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Eicosanoid production varies by sex in mesenteric ischemia reperfusion injury

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Abstract

Intestinal ischemia/reperfusion (I/R)-induced injury is an inflammatory response with significant morbidity and mortality. The early inflammatory response includes neutrophil infiltration. However, the majority of rodent studies utilize male mice despite a sexual dimorphism in intestinal I/R-related diseases. We hypothesized that sex may alter inflammation by changing neutrophil infiltration and eicosanoid production. To test this hypothesis, male and female C57Bl/6 mice were subjected to sham treatment or 30 min intestinal ischemia followed by a time course of reperfusion. We demonstrate that compared to male mice, females sustain significantly less intestinal I/R-induced tissue damage and significant LTB₄. male mice release PGE₂. Finally, treatment with a COX-2 specific inhibitor, NS-398, attenuated I/R-induced injury, total peroxidase level, and PGE₂ production in males, but not in similarly treated female mice. Thus, I/R-induced eicosanoid production and neutrophil infiltration varies between sexes suggesting that distinct therapeutic intervention may be needed in clinical ischemic diseases.

Keywords

Prostaglandins; leukotrienes; male; female; inflammation

1. Introduction

Ischemia-reperfusion (I/R) injury poses a serious clinical therapeutic problem during multiple conditions including surgery, myocardial infarction and stroke, gastrointestinal dysfunction, cerebral and hepatic diseases, systemic inflammatory response syndrome, and organ transplantation. Compared to other internal organs, the intestine appears exquisitely sensitive to I/R injury [1]. Ischemia induces local tissue injury, which is significantly

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SDF provided SDF and MMW participated in study conception and design, data analysis, and interpretation of the data. MMW and JMR acquired data. All authors revised and approved the final manuscript.

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Data availability statement

The data sets used and/or analyzed during the current research are available from the corresponding author on reasonable request.

Declaration of competing interest

None of the authors has any potential financial conflict of interest related to this manuscript.

magnified during reperfusion. The local intestinal inflammatory response may progress to a systemic inflammatory response in remote organs including the lungs. Reperfusion-induced inflammation includes complement, cytokines, and cellular influx by increased adhesion molecules generated both locally and systemically in the blood [2–4]. In mice, intestinal ischemia also induces neutrophil infiltration and secretion of cytokines and eicosanoids leading local mucosal injury [5–11].

Numerous clinical studies of I/R-related conditions support the importance of sex-associated differences [12–14]. After myocardial infarction, women generally have a higher in-hospital mortality than men and tend to develop heart failure [15]. The operative mortality of women after coronary artery bypass grafting surgery is 2–3 times higher than men [16]. Although the prevalence of ischemic heart disease is 1.8% higher in men than in women [17], the hospital discharges for cardiovascular diseases of men were higher than women during the same time period in the U.S [18]. Together, these data suggest that sex may be an independent factor and affect the clinical treatment. Quantitative trait locus expression analysis of patients with ischemia also indicated that genomic sex differences affect myocardial gene expression and cell type enrichment analysis demonstrated sex-bias in different proportions of specific cell types [12]. Finally, females present distinct symptomology compared to males in response to myocardial I/R, also suggesting sex as an independent risk factor for I/R [19].

Despite the systematic differences between women and men in the I/R injuries, women remain underrepresented in clinical research studies [20–22]. In addition, the mouse model of intestinal I/R was established primarily using male mice, as female mice show decreased injury compared to male mice in multiple forms of I/R including, hemorrhage [23], myocardial [24], kidney [25], and intestine [26], and sepsis [25]. Although I/R injury in female mice is attenuated or delayed, the specific protective mechanisms were not defined [27–29]. Recent studies demonstrated that female sex hormones could reduce pro-inflammatory cytokines production in inflammatory bowel disease [30], reduce inflammation in heart and kidney I/R injuries [31, 32] and preserve endothelial integrity [33].

