130	0.492 ± 0.034	0.344 ± 0.062	0.275 ± 0.019	0.438 ± 0.114	0.280 ± 0.037
40:7	0.149 ± 0.043	0.236 ± 0.016	0.703 ± 0.148	0.517 ± 0.015	0.008 ± 0.001	0.004 ± 0.001	0.025 ± 0.006	0.023 ± 0.004
40:6	0.630 ± 0.174	1.028 ± 0.054	2.587 ± 0.536	2.265 ± 0.075	0.201 ± 0.037	0.138 ± 0.012	1.833 ± 0.677	0.840 ± 0.088
40:5	0.108 ± 0.039	0.201 ± 0.008	0.297 ± 0.073	0.329 ± 0.008	0.054 ± 0.013	0.048 ± 0.003	0.316 ± 0.089	0.191 ± 0.008

Table 2.3 C57B1/6 and *Rag-1*$^{-/-}$ mice maintain similar intestinal acyl chains within the lipid classes.

Lipids were extracted from mid-jejunal intestinal sections from C57B1/6 (B6) and *Rag-1*$^{-/-}$ (Rag) Sham treated mice. ESI-MS/MS analysis determined mol% of the fatty acid chains for the major lipid classes.

The mean ± SEM of 5 to 9 mice.

IR-induced Cox 2-mediated prostaglandin E_2 expression requires antibody

The formation of PGE_2 requires multiple enzymatic activities, including activation of the acyl hydrolase, phospholipase A_2 (PLA_2) which releases free AA. Subsequently, activation of cyclooxygenase converts free AA to PGH_2 which in turn is converted to PGE_2. Previous studies have correlated IR-induced inflammation and tissue damage with increased PGE_2 production in wildtype mice after 2 h reperfusion (Fleming et al., 2002b; Miner et al., 1999a; Rehrig et al., 2001b). In addition, we previously demonstrated that inhibition of Cox 2 and subsequent PGE_2 production resulted in attenuated IR-induced intestinal damage (Moses et al., 2009). Therefore, we quantified intestinal PGE_2 production in wildtype and *Rag-1*$^{-/-}$ mice at 2 h post ischemia. As in previous studies, we observed that wildtype IR treated mice had significantly increased PGE_2 concentrations compared to the Sham treatment group (Fig. 6A). In contrast, PGE_2 concentrations were not significantly increased in response to IR in *Rag-1*$^{-/-}$ mice. Administration of wildtype Ab prior to intestinal IR induced significant concentrations of intestinal PGE_2 in *Rag-1*$^{-/-}$ mice within 120 min post ischemia. The observed difference in PGE_2 production, despite similar levels of free AA, in wildtype and Ab-deficient mice, suggest that Ab are required for IR-induced PGE_2 production from AA.

To better understand the Ab requirement, we compared the IR-induced expression of Cox 2 mRNA in wildtype and *Rag-1*$^{-/-}$ mice. Cox 2 expression was rapidly increased in response to IR in wildtype mice (Fig. 6B) and the elevated expression was maintained for 2 h post ischemia. In contrast, Cox 2 expression was not elevated at any time point in similarly treated *Rag-1*$^{-/-}$ mice (Fig. 6B). Importantly, Cox 2 mRNA expression increased in *Rag-1*$^{-/-}$ mice which were pretreated with Ab purified from wildtype mice. These data suggest that Ab have a role in Cox 2 activation.

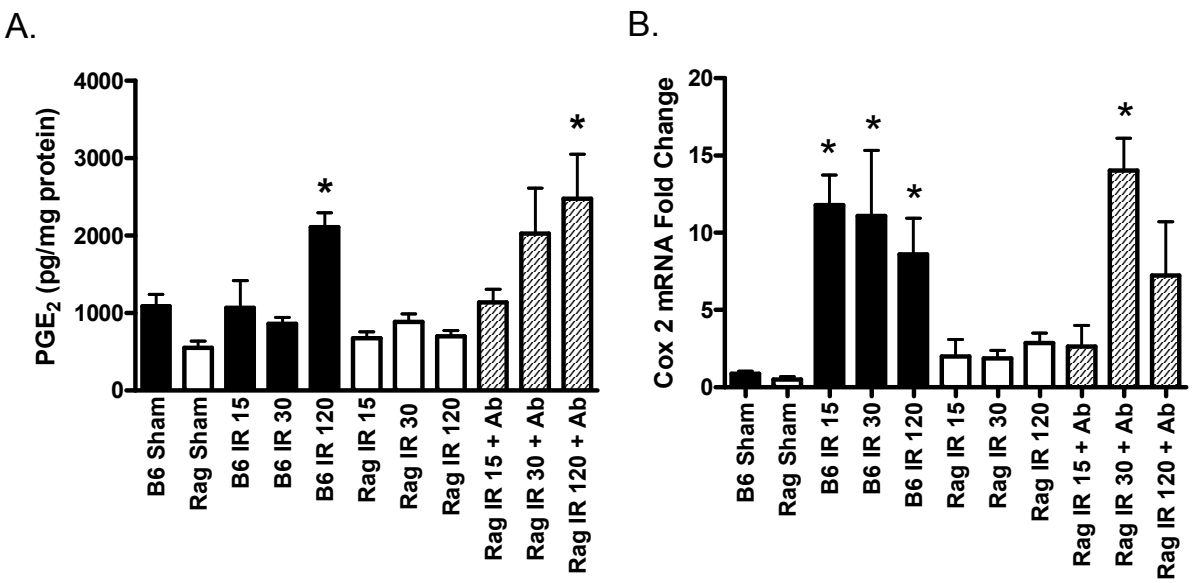


Figure 2.6 Antibody is required for IR-induced Cox 2-mediated PGE_2 expression. C57Bl/6 (solid bars) or *Rag-1*$^{-/-}$ mice (open bars) or *Rag-1*$^{-/-}$ mice treated with 100 µg purified Ab (striped bars) were subjected to Sham or IR treatment prior to collection of mid-jejunal tissue at 15, 30 or 120 min post ischemia. (A) Ex vivo intestinal PGE_2 production was determined by enzyme immunoassays. Each bar represents the average ± SEM with 5 to 9 animals per group. (B) RT-PCR determined intestinal Cox 2 expression from additional intestinal sections. The fold change was determined by the ΔCt method after normalization to 18s rRNA. Each bar represents the average ± SEM with 3 to 8 animals per group. One-way ANOVA with Newman-Keuls post-hoc test was used to determine significance; (*) indicates $p < 0.05$ compared to Sham treatment.

Discussion

Using ESI-MS/MS, we determined the phospholipid profile of the mid-jejunum of Sham and IR treated mice. We showed that IR induces an increase in intestinal lysoPC and free AA in wildtype and *Rag-1*$^{-/-}$ mice. Despite this increase in both strains of mice, *Rag-1*$^{-/-}$ mice did not exhibit Cox 2-mediated PGE_2 production and intestinal damage as did wildtype mice. These data indicate that the production of lysoPC and free AA occurs early in the IR-induced process of cellular injury. In addition, Ab present in wildtype mice and lacking in *Rag-1*$^{-/-}$ mice are

required for Cox 2-mediated PGE_2 production but not for the IR-induced intestinal lipid alterations.

Phosphatidylcholine, PI, PS, PE and SM were the main lipid classes present in both wildtype and *Rag-1*$^{-/-}$ mice after Sham treatment (Fig. 1 and Table 2). Similar results were obtained by ESI-MS/MS analysis of untreated mouse and rat intestines (Hicks et al., 2006; Tyurina et al., 2008). Hicks et al. found the prominent PC peaks in the rat intestine were composed of 16:0-18:2 PC species (Hicks et al., 2006). These data correlate with the current data, which indicate the most prominent PC species was 34:2 (Fig. 1). A recent study by Braun et al. also showed that normal mouse jejunum expressed a 496 Da and 524 Da lysoPC in the highest concentrations (Braun et al., 2009). These masses correlate with the predominant 16:0 and 18:0 lysoPC species found in Sham-treated animals (Fig.4). This is consistent with formation 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.

Previously, there has been limited use of ESI-MS/MS to study lipids in intestinal disease with variable results depending on the disease model. Glycerophospholipids were not significantly changed in response to 15 Gy of total body irradiation (Tyurina et al., 2008). However, using MS/MS, Ehehalt et al. found that the intestinal mucus of ulcerative colitis patients contained significantly less of the protective glycerophospholipids PC and lysoPC with no significant differences in the molecular species between diseased and normal subjects (Ehehalt et al., 2004). Similarly, Braun et al. recently showed that PC was significantly lower in ulcerative colitis patients but not in patients with Crohn's disease (Braun et al., 2009). Correlating with this finding, the lysoPC to PC ratio also increased (Braun et al., 2009). The present study showed that IR increased lysoPC with a concomitant decrease in PC (Fig. 3A, B). Others found that PLA_2 activity and the subsequent lysoPC:PC ratio increased in response to IR, although these studies involved two h ischemia and five min reperfusion (Otamiri et al., 1987). In addition, we found the lysoPC increase was specifically in the 16 and 18 carbon molecular species (Fig. 5). These data correlate with previous studies indicating that lysoPC increases the intestinal permeability and PLA_2 activity as a result of intestinal IR (Cunningham et al., 2008). In addition to increased lysoPC levels following IR treatment, we found increased intestinal free AA levels. Similar significantly increased free AA levels occurred in the inflamed intestinal mucosa of patients with Crohn's disease when compared to non-inflamed mucosa (Morita et al.,

1999). Thus, some, but not all, forms of intestinal damage alter the lipid profile by changing either the lipid classes or molecular species.

It appears as though Ab are essential for the release of PGE_2 and that PGE_2 production contributes to IR-induced damage. Cox 2-mediated PGE_2 production has been correlated with IR-induced damage via use of selective Cox 2 inhibitors (Arumugam et al., 2003; Moses et al., 2009; Sato et al., 2005). Previous studies also indicate that treatment of complement receptor 1 and 2 deficient mice with anti-phospholipid Ab induces damage and eicosanoid production similar to that of wildtype mice (Fleming et al., 2004a), further supporting the role of Ab involvement in cellular damage. The specific role of Ab in PGE_2 production is currently unknown. Early studies showed that intestinal smooth muscle cells release PGE_2 in response to IL-1 and LPS (Longo et al., 1998). In addition, recent studies indicate that in the absence of the LPS receptor, TLR4 significantly attenuated intestinal damage and Cox 2 expression (Moses et al., 2009). It is possible that Ab recognize a lipid moiety expressed on the cell surface or bound to a surface receptor, triggering Cox 2 expression which leads to formation of PGE_2 (Fig. 6).

Although Ab appear important for Cox 2 catalyzed formation of PGE_2, Ab are not required for cleavage of PC to lysoPC. This is indicated by the fact that after IR, *Rag-1*$^{-/-}$ mice express similar quantities of lysoPC as wildtype mice without elevated PGE_2 and subsequent tissue damage. The specific phospholipase which produces lysoPC in the intestines during IR is currently unknown. Previous studies demonstrate a role for PLA_2 and subsequent AA formation in glomerular epithelial cell damage due to the complement complex C5b-9 (Cohen et al., 2008). In vitro studies also indicate that calcium independent PLA_2 induces damage in cardiomyocytes following simulated IR (Andersen et al., 2009; Lawrence et al., 2003). The contribution of soluble PLA_2 ($sPLA_2$) is controversial. Inhibitory studies suggested that $sPLA_2$ is critical in intestinal IR induced lung damage at two to eight h post reperfusion (Koike et al., 2000; Kostopanagiotou et al., 2008) and may play a role in intestinal damage at three h post reperfusion (Arumugam et al., 2003). Our data indicate that lysoPC 16:0 and lysoPC 18:0 are rapidly increased in the intestine in response to IR and previous studies indicated that these lipids uncompetitively inhibit $sPLA_2$ expression (Cunningham et al., 2008). Thus, it is possible that multiple PLA_2s are responsible for cleavage of PC to lysoPC.

In summary, using ESI-MS/MS, we have shown that wildtype C57Bl/6 and antibody deficient *Rag-1*$^{-/-}$ mice have conserved lipid profiles. In response to intestinal IR, both strains of

mice show increased levels of lysoPC. However, *Rag-1*$^{-/-}$ mice produced significantly less Cox 2-mediated PGE_2 compared to wildtype mice. With the injection of wildtype Ab into *Rag-1*$^{-/-}$ mice, Cox 2 mRNA expression and PGE_2 concentrations were restored to similar levels observed in wildtype mice. Thus, Ab play a role in inducing intestinal damage and identification of the specific lipid antigens recognized may provide improved therapeutics for the prevention of tissue damage associated with ischemia.

Chapter 3 - TLR9 is dispensable for intestinal ischemia/reperfusion-induced tissue damage

Abstract

The mortality rate due to intestinal ischemia/reperfusion (IR) remains at 60 to 80%. As Toll-like receptor (TLR) 4 has been shown to be critical for IR injury in several organs, including the intestine, and TLR9 is necessary for IR-induced damage of the liver, we investigated the hypothesis that TLR9 is involved in intestinal IR-induced damage. Wildtype (C57Bl/6) and *TLR9*$^{-/-}$ mice were subjected to intestinal IR or Sham treatment. Several markers of damage and inflammation were assessed, including mucosal injury, eicosanoid production, cytokine secretion and complement deposition. Although IR-induced injury was not altered, PGE_2 production was decreased in *TLR9*$^{-/-}$ mice. Attenuated PGE_2 production was not due to differences in percentage of lipids or Cox 2 transcription. The data indicate that TLR9 is not required for IR-induced injury or inflammation of the intestine.

Introduction

Intestinal ischemia/reperfusion (IR), while not as common as myocardial or cerebral IR, is associated with a 60 to 80% mortality rate (Clark and Coopersmith, 2007; Deitch, 2001; Leaphart and Tepas, 2007). Mesenteric ischemia induces cellular damage which is exacerbated upon reperfusion resulting in tissue injury and often systemic inflammation and death. Reperfusion initiates an excessive inflammatory response involving complement activation and cytokine and eicosanoid production which recruit inflammatory neutrophils and macrophages (reviewed in (Fleming and Tsokos, 2006)).

In response to hypoxia, cells express cryptic antigens similar to those expressed on the plasma membrane of apoptotic cells (Holers and Kulik, 2007; Weiser et al., 1996). During reperfusion, naturally occurring antibodies (Ab) recognize newly expressed antigens, triggering complement activation and the innate immune response. Identification of naturally occurring monoclonal Ab which result in damage when administered to IR-resistant, Ab-deficient *Rag-1*$^{-/-}$ mice have suggested the neo-antigens include DNA, non-muscle myosin and cardiolipin

(Fleming et al., 2004a; Keith et al., 2007; Weiser et al., 1996; Williams et al., 1999a; Zhang et al., 2004; Zhang and Carroll, 2007). Additionally, oxygen deprivation is known to cause membrane lipid alterations and results in the liberation of arachidonic acid and subsequent production of eicosanoids. We have previously shown that prostaglandin E_2 (PGE_2) is necessary but not alone sufficient for tissue damage (Moses et al., 2009; Sparkes et al., 2010). Leukotriene B_4 (LTB_4) is chemotactic for neutrophils, which are also involved in IR-induced damage (Simpson et al., 1993).

