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Intestinal lipid alterations occur prior to antibody-induced prostaglandin E2 production in a mouse model of ischemia/reperfusion

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Summary

Ischemia/reperfusion (IR) induced injury results in significant tissue damage in wild-type, but not antibody-deficient, Rag-$1^{-/-}$ mice. However, Rag-$1^{-/-}$ mice sustain intestinal damage after administration of wild-type 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 induced 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 (wild-type) and Rag-$1^{-/-}$ mice, IR led to Cox-2 activation and prostaglandin E_2 (PGE_2) production in wild-type, but not in the antibody-deficient, Rag-$1^{-/-}$ mice. Administration of wild-type 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.

1. Introduction

Ischemia, a condition in which a lack of oxygen and nutrients results in severe inflammation and cellular damage, is a common medical pathology [1, 2]. Ischemic cells undergo biological and chemical changes including activation of numerous proteases and lipases which induce tissue damage [3]. Subsequent reperfusion to the ischemic region results in far greater injury than observed as a result of ischemia alone [1, 2, 4]. The intestines are thought to be the organ most sensitive to reperfusion-induced damage [2, 5]. Mesenteric reperfusion damage is associated with multiple organ failure, resulting in a mortality rate ranging from 60 – 80% in humans [5-7]. 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 [8-10] or complement blockade [11-13] 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 wild-type mice. Indeed, Ab play a critical role in both neutrophil recruitment and complement activation [14, 15]. After administration of two monoclonal Ab, one recognizing phospholipids and another recognizing a phospholipid-binding protein, these damage-resistant mice sustain inflammation and intestinal injury at levels seen in wild-type mice [14, 15]. 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 (ESI)-tandem mass spectrometry (MS/MS) to determine the relative prevalence of 10 glycerophospholipid classes in normal rat intestine [16]. Another study showed that phosphatidylcholine (PC) and lysophosphatidylcholine (lysoPC) were decreased in the intestinal mucus of ulcerative colitis patients [17, 18]. Using lipidomics, a recent study found that normal mouse duodenum and jejunum contain the highest concentrations of PC and lysoPC among the intestinal segments [18]. Although intestinal lipid changes in the IR model were not investigated in previous studies, the data indicate that lipidomics is a useful tool for identification of intestinal lipid changes associated with disease. ESI-MS/MS may provide a means for detection of the IR-induced lipid antigen. In the current study, we test the hypothesis that mesenteric IR alters intestinal lipid composition and examine the role of Ab in IR-induced lipid changes and subsequent tissue injury.

2. Methods

2.1 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 (Purina Lab Diet 5001) 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.

2.2 Intestinal Ischemia Reperfusion

Mice were subjected to IR as described previously [15]. Briefly, mice were anaesthetized by an intraperitoneal injection of 8 mg/kg xylazine and 16 mg/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 1-2 cm long jejunal sections (~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 IV injection of 100 μg of purified Ab 15-20 min prior to occlusion of the superior mesenteric artery. All tissues collected were assayed in a blinded manner.

2.3 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* [19]. 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 75-150 villi in a 2 cm section were averaged to determine the injury score for that mouse.

2.4 Lipid Extraction

Frozen 2 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 [20, 21]. A portion of the homogenized tissue was retained for bicinchoninic acid (BCA, Pierce) protein analysis. The remaining tissue (80-150 mg) was suspended in 1 ml cold water. One ml chloroform and 3 ml methanol were added and the sample was shaken vigorously. An additional 1 ml chloroform and 1 ml water were added and the sample was centrifuged at 4000 rpm for 5 min at 4ºC. The lower (organic) layer was removed and 1 ml chloroform added to the aqueous layer. The sample was again centrifuged and the process repeated for a total of three chloroform extractions. The organic layers from each extraction were pooled and the combined extract washed with 1 ml water. The final extract was submitted for mass spectrometry analysis.

2.5 Mass Spectrometry Analysis

An automated ESI-MS/MS approach was used and data acquisition and analysis carried out at the Kansas Lipidomics Research Center as described previously [22, 23] with

modifications. Solvent was evaporated from the extracts and each sample was dissolved in 1 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 [24], were added to the sample to be analyzed 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 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). 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/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, $[M + NH_4]^+$ in positive ion mode with NL 277.0; PS, $[M + H]^+$ in positive ion mode with NL 185.0; PA, $[M + NH_4]^+$ in positive ion mode with NL 115.0; PG, $[M + NH_4]^+$ 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. SM was determined from the same mass spectrum as PC (precursors of m/z 184 in positive mode) [25, 26] 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 diacyl standards as other PC and PE species, and no response factors were applied. This approach may result in under-representation of ether-linked molecular species [27]. The scan speed was 50 or 100 u per sec. The collision gas pressure was set at 2 (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, +24 V for PG, and –57 V for lysoPG. Declustering potentials were +100 V, except for lysoPG, which was –100 V. Entrance potentials were +15 V for PE, +14 V for PC (and SM), PI, PA, PG, and PS, and –10 V for lysoPG. Exit potentials were +11 V for PE, +14 V for PC (and SM), PI, PA, PG, and PS, and –14 V for lysoPG. 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 collision gas was "low", 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 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 intestinal proteins and expressed as nmol lipid class/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.

2.6 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 [4, 12, 28]. Briefly, the intestinal section was minced in oxygenated Tyrodes buffer (Sigma), washed 3 times to remove feces and incubated for 20 min at 37ºC in freshly oxygenated Tyrodes 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.