Arachidonic acid, cleaved from membrane phospholipids by activated phospholipase A2 during ischemia, is metabolized into prostaglandins E_2 (PGE_2) by cyclooxygenase-2 (COX-2) [34–37]. COX-2 is expressed at low levels constitutively and is strongly expressed induced by multiple stimuli [38]. In addition, COX-2 plays a predominant role in prostaglandins (PGs) production in the pathophysiological processes, such as ischemic injury [39–41]. A5-LOX also metabolizes arachidonic acid into leukotriene B_4 (LTB_4). Additional literature indicates that LTB_4 recruits neutrophils in response to I/R by chemotaxis [11, 42].

Understanding the molecular mechanisms of female attenuated or delayed I/R injury will provide insight into the sexual dimorphism of intestinal I/R and may allow the development of sex-specific therapies to reduce the injuries. To test the hypothesis that male and female I/R-induced damage is mechanistically different, we examined the kinetics of intestinal I/R-induced mediators and pathology in C57BL/6 female and male mice subjected to 30 min

ischemia followed by 15 min, 30 min, 60 min, and 120 min reperfusion. Our data indicate that sex differences alter the mechanisms of intestinal I/R injury. Specifically, males produce PGE₂ via the COX-2 pathways in response to I/R injury, while under similar conditions, female mice use A5-LOX to produce LTB₄.

2. Materials and methods

2.1. Mice

C57Bl/6J mice were purchased from Jackson Laboratory and bred in the Division of Biology at Kansas State University, Manhattan, KS. All mice were allowed free access to food and water, and maintained under 12 h light/dark cycles in specific pathogen free conditions (*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). All studies were approved by the Institutional Animal Care and Use Committee and conducted in compliance with the Animal Welfare Act and other federal statutes and regulations concerning animals and experiments involving animals.

2.2. Ischemia and Reperfusion Procedure

Animals were subjected to I/R, as previously described [39]. Briefly, isoflurane (2–3%) anesthetized mice were subjected to a midline laparotomy and covered with a moistened surgical gauze to prevent desiccation. After a 30 min equilibration period, the superior mesenteric artery was identified, isolated, and a small vascular clamp (Roboz Surgical Instruments) applied to induce ischemia. Ischemia was confirmed by the blanching of the intestine and the absence of pulsations distal to the clamp. After 30 min of ischemia, the clamp was removed and the intestines were reperfused for 15, 30, 60, or 120 min. Reperfusion was confirmed by the return of pinkish color to the intestine. All mice received buprenorphine (0.075 mg/kg) for pain. Sham treated animals underwent the same procedure with manipulation of the intestines but without clamping. After reperfusion, the mice were deeply anesthetized, bled for serum collection and euthanized. Then beginning approximately 10 cm distal to the gastroduodenal junction, five sections of the mid-jejunum (2 cm each) were collected for histological and other analyses. All procedures were performed with all animals breathing spontaneously and anesthetized mice were kept on a 37°C water-circulating heating pad to maintain body temperature. In some studies, 17 h prior to surgery mice were injected intraperitoneally with NS-398 (0.25 mg/mouse), a cyclooxygenase-2-specific inhibitor (Sigma-Aldrich) prepared as indicated in the company's instructions. A second dose of NS-398 was injected intravenously 3 h prior to I/R surgery.

2.3. Histology and Immunohistochemistry

Immediately after removal, a 2 cm mid-jejunum tissue section was fixed in 10% buffered formalin phosphate, embedded in paraffin, cut transversely (8 µm), and H&E stained. The mucosal injury score (SMI) was graded based on a six-tiered scale modified from Chiu et al [43, 44] Briefly, the average intestinal mucosal injury was calculated by the average of two well trained, blinded observers. Each observer graded 90–150 villi from 0–6. Normal villi were assigned a score of zero; a score of 1 was assigned when the villi tip was distorted; villi