Recent studies indicate a significant role for Toll-like receptors (TLRs) in IR-induced tissue damage and inflammation (Moses et al., 2009; Pope et al., 2010). As pathogen-associated molecular pattern receptors, TLRs recognize distinct microbial components. Although TLRs recognize commensal microflora to maintain intestinal homeostasis (Rakoff-Nahoum et al., 2004), activation of these pathogen recognition receptors also induces inflammation following tissue damage (Mollen et al., 2006). As a regulator of complement activation, TLR4 is required for IR-induced tissue injury and inflammation in the intestine, kidney, brain, lung and heart (Gao et al., 2009b; Li and Cherayil, 2004; Moses et al., 2009; Takeishi and Kubota, 2009; Victoni et al., 2010; Wu et al., 2007; Yang et al., 2008a). TLR9 has been shown to be critical in liver IR (Bamboat et al., 2010; Huang et al., 2011). Upon activation, most TLRs, including TLR4 and TLR9, signal through the common MyD88 pathway. Recently, we demonstrated that MyD88 is necessary for intestinal IR-induced tissue damage (Moses et al., 2009) and that both TLR4 and MyD88 are critical for PGE_2 production and the inflammatory response.

TLR9 localizes to endosomal and lysosomal compartments, where it can recognize internalized ligand. In addition to bacterial CpG DNA, TLR9 recognizes self DNA, particularly histones and mitochondrial DNA (Huang et al., 2011; Oka et al., 2012). As IR-induced injury involves both cellular damage and death, self DNA is released into the extracellular environment for uptake by macrophages and other cells. Furthermore, anti-DNA and anti-histone monoclonal Ab restored intestinal IR-induced injury in *Rag-1*$^{-/-}$ mice (Fleming et al., 2004a). Although TLR9 is a key component for IR-induced liver damage, its role in intestinal IR is not clear. It is possible that TLR9 regulates complement activation, PGE_2 production or other critical components in IR-induced injury. We hypothesized that TLR9 is critical to IR-mediated intestinal damage. We tested the hypothesis by subjecting C57Bl/6 and *TLR9*$^{-/-}$ mice to intestinal IR and examined several markers of intestinal tissue damage, including complement deposition, eicosanoid

production and cytokine secretions, in both *TLR9*$^{-/-}$ and wildtype mice. Contrary to expectations, TLR9 appears to be dispensable in intestinal IR-induced tissue injury.

Methods

Mice

TLR9$^{-/-}$ mice were obtained from S. Akira (Osaka University, Osaka, Japan) and bred as homozygote deficient mice along with C57Bl/6 mice (wildtype control) (Jackson Laboratory, Bar Harbor, ME) in the Division of Biology at Kansas State University with free access to food and water. All mice were backcrossed to the C57Bl/6 background for at least nine generations and maintained as specific pathogen free (Helicobacter species, mouse hepatitis virus, minute virus of mice, mouse parvovirus, Sendai virus, murine norovirus, Mycoplasma pulmonis, Theiler's murine encephalomyelitis virus, and endo- and ecto-parasites). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and was approved by the Institutional Animal Care and Use Committee at Kansas State University.

Ischemia/Reperfusion

Animals were subjected to IR using a protocol similar to that of previous studies (Moses et al., 2009). Briefly, a laparotomy was performed on ketamine (16 mg per kg) and xylazine (80 mg per kg) anesthetized mice with buprenorphine (0.06 mg per kg) administered for pain. After a 30 min equilibration period, ischemia was induced by applying a small vascular clamp (Roboz Surgical Instruments, Gaithersburg, MD) to the isolated superior mesenteric artery. Blanching of the intestine and absence of pulsations distal to the clamp confirmed ischemia. Covering the bowel with warm normal saline moistened surgical gauze prevented desiccation. After 30 min of ischemia, the clamp was removed allowing two h mesenteric reperfusion, confirmed by color change of the bowel and the return of pulsatile flow to the superior mesenteric artery. Some experiments reconstituted *Rag-1*$^{-/-}$ mice by intravenous injection of 200 µg of Protein L purified Ab from *TLR9*$^{-/-}$ or wildtype (C57Bl/6) mice at the time of laparotomy. Sham treated animals underwent the same surgical intervention except for vessel occlusion. All procedures were

performed with the animals breathing spontaneously and body temperature maintained at 37°C using a water circulating heating pad. Additional ketamine and xylazine was administered as needed and immediately prior to sacrifice. After sacrifice, two cm sections of the small intestine 10 cm distal to the gastroduodenal junction were harvested for histologic evaluation and eicosanoid determination.

Histology and Immunohistochemistry

Mid-jejunal specimens were promptly fixed in 10% buffered formalin phosphate prior to being embedded in paraffin, sectioned transversely (8 μm) and H & E stained. The mucosal injury score was graded on a six-tiered scale defined by Chiu et al. (Chiu et al., 1970). Briefly, the average damage score of the intestinal section (75 to 150 villi) was determined after grading each villus from 0 to 6. Normal villi were assigned a score of 0; villi with tip distortion were assigned a score of 1; a score of 2 was assigned when Guggenheims' spaces were present; villi with patchy disruption of the epithelial cells were assigned a score of 3; a score of 4 was assigned to villi with exposed but intact lamina propria with epithelial sloughing; a score of 5 was assigned when the lamina propria was exuding; last, villi that displayed hemorrhage or were denuded were assigned a score of 6. Photomicrographs were obtained from H & E stained slides using a 20X, 0.5 Plan Fluor objective on Nikon 80i microscope and images acquired at room temperature using a Nikon DS-5M camera with DS-L2 software (Nikon, Melville, NY).

An additional two cm intestinal section was immediately snap-frozen in O.C.T. freezing medium and 8 μm sections were transversely cut and placed on slides for immunohistochemistry. Nonspecific antigen binding sites were blocked via treatment with a solution of 10% donkey sera in phosphate buffered saline (PBS) for 30 min. After washing in PBS, the tissues were incubated with primary Ab overnight at 4°C. The C3 deposition, IgM and F4/80 expression on the tissue sections was detected by staining with a purified rat anti-mouse C3 (Hycult Biotechnologies, Plymouth Meeting, PA) or anti-IgM Ab (eBioscience, San Diego, CA) or anti-F4/80 Ab (eBioscience) followed by a Texas-red conjugated donkey anti-rat IgG secondary Ab (Jackson Immunoresearch, West Grove, PA). Each experiment contained serial sections stained with the appropriate isotype control Abs. All slides were mounted with ProLong Gold (Invitrogen, Grand

Island, NY). A blinded observer obtained images at room temperature using a Nikon Eclipse 80i microscope equipped with a CoolSnap CF camera (Photometrics, Tucson, AZ) and analyzed using Metavue software (Molecular Devices, Sunnyvale, CA).

Eicosanoid and Cytokine Determination

The ex vivo generation of eicosanoids in small intestine tissue was determined as described previously (Sjogren et al., 1994). Briefly, fresh mid-jejunum sections were minced, washed and resuspended in 37°C oxygenated Tyrode's buffer (Sigma, St. Louis, MO). After incubating for 20 min at 37°C, supernatants were collected and supernatants and tissue were stored at -80°C until assayed. The concentration of LTB_4 and PGE_2 were determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). Cytokine analysis of the same intestinal supernatants was determined using a Milliplex MAP immunoassay kit (Millipore, Billerica, MA) and read on a Milliplex Analyzer (Millipore, Billerica, MA). The tissue protein content was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) adapted for use with microtiter plates. Eicosanoid and cytokine production was expressed per mg protein per 20 min.

Lipid Extraction

Lipids were extracted as described previously (Sparkes et al., 2010). Briefly, after Sham or IR treatment, intestinal sections (jejunum) were snap-frozen in liquid nitrogen. Samples were ground into a fine powder using a liquid nitrogen-cooled stainless steel mortar and pestle. Samples were transferred to glass tubes that had been washed with a cation/phosphate-free liquid detergent (Contrex, Decon Labs, King of Prussia, PA). One ml chloroform and two ml methanol were added and tubes shaken vigorously. An additional one ml chloroform and one ml distilled water were added and tubes shaken vigorously again. Tubes were centrifuged at 4000 rpm for five min at 4°C. The organic layer was transferred to a clean glass tube and one ml chloroform added to the aqueous phase. Twice more, the tubes were shaken, centrifuged and organic layer removed. Distilled water (0.5 ml) was added to the combined organic layers for each sample; the tubes were shaken and centrifuged once more. The organic layer was submitted to the Kansas Lipidomics Research Center for mass spectrometry analysis.

Mass Spectrometry

Mass spectrometry analysis was carried out similar to that described previously (Sparkes et al., 2010). An automated electrospray triple quadrupole mass spectrometry (ESI-MS/MS) approach was used and data acquisition and analysis carried out at the Kansas Lipidomics Research Center as described previously with modifications (Bartz et al., 2007; Devaiah et al., 2006). Solvent was evaporated from the extracts and each was dissolved in one ml of chloroform. Precise amounts of internal standards, obtained and quantified as previously described (Welti et al., 2002), were added to each sample to be analyzed. The sample and internal standard mixture were combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.2 ml. These unfractionated lipid extracts were introduced by continuous infusion into the ESI source of a triple quadrupole MS (API 4000, Applied Biosystems, Foster City, CA) using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 µl/min.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: phosphatidylcholine (PC), sphingomyelin (SM) and lysoPC, [M + H]+ ions in positive ion mode with Precursor of 184.1 (Pre 184.1); Phosphatidylethanolamine (PE) and lysoPE, [M + H]+ ions in positive ion mode with Neutral Loss of 141.0 (NL 141.0); phosphatidylinositol (PI), [M + NH4]+ in positive ion mode with NL 277.0; phosphatidylserine (PS), [M + H]+ in positive ion mode with NL 185.0; phosphatidic acid (PA), [M + NH4]+ in positive ion mode with NL 115.0; phosphatidylglycerol (PG), [M + NH4]+ in positive ion mode with NL 189.0; lysoPG, [M – H]- in negative mode with Pre 152.9; and free fatty acids (i.e., free arachidonic acid, (AA)), [M - H]- in negative mode with single stage MS analysis. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode.

The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software (Applied Biosystems, Carlsbad, CA). The lipids in each class were quantified in comparison to the two internal standards of that class. The first and typically every 11th set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these

spectra were identified and molar amounts calculated in comparison to the internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the "internal standards only" spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. An "internal standards only" sample was included for each set of 10 samples. Finally, the data were expressed as percent of the signal (intensity) of total lipid analyzed after the described normalization to internal standards. Each class of lipid was also normalized to intestinal proteins and expressed as nmol lipid class per mg protein.

Real-time PCR

Total RNA was isolated from the jejunum, liver and spleen using TRIzol reagent (Invitrogen, Grand Island, NY) according to manufacturer's instructions. RNA integrity and genomic DNA contamination was assessed using a BioAnalyzer (Agilent, Santa Clara, CA) and the quantity was determined by Nanodrop (Nanodrop 1000 spectrophotometer) evaluation. Only samples with no measurable DNA contamination and RIN values greater than 7.0 were used for cDNA synthesis. Total RNA (two μg) was reverse transcribed using RevertAid first strand cDNA synthesis kit (Fermentas, Glen Burnie, MD) using random primers. Quantitative real-time PCR was performed in 25 μl volumes using a Mini-Opticon real time thermal cycler (Bio-Rad, Hercules, CA) and B-R SYBR Green Supermix for iQ reagent (Quanta Biosciences, Gaithersburg, MD) using the following protocol: 3 min at 95°C; 50 cycles of 10 sec at 95°C, 20 sec at T_m, 10 sec at 72°C; melt curve starting at 65°C, increasing 0.5°C every 5 sec up to 95°C. Primer sequences for Cox 2 were forward: 5'ATCCTGCCAGCTCCACCG and reverse: 5'TGGTCAAATCCTGTGCTCATACAT with T_m 54°C. After amplification, gene of interest Ct values were normalized to 18s rRNA (forward: 5'GGTTGATCCTGCCAGTAGC and reverse: 5'GCGACCAAAGGAACCATAAC with T_m 58°C) and then ΔΔCt fold change relative to Sham-treated wildtype mice was determined as described previously (Zhao et al., 2008). Melt-curve analysis of the PCR products ensured amplification of a single product.

Statistical Analysis

Data are presented as mean ± SEM and were compared by one-way analysis of variance with post hoc analysis using Newman-Kuels test (Graph Pad/Instat Software Inc., Philadelphia, PA) unless otherwise specified. The difference between groups was considered significant when $p < 0.05$.

Results

TLR9 deficiency does not protect from intestinal ischemia/reperfusion-induced injury

Previous studies indicated that MRL/lpr autoimmune mice sustain greater tissue damage than wildtype mice following intestinal IR (Fleming et al., 2004a). In addition, administration of anti-DNA or anti-histone Ab (also found in Systemic Lupus Erythematosus) resulted in intestinal injury in Ab-deficient *Rag-1*$^{-/-}$ mice after IR treatment (Fleming et al., 2004a). As TLR9 recognizes CpG DNA, we hypothesized that intestinal IR-induced damage would be attenuated in *TLR9*$^{-/-}$ mice. We tested the hypothesis by subjecting *TLR9*$^{-/-}$ mice to intestinal Sham or IR treatment. As indicated in Figure 1, Sham treatment did not result in injury in either strain of mice with injury scores of 0.35 ± 0.07 in wildtype and 0.64 ± 0.10 in *TLR9*$^{-/-}$ mice (Fig. 1A-C). As expected, after IR, wildtype C57Bl/6 mice sustained significant tissue injury (Fig 1A, D). To our surprise, the injury sustained by the *TLR9*$^{-/-}$ mice (2.02 ± 0.21) was not significantly different from wildtype (2.52 ± 0.27) (Fig. 1A, E), suggesting that TLR9 is not required for intestinal IR-induced injury.

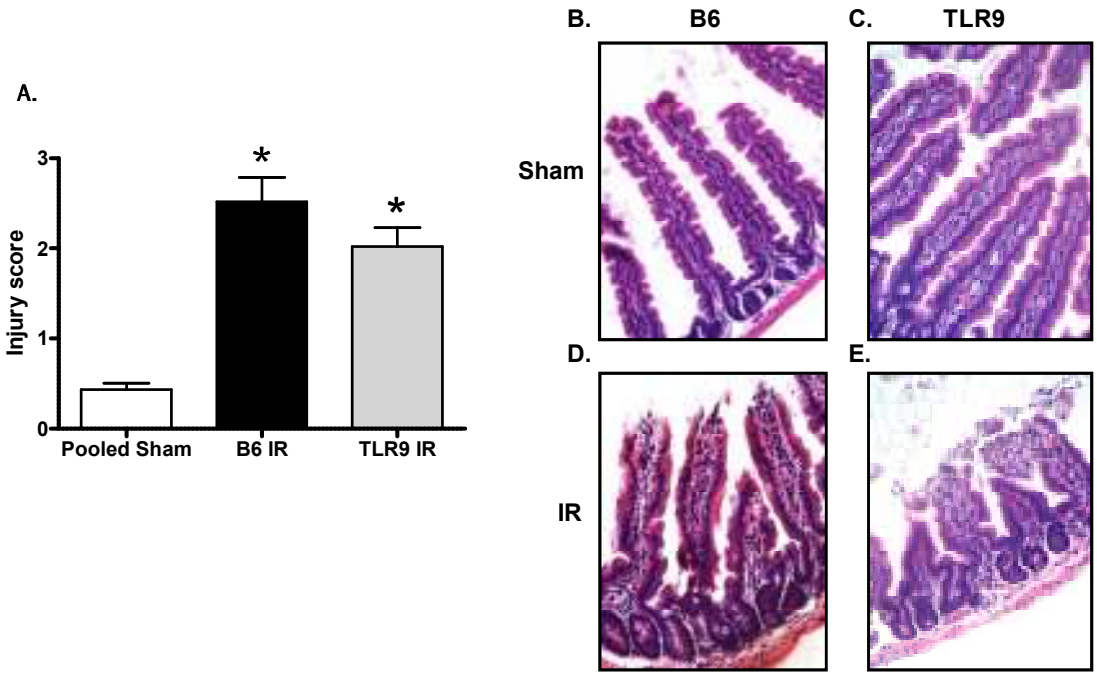


Figure 3.1 *TLR9*$^{-/-}$ mice sustain intestinal injury following ischemia/reperfusion. Wildtype and *TLR9*$^{-/-}$ mice were subjected to Sham or intestinal IR treatment. Intestinal sections were collected and formalin fixed following 30 min ischemia and 120 min reperfusion. (A) intestinal injury scores as determined by H&E stained intestinal sections and described in Methods; (B-E) representative images of H&E stained intestinal sections; each bar represents 10-11 animals; * indicates $p<0.05$ compared to Sham treatment.