2.7 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). 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 rRNA, relative gene expression between treatment groups was determined using the comparative ΔCt method. 18s rRNA was selected as the internal standard based on preliminary studies indicating no significant differences in the 18s rRNA quantities between treatment groups. The fold change in Cox-2 mRNA was determined relative to the Cox-2 mRNA level of the C57Bl/6 (wild-type) Sham-treated control group after normalization. . 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).

2.8 Antibody Purification

Protein L bead columns (Pierce) were used to purify all antibody isotypes of Immunoglobulins (Ig) from C57Bl/6 sera. Multiplex analysis indicated that IgM, IgG1, IgG2b and IgG3 were present in similar proportions to those 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^{o}C overnight in phosphate buffered saline

(PBS). The PBS was changed twice during dialysis. Dialyzed fractions were concentrated to 1 mg per ml using Centriplus concentrators (Millipore).

2.9 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 $p < 0.05$.

3. Results

3.1 Lipidomic analysis of the molecular composition of intestinal phospholipids, sphingomyelin, and free arachidonic acid in Sham-treated wild-type mice

Using ESI-MS/MS, characterization of the jejunal polar glycerophospholipids, sphingomyelin, and free arachidonic acid (AA) of Sham-treated wild-type (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) classes, are the major phospholipids of the jejunum making up 56 mol% and 18 mol% respectively of the classes analyzed in C57Bl/6 wild-type mice (Fig. 1A). Phosphatidylinositol (PI), sphingomyelin (SM), and phosphatidylserine (PS) represented 8, 10, and 6 mol% respectively, while phosphatidic acid (PA), phosphatidylglycerol (PG), monoacyl PG (lysoPG) and free AA were present in lesser amounts (Fig. 1A).

The fatty acyl compositions of the various jejunal phospholipid classes were different (Fig. 1B). In the PC class, species containing a total of 34 and 36 fatty acyl carbons were most abundant, while 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 AA, which is also known as 20:4. 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).

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

Rag-1$^{-/-}$ mice sustain significantly less IR-induced mesenteric injury than C57Bl/6 wild-type mice at 2 h post ischemia [15]. To verify this finding and examine the extent of injury at earlier time points of reperfusion, Rag-1$^{-/-}$ mice and wild-type 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 wild-type 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). Wild-type mice sustained significant damage compared to the Sham-treated mice by 15 min reperfusion (Fig. 2A, B). The damage increased slightly by 2 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 wild-type Ab to Rag-1$^{-/-}$ mice results in significantly increased intestinal damage by 15 min post ischemia (Fig. 2A). The data indicate that antibody-mediated intestinal damage occurs within 15 min after reperfusion begins in wild-type mice. The damage was further increased by 2 h post ischemia (Fig. 2A, F).

3.3 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 in the IR group than in the Sham group at 30 min of reperfusion after ischemia (Fig. 3A). LysoPG also rose over time with significant increases in the IR group

compared to the Sham group at 30 and 120 min post-ischemia (Fig. 3A). PC levels decreased upon reperfusion, with significant differences in the IR compared to the 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 5.8 ± 0.43 (SEM) mol% compared to 2.6 ± 0.53 (SEM) 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 AA levels were increased by IR treatment at 15 min post ischemia (Fig. 3C and 4A). By 30 min reperfusion, free AA levels in Sham-treated wild-type mice were 1.5 mol%, while free AA levels of IR- treated wild-type mice were 4.6 mol%. The elevated free AA was maintained at 120 min post ischemia. Taken together, the 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.

3.4 Jejunal lipid composition is similar between Rag-1 $^{-/-}$ mice and wild-type mice during mesenteric reperfusion

Rag-1$^{-/-}$ mice are naturally resistant to IR-induced damage. Examination of total jejunal lipid amounts between mouse strains revealed no significant differences (Table 1). Additionally, comparison of intestinal lipid class composition of Sham-treated mice (Table 2) showed no significant differences between wild-type and Rag-1$^{-/-}$ mice. Acyl composition within classes was also similar between wild-type and Rag-1$^{-/-}$ Sham-treated mice (Table 3). Because Rag-1$^{-/-}$ mice sustain less IR-induced intestinal damage as compared to wild-type mice, we tested the hypothesis that changes in lipid composition in response to IR would differ between strains. Results indicated that total lipid amount and composition from mid-jejunal samples collected at 15, 30 and 120 min post ischemia did not display differences between the strains of mice at any

time of reperfusion examined (Table 1 and Fig. 5). The major change occurring in response to IR in the measured phospholipids of the Rag-$1^{-/-}$ mice, as in the wild-type mice, was the production of lysoPC species (Fig. 5). As in wild-type (C57Bl/6) mice, free AA levels increased in response to IR in the Rag-$1^{-/-}$ mice (Fig. 5), with Rag-$1^{-/-}$ free AA levels at 120 min after reperfusion at 1.5 mol% in Sham-treated and 4.5 mol% in the IR group.