with Guggenheims' spaces were scored 2; a score of 3 was assigned to villi with small regions of disruption in the epithelial cells; villi with large regions of exposed but intact lamina propria with epithelial sloughing were scored 4; villi with extruding lamina propria were assigned to a score of 5; a score of 6 was assigned when the villi displayed hemorrhage or were denuded. Photomicrographs were obtained from H&E stained slides by utilizing a 40X, 0.5 Plan Fluor objective on Nikon 80i microscope. Images were acquired by using a Nikon DS-5M camera with the DS-L2 software at room temperature (Nikon). Additional 2 cm intestinal sections were immediately snap frozen in optimal cutting temperature (O.C.T) freezing medium and serial 8 μ m transverse sections were placed on the slides for future immunohistochemistry, as described previously [45]. Briefly, slides were fixed using acetone, and followed by incubating with 10% sera in phosphate buffered saline (PBS) for 30 min to block nonspecific binding. After PBS washes, the intestinal sections were incubated with primary antibodies in the dark for 1h at room temperature or ON at 4 °C. The F4/80 and myeloperoxidase (MPO) deposition on the tissue was detected by staining with a rat-anti-mouse anti-F4/80 (Biolegend) Alexa Fluor 594 or purified rabbit-anti-MPO (Spring Bioscience) followed by a Texas-red conjugated donkey-anti-rat IgG secondary antibody (Jackson ImmunoResearch). All experiments contained serial sections stained with appropriate isotype control antibodies. Each slide was mounted with ProLong Gold (Invitrogen). Four to eight images per section were acquired by a blinded observer at room temperature by utilizing a Nikon eclipse 80i microscope equipped with a CoolSnap CF camera (Photometrics) and analyzed by using Metavue software (Molecular Devices). Densitometry used ImageJ (<https://imagej.nih.gov/ij/>)

2.4. Ex vivo Eicosanoid, Cytokine, and Peroxidase Determination

The ex vivo generation of eicosanoids and cytokines in the small intestine tissue section was detected using a modified method previously described [46]. Immediately after removal, a 2 cm intestinal section was minced, and washed in cold Tyrode buffer and resuspended in 37°C freshly oxygenated Tyrode buffer (Sigma-Aldrich). After incubation at 37°C for 20 min, the supernatants were collected. Levels of LTB₄ and PGE₂ were determined using enzyme immunoassay kits (Cayman Chemical). Analysis of cytokines present in the same intestinal supernatants was determined by using a Milliplex MAP immunoassay kit (Millipore) that was analyzed on a Milliplex Analyzer (Millipore). Supernatants generated for eicosanoids and cytokines were also used to determine peroxidase activity by measuring oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) as described previously[11]. Protein content was determined using the bicinchoninic acid (Pierce) assay adapted for use with microtiter plates. All eicosanoid and cytokines concentrations were standardized per mg protein per 20 min.

2.5. MPO activity and C5a level determination by ELISA

Blood was collected from mice after intestinal I/R, allowed to clot on ice at least 30 min and whole serum collected by centrifugation for 20 min at approximately 1000×g. Serum MPO activity and serum C5a level were determined based on the sandwich ELISA principle. The mouse MPO and C5a capture antibody, detection antibody, standard, and Streptavidin-HRP were purchased from R&D SYSTEMS.

2.6. Quantitative Real-time PCR

One 2 cm jejunal section was snap froze in liquid nitrogen and stored at -80°C until used for RNA analysis. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. RNA quantity and quality was determined by using the Nano drop (Thermo Fisher Scientific). Total RNA ($1\text{ }\mu\text{g}$) was reverse transcribed into cDNA by using aScript first strand cDNA synthesis kit (Quanta Biosciences). Quantitative real-time PCR analysis was performed in $25\text{ }\mu\text{l}$ total volume using an Applied Biosciences StepOnePlus thermocyclers (Thermo Fisher) with Perfecta SYBR Green Fastmix reagent (Quanta Biosciences) using the following protocol: 3 min at 95°C ; 50 cycles of 10 sec at 95°C , 20 sec at T_m (Table 1), 10 sec at 72°C ; melt curve start at 60°C , increasing by 0.5°C up to 95°C . After amplification, relative gene expression was normalized to 18s rRNA values, and $\Delta\Delta\text{Ct}$ fold change relative to sham-treated mice was determined.