TLR9 deficiency attenuates prostaglandin production.

Previous studies indicated that Cox 2-mediated PGE_2 production is required for IR-induced tissue damage and that the signal is transduced via MyD88 (Moses et al., 2009; Pope et al., 2010). As TLR9 also signals through MyD88, we examined the intestinal eicosanoid production in response to IR in the *TLR9*$^{-/-}$ mice. Similar to intestinal damage, Cox 2 transcript was elevated in response to IR in wildtype and *TLR9*$^{-/-}$ mice (Fig. 2A). Although IR resulted in an increase of PGE_2 production in the *TLR9*$^{-/-}$ mice, it was attenuated in comparison to wildtype PGE_2 levels (Fig. 2B). To determine if the decreased PGE_2 levels were specific for PGE_2 or common to all eicosanoids, we examined the secretion of LTB_4 which recruits neutrophils. Neither the production of the chemotactic eicosanoid LTB_4 nor peroxidase secretion from the intestine was

significantly different between the strains (Fig. 2C and data not shown). Thus, it appears that neutrophils may be recruited normally but PGE_2 production is decreased.

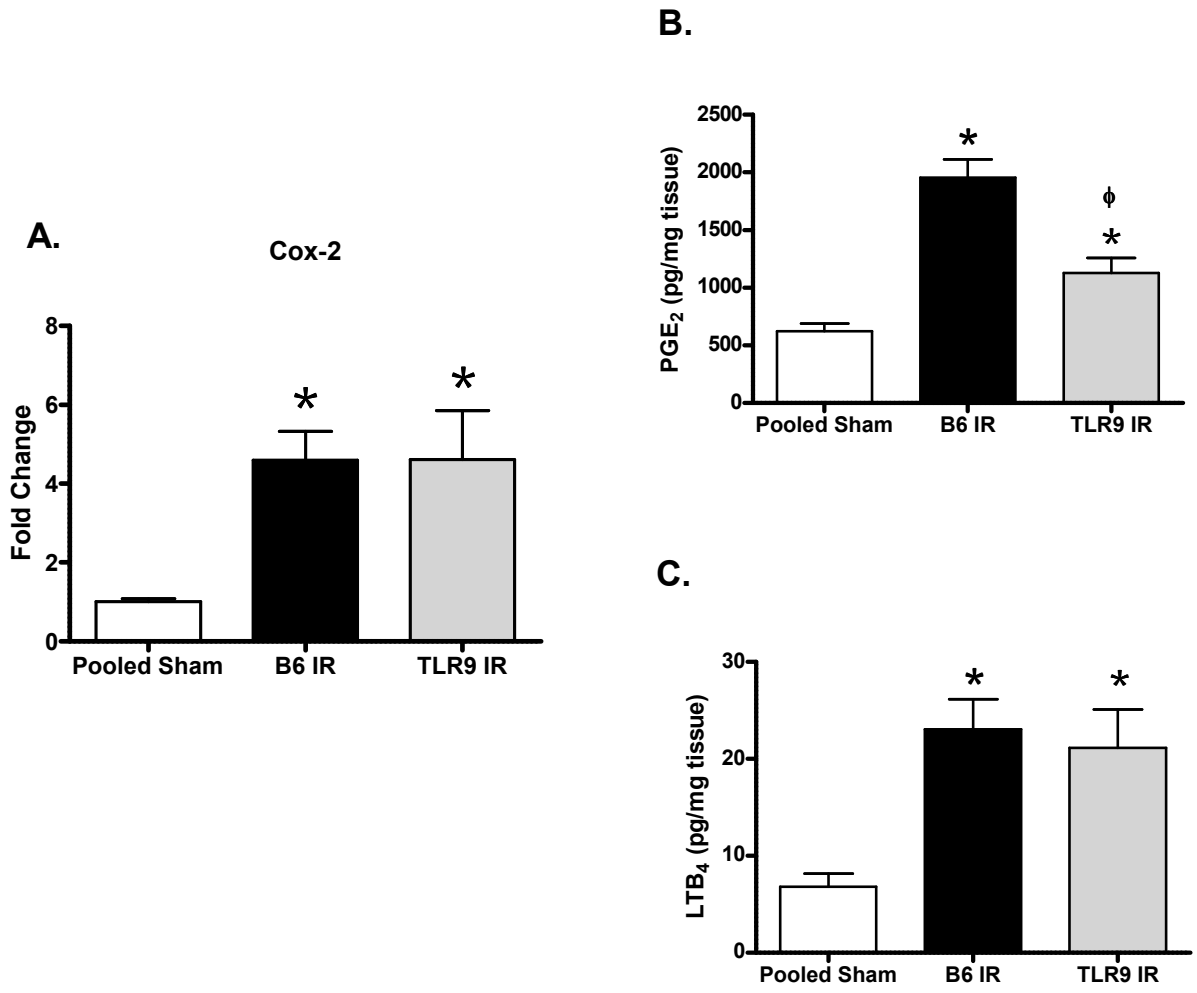


Figure 3.2 The absence of TLR9 attenuates intestinal PGE_2 production but not LTB_4 production.

Wildtype and *TLR9*$^{-/-}$ mice were subjected to Sham or intestinal IR treatment. Intestinal sections were collected following 30 min ischemia and 120 min reperfusion. (A) RNA was extracted from snap-frozen intestinal sections and fold change in Cox 2 transcript determined by real-time PCR using ΔΔCt values; (B-C) Intestinal sections were minced in oxygenated Tyrode's buffer and incubated at 37°C for 20 min. Supernatants were collected for PGE_2 (B) and LTB_4 (C) quantitation by EIA; values are expressed as pg eicosanoid secreted per mg protein per 20 min; each bar represents 5 to 6 animals (A) or 8 to 11animals (B-C); * indicates $p < 0.05$ compared to Sham treatment and Φ indicates $p < 0.05$ compared to wildtype IR.

TLR9 deficiency does not alter cellular lipid composition or metabolism following IR.

As eicosanoids are derived from the fatty acid arachidonic acid, we examined the lipid profiles of both wildtype and *TLR9*$^{-/-}$ mice by mass spectrometry. The relative intestinal lipid composition between Sham treated wildtype and *TLR9*$^{-/-}$ mice was not different (Table 1). However, the relative amounts of several classes of phospholipids did change with IR treatment (Table 1). The relative amount of PC decreased from 25.07 ± 1.88 to 13.41 ± 1.66 after IR treatment in the wildtype mice and from 25.98 ± 4.16 to 14.05 ± 1.83 in the *TLR9*$^{-/-}$ mice. PE similarly decreased in both strains of mice following IR treatment (wildtype: 14.15 ± 1.35 to 7.81 ± 1.56; *TLR9*$^{-/-}$: 15.31 ± 3.44 to 7.47 ± 1.28). In contrast, IR treatment resulted in an increase in the relative amount of free arachidonic acid in the intestine. Free arachidonic acid increased from 2.67 ± 0.34 to 7.62 ± 0.89 in the wildtype mice and from 2.59 ± 0.53 to 7.49 ± 0.88 in the *TLR9*$^{-/-}$ mice. We previously identified specific lipid species, that upon degradation, would likely result in release of free arachidonic acid (Sparkes et al., 2010). The changes in these specific lipid species as a result of IR were similar between wildtype and *TLR9*$^{-/-}$ mice, except for the PC species where a greater decrease from Sham levels occurred in the wildtype mice subjected to IR than the *TLR9*$^{-/-}$ mice subjected to IR (Fig. 3A). The relative amount of free arachidonic acid released as a result of IR-induced injury was comparable between strains (Fig. 3B).

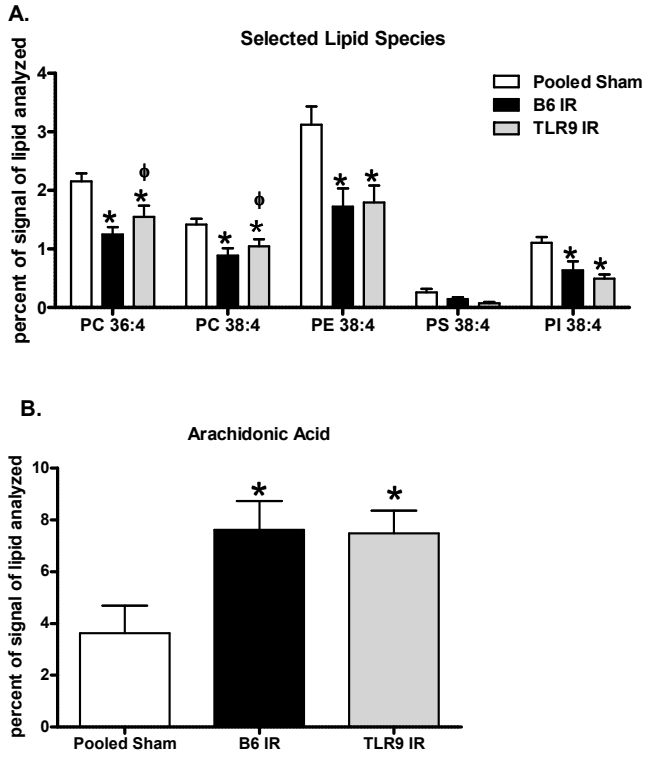


Figure 3.3 Arachidonic acid release is TLR9-independent.

Extracted intestinal lipids from Sham and IR treated wildtype and *TLR9*$^{-/-}$ mice were quantitated by ESI-MS/MS. (A) percent of signal of lipids analyzed for selected phospholipids as determined by mass spectrometry; (B) percent of signal of lipids analyzed for free arachidonic acid as determined by mass spectrometry; each bar represents 5 to 8 animals; * indicates $p < 0.05$ compared to Sham treatment.

TLR9$^{-/-}$ mice produce an antibody repertoire conducive to damage.

Previous studies indicated that the appropriate Ab repertoire is required for recognition of IR-induced neoantigens and complement activation (Fleming et al., 2002b; Williams et al., 1999b; Zhang et al., 2008). In addition, Ab appeared to be required for IR-induced PGE_2 production (Sparkes et al., 2010). To verify that *TLR9$^{-/-}$* mice have the proper Ab repertoire, we injected Ab-deficient, IR-resistant *Rag-1$^{-/-}$* mice with purified Ab obtained from wildtype or *TLR9$^{-/-}$* mice and subjected the mice to IR. As indicated in Figure 4, after IR, addition of Ab purified from wildtype mice induced significant intestinal damage and eicosanoid production in *Rag-1$^{-/-}$* mice. Similarly, *Rag-1$^{-/-}$* mice administered Ab from *TLR9$^{-/-}$* mice sustained significant intestinal damage in response to IR (Fig. 4A). In addition, eicosanoid production was at wildtype levels in *TLR9$^{-/-}$* Ab reconstituted *Rag-1$^{-/-}$* mice after IR treatment (Fig. 4 B, C). Thus, the Ab repertoire of *TLR9$^{-/-}$* mice is sufficient to induce intestinal damage, PGE_2 production and inflammation in response to IR.

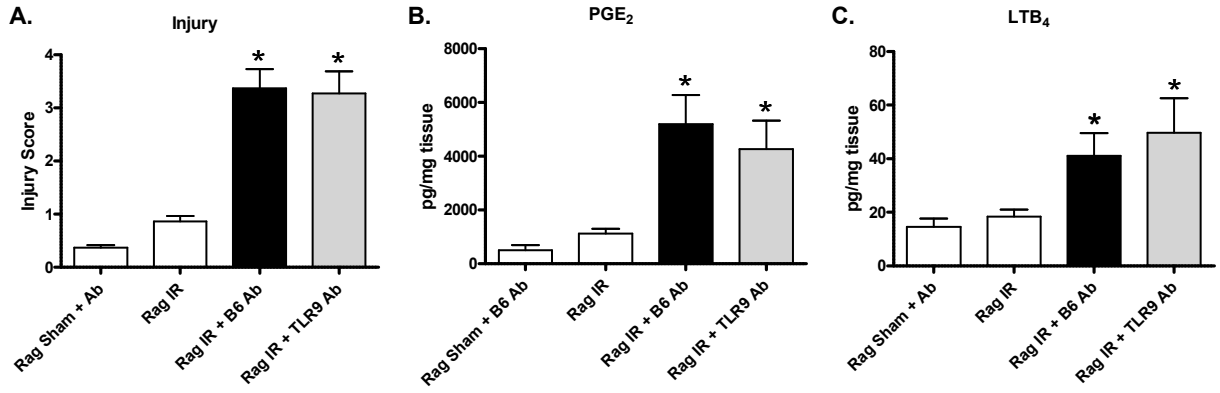
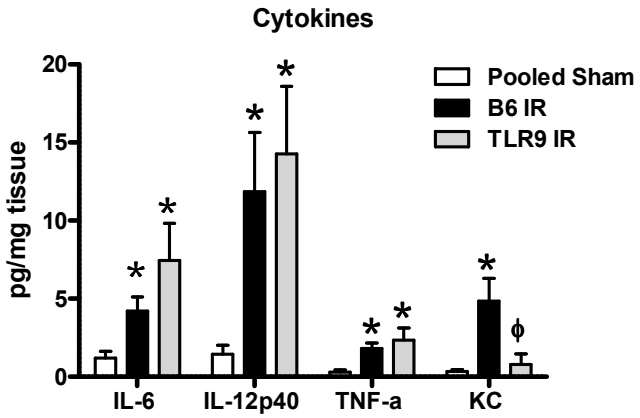


Figure 3.4 Antibodies produced by *TLR9$^{-/-}$* mice are sufficient to induce intestinal injury following IR.

Rag-1$^{-/-}$ mice were administered antibodies from wildtype or *TLR9$^{-/-}$* mice prior to Sham or IR treatment. (A) Intestinal injury scores were determined from H & E stained intestinal sections as described in Methods; (B-C) Intestinal sections were minced in oxygenated Tyrode's buffer and incubated at 37°C for 20 min. Supernatants were collected and assayed for PGE_2 (B) and LTB_4 (C) by EIA. Values are expressed as pg eicosanoid secreted per mg protein per 20 min. Each bar represents 4 to 7 animals; * indicates $p < 0.05$ compared to Sham treatment.

Complement deposition and inflammatory cytokine production are TLR9-independent.

Similar to eicosanoid production and previous studies with *TLR4*$^{-/-}$ or *MyD88*$^{-/-}$ mice (Pope et al., 2010), intestinal production of IL-6, IL-12p40 and TNFα increased in response to IR in both wildtype and *TLR9*$^{-/-}$ mice (Fig. 5). Similar to myocardial ischemia, intestinal IR induced significant KC and MCP-1 (CCL2) production in wildtype mice that was significantly reduced in *TLR9*$^{-/-}$ mice (Fig. 5 and data not shown). These findings are in contrast with previous reports of IR in the liver, where IL-6 and TNFα levels were significantly lowered in *TLR9*$^{-/-}$ mice (Bamboat et al., 2010).