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

The formation of PGE_2 requires multiple enzymatic activities, including an acyl hydrolase, phospholipase A_2 (PLA_2) which releases free AA. Subsequently, 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 wild-type mice after 2 h reperfusion [4, 12, 28]. Previous studies, using inhibitors of Cox-2, demonstrated that PGE_2 is required for IR-induced inflammation and tissue damage in wild-type mice after 2 h reperfusion [29]. Therefore, we quantified intestinal PGE_2 production in wild-type and Rag-$1^{-/-}$ mice at 2 h post ischemia. As in previous studies, we observed that wild-type 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 wild-type 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 wild-type and antibody-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 wild-type and Rag-$1^{-/-}$ mice. Cox-2 expression was rapidly increased in

response to IR in wild-type 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, Cox2 mRNA expression increased in Rag-$1^{-/-}$ mice which were pretreated with Ab purified from wild-type mice. These data suggest that Ab have a role in Cox-2 activation.

4. 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 wild-type 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 wild-type 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 wild-type 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 wild-type 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 [16, 30]. Hicks *et al* found the 16:0-18:2 was a prominent PC species in rat intestine [16]. These data are consistent 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 [18]. These masses correlate with the $[M + H]^+$ adducts of the predominant 16:0 and 18:0 lysoPC species in Sham-treated animals (Fig.4). This is consistent with formation of lysoPCs by a lipase acting at the sn2 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 [30]. 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 [17]. Similarly, Braun *et al* recently showed that PC levels were significantly lower than normal in ulcerative colitis patients but not in patients with Crohn's disease [18]. Correlating with this finding, the lysoPC to PC ratio also increased [18]. The present study showed that IR increased lysoPC with a concomitant decrease in PC (Fig. 3A, B). Others have found that PLA_2 activity and the subsequent lysoPC:PC ratio increased in response to IR, although these studies involved 2 h ischemia and 5 min reperfusion [31]. In addition, we found the lysoPC increase was specifically in the 16 and 18 carbon molecular species (Fig. 4). Lysophosphosphatidylcholine tends to form non-bilayer (micelle) structures and can destabilize lipid bilayers. [32]. Specifically, during intestinal IR lysoPC contributes to intestinal permeability [33]. 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 [35]. 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 or PLA2 inhibitors [29, 36, 37]. 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 wild-type mice [15], 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 [38]. In addition, recent studies indicate that, in the absence of the LPS receptor, TLR4 significantly attenuated intestinal damage and Cox-2 expression [29]. 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 quantities of lysoPC similar to those of wild-type 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 [39]. In vitro studies also indicate that calcium independent PLA_2 induces damage in cardiomyocytes following simulated IR [40, 41]. 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 2-8 h post reperfusion [42, 43] and may play a role in intestinal damage at 3 h post reperfusion [37]. 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 [33]. 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 wild-type 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 wild-type mice. With the injection of wild-type antibodies into

Rag-$1^{-/-}$ mice, Cox-2 mRNA expression and PGE_2 concentrations were restored to levels similar to those observed in wild-type mice. Thus, antibodies play a role in inducing intestinal damage. Identification of the specific lipid antigens may provide improved therapeutics for the prevention of tissue damage associated with ischemia.

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Figure Legends

Figure 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 jejunal glycerophospholipids, sphingomyelin and free arachidonic acid and (B) fatty acyl compositions of the major phospholipid classes. Each bar represents the mean ± SEM of 10 mice.

Figure 2: Rag-1$^{-/-}$ mice sustain significantly less IR-induced intestinal damage than wild-type 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 *Materials and 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 wild-type 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 wild-type.

Figure 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 10 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 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 5-10 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 $p < 0.05$.

Figure 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 4 to 5 mice per group. None of the values are significantly different than the others at $p < 0.05$.

Figure 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 4 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.

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

Total lipid nmol/mg protein (mean ± SEM)[1]	Sham	IR 15	IR 30	IR 120
B6	241 ± 85	133 ± 13	105 ± 23	152 ± 36
Rag	110 ± 13	81 ± 17	94 ± 14	129 ± 4

[1] Each value is the nmol total lipids per mg tissue analyzed and is a mean of 4 to 8 mice per time point. None of the values are significantly different than the others.

Table 2. C57Bl/6 and Rag-1$^{-/-}$ mice have similar intestinal lipid class compositions.

Lipid class[1]	B6 (mol% ± SEM)[2]	Rag (mol% ± SEM) [2]
Lyso PC	2.65 ± 0.53	2.20 ± 035
PC	52.92 ± 0.72	51.90 ± 0.80
ePC	2.50 ± 0.09	2.48 ± 0.09
SM	7.8 ± 0.43	7.98 ± 0.23
Lyso PE	0.34 ± 0.04	0.25 ± 0.04
PE	18.16 ± 0.52	17.69 ± 0.29
ePE	3.27 ± 0.21	3.65 ± 0.27
PI	5.2 ± 0.19	6.00 ± 0.30
PS	4.45 ± 0.42	4.22 ± 0.40
PA	0.70 ± 0.12	0.60 ± 0.10
Lyso PG	0.01 ± 0.00	0.02 ± 0.00
PG	1.36 ± 0.13	1.40 ± 0.16
Free AA	1.50 ± 0.13	1.46 ± 0.22

[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.

[2]The mean ± SEM of 5 to 9 mice.

Table 3. C57B1/6 and Rag-1$^{-/-}$ mice maintain similar intestinal acyl chains within the lipid classes[1].