2.7. Statistical Analysis

Data are presented as mean $\pm$ the standard error of the mean (SEM) and were compared by unpaired t-test with Mann-Whitney *post hoc* analysis (Graph Pad/Instant Software Inc). Differences ($P < 0.05$) between groups were considered significant.

3. Results

3.1. Higher intestinal I/R-induced tissue damage and inflammation in male mice

Previous studies indicated that the prevalence of ischemic heart disease is higher in men than in women with 8.3% of cardiovascular deaths in men vs. 6.1% in women in 2008 [47], and intestinal I/R-induced injury in female mice is attenuated or delayed in multiple forms of I/R and hemorrhage [28, 29, 48]. It is likely the time course of the I/R damage differs between sexes. To test this hypothesis, we subjected both male and female wild-type, C57Bl/6 mice to 30 min ischemia, followed by 15 min, 30 min, 60 min, and 120 min reperfusion, and evaluated intestinal epithelial damage. Sham treatment was not significantly different at various time points of the same gender of mice. Thus, the scores of damage and subsequent analyses were pooled by gender for sham treatment only. As expected, after I/R, male mice sustained significant tissue damage (Fig. 1A). Although female mice also sustained significant intestinal damage, the damage was significantly lower than that sustained by male mice at a similar time points (Fig. 1A). Thus, these data indicate that intestinal I/R-induced significant tissue injury in both sexes, but male mice sustained significantly higher mucosal tissue damage than females throughout the time course of intestinal I/R.

Intestinal I/R-induced tissue damage is accompanied by inflammation with significantly elevated levels of total intestinal peroxidase [11], myeloperoxidase [49] and cytokines [39]. Myeloperoxidase (MPO), one of the most abundant enzymes released by activated neutrophils, is an inflammatory molecule common ischemic tissue [50]. We hypothesized that total tissue peroxidase and specifically myeloperoxidase would be increased after intestinal I/R, but the dynamic change may differ between male and female mice. As expected in a time course of I/R, total peroxidase in males was significantly increased compared to sham treated animals (Fig. 1B). In contrast, the total tissue peroxidase was not significantly increased at any time point examined in female mice (Fig 1B). After I/R, both

males and females had significantly elevated serum MPO level compared with their corresponding shams (Fig. 1C). In addition, serum MPO level in males was slightly higher than it was in females during all I/R time course, with significance at 15 min, 30 min, and 120 min (Fig 1C). As complement plays a significant role in intestinal I/R tissue damage [51], we examined neutrophil chemotactic C5a as well. After I/R at 30 and 60 min, males had significantly decreased serum C5a level compared with their corresponding shams (Fig. 1D). In contrast, in female mice the serum C5a level did not increase during I/R compared to its corresponding sham treatment (Fig. 1D). Importantly, between males and females within the same treatment, males had significantly higher level of C5a. Together, the intestinal injury, total peroxidase and MPO and C5a indicate that compared to females, males have higher levels of tissue damage and inflammation during intestinal I/R, which begins earlier. These data suggest the inflammatory response to intestinal I/R differs between males and females.

3.2. Male mice recruit more neutrophils and macrophages during intestinal I/R

MPO and F4/80 identify infiltration of neutrophil and macrophage, respectively. To examine differences between neutrophil and macrophage infiltration in males and females after intestinal I/R, we performed immunohistochemistry to examine MPO and F4/80 expression. In addition, MPO and F4/80 staining was significantly increased in I/R treated intestines compared to sham treatment. Importantly, in response to I/R, MPO and F4/80 staining was significantly higher in male intestines compared to female intestines (Fig. 2A, 2B). Taken together, intestinal I/R induces neutrophil and macrophage infiltration in both sexes but I/R induces more neutrophil and macrophage infiltrates in males than females. Together these data indicate the inflammatory infiltrate differs between sexes.