3.5 TLR9 deficiency does not affect cytokine secretion.

Wildtype and *TLR9*$^{-/-}$ mice were subjected to Sham or IR treatment. Intestinal sections were minced in oxygenated Tyrode's buffer and incubated at 37°C for 20 min. Supernatants were collected and assayed for cytokine secretion. Values are expressed as pg cytokine secreted per mg protein per 20 min. Each bar represents 4 to 8 animals. * indicates $p < 0.05$ compared to Sham treatment.

Because Ab activation of complement C3 is required for IR-induced intestinal damage, C3 and IgM deposition within the intestinal villi were determined by immunohistochemistry. As indicated in Figure 6, neither C3 nor IgM were deposited in response to Sham treatment. However, significant deposits of both IgM and C3 were present in wildtype and *TLR9*$^{-/-}$ mice after mesenteric IR. Furthermore, macrophages were identified in both wildtype and *TLR9*$^{-/-}$ IR-treated tissues, as detected by F4/80 staining (Fig. 6). Both the immunohistochemistry staining and cytokine data support the conclusion that intestinal macrophages are not affected by the presence or absence of TLR9 in the context of IR.

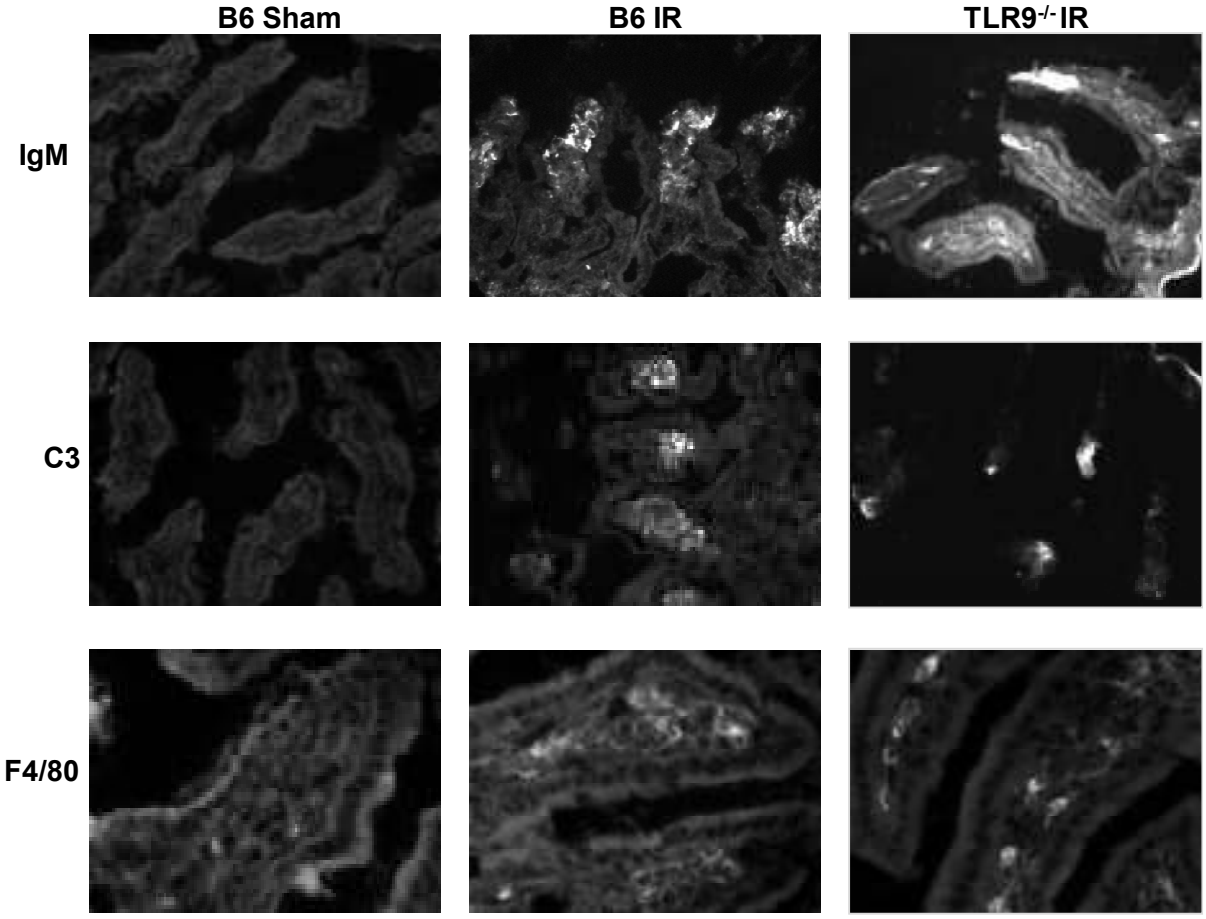


Figure 3.6 IR-induced complement and IgM deposition occurs in both C57Bl/6 and *TLR9*$^{-/-}$ mice.

Wildtype and *TLR9*$^{-/-}$ mice were subjected to Sham or IR treatment. Intestinal sections were frozen in O.C.T. and serial sections cut for immunohistochemistry. Antibody staining for IgM deposition, C3 deposition and F4/80+ macrophages was performed as described in *Methods*. Original magnification was 200x. Each photomicrograph is representative of 5 to 6 photos per treatment in each of 3 to 4 experiments.

Discussion

This is the first study, to our knowledge, to examine the role of TLR9 in intestinal IR-induced injury. As TLR9 is critical to IR-induced liver damage, we hypothesized that intestinal IR would also require TLR9. Surprisingly, *TLR9*$^{-/-}$ mice sustained significant tissue damage and inflammation following intestinal IR. Mucosal injury scores and the production of eicosanoids and cytokines were very similar between *TLR9*$^{-/-}$ and wildtype mice. In addition, it was verified that *TLR9*$^{-/-}$ mice produce an Ab repertoire conducive to injury. The intestinal lipids and release of arachidonic acid as well as complement deposition mimic that observed for wildtype mice. These data support the conclusion that TLR9 is not required for intestinal tissue damage or inflammation following IR.

TLRs, while originally identified as pathogen-associated molecular pattern receptors, recognize various self-ligands. As IR-induced tissue injury results in cellular damage and apoptosis, several self-ligands are released and available for TLR recognition. TLR4 has been shown to be involved in mediating IR-induced injury in several organs. We have previously shown TLR4 to be involved in intestinal IR, as TLR4 mutant mice had attenuated injury and eicosanoid and cytokine secretion (Moses et al., 2009; Pope et al., 2010). Furthermore, TLR4 mutant mice were protected from liver injury in a model of liver IR (Zhai et al., 2004). Additionally, TLR2 has been implicated in IR-induced damage to the kidney (Shigeoka et al., 2007).

Limited data exists regarding the role of TLR9 in sterile inflammation and injury models. However, recent studies have demonstrated involvement of TLR9 in the pathogenesis of tissue injury and inflammation of the liver in both an IR model (Huang et al., 2011) and a hemorrhagic-shock and trauma model (Gill et al., 2011). In addition, inhibitory CpG oligodeoxynucleotides (ODN) attenuated liver IR-induced injury (Wang et al., 2012). Oka, et al. found *TLR9*$^{-/-}$ mice to experience attenuated injury and less severe cardiac failure following transverse aortic constriction (Oka et al., 2012) and inhibitory CpG ODN attenuated cardiac IR, suggesting a role for TLR9 (Mathur et al., 2011). In contrast, there are conflicting data on the role of TLR9 in brain IR. The absence of TLR9 in brain IR was not protective (Hyakkoku et al., 2010) but CpG ODN was protective when administered prior to IR (Stevens et al., 2008). These conflicting data may be resolved by a current study which indicates that complement receptor 2 may function as a receptor for DNA in addition to TLR9 (Asokan et al., 2013). Similar to brain IR, our data

indicate that *TLR9*$^{-/-}$ mice are not protected from injury following intestinal IR. It is currently unknown if CpG ODN attenuates intestinal IR; however, complement receptor 2 deficient mice are protected from IR-induced tissue damage (Fleming et al., 2002a). These data provide another mechanism by which IR-induced injury differs between organs. This is an important finding as the field continues to uncover contributing factors to IR-induced damage and the differences between organs.

We previously demonstrated that tissue damage requires PGE_2 production and the PGE_2 production requires the appropriate Ab repertoire (Moses et al., 2009; Sparkes et al., 2010). It is interesting to note that PGE_2 was secreted but levels were attenuated in the *TLR9*$^{-/-}$ mice. However, the attenuated PGE_2 production was not due to an improper Ab repertoire, as intestinal sections from *Rag-1*$^{-/-}$ mice administered Ab from *TLR9*$^{-/-}$ mice secreted similar concentrations of PGE_2 as *Rag-1*$^{-/-}$ mice receiving Ab from wildtype mice. Thus, *TLR9*$^{-/-}$ mice contain the proper Ab repertoire to induce normal PGE_2 production.

PGE_2 is produced from free arachidonic acid after conversion by Cox 2. It is possible that TLR9 influences the production or release of arachidonic acid to promote eicosanoid production. Our data indicate that Cox 2 transcription is normal despite the fact that previous studies indicated that CpG DNA stimulates Cox 2 production (Yeo et al., 2003). Other studies indicate that other TLR agonists also stimulate Cox 2 (Fukata et al., 2006). Thus, IR-induced PGE_2 production does not require TLR9 stimulation of Cox 2. It is possible that arachidonic acid is not released in the absence of TLR9 stimulation. Our data indicate that the relative amount of free arachidonic acid is the same between wildtype and *TLR9*$^{-/-}$ mice (and *Rag-1*$^{-/-}$ mice, data not shown). However, *TLR9*$^{-/-}$ mice do have significantly lower levels of arachidonic acid per mg protein (data not shown). Conflicting reports exist in the literature regarding arachidonic acid release following TLR9 stimulation. Lee, et al. showed an increase in arachidonic acid release from RAW264.7 cells after stimulation with CpG ODN and that TLR9 knockdown reduced the amount of arachidonic acid released from the stimulated cells (Lee et al., 2007). However, Ruiperez, et al. found no release of arachidonic acid from RAW264.7 or P388D1 cells when stimulated with synthetic ODN 1826 (Ruiperez et al., 2009). Thus, further investigation on the effects of TLR9 stimulation-induced release of arachidonic acid is needed.

Previous studies indicated that anti-DNA and anti-histone mAb restored injury in the *Rag-1*$^{-/-}$ mice, suggesting that DNA acted as a neo-antigen. Our data indicate that while TLR9 is

involved in IR-induced injury of some organs, it is not vital for injury of the intestine. A recent study suggests that in addition to TLR9, complement receptor 2 functions as a receptor for DNA (Asokan et al., 2013). Thus, CR2 or other DNA receptors may be recognized by naturally occuring Ab to promote tissue damage and inflammation in the absence of TLR9.

Chapter 4 - A role for phospholipid scramblase 1 in hypoxia and re-oxygenation-induced endothelial damage

Abstract

Ischemia and reperfusion (IR) of the intestine is highly lethal due to local and systemic inflammatory effects. Previous studies have established a role for innate immune cells and naturally occurring antibodies (Ab) in intestinal IR-induced pathology. Furthermore, administration of two monoclonal Ab that recognize phospholipids and the phospholipid binding protein β_2-glycoprotein I (β_2-GPI) restores tissue damage to wildtype levels in IR-resistant, Ab-deficient *Rag-1*$^{-/-}$ mice. These and other data indicate the involvement of a lipid or lipid-like moiety in mediating IR-induced damage. We hypothesized that phospholipid scramblase 1 (PLSCR1), a protein that regulates bilayer asymmetry, is involved in altering the phospholipids of endothelial cells during hypoxia, a component of ischemia, leading to β_2-GPI binding and subsequent inflammatory responses. Using an in vitro cell culture model, we completed the first comprehensive study of PLSCR1 transcription, protein expression and activity under conditions of hypoxia and re-oxygenation. PLSCR1 is involved in exposing phosphatidylserine in response to oxygen stress, leading to β_2-GPI binding and other cellular responses.

Introduction

Interruption of blood flow to tissues and organs occurs frequently within a medical context (Mallick et al., 2004). Ischemia, the absence of blood flow, restricts oxygen and nutrient supply to cells. Cellular damage is initiated during this period of deprivation, and the damage is further magnified by the return of blood flow, termed reperfusion (Fleming et al., 2002a; Mallick et al., 2004). Ischemia/reperfusion (IR)-induced injury accompanies several conditions including stroke, myocardial infarction, organ transplantation and many other surgical procedures (Kiang and Tsen, 2006; Lopez-Neblina et al., 2005; Mallick et al., 2004). Intestinal IR is associated with a 70 to 80% mortality rate, as both local and systemic damage occurs (Lock, 2001). Several studies have confirmed the involvement of the immune system in perpetuating IR-induced injury. The pathology includes a requirement of antibodies (Ab), complement activation and

endothelial damage resulting in a vast influx of neutrophils (Crawford et al., 1988; Eror et al., 1999b; Fleming et al., 2004a; Hernandez et al., 1987; Hill et al., 1992b; Rehrig et al., 2001a; Simpson et al., 1993; Williams et al., 1999a).

While administration of the entire repertoire of Ab from wildtype mice restores IR-induced injury in otherwise protected *Rag-1*$^{-/-}$ mice, administration of a combination of two monoclonal Ab produces the same effect (Fleming et al., 2004a). Phospholipids appear to be involved in the pathology as one of the Ab directly binds phospholipids (FA3) and the other (FC1) binds a serum protein that can bind cell membranes. A prior study investigated the intestinal phospholipid composition of wildtype and *Rag-1*$^{-/-}$ mice using mass spectrometry (Sparkes et al., 2010). An additional study utilized the same methods for analysis of *TLR9*$^{-/-}$ mice (Slone et al., 2012). While no statistical differences in intestinal phospholipids were found between the strains of mice, the phospholipid compositions were altered by IR treatment (Slone et al., 2012; Sparkes et al., 2010).

Production of prostaglandin E_2 (PGE_2), a strong vasodilator and mediator of vascular permeability, is necessary, although not sufficient, for IR-induced injury (Moses et al., 2009). Numerous studies have reported an increase in PGE_2 production following re-oxygenation of an oxygen deprived tissue. In vivo IR studies of the intestine (Moses et al., 2009; Sparkes et al., 2010) and cerebrum (Candelario-Jalil et al., 2003; Kishimoto et al., 2010) have demonstrated an increase in PGE_2 levels, as have in vitro hypoxia studies with neonatal dermal cells (Watkins et al., 2004). However, the specific cell types involved in the production of PGE_2 during IR is unknown. It is known that hypoxia stimulates transcription of the inducible cyclooxygenase (Cox) isoform, Cox 2, which converts arachidonic acid to prostaglandins in endothelial cells (Schmedtje et al., 1997).

One of the damage-inducing Ab, FC1, recognizes surface-bound β_2-glycoprotein I (β_2-GPI) (Monestier et al., 1996). β_2-GPI is a serum protein that circulates in a closed, ring-like conformation, but adopts an open, J-shaped conformation upon binding to anionic phospholipids (Agar et al., 2010). This conformational change exposes a neoantigen recognized by Ab. β_2-GPI is involved in intestinal IR-induced injury, as administration of peptides derived from the phospholipid binding domain inhibit both β_2-GPI binding and tissue damage (Fleming et al., 2010).