	PC		PE		PI		PS	
Acyl chains[2]	**B6[3]**	**Rag[3]**	**B6**	**Rag**	**B6**	**Rag**	**B6**	**Rag**
32:0	1.22 ± 0.05	1.20 ± 0.03	0.01 ± 0.00	0.01 ± 0.00	ND[4]	ND	0.00 ± 0.00	0.00 ± 0.00
34:2	15.32 ± 0.96	13.97 ± 0.78	0.88 ± 0.06	0.76 ± 0.06	0.42 ± 0.02	0.49 ± 0.04	0.03 ± 0.00	0.02 ± 0.00
34:1	2.39 ± 0.22	2.18 ± 0.15	0.18 ± 0.01	0.16 ± 0.01	0.13 ± 0.01	0.17 ± 0.02	0.03 ± 0.00	0.02 ± 0.00
34:0	0.27 ± 0.02	0.25 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	ND	ND	0.01 ± 0.00	0.01 ± 0.00
36:5	0.99 ± 0.08	1.00 ± 0.07	0.15 ± 0.01	0.13 ± 0.01	0.04 ± 0.00	0.06 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
36:4	5.05 ± 0.16	5.26 ± 0.11	0.64 ± 0.02	0.60 ± 0.01	0.49 ± 0.04	0.62 ± 0.04	0.01 ± 0.00	0.01 ± 0.00
36:3	3.63 ± 0.11	3.50 ± 0.08	0.83 ± 0.03	0.72 ± 0.03	0.23 ± 0.01	0.26 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
36:2	11.92 ± 0.34	12.86 ± 0.50	4.46 ± 0.22	4.17 ± 0.18	0.67 ± 0.03	0.70 ± 0.04	1.31 ± 0.10	1.29 ± 0.10
36:1	0.94 ± 0.08	0.94 ± 0.08	0.41 ± 0.02	0.44 ± 0.01	0.07 ± 0.00	0.07 ± 0.00	0.54 ± 0.07	0.51 ± 0.08
38:6	1.48 ± 0.05	1.48 ± 0.03	0.96 ± 0.03	0.87 ± 0.02	0.09 ± 0.01	0.10 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
38:5	1.52 ± 0.05	1.69 ± 0.03	1.06 ± 0.04	1.09 ± 0.04	0.28 ± 0.01	0.32 ± 0.02	0.05 ± 0.00	0.06 ± 0.00
38:4	2.94 ± 0.09	3.27 ± 0.09	3.71 ± 0.11	3.95 ± 0.10	2.36 ± 0.08	2.69 ± 0.12	0.44 ± 0.04	0.47 ± 0.04
38:3	0.72 ± 0.04	0.77 ± 0.04	0.49 ± 0.04	0.49 ± 0.03	0.25 ± 0.01	0.28 ± 0.02	0.31 ± 0.04	0.28 ± 0.04
40:7	0.25 ± 0.01	0.24 ± 0.02	0.58 ± 0.02	0.52 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
40:6	0.98 ± 0.05	1.03 ± 0.05	2.35 ± 0.12	2.27 ± 0.07	0.14 ± 0.01	0.14 ± 0.01	0.95 ± 0.11	0.84 ± 0.09
40:5	0.18 ± 0.01	0.20 ± 0.01	0.34 ± 0.02	0.33 ± 0.01	0.04 ± 0.00	0.05 ± 0.00	0.21 ± 0.02	0.19 ± 0.01

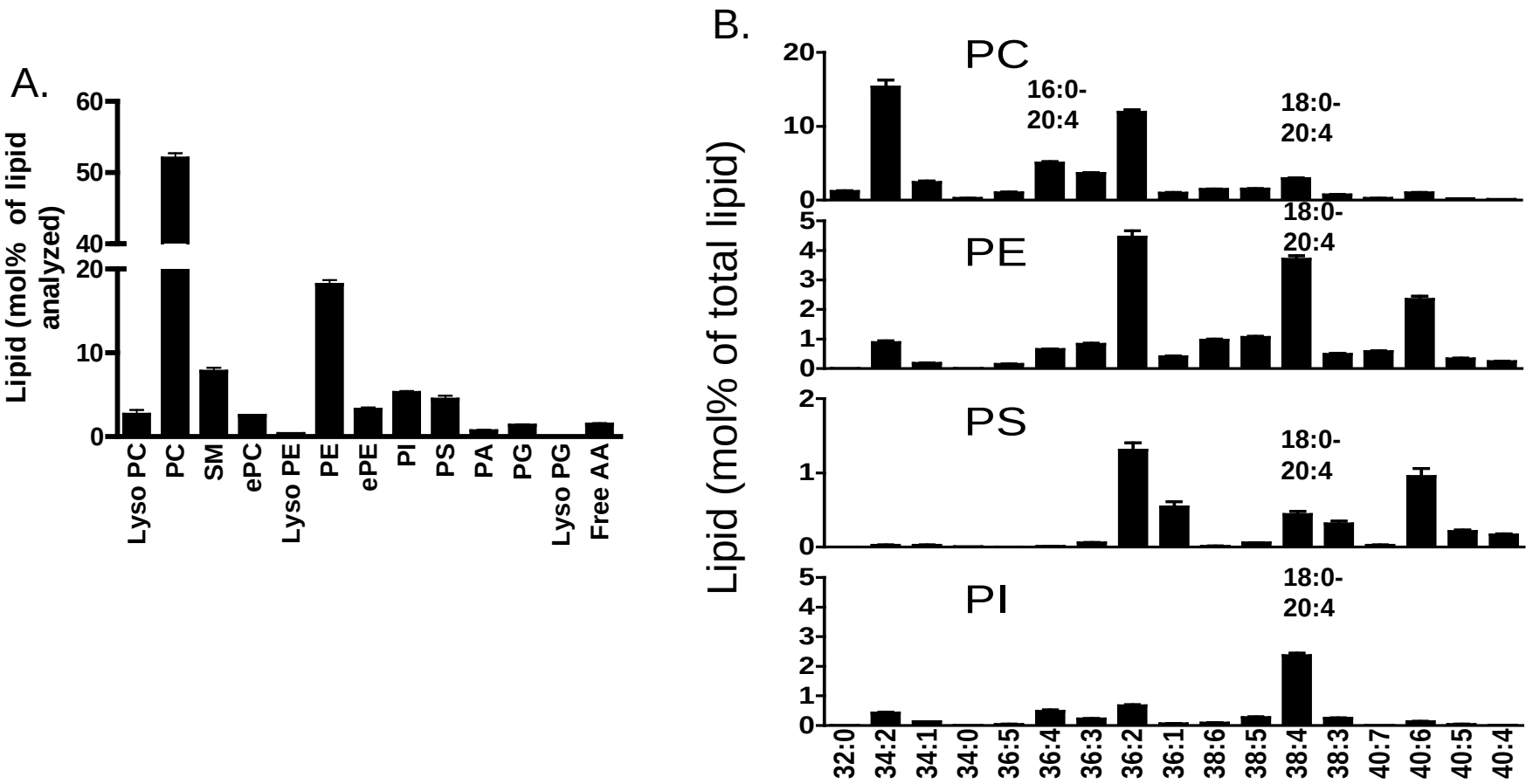
[1].Lipids were extracted from mid-jejunal intestinal sections from C57B1/6 (B6) and Rag-1$^{-/-}$ (Rag) Sham treated mice. ESI-MS/MS analysis determined mol% of the fatty acid chains for the major lipid classes.