As the cellular influx induces significant cytokine secretion, which further contributes to the inflammatory response to I/R, we examined ex vivo cytokine production (Fig. 3A–F). Intestines from both sexes secreted similar levels of IL-12p40 and p70, CXCL1 (KC) and TNF- α . However, female intestinal IL-6 and IL-10 secretion were significantly higher at 15 and 30 min post ischemia, respectively. The increased IL-10 early during reperfusion suggests that female intestines maintain an anti-inflammatory response longer compared to male intestines.

3.3. Female mice produce LTB₄ whereas male mice produce PGE₂

We previously demonstrated pivotal roles in intestinal I/R for the arachidonic acid metabolites, PGE₂ and LTB₄ [39]. LTB₄ induces neutrophil chemotaxis [52, 53] while COX-2 production of PGE₂ regulates hypoxia-inducible factor-1 α (HIF-1 α), a master regulator of oxygen homeostasis [54]. Thus, we performed RT-PCR to determine if expression of HIF-1 α , COX-2 and A5-LOX in the intestinal tissue from male and female mice differed (Fig. 4A–C). As indicated in Figure 4A, sham-treated intestines from females expressed increased HIF-1 α compared to those from males. In response to I/R, male intestines expressed significantly more HIF-1 α at 30 min I/R (Fig. 4A), while HIF-1 α expression changed little when I/R was compared to sham treatment in female intestines. These data suggest that female intestines may be more sensitive to handling than male intestines. Similar to HIF-1 α expression, males expressed significant levels of COX-2 at

early time points (15 and 30 min) I/R (Fig. 4B), while female expression of COX-2 increased at 60 min (Fig. 4B). Surprisingly, intestines from female mice expressed significantly increased levels of A5-LOX from 15min to 120 min I/R and the level of A5-LOX was significantly higher than males at 15 min I/R (Fig. 4C).

As demonstrated previously, intestinal I/R also increases the chemotactic eicosanoids, LTB₄ and PGE₂ in wild-type mice [11, 46]. To determine alteration of LTB₄ and PGE₂ production in all time points in male and female mice, we detected ex vivo generation of LTB₄ and PGE₂. Similar to previous studies, ex vivo intestinal sections from I/R-treated male mice released significantly more PGE₂ at 120 min I/R. PGE₂ production by female intestines after I/R was not elevated compared to female sham mice (Fig. 5A). In contrast, intestines from female mice consistently produced increased LTB₄ from 15 min I/R to 120 min I/R, while male mice maintained the same level of LTB₄ compared to male sham mice (Fig. 5B). Taken together, after intestinal I/R, arachidonic acid is converted to LTB₄ in female intestines whereas male intestines produce PGE₂.

3.4. Male but not female mice are protected with COX-2 inhibitor, NS-398

The dichotomous effect of mesenteric I/R-induced production of LTB₄ and PGE₂ raised the question of whether COX-2 plays different roles females and males in I/R-induced tissue damage and eicosanoids production. To explore the possibility that the inhibition of COX-2 would produce distinct protective effects in males and females during intestinal I/R, we treated both sexes of mice with a COX-2 specific inhibitor, NS-398, and evaluated injury, peroxidase and PGE₂ and LTB₄ production. As expected, treatment with NS-398 significantly reduced ischemic intestinal injury in males compared with non-treated males (Fig. 6A). As peroxidase is a critical catalytic step of COX-2 [55], treatment with NS-398 also decreased total peroxidase concentrations significantly in males after I/R compared to untreated I/R male mice (Fig. 6B). Peroxidase levels in females increased significantly after NS-398 treatment without changing the injury level (Fig. 6B). Importantly, after I/R NS-398 treatment decreased PGE₂ production in males, but did not alter production in females (Fig. 6C). NS-398 treatment did not affect LTB₄ production in either sexes after I/R (Fig. 6D). Taken together, NS-398 inhibits tissue damage, total peroxidase level, and PGE₂ production in male intestines after I/R, but not in intestines from females. These data demonstrate a sexual dimorphism in I/R-induced arachidonic acid metabolism.