The eukaryotic cell membrane is a mosaic of phospholipids, glycolipids, cholesterol and other lipid moieties in addition to the many proteins that associate with the membrane. The lipid bilayer is asymmetric, with the majority of choline containing phospholipids in the outer leaflet and most of the anionic phospholipids in the inner or cytosolic leaflet (reviewed in (Daleke, 2003; Fadeel and Xue, 2009)). While lipid bilayers are dynamic and continuously undergoing slight modifications, certain stimuli can induce major changes in the organization of the bilayer. A common end result of bilayer disruption is the exposure of phosphatidylserine (PS), an anionic phospholipid, on the outer leaflet of the cell membrane which marks the cell for apoptosis and/or coagulation (reviewed in (Fadeel et al., 2010; Zwaal and Schroit, 1997)).

Three classes of proteins are responsible for maintaining the asymmetry of the phospholipid bilayer under quiescent conditions (reviewed in (Contreras et al., 2010)). Two of these protein classes, flippases and floppases, require ATP for phospholipid transport, whereas scramblases are ATP-independent, alternatively responding to increased cytosolic calcium concentrations ((Basse et al., 1996; Williamson et al., 1995; Williamson et al., 1992), reviewed in (Contreras et al., 2010)) or acidic pH (Stout et al., 1997). The scramblases are a very likely candidate for involvement in hypoxia-induced phospholipid changes as hypoxia treatment results in ATP depletion (Arnould et al., 1992; Bouaziz et al., 2002), increased acidity (Hattori et al., 2001) and increased concentrations of intracellular calcium ((Aono et al., 2000; Arnould et al., 1992), reviewed in (Toescu, 2004)). While four scramblase genes have been identified, each of the resulting proteins localizes to a specific cellular compartment with phospholipid scramblase 1 (PLSCR1) found in the plasma membrane (Zhou et al., 1997).

The current study investigates the hypothesis that endothelial cells are key mediators of the inflammatory response observed following oxygen deprivation. Furthermore, this response may be initiated by PLSCR1-mediated lipid scrambling allowing for β_2-GPI binding and subsequent signaling. We report findings on the transcription, expression and activity of PLSCR1 under hypoxic conditions. The effects of hypoxia and re-oxygenation on β_2-GPI binding, phospholipid changes and downstream inflammatory markers in endothelial cells are also demonstrated. Our results confirm that endothelial cells contribute to the inflammatory response observed following a period of hypoxia and are likely intimately involved in the tissue damage observed following IR. Furthermore, PLSCR1 appears to play a role in facilitating early phospholipid changes in endothelial cells which ultimately result in tissue damage.

Materials and Methods

Cells

The mouse (C57Bl/6) endothelial cell line, MS1 (ATCC, Manassas, VA), was grown and maintained in Dulbecco modified Eagle medium (DMEM) (Gibco, Grand Island, NY) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10% Opti-MEM (Gibco) and one percent Gluta-MAX (Gibco) in a humidified five percent CO_2 incubator.

Hypoxia

Cells were seeded ($3x10^6$ for lipid and PGE_2 analysis, $1x10^6$ for RNA extraction and $1x10^7$ for western blot) on tissue culture plates 12 to 18 h prior to hypoxia treatment. At time of experiment, media was replaced with media that had been de-oxygenated, either by five min of bubbling with a 0.989% O_2, 5.070% CO_2, 93.941% N_2 gas mixture (referred to as one percent oxygen) or placing the media under vacuum for 15 min in a sealed flask. Plates of cells were transferred to a hypoxia chamber (modular incubator chamber, Billups-Rothenburg Inc., Del Mar, CA) and the chamber purged with the same gas mixture at 20 L per min for 20 min. The chamber was held at 37°C for two h. After two h of hypoxia, the cells were removed from the hypoxia chamber, the hypoxic media was replaced with normoxic media and cells placed into normoxic culture conditions (37°C humidified incubator with five percent CO_2). The cells were then collected 15, 30 or 60 min later. Normoxic treated cells were subjected to the same treatment with replacement of normoxic media at the beginning of the experiment.

Prostaglandin E_2

Following normoxic or hypoxic treatment, supernatants were collected from cells and stored at -80°C until assay. PGE_2 concentration was determined with a PGE_2 Express EIA Kit (Cayman Chemical, Ann Arbor, MI) and expressed as pg per ml per $3x10^6$ cells.

Lipid Extraction

Lipids were extracted using a method similar to that described previously (Sparkes et al., 2010). Briefly, after normoxic or hypoxic treatment, media was removed and the cells lysed with distilled water. Cells were then collected from the tissue culture plates and lysates stored at

-80°C until lipid extraction. Lysates were thawed, vortexed and transferred to glass tubes that had been washed with a cation/phosphate-free liquid detergent (Contrex, Decon Labs, King of Prussia, PA) to eliminate contamination. One ml chloroform and two ml methanol were added and tubes shaken vigorously. An additional one ml chloroform and one ml distilled water were added and tubes shaken vigorously again. Tubes were centrifuged at 4000 rpm for five min at 4°C. The organic layers were transferred to clean glass tubes and one ml chloroform added to the aqueous phase. The tubes were again shaken, centrifuged and organic layers removed two more times. Distilled water (0.5 ml) was added to the combined organic layers from each sample, and each was centrifuged once more. The samples (organic layers) were submitted to the Kansas Lipidomics Research Center for analysis by mass spectrometry.

Mass Spectrometry

An automated ESI-MS/MS approach was used and data acquisition and analysis carried out at the Kansas Lipidomics Research Center as described previously with some modifications (Bartz et al., 2007; Devaiah et al., 2006). Solvent was evaporated from the extracts and each sample was dissolved in one ml of chloroform. Precise amounts of internal standards, obtained and quantified as previously described (Welti et al., 2002), were added to the sample to be analyzed. The sample and internal standard mixture were combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.2 ml. These unfractionated lipid extracts were introduced by continuous infusion into the ESI source of a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA) using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 μL per min.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: phosphatidylcholine (PC), sphingomyelin (SM) and lysoPC, $[M + H]^+$ ions in positive ion mode with Precursor of 184.1 (Pre 184.1); phosphatidylethanolamine (PE) and lysoPE, $[M + H]^+$ ions in positive ion mode with Neutral Loss of 141.0 (NL 141.0); phosphatidylinositol (PI), $[M + NH_4]^+$ in positive ion mode with NL 277.0; phosphatidylserine (PS), $[M + H]^+$ in positive ion mode with NL 185.0; phosphatidic acid (PA), $[M + NH_4]^+$ in positive ion mode with NL 115.0; PG, $[M + NH_4]^+$ in positive ion mode

with NL 189.0; lysophosphatidylglycerol (lysoPG), $[M - H]^-$ in negative mode with Pre 152.9; and free fatty acids (i.e., free arachidonic acid, (AA)), $[M - H]^-$ in negative mode with single stage MS analysis. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode.

The background of each spectrum was subtracted, the data were smoothed and peak areas integrated using a custom script and Applied Biosystems Analyst software (Applied Biosystems, Carlsbad, CA). The lipids in each class were quantified in comparison to the two internal standards of that class, except PI and AA, in which single standards were employed. The first and typically every 11^{th} set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the "internal standards only" spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each "internal standards only" set of spectra were used to correct the data from the following 10 samples. Finally, the data were expressed as mole percent of total lipid analyzed. Each class of lipid was also normalized to the sample protein content and expressed as nmol lipid class per mg protein.

Real-Time PCR

Following normoxic or hypoxic treatment, media was removed and cells washed once with tissue culture phosphate buffered saline (TCPBS; 136.8 mM NaCl, 1.47 mM KH_2PO_4, 2.68 mM KCl, 8.58 mM $Na_2HPO_4*7H_20$). Cells were collected in one ml of TRIzol Reagent (Invitrogen, Carlsbad, CA) and total RNA extracted according to manufacturer's protocol. Complementary DNA was reverse transcribed from two µg extracted RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD) and made according to manufacturer's protocol using random hexamers. Quantitative real-time PCR was used to assess mRNA transcript levels. Either Maxima SYBR (Fermentas) or PerfeCTa SYBR Green FastMix (Quanta BioSciences, Gaithersburg, MD) was used as master mix (25 µl total volume). Primers used are listed in Table 1 and were obtained from Integrated DNA Technologies (Coralville, IA). Genes of interest were normalized to 18s rRNA of each sample and fold change determined in relation to normoxic controls.

Table 4.1 qRT-PCR Primer Sequences

[a]Annealing temperature

[b]Ribosomal RNA; house-keeping gene to which genes of interest were normalized

Gene	Tm °C[a]	Primer Sequence
18s[b]	58	FWD: GGTTGATCCTGCCAGTAGC
		REV: GCGACCAAAGGAACCATAAC
VEGF	57	FWD: AGAGCAACATCACCATGCAG
		REV: TTTCTTGCGCTTTCGTTTTT
Flt-1	56	FWD: TATAAGGCAGCGGATTGACC
		REV: TCATACACATGCACGGAGGT
COX-1	56	FWD: AAGGAGTCTCTCGCTCTGCTTT
		REV: TCTCAGGGATGGTACAGTTGGG
COX-2	58	FWD: ATCCTGCCAGCTCCACCG
		REV: TGGTCAAATCCTGTGCTCATACAT
PLSCR1	56	FWD: TAGCTGCTGTTCCGACATTG
		REV: ACAAGCACCAAGCATCACAG
PLSCR3	57	FWD: GTTCACCATCTCCAGGCAGT
		REV: TAAGGGAAGGGTGGTGCTTG
Atp8a1 (flippase)	56	FWD: GTGTTTTGCTGTGGCTGAGA
		REV: ATGGTTTCAGGCACTTGGTC
Abcb1a (floppase)	56	FWD: GGCTTACAGCCAGCATTCTC
		REV: CCAGCTCACATCCTGTCTCA

Western Blot

Cell lysates were prepared after various lengths of normoxia following two h hypoxia as well as from normoxic controls. Cells were lysed and stored in a buffer of 10 mM Tris-HCl, pH 7.0, 200 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Nonidet P40 with the following protease inhibitors: AEBSF ($2x10^{-7}$ M), aprotin (1 μg per ml), leupetin (10 μg per ml), pepstatin (2.5 μg per ml), calpain inhibitor (17 μg per ml), chymostatin (2.5 μg per ml), antipain (2.5 μg per ml). Protein

concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL) adapted for microtiter plates and 60 μg protein loaded per well on a 10% SDS gel. Following transfer, the membrane was blocked and probed with rabbit anti-PLSCR1 Ab (Proteintech Group, Chicago, IL) at a 1:1000 dilution or mouse anti-α-tubulin Ab (GenScript, Piscataway, NJ) at a 1:10,000 dilution. Peroxidase conjugated goat anti-rabbit IgG (Thermo Scientific, Rockford, IL) and peroxidase conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary Ab at a dilution of 1:2,500. Image J (NIH) was used to quantify results and the average of five independent sets of lysates are presented.

Immunohistochemistry

Cells were seeded ($1.5x10^5$ per chamber) on eight chamber glass slides (BD Falcon, Franklin Lakes, NJ) and allowed to adhere for two to three h. Media was replaced with serum free de-oxygenated mediua, as described above, and slides placed in hypoxia chamber for two h or remained in normal culture conditions as normoxic controls. Following hypoxia, cells were provided with fresh media and re-oxygenation allowed for one h. Cells were then fixed with methanol and blocked with 10% normal donkey sera for 30 min at 37°C. Cells were stained with goat anti-human β_2-GPI Ab (Bethyl Laboratories, Montgomery, TX) at a concentration of one mg per ml overnight at 4°C. Fitc-conjugated donkey anti-goat IgG Ab (Jackson ImmunoResearch Laboratories) was used as secondary Ab. Appropriate isotype control Ab were used and slides mounted with Prolong Gold containing DAPI (Invitrogen). A Nikon eclipse 80i microscope with CoolSnap CF camera (Photometrics, Tucson, AZ) and Metavue software (Molecular Devices, Sunnyvale, CA) were used to obtain images at room temperature.

Labeled Lipid Experiments

Cells were seeded (1 to $1.5x10^5$) on glass coverslips and allowed to adhere for two h. During this time, a solution containing NBD-labeled phosphatidylserine (NBD-PS) (Avanti Polar Lipids, Alabaster, AL) (1 μM lipid, 5.5 mM dextrose in TCPBS) was added at a dilution of 1:100 to the media to allow for lipid incorporation. The thiol modifying chemical N-ethylmaleimide (NEM) (Alfa Aesar, Ward Hill, MA) was added to coverslips at a concentration of five mM for the final 30 min. The inhibitor NEM was removed and cells treated with hypoxia as described above. Following hypoxic or normoxic treatment, cells were rinsed with TCPBS and fixed with ice-cold methanol for three min. Trypan blue (0.4%) was added to selected coverslips for 30 sec to

quench fluorescence in the outer leaflet. After additional TCPBS washes, coverslips were mounted on glass slides with Prolong Gold containing DAPI (Invitrogen). Parallel experiments were performed without the inhibitor NEM. A Nikon eclipse 80i microscope with CoolSnap CF camera (Photometrics) and Metavue software (Molecular Devices) were used to obtain images at room temperature.

siRNA Transfection

Transfection protocol provided by manufacturer was followed (Santa Cruz Biotechnology, Inc., Dallas, TX). Briefly, cells were seeded into 6-well plates at a density of $2x10^5$ per well. Five µl siRNA duplex and five µl siRNA transfection reagent were used to prepare solutions A and B, respectively. Solutions A and B were mixed and allowed to incubate at room temperature for 30 min prior to addition to the cells. Cells were exposed to transfection medium for six h prior to addition of serum-containing media. Cells were assayed approximately 24 h later. A fraction of the cells from each transfected well was collected in Trizol reagent and knock-down was assessed by real-time PCR as described above. The other portion of each well was used for labeled lipid experiments as described above.

Statistics

Data are presented as mean ± SEM and significance ($p < 0.05$) determined by one-way ANOVA with Newman-Keuls post hoc analysis (GraphPad Prism 5, GraphPad Software, Inc., La Jolla, CA) unless otherwise noted.

Results

Two hours of hypoxia treatment induces transcription of hypoxia inducible factor-regulated genes.

To determine an appropriate length of hypoxia treatment for the following studies, an endothelial cell line, MS1, derived from a C57Bl/6 mouse was subjected to hypoxia for several hours. Cell viability and transcription of genes regulated by hypoxia inducible factor were assessed. We examined transcript levels of vascular endothelial growth factor (VEGF) and Flt-1 (VEGFR1) as markers for hypoxia since transcription of these genes is induced by hypoxia (Gerber et al.,

1997; Levy et al., 1995; Liu et al., 1995; Namiki et al., 1995). Exposure to one percent O_2 for two h significantly increased the transcript level of VEGF (1.00 ± 0.19 normoxia vs 2.11 ± 0.30 hypoxia) and Flt-1 (1.00 ± 0.28 normoxia vs 2.50 ± 0.21 hypoxia) as determined by qRT-PCR. At two h of hypoxia, cell viability was approximately 88%, but dropped sharply as length of hypoxic treatment increased further (data not shown). Thus, a hypoxic period of two h was used for the remainder of the studies.

β_2-glycoprotein I binds hypoxic endothelial cells

β_2-GPI is a serum protein that binds negatively charged lipids and when bound exposes a neo-antigen. We have previously demonstrated that β_2-GPI binds endothelium following a period of hypoxia and re-oxygenation (Fleming et al., 2010). To confirm our previous results, endothelial cells were subjected to hypoxia treatment for two h followed by one h re-oxygenation prior to analysis by immunohistochemistry. As shown in Figure 1, anti-β_2-GPI Ab stained the hypoxia-treated cells to a much greater extent than the normoxic control cells. This suggests that oxygen stress results in exposure of negatively charged phospholipids on the outer leaflet of the bilayer as the cells were not permeabilized.