[2] Total acyl carbons : total double bonds

[3]The mol% mean ± SEM of 5 to 9 mice.

[4] Not determined

Figure 1


A.
Lipid (mol% of lipid analyzed)
60
50
40
20
10
0
Lyso PC
PC
SM
ePC
Lyso PE
PE
ePE
PI
PS
PA
PG
Lyso PG
Free AA
B.
Lipid (mol% of total lipid)
PC
16:0-20:4
18:0-20:4
PE
18:0-20:4
PS
18:0-20:4
PI
18:0-20:4
32:0
34:2
34:1
34:0
36:5
36:4
36:3
36:2
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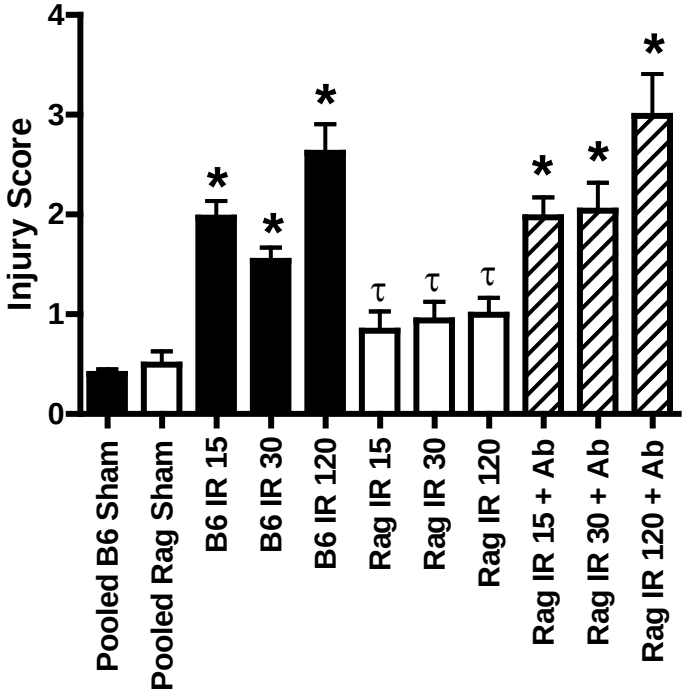
38:6
38:5
38:4
38:3
40:7
40:6
40:5
40:4

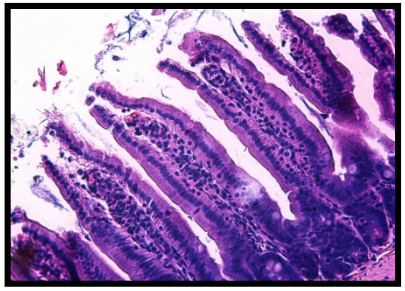
Figure 2

A.



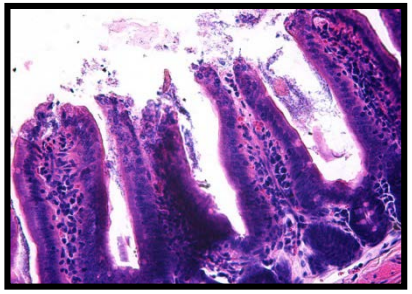
B.

B6 IR 15 min



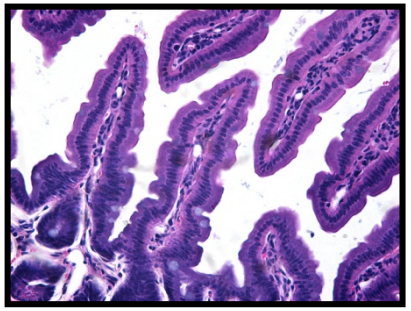
C.