4. Discussion

Differences in sex play a critical role in the outcome of an I/R event in both experimental and clinical studies. Males appear to sustain increased tissue damage but females sustain greater mortality and longer hospital stays after an ischemic insult [19, 56–58]. However, our understanding of sex-related time course and mechanisms of I/R-induced injury was unclear, largely due to the lack of experimental modeling. While males are larger and appear to sustain more damage in response to kidney, intestine and heart IR [25, 26, 59, 60], we demonstrate that sex also impacts intestinal I/R-induced tissue injury by changing neutrophil recruitment, and eicosanoid production.

Our data demonstrate that both male and female mice have significantly increased injury after 30 min ischemia followed by as little as 15 min reperfusion and was sustained through 120 min reperfusion. Previous studies indicate that females are more resistant to oxygen deprivation than males [61, 62]. In addition, increased tolerance of females to I/R damage was observed in rabbits, rats, mice, and dogs [59, 63–65]. In agreement with the above studies, males sustained increased histological damage compared to females throughout the reperfusion period.

Following I/R, intestines from female mice produced a marked increase of LTB₄ with little increase in PGE₂. In contrast, intestines from male mice produced little LTB₄ and significant amounts of PGE₂. This observation is consistent with prior studies showing in vivo sex differences in leukotriene biosynthesis with males producing more PGE₂ [66] and females producing more LTB₄ [67, 68]. Furthermore, previous studies found significant HIF-1 differences between male and female cardiac responses to chronic hypoxia [69] similar to the current data in response to intestinal I/R (Fig. 4A). Specifically, similar hypoxic conditions induced significantly higher HIF-1 α expression in male mouse intestines compared to intestines from female mice (Fig. 4A). Our data also indicate that during intestinal I/R, female intestines use A5-LOX to upregulate LTB₄ production, rather than inducing COX-2 and PGE₂ production. This suggests that attenuating inflammation during I/R in females may require a LTB₄ inhibitor rather than PGE₂ inhibitor. In a trauma model using male mice, the COX-2 inhibiting drug, NS-398, decreased PGE₂ production to approximate levels found in females [66]. Another study found the A5-LOX product (5 lipoxigenase) is critical to increasing cutaneous wound healing of female mice [70]. It is possible that LTB₄ has a similar role in intestinal I/R. In the trauma study, PGE₂ production remained unchanged in females treated with or without NS-398 [66]. Importantly, our current studies correlate with previous studies that demonstrated that NS-398 attenuated PGE₂ production and intestinal injury in males in response to I/R [39, 71]. However, our current studies also show that peroxidase levels in female intestines increased significantly after NS-398 treatment suggesting a compensatory effect may occur. Although females do not upregulate the COX-2 pathway during I/R, the inhibition of COX-2 appears to increase other pathways such as neutrophil infiltration in females. Taken together, our data indicate that in response to I/R, male mice sustain significant injury which appears to require COX-2-mediated PGE₂ production while females appear to use the A5-LOX to produce LTB₄.