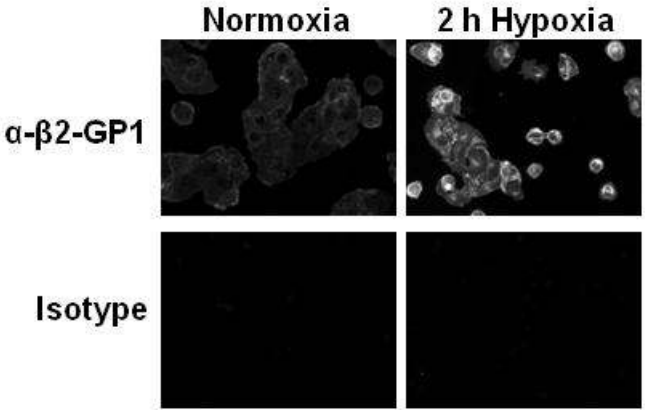


Figure 4.1 β_2-GPI binds endothelium following hypoxia treatment.
MS1 cells were grown on chamber slides, subjected to 2 h hypoxia (1% O_2) followed by 60 min normoxia or left in normal culture conditions, fixed and stained for β_2-GPI binding or negative control (isotype). Images (40x) are representative of 3 independent experiments with 4 to 6 pictures per treatment group per experiment.

Re-oxygenation of endothelial cells generates lipids involved in signaling

The β_2-GPI binding studies led us to investigate the phospholipid and free fatty acid composition of the endothelial cells. ESI-MS/MS was used to analyze cells treated with two h hypoxia alone, two h hypoxia followed by varying lengths of re-oxygenation and cells remaining in normal culture conditions. While the overall phospholipid composition was not changed after treatment (Table 2), a significant increase in lysophosphatidylcholine (lysoPC) was found during the re-oxygenation period (Fig. 2A). Phosphatidylcholine did not significantly decrease, however the abundance of this phospholipid could easily mask small changes in quantity (Fig. 2B).

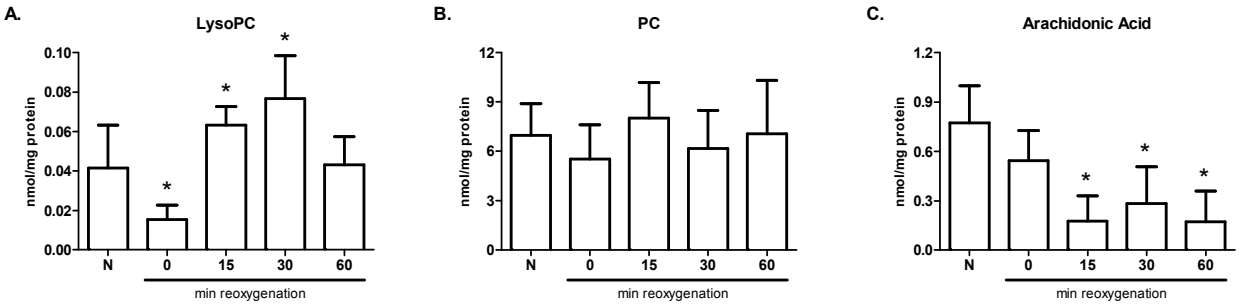


Figure 4.2 Phospholipid and arachidonic acid contents change with hypoxia treatment. (A, B, C) MS1 cells were subjected to 2 h hypoxia (1% O_2) followed by 0, 15, 30 or 60 min normoxia or left in normal culture conditions (N). Lipid content was determined by ESI-MS/MS and normalized to protein content of each sample. N = 5 to 9 samples per bar. * indicates $p < 0.05$ compared to N.

We next evaluated the free fatty acid content of the cells, with a special interest in free arachidonic acid. A source of free arachidonic acid is its cleavage from a phospholipid by a phospholipase. The increased lysoPC observed with re-oxygenation (Fig. 2A) implies phospholipase activity during this period. Mass spectrometry indicated a decrease in the free arachidonic acid content of the cells with re-oxygenation (Fig. 2C), suggesting possible conversion of newly released arachidonic acid to eicosanoids, such as PGE_2.

Table 4.2 Lipid composition of MS1 endothelial cells with hypoxia and re-oxygenation treatment.

[a]Lipids were extracted from MS1 endothelial cells after being subjected to 2 h hypoxia ± varying lengths of re-oxygenation or normoxic culture conditions and subjected to analysis by mass spectrometry. Each treatment group includes 6 to 9 individual samples.

[b]Normoxic controls

[c]2 h hypoxia + 0 min re-oxygenation

[d]2 h hypoxia + 15 min re-oxygenation

[e]2 h hypoxia + 30 min re-oxygenation

[f]2 h hypoxia + 60 min re-oxygenation

[g]Data are presented as nmol lipid per mg protein after normalization of signal to internal standards of each lipid class. Mean and SEM are reported. * indicates a significant difference ($p < 0.05$) from normoxic controls.

Lipid class[a]	Normoxia[b]	0 min[c]	15 min[d]	30 min[e]	60 min[f]
LysoPC	0.04 ± 0.01[g]	0.02 ± 0.00*	0.06 ± 0.00*	0.08 ± 0.01*	0.04 ± 0.01
PC	7.80 ± 0.90	7.82 ± 1.31	8.42 ± 1.21	8.95 ± 1.32	8.59 ± 1.33
SM	1.06 ± 0.20	1.18 ± 0.45	1.34 ± 0.41	1.70 ± 0.46	1.42 ± 0.42
LysoPE	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.00
PE	1.28 ± 0.32	1.61 ± 0.67	2.10 ± 0.67	2.50 ± 0.74	2.18 ± 0.83
PI	0.33 ± 0.09	0.38 ± 0.19	0.38 ± 0.14	0.54 ± 0.18	0.37 ± 0.14
PS	0.21 ± 0.05	0.31 ± 0.11	0.30 ± 0.09	0.47 ± 0.10	0.34 ± 0.09
PA	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
PG	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.02	0.07 ± 0.02

Hypoxia and re-oxygenation promotes increased transcription of the Cox enzymes and production of PGE_2

The lipid findings, particularly the decrease in free arachidonic acid, led us to examine the Cox enzymes and production of PGE_2, as PGE_2, a modulator of inflammation, is a necessary component for intestinal IR-induced damage (Moses et al., 2009). Accordingly, the PGE_2 concentration in the intestine increases significantly following IR of the intestine, while the PGE_2

concentration is minimal in healthy tissue (Fleming et al., 2010; Miner et al., 1999b; Moses et al., 2009; Rehrig et al., 2001a; Sparkes et al., 2010). The Cox enzymes are required in the conversion of arachidonic acid to prostaglandins and we hypothesized that transcription of the enzymes as well as PGE_2 production would increase with hypoxia.

We used qRT-PCR to determine the change in transcription of two Cox isofoms in normoxia and hypoxia/re-oxygenation treated cells. The transcript number of Cox 2, the inducible isoform, was significantly elevated during the hypoxic period and continued to rise with a peak at 30 min re-oxygenation (Fig. 3A). Surprisingly, a significant increase in Cox 1, considered the constitutive isoform, was also observed and followed the trend of Cox 2 (Fig. 3B). Furthermore, the fold change of Cox 1 exceeded that of Cox 2 (Fig. 3A, B). These data indicate that both isoforms may be involved in prostaglandin regulation during periods of hypoxic stress.

We continued to investigate our hypothesis by quantifying PGE_2 production. A time course was established to determine if PGE_2 was produced during the hypoxic period or only upon re-oxygenation and to identify the time point of maximal response. The endothelial cells significantly up-regulated production of PGE_2 upon re-oxygenation, but no change was seen during the hypoxic period (Fig. 3C). As early as 15 min post-hypoxia the cells significantly increased PGE_2 production compared to control cells maintained in normal culture conditions. Based on our sampling time points, the production of PGE_2 peaked at 30 min post-hypoxia. At this time point, the hypoxia treated cells showed an almost 1,800% increase (3,391 ± 575 pg per ml at 30 min re-oxygenation versus 179 ± 33 pg per ml for normoxic controls) in PGE_2 production versus normoxia treated cells. The time course of Cox transcription and PGE_2 production correlate, in that transcription of Cox enzymes is increased during hypoxia while a significant increase in PGE_2 is not observed until 15 min into the re-oxygenation period (Fig. 3).

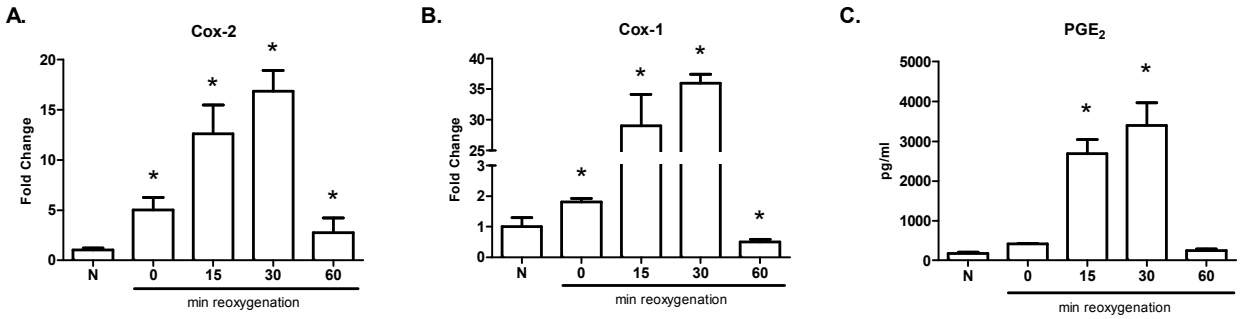


Figure 4.3 Transcription of Cox enzymes and PGE_2 production increases with hypoxia treatment.

(A, B, C) MS1 cells were subjected to 2 h hypoxia (1% O_2) followed by 0, 15, 30 or 60 min normoxia or left in normal culture conditions (N). (A, B) Fold change in transcription was determined by qRT-PCR. Cox 2 (A) and Cox 1 (B) results were normalized to corresponding 18s rRNA and compared to normoxic samples. N = 4 to 5 samples per bar. * indicates $p < 0.05$ compared to N. (C) PGE_2 concentration of supernatants was determined by EIA. N = 3 to 6 samples per bar. * indicates $p < 0.05$ compared to N.

Recovery from oxygen deprivation selectively up-regulates transcription of PLSCR1

Phospholipids are involved in facilitating both β_2-GPI binding and the arachidonic acid cascade; however the mechanism by which the phospholipids change in hypoxic conditions is unknown. Several enzymes play a role in maintaining the asymmetry of the lipid bilayer. We hypothesized that PLSCR1 is involved in mediating the lipid changes that occur with hypoxia and re-oxygenation. Analysis by qRT-PCR revealed a significant increase in PLSCR1 transcript in endothelial cells recovering from hypoxia treatment compared to the normoxic control cells (Fig. 4A). Additional phospholipid transporting proteins were examined to verify that the response of PLSCR1 to hypoxia and re-oxygenation is specific. Transcription of PLSCR3, which localizes to the mitochondrial membrane, did not change with hypoxia, but decreased upon re-oxygenation (Fig. 4C). Similarly, two h of hypoxia alone did not alter transcription of representative flippase (Atp8a1) and floppase (Abcb1a) proteins, whereas re-oxygenation decreased transcription (Fig. 4B, D).

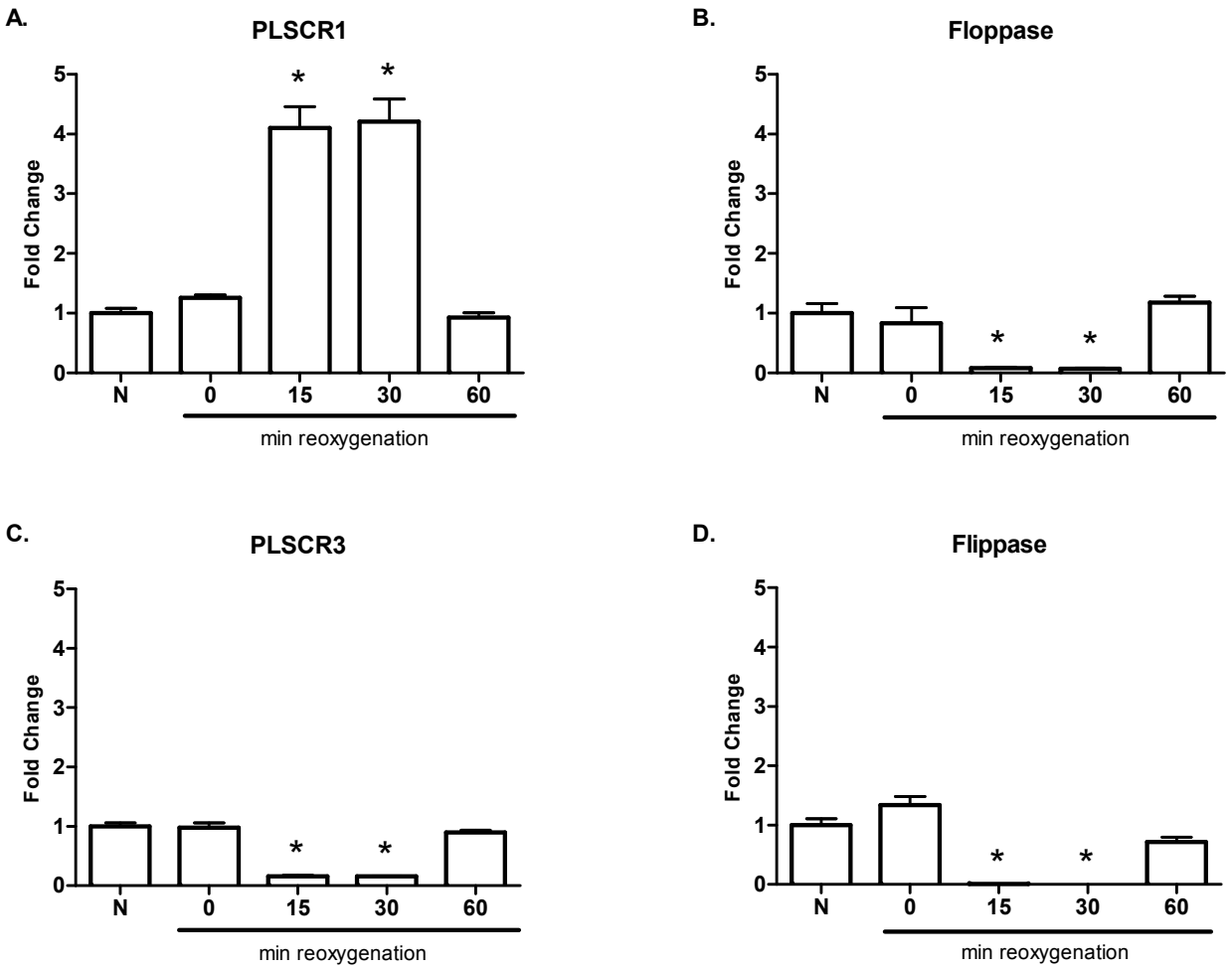


Figure 4.4 Hypoxia selectively increases transcription of PLSCR1.

(A, B, C, D) MS1 cells were subjected to 2 h hypoxia (1% O_2) followed by 0, 15, 30 or 60 min normoxia or left in normal culture conditions (N). Fold change in transcription of PLSCR1 (A), floppase Abcb1a (B), PLSCR3 (C) and flippase Atp8a1 (D) was determined by qRT-PCR. Genes of interest were normalized to corresponding 18s rRNA and compared to normoxic samples. N = 4 to 5 samples per bar. * indicates $p < 0.05$ compared to N.