B6 IR 120 min



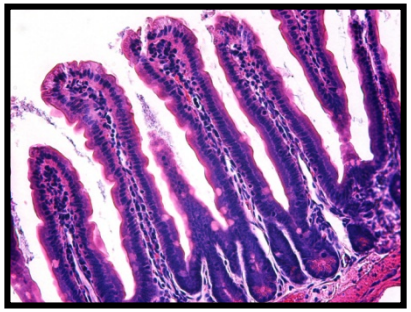
D.

Rag IR 15 min



E.

Rag IR 120 min



F.

Rag IR 120 min + B6 Ab

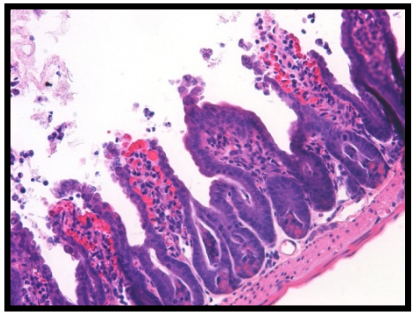
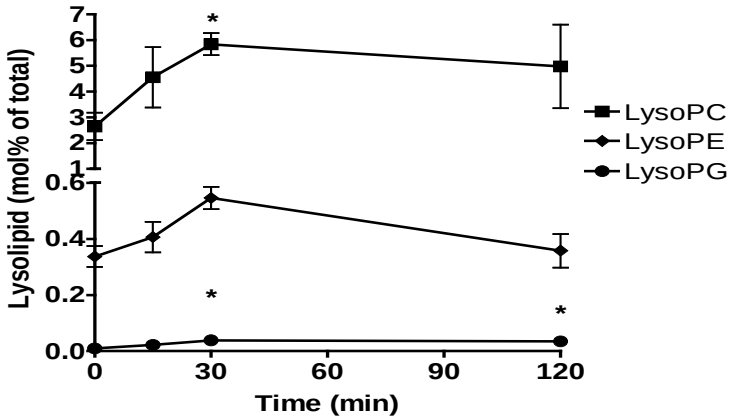
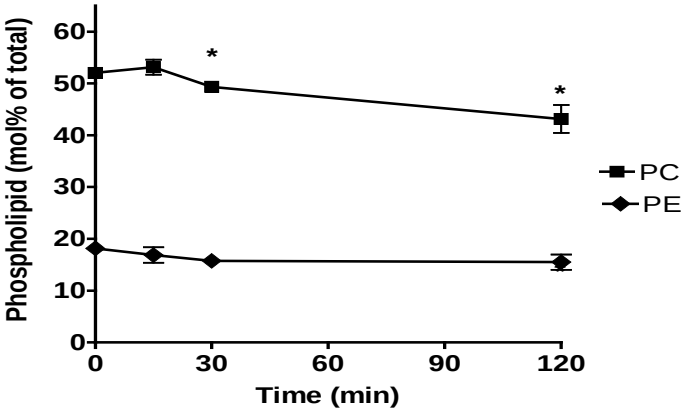


Figure 3

A.


Lysolipid (mol% of total)
7
6
5
4
3
2
1
0.6
0.4
0.2
0.0
*
*
*
0
30
60
90
120
Time (min)
LysoPC
LysoPE
LysoPG

B.


Phospholipid (mol% of total)
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50
40
30
20
10
0
*
*
0
30
60
90
120
Time (min)
PC
PE

C.

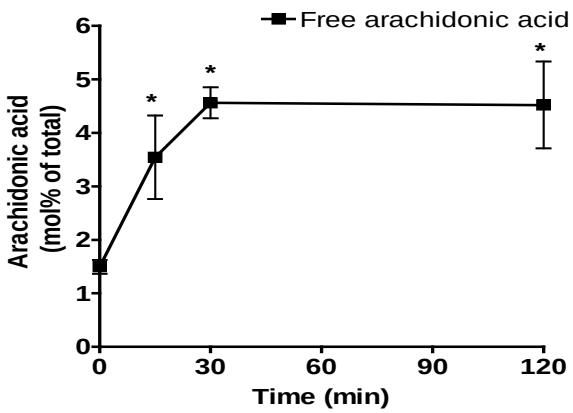

Free arachidonic acid
Arachidonic acid (mol% of total)
6
5
4
3
2
1
0
*
*
*
0
30
60
90
120
Time (min)