Myeloperoxidase, an abundant enzyme released by tissue-infiltrating neutrophils correlates with tissue injury in both acute and chronic inflammation and plays a critical role in organ damage after renal, myocardial, hepatic and intestinal I/R injury as well as other forms of tissue injury including end stage renal disease [72–75] [76, 77]. C5a is generated via complement pathway activation, and acts as a potent chemoattractant for recruiting neutrophils to the sites of injury or inflammation[78]. Our data demonstrate that females produce significantly less myeloperoxidase and C5a than males at both the early and late stages of I/R. However, additional studies are needed. While MPO is a potent neutrophilic mechanism of tissue damage, other studies indicate that TLR9 mediated neutrophil extracellular traps (NETs) create significant damage in hepatic I/R of male mice [76]. However, there is no sex dimorphism in NETs during end stage renal disease. Thus, future studies should compare mechanistic differences between sexes as therapeutic targets. Mouse

studies demonstrate that TLRs including TLR4 [45] and TLR9 [76] are critical to induce PGE2 or NETs. Thus, TLRs may provide therapeutic targets for I/R damage but female mice should be examined as well [45, 76]. Importantly, therapeutics should normalize both male and female differences as cetirizine in allergic inflammation [84].

F4/80+ macrophages, also participate in tissue injury after hepatic, myocardial, cerebral, and intestinal I/R [79–83]. In addition, intestines from male mice express higher levels of F4/80+ macrophages after I/R compared to intestines from female mice. Male neutrophils showed greater pro-inflammatory phenotype polarization, while female neutrophils displayed lower inflammatory markers and were polarized to the anti-inflammatory phenotype [85]. This suggests a longer and more sustained I/R-induced inflammatory cell infiltration in intestines from male than female mice. Similarly, a previous study indicated that during myocardial infarction, the leukocyte ratios and wound healing kinetics benefit females by preventing the excessive myocardial infarction-induced inflammation [86]. In agreement with these studies, a recent publication reported that during myocardial infarctions, males have a higher neutrophil abundance than females [85]. Importantly, clinical gender differences exist in the immune cell changes in a multitude of diseases including allergic rhinitis where females rapidly induced a monocytic response compared to a significant eosinophilic decrease in males [84]. A complete profile of the cellular responses between males and females remains to be accomplished.

I/R is considered an inflammatory disease which induces pro-inflammatory cytokines and chemokines produced from monocytes or damaged intestine by I/R [87]. Pro-inflammatory TNF is produced in response to myocardial ischemia and lung ischemia/reperfusion [87, 88]. Our results demonstrate little TNF- α production in response to I/R and importantly, no differences between sexes suggesting that the pro-inflammatory cytokine was not instrumental in the sexual dimorphism. IL-6 is a unique pleiotropic cytokine displaying both pro-inflammatory and anti-inflammatory properties depending on the type of injury and the target cell type [89]. Importantly, females with an overall lower mortality rate in myocardial infarctions also resolved increased plasma IL-6 faster than males [85]. These data correlate with the current data demonstrating that females secrete more IL-6 after I/R and reports that IL-6 mediates anti-inflammatory effects to protect intestine from I/R injury [90]. IL-10, an anti-inflammatory cytokine inhibits the production of pro-inflammatory cytokines and reduces inflammation-mediated tissue damage in both non-infectious [91, 92] and infectious [93, 94] causes of inflammatory responses. Our data also demonstrate that females produce increased IL-10, an anti-inflammatory cytokine, after 30 min reperfusion. One explanation for this dichotomy of cytokines (IL-6 and IL-10) in females is to balance or inhibit excessive pro-inflammatory cytokine production to protect the intestine and surrounding tissue from damage.

5. Conclusion

This study provides in vivo evidence that males and females respond differently to intestinal I/R-induced injury with distinct inflammatory differences. Specifically, compared to male mice, females secrete significantly more LTB₄, less PGE₂ and more anti-inflammatory cytokines. Thus, this study demonstrates specific sex differences and suggests that

understanding the different mechanisms will be critical for development of appropriate medications or medical treatments.