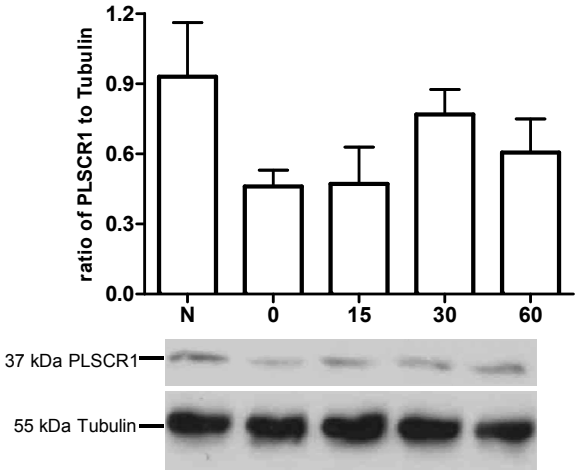
Hypoxia alters PLSCR1 protein expression

Our qRT-PCR data suggested increasing PLSCR1 protein levels may also increase with re-oxygenation. To confirm an increase in PLSCR1 protein, whole-cell lysates were prepared from hypoxia-treated and normoxic control endothelial cells. Western blot analysis indicated a

decrease in PLSCR1 protein levels with two h of hypoxia treatment compared to normoxic controls (Fig. 5). Protein levels tended to increase back towards normoxic levels as re-oxygenation proceeded (Fig. 5). Taken together, our qRT-PCR and Western blot data indicate that PLSCR1 protein decreases during the hypoxic period, while re-oxygenation induces an up-regulation in transcription and subsequent protein expression within 15 to 30 min (Fig. 4A, 5). These data suggest that PLSCR1 is sensitive to oxygen tension and may be involved in the phospholipid changes that occur in response to hypoxia and re-oxygenation.

Figure 4.5 PLSCR1 protein decreases with hypoxia in endothelial cells.

MS1 cells were subjected to 2 h hypoxia (1% O_2) followed by 0, 15, 30 or 60 min normoxia or left in normal culture conditions (N). Whole-cell lysates were prepared and run on SDS-PAGE followed by immunoblotting for PLSCR1 and the control protein tubulin. A representative blot is shown. Image J software (NIH) was used to quantify bands and a ratio of PLSCR1 to tubulin was determined. The average of 5 independent sets of lysates is presented.



Hypoxia and re-oxygenation increases activity of PLSCR1

Changes in transcription and total protein do not necessarily correlate with protein activity. Thus, we assayed for PLSCR1 activity under conditions of hypoxia and re-oxygenation. NBD-PS was allowed to incorporate into endothelial cell membranes and flippase activity chemically inhibited with NEM. Excess labeled lipid was removed prior to hypoxia or parallel normoxia treatment to determine loss of fluorescent signal due to PLSCR1 activity. As shown in Figure 6, NBD-PS was readily incorporated into the cell membranes and no change in total (inner leaflet and outer leaflet) NBD-PS was observed after hypoxia or re-oxygenation (Fig. 6A). However, quenching of the NBD-PS in the outer leaflet by trypan blue revealed a statistically significant decrease in inner leaflet NBD-PS in all treatments (Fig. 6B, C). Furthermore, PLSCR1 activity is

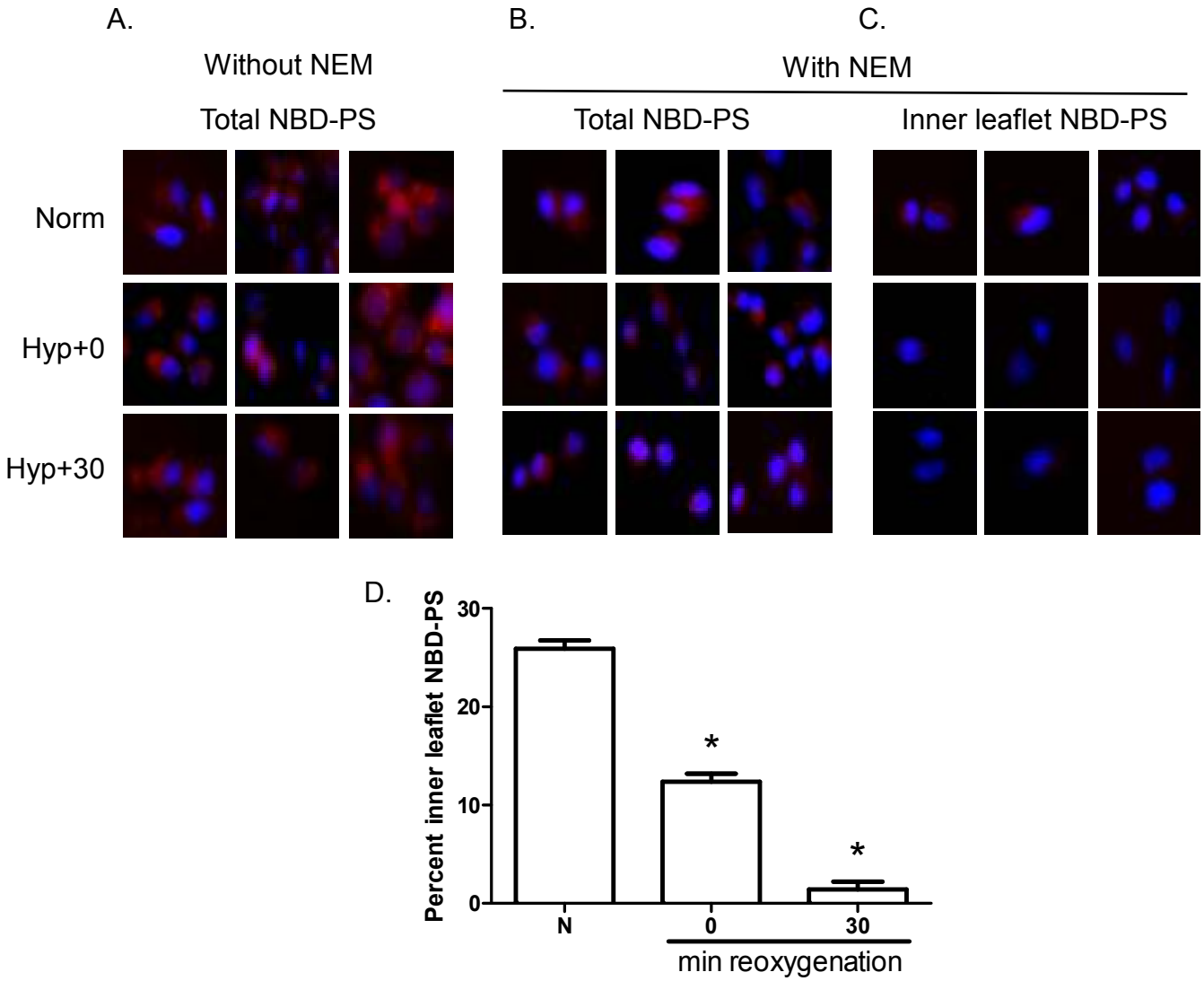


Figure 4.6 PLSCR1 activity increases during hypoxia and re-oxygenation.

(A, B, C) Prior to normoxia (top row) or 2 h hypoxia (1% O_2) ± 30 min re-oxygenation treatment, NBD-PS was incorporated into MS1 cell membranes for 2 h with (B, C) or without (A) NEM treatment to inhibit flippase and floppase activity . Trypan blue was added to some of the samples to quench fluorescence in the outer leaflet (C). NBD-PS fluorescence is presented in red and nuclei are shown in blue. (A) represents NBD-PS in both the inner and outer leaflets in the absence of NEM, (B) represents NBD-PS in both the inner and outer leaflets in the presence of NEM, and (C) represents NBD-PS in the inner leaflet only in the presence of NEM. Images (80x) are representative of 3 independent experiments with 3 to 6 pictures per treatment per experiment. Image J software (NIH) was used to quantify florescence. Percent fluorescence remaining in inner leaflet after treatment (in the presence of NEM) is presented with 12 to 18 cells per treatment analyzed (D). * indicates $p < 0.05$ compared to normoxia (N).

significantly increased by hypoxia and re-oxygenation, as the percent NBD-PS in the inner leaflet significantly decreases with hypoxia and re-oxygenation in comparison to normoxia, indicating loss of NBD-PS from the inner leaflet (Fig. 6D). These data suggest that during hypoxia, PLSCR1 activity increases and translocates NBD-PS to the outer leaflet where it is quenched by trypan blue. PLSCR1 activity remains high during re-oxygenation as it continues to translocate NBD-PS.

Knock down of PLSCR1 abrogates PS movement during hypoxia

To demonstrate a specific role of PLSCR1, the labeled lipid experiments were repeated after PLSCR1 expression was knocked down with siRNA. We hypothesized that if PLSCR1 was transporting NBD-PS across the membrane, knock down would limit the movement of NBD-PS and no difference would be observed in inner leaflet fluorescence across the treatments. Transfection and knock down of PLSCR1 was successful (Fig. 7). Similar to the untreated cells in the previous experiment (Fig. 6), hypoxia treatment did not alter the total fluorescent signal in the control cells (Fig. 8A). However, the inner leaflet fluorescent signal decreased with hypoxia treatment suggesting movement from inner leaflet to outer leaflet by PLSCR1 (Fig. 8B). Importantly, no change in inner leaflet fluorescence occurred with hypoxia or hypoxia and re-oxygenation when PLSCR1 was knocked down (Fig. 8D). No net movement of the lipid was detected in the absence of PLSCR1 (Fig. 8D). The data collected from the labeled lipid

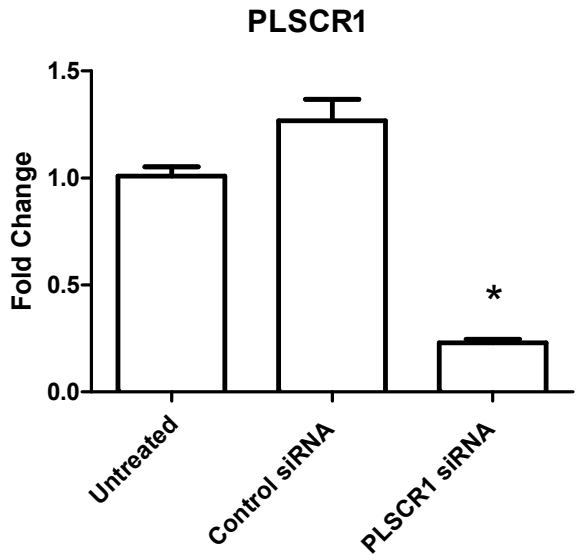


Figure 4.7 Knock down of PLSCR1. MS1 cells were transfected with PLSCR1 siRNA, control siRNA or left untreated. Cells were transfected for 6 h and collected in Trizol 24 h later. RNA was isolated, cDNA synthesized and fold change in transcription of PLSCR1 was determined by qRT-PCR. Values were normalized to corresponding 18s rRNA and compared to untreated samples. N = 10 samples per bar. * indicates $p < 0.05$ compared to untreated.

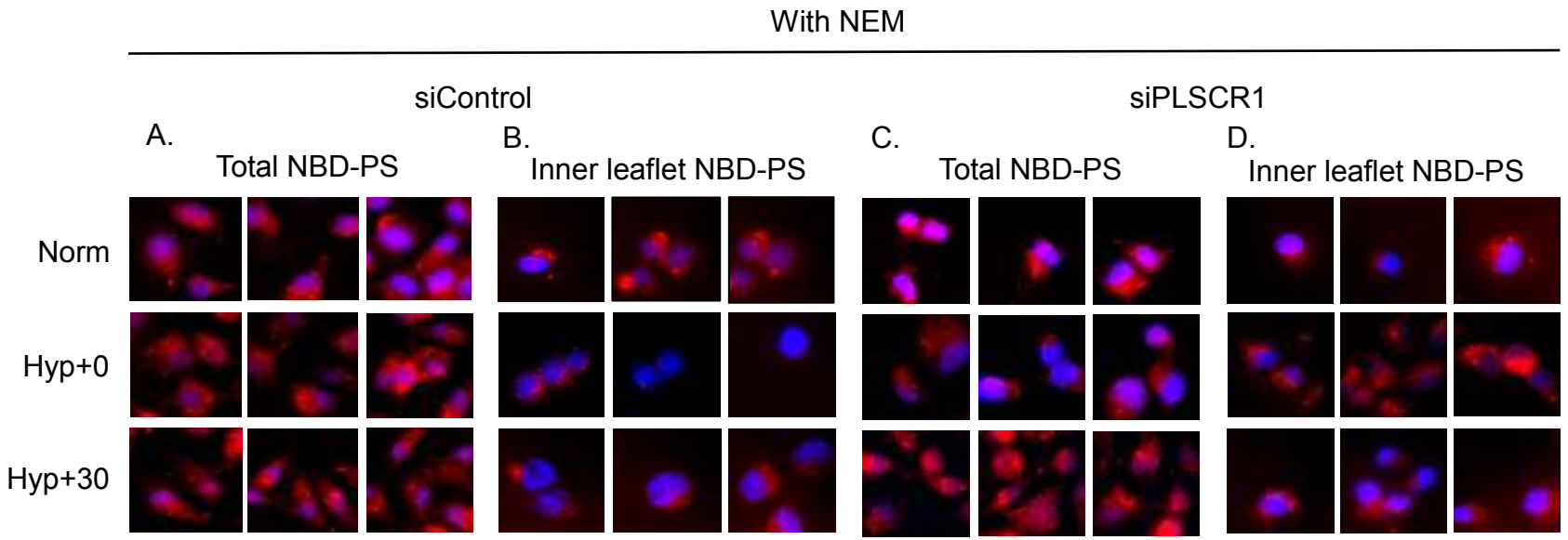
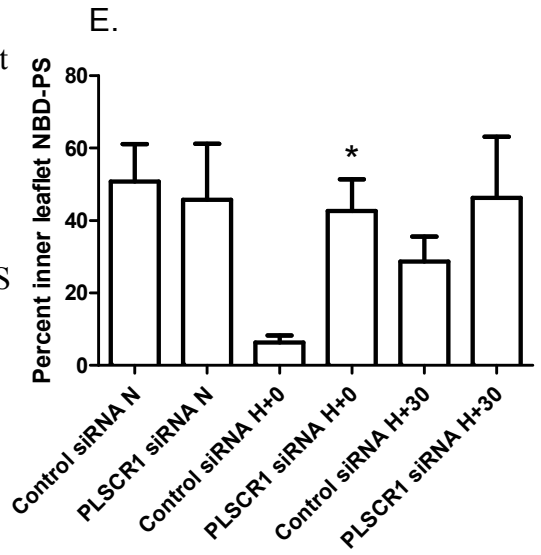


Figure 4.8 Hypoxia activates PLSCR1.

(A, B, C, D) Prior to normoxia (top row) or 2 h hypoxia (1% O_2) ± 30 min re-oxygenation treatment, NBD-PS was incorporated into MS1 cell membranes for 2 h with NEM treatment to inhibit flippase and floppase activity. Trypan blue was added to some of the samples to quench fluorescence in the outer leaflet (B, D). NBD-PS fluorescence is presented in red and nuclei are shown in blue. (A) represents NBD-PS in both the inner and outer leaflets and (B) in inner leaflet only of cells transfected with control siRNA, (C) represents NBD-PS in both the inner and outer leaflets and (D) in inner leaflet only of cells transfected with PLSCR1 siRNA. Images (80x) are representative of 5 independent experiments with 3 to 6 pictures per treatment per experiment. Image J software (NIH) was used to quantify florescence (E). Percent fluorescence remaining in inner leaflet after treatment is presented with 12 to 18 cells per treatment analyzed (E). * indicates $p < 0.05$ compared to respective control.



experiments suggests that PLSCR1 activity is increased during hypoxia and re-oxygenation allowing for the exposure of negatively charged phospholipids, such as PS, on the outer leaflet of the bilayer.