Figure 4

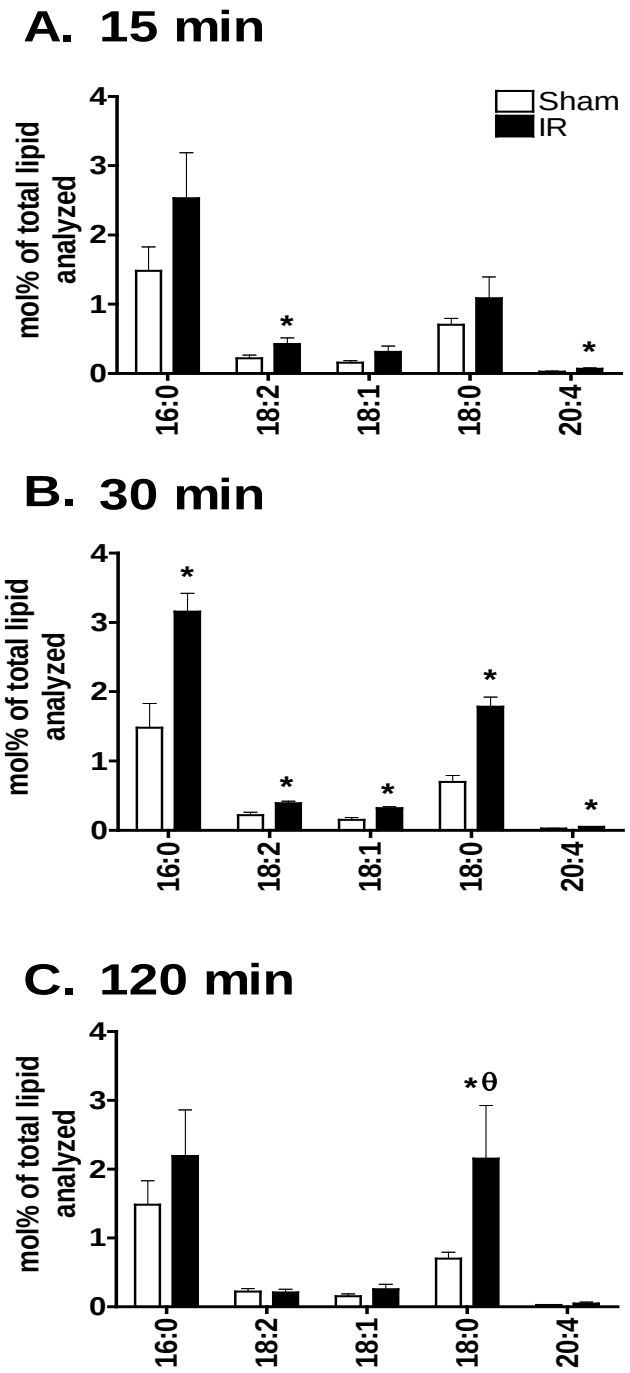

A. 15 min
Sham
IR
mol% of total lipid analyzed
0
1
2
3
4
*
*
16:0
18:2
18:1
18:0
20:4
B. 30 min
mol% of total lipid analyzed
0
1
2
3
4
*
*
*
*
*
16:0
18:2
18:1
18:0
20:4
C. 120 min
mol% of total lipid analyzed
0
1
2
3
4
*θ
16:0
18:2
18:1
18:0
20:4