Discussion

Oxygen deprivation, whether acute or chronic, results in several changes at the cellular level. Stabilization of the hypoxia inducible factor 1α (HIF-1α) subunit allows for HIF activity, which alone influences the transcription of more than 150 genes (reviewed in (Majmundar et al., 2010; Schofield and Ratcliffe, 2004)). It is well established that inflammation contributes to IR–induced intestinal pathology. However, the specific cell types involved are unclear. We hypothesized that endothelial cells are key mediators of the inflammatory response observed following the oxygen deprivation that occurs with ischemia. Specifically, we hypothesized that lipid scrambling by PLSCR1 allows for binding of β_2-GPI, a serum protein, to endothelial cells and elicits cellular responses following hypoxia and re-oxygenation. In this study, we demonstrate that PLSCR1 transcription, expression and activity are altered by hypoxia and re-oxygenation in endothelial cells. In addition, we confirmed that β_2-GPI binds hypoxia treated, but not normoxic, endothelial cells (Fleming et al., 2010) further supporting the hypothesis that β_2-GPI is intimately involved in IR-induced injury. Endothelial cells produce and secrete vast quantities of PGE_2, a vasodilator and driver of cellular permeability, in a short time period following relief of hypoxic insult, another similarity to intestinal IR pathology.

Preliminary studies were required to determine the optimal length of hypoxia treatment. Our goal was to ensure hypoxic conditions while preserving a high level of cell viability. Endothelial cells are more sensitive to hypoxia than other cell types (data not shown). We found that two h of hypoxia at one percent O_2 resulted in increased transcription of VEGF and Flt-1, genes directly induced by HIF (Gerber et al., 1997; Liu et al., 1995; Namiki et al., 1995), providing evidence that a state of hypoxia was attained. Exposure of the endothelial cells to hypoxia for greater than two h significantly decreased cell viability (data not shown). These results are in agreement with Michiels et al. who also found two h of hypoxia to be optimal for primary human umbilical vein endothelial cells (Michiels et al., 1993). As PGE_2 production was significantly up-regulated following hypoxia treatment, similar to the response observed from ex

vivo intestinal tissue after IR (Fleming et al., 2010; Moses et al., 2009; Sparkes et al., 2010), we continued our studies exposing endothelial cells to two h hypoxia.

This is the first comprehensive examination of the effects of hypoxia on PLSCR1 of which we are aware. A study by Rami et al. performed immunohistochemical staining for PLSCR1 in human ischemic brain samples and found an increase in PLSCR1 protein in the ischemic samples versus controls (Rami et al., 2003). Our data indicate a decrease in protein levels during a period of acute hypoxia (Fig. 5) but a significant increase in transcription upon re-oxygenation (Fig. 4A). Importantly, the Rami study focused on the neurons of the brain while our study examined endothelial cells in culture; thus the differences in cell type and insult may resolve the apparent discrepancy. An additional factor to consider is the potential of PLSCR1 to be released from the membrane. The decreased protein expression found with hypoxia treatment (Fig. 5) could be due to shedding of the protein into the culture media as was recently described for PLSCR3 (Inuzuka et al., 2013).

PLSCR1 is a transmembrane protein when palmitylated (Wiedmer et al., 2003); it facilitates scrambling of the lipid bilayer upon an increase in intracellular calcium or other polycations (Bucki et al., 2000). Unlike flippases and floppases, PLSCR1 activity often abolishes membrane asymmetry resulting in exposure of phosphatidylethanolamine (PE) and PS on the outer leaflet (reviewed in (Contreras et al., 2010)). Externalization of PS is a hallmark of apoptosis (reviewed in (Fadeel et al., 2010; Ravichandran, 2011)), however, transient exposure of PS is observed upon cellular activation, coagulation (Zwaal and Schroit, 1997) and membrane blebbing (Kirov et al., 2012). β_2-GPI, a serum protein, binds negatively charged phospholipids and has an affinity for PS. Thus, several events may lead to β_2-GPI binding to the endothelium.

The role of PLSCR1 in PS translocation appears to vary depending on the context or purpose of the PS exposure. Initial studies of calcium activated, PLSCR1 activation demonstrated that PLSCR1 translocated PS across the membrane but was not required for apoptosis-induced PS translocation or PS-induced coagulation when expressed on white blood cells or platelets, respectively (reviewed in (Bevers and Williamson, 2010)). Ory et al. demonstrated that although PLSCR1 is not required for exocytosis by lung epithelial cells, re-internalization of the vesicular membranes requires PLSCR1(Ory et al., 2013). Recent data suggest that interferon-α activation of PLSCR1 may play a role in PS exposure and apoptosis as ovarian cancer cell apoptosis increased when PLSCR1 expression was decreased (Kodigepalli et

al., 2013). Our data demonstrates that hypoxia induces PS expression on the outer leaflet (Fig. 6) in a PLSCR1 dependent manner (Fig. 7). Together, these studies support the hypothesis that PLSCR1 is activated during hypoxia and facilitates the movement of phospholipids between leaflets. Importantly PS expression as a result of IR-induced injury does not result in apoptosis but rather necrosis due to complement mediated cell death (Zhang et al., 2013). Thus further studies are needed to determine if complement mediated cell death requires PLSCR1.

Recent studies examined PLSCR1 levels in autoimmune patients, including Antiphospholipid Syndrome (APS) and Systemic Lupus Erythematosus (SLE) patients. Transcription of PLSCR1 was found to be increased in blood monocytes of APS and SLE patients versus controls (Amengual et al., 2012; Suzuki et al., 2010). Interestingly, β_2-GPI is a common antigen for APS and SLE patients ((McNeil et al., 1990), reviewed in (Alessandri et al., 2011)). Similarly, our data indicate increased PLSCR1 transcription (Fig. 4A) and increased binding of β_2-GPI (Fig. 1) to endothelial cells with hypoxia and re-oxygenation treatment. Furthermore, inflammation is common to APS, SLE and IR-induced injury, supporting the hypothesis that PLSCR1-promoted β_2-GPI deposition is an early pathogenic change.

ESI-MS/MS analysis revealed few changes in phospholipid composition of the endothelial cells with hypoxia treatment. LysoPC did increased with re-oxygenation (Fig. 2A and Table 2), a trend also observed with hydrogen peroxide treatment of a human endothelial cell line (Yang et al., 2011). Perhaps lysoPC production is a response coupled to oxidative damage, as both treatments result in such damage. Additionally, changes in arachidonic acid were observed when endothelial cells were exposed to hydrogen peroxide (Yang et al., 2011).

The Cox 1 isoform has traditionally been considered the constitutively active Cox enzyme, with inflammatory signals up-regulating the Cox 2 enzyme. Our data indicate a transcriptional up-regulation of both isoforms (Fig. 3). Additionally, the fold increase in transcription of Cox 1 is more than double that of Cox 2 (36.0 + 1.4 vs 16.8 + 2.1 at 30 min post-hypoxia). North et al. similarly found an increase in Cox 1 protein, but not Cox 2, 15 min following exposure of primary pulmonary endothelial cells to low oxygen tension (North et al., 1994). These results suggest that both isoforms may be involved in downstream effects of hypoxic stress.

A time course tracking the effect of re-oxygenation on PGE_2 production and secretion by endothelial cells revealed a peak at 30 min re-oxygenation following two h hypoxia (Fig. 3C).

Previous in vivo studies which followed a similar time course found the maximum PGE_2 response at two h post-ischemia (Sparkes et al., 2010). Several factors may contribute to this difference in the timing of maximal PGE_2 production and secretion. The current study examined a single cell type (endothelium) while whole intestinal tissue comprised of several cell types (including epithelium and smooth muscle which are known to produce PGE_2 (Carew and Thorn, 2000; Longo et al., 1998)) was assessed in the previous study. Additionally, the hypoxic period in the current study was two h versus 30 min ischemia in vivo. Data from an in vitro study with bovine aortic endothelial cells and human umbilical vein endothelial cells complements our data (Fig. 3C) as an increase in total prostaglandins was only observed after re-oxygenation, but not immediately following a hypoxic period of two and a half h (Oudot et al., 1998).

Several factors that contribute to IR-induced injury have been identified; however, few effective therapies are currently available. We determined the cell type that contributes to early pathology and PGE_2 production in relation to intestinal IR. Our work demonstrates that molecular events occurring in endothelial cells are relevant to the perpetuation of tissue injury. Lipid scrambling during hypoxia, via PLSCR1, allows for binding of the serum protein β_2-GPI upon re-oxygenation (reperfusion) which in turn can trigger activation of the complement cascade. Additionally, lipid metabolism, including the generation of arachidonic acid and subsequent conversion to PGE_2 via Cox enzymes, occurs. Thus, multiple pathways all leading to cellular damage and inflammation begin with the endothelial cells. Our study also suggests that PLSCR1 may be a novel therapeutic target for IR-induced injury.

Chapter 5 - Conclusions

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. This work contributes to the knowledge regarding early events in intestinal IR-induced pathogenesis with an ultimate goal of reducing associated tissue damage and death.

The work presented here supports the hypothesis that endothelial cells are critically involved in intestinal IR-induced injury. Several of the responses observed with in vivo experiments are replicated by endothelial cells in vitro. 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_2, another required component for IR-induced damage, is not increased. 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.

TLRs are involved in the pathogenesis of IR-induced injury in many organs. TLR4 has been studied extensively and is an important factor for IR-induced damage to a large number of organs (Gao et al., 2009a; Moses et al., 2009; Takeishi and Kubota, 2009; Victoni et al., 2010; Wu et al., 2007; Yang et al., 2008b). Similarly, TLR2 is known to contribute to injury in several organs; whereas, much less is known about the role of other TLRs in the context of IR. Several studies have demonstrated the protective effect of TLR4 or TLR9 deficiency in the context of hepatic IR (Bamboat et al., 2010; Huang et al., 2011). As TLR4 is known to contribute to intestinal IR-induced damage, the role of TLR9 in the intestine was assessed. Contrary to expectations, intestinal IR-induced injury is not affected by TLR9. The same degree of tissue damage occurs in *TLR9*$^{-/-}$ as in wildtype mice (Slone et al., 2012). These results illustrate the differences in the pathogenesis of IR-induced injury in different organs. TLR4 resides in the

cellular membrane while TLR9 remains intracellular. Thus, a TLR4 ligand only needs to contact the cell but TLR9 ligands must be phagocytized for recognition. Although the intestine contains a great number of resident macrophages, perhaps the Kuppfer cells of the liver are more efficient in phagocytizing apoptotic and necrotic cells. Ligand recognition by TLR9 may be a relatively more important aspect of innate immunity in Kuppfer cells due to their location and function.

Mass spectrometry was used to compare the intestinal lipid profiles of polar lipids and free fatty acids between different strains of mice. This endeavor led to the first published characterization of the intestinal lipid profile of wildtype mice. IR treatment resulted in considerable changes in the lipid composition; however these alterations were largely independent of genetic background as intestinal lipid profiles did not differ significantly between strains of mice, C57Bl/6, *Rag-1*$^{-/-}$, *TLR9*$^{-/-}$, *TLR2*$^{-/-}$, *CR2*$^{-/-}$. Importantly, the levels of lysolipids increased, especially lysoPC and lysoPE, as did the levels of arachidonic acid. Release of arachidonic acid 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. Following exposure of negatively charged phospholipids, such as PS, circulating β_2-GPI is able to deposit on the cellular membranes of endothelial cells. The conformational change that occurs in β_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.

This proposed cascade in the pathogenesis of IR-induced injury is supported by experimental results in several strains of mice. For example, antibody-deficient *Rag-1*$^{-/-}$ mice do not experience IR-induced tissue damage. The lipid changes that occur in *Rag-1*$^{-/-}$ mice are consistent with wildtype mice and β_2-GPI levels are comparable. However, since antibodies are lacking, the cascade does not continue and damage does not occur.

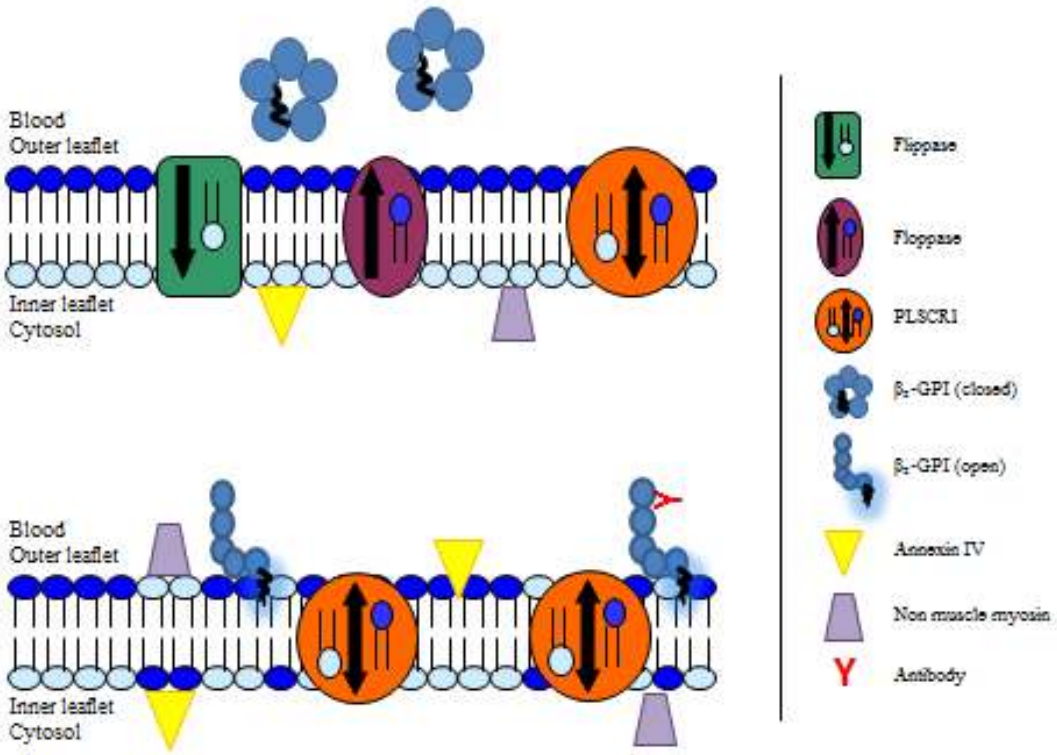


Figure 5.1 Proposed model of early events in the pathogenesis of IR-induced injury. During normoxia, the asymmetry of the bilayer is maintained by flippases, floppases and scramblases. The neo-antigen β2-GPI circulates in a closed conformation and annexin IV and non-muscle myosin, additional neo-antigens, 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, β2-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.

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, β_2-GPI. Experiments described in Chapter 4 investigated the relationship between the activation of PLSCR1, a transmembrane protein, and membrane changes that promote binding of β_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, 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 β2-GPI to endothelial cells following hypoxia in the absence of PLSCR1 will further clarify the potential value in pursuing PLSCR1 as a therapeutic target (Fig. 5.1). Hypothesized to be one of the first cellular responses to hypoxia, PLSCR1 may become an important target for the development of IR-related therapeutics.

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