Figure 5

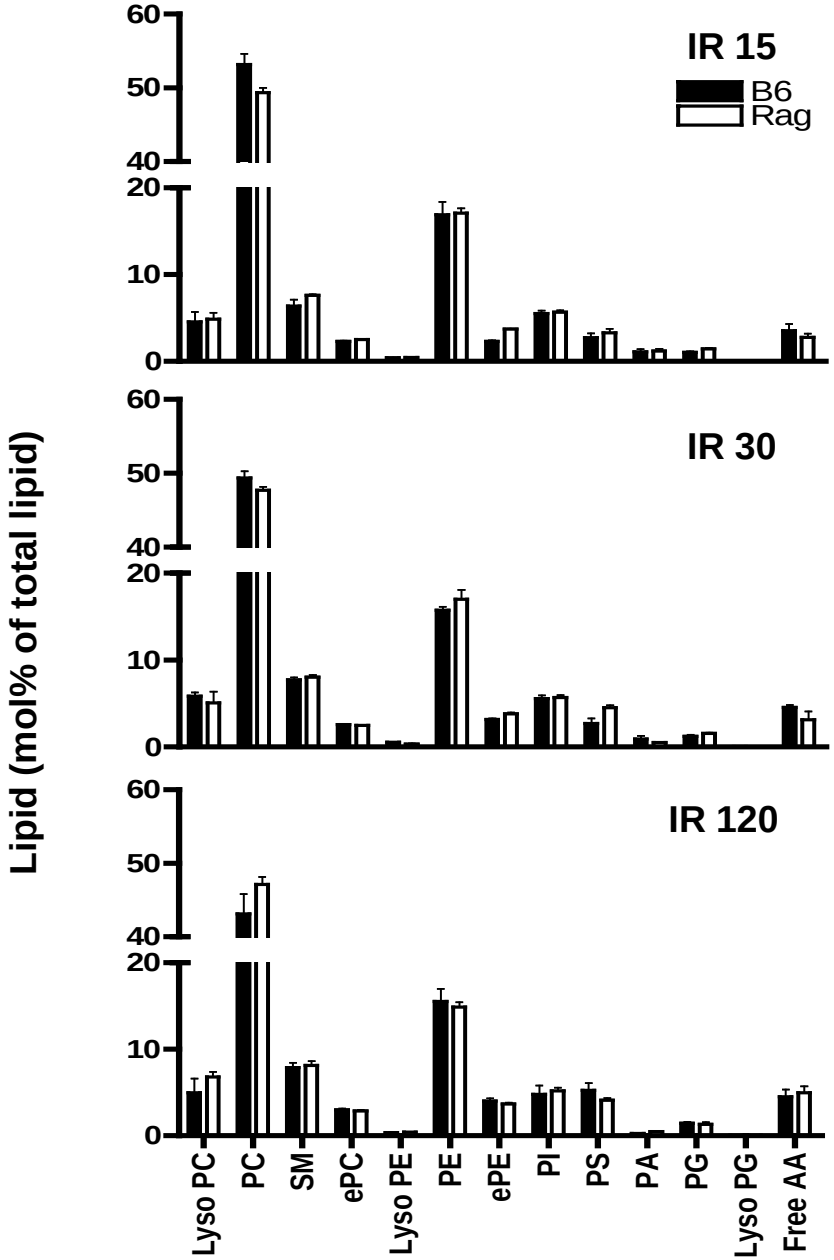
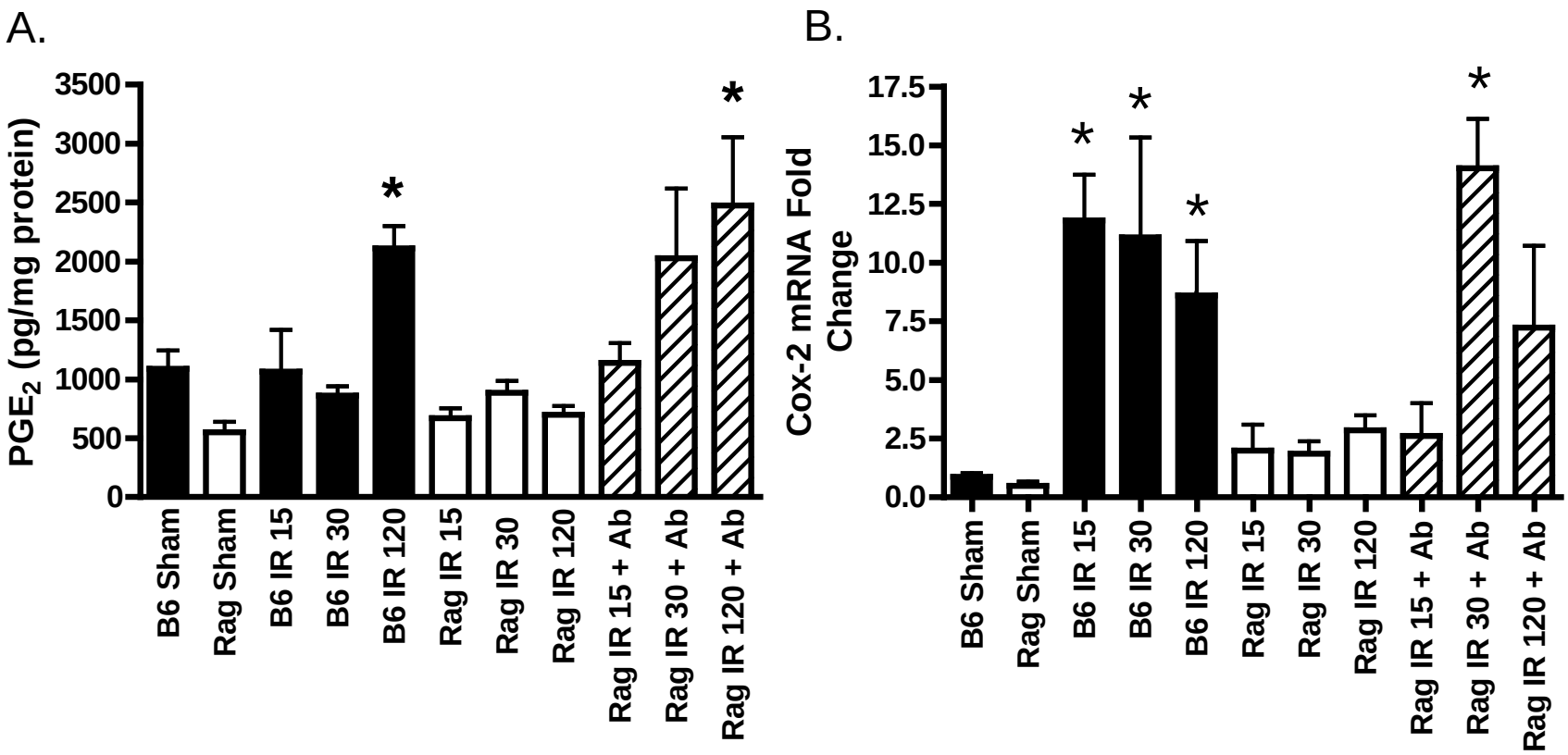

IR 15
B6
Rag
IR 30
IR 120
Lipid (mol% of total lipid)
60
50
40
20
10
0
Lyso PC
PC
SM
ePC
Lyso PE
PE
ePE
PI
PS
PA
PG
Lyso PG
Free AA

Figure 6


A.
PGE2 (pg/mg protein)
3500
3000
2500
2000
1500
1000
500
0
*
*
B6 Sham
Rag Sham
B6 IR 15
B6 IR 30
B6 IR 120
Rag IR 15
Rag IR 30
Rag IR 120
Rag IR 15 + Ab
Rag IR 30 + Ab
Rag IR 120 + Ab
B.
Cox-2 mRNA Fold Change
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
*
*
*
B6 Sham
Rag Sham
B6 IR 15
B6 IR 30
B6 IR 120
Rag IR 15
Rag IR 30
Rag IR 120
Rag IR 15 + Ab
Rag IR 30 + Ab
Rag IR 120 + Ab