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Abbreviations

I/R	ischemia reperfusion
LTB₄	leukotriene B ₄
PGE₂	prostaglandins E ₂
COX-2	cyclooxygenase-2
5-LOX	5-lipoxygenase
PGs	prostaglandins
HIF-1α	hypoxia-inducible factor-1 α
MPO	myeloperoxidase
SMI	mucosal injury score
TMB	3,3',5,5'-tetramethylbenzidine

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Highlights

- Males sustain more intestinal I/R-induced tissue injury than females.
- Male mice recruit more inflammatory cells in response to I/R.
- Male intestines secrete increased PGE₂, while female intestines secrete LTB₄.
- COX-2 inhibitor attenuates I/R-induced intestinal damage in male but not female mice.

Figure 1. Intestinal I/R-induced tissue damage and inflammation differs between male and female mice.

(A) C57Bl/6J mice were subjected to sham or 15, 30, 60, or 120 min intestinal I/R treatment and mid-jejunal sections H&E stained and scored for mucosal injury (75–150 villi per section); (B) Total peroxidase levels were determined by measuring oxidation of 3,3',5,5'-tetramethylbenzidine (TMB); (C, D) Serum myeloperoxidase (MPO) and C5a were measured with sandwich ELISA. (E) Representative microphotographs of H&E stained sections. Bar = 40 μ m. *, Significant difference between I/R treatment and sham within the same sex ($P < 0.05$), as determined by unpaired t-test; **, Significant difference between sexes with the same treatment ($P < 0.05$). Each bar is representative of 6–10 animals per group.

Figure 2. Intestine tissue myeloperoxidase (MPO) and F4/80 staining differs between sexes. C57Bl/6J mice were subjected to sham and I/R treatment with 120 min reperfusion. Intestinal sections were stained for (A) MPO and (B) F4/80 by immunohistochemistry. Bar = 40 μ m. Densitometry was performed for each animal using ImageJ. Microphotographs (400x) are representative of at least 3–4 animals stained in at least 3 independent experiments.

Figure 3. Cytokine production in different sexes after intestinal I/R.

C57Bl/6J mice were subjected to sham and I/R with 15, 30, 60 or 120 mins of reperfusion.

Intestinal sections were analyzed ex vivo for (A) IL-6, (B) IL-10, (C) IL-12p40, (D) IL-12p70, (E) CXCL1(MKC), and (F) TNF production and normalized to a per mg tissue protein; *, Significant difference between I/R treatment and sham within the same sex ($P < 0.05$), as determined by unpaired t-test; **, Significant difference between sexes with the same treatment ($P < 0.05$). Each bar is representative of 5–10 animals per group.

Figure 4. Intestinal I/R-induced HIF-1 α , COX-2, and A5-LOX transcription differs between sexes.

C57Bl/6J mice were subjected to sham and I/R treatment with 15, 30, 60 or 120 min of reperfusion. Mid-jejunal expression of (A) HIF-1 α , (B) COX-2 and (C) A5-LOX, RNA was determined by RT-PCR analysis. Samples were normalized to 18S followed by fold change compared to Sham treatment. *, Significant difference between I/R treatment and sham within the same sex ($P < 0.05$), as determined by unpaired t-test; **, Significant difference between sexes with the same treatment ($P < 0.05$). Each bar is representative of 5–10 animals per group.

Figure 5. Ex vivo production of PGE₂ and LTB₄ differs between sexes over reperfusion times. C57Bl/6J mice were subjected to sham and I/R treatment with distinct 15, 30, 60, or 120 min reperfusion. Intestinal sections were analyzed ex vivo for (A) PGE₂ and (B) LTB₄ production and standardized to mg total tissue protein. *, Significant difference between I/R treatment and sham within the same sex ($P \leq 0.05$), as determined by unpaired t-test; **, Significant difference between sexes with the same treatment ($P \leq 0.05$). Each bar is representative of 5–10 animals per group.

Figure 6. COX-2 inhibition attenuates intestinal I/R-induced tissue damage in male but not female mice.

(A) C57Bl/6J mice were subjected to sham or 120 min intestinal I/R treatment with or without administration of COX-2 inhibitor, NS-398. After 120 min reperfusion, mid-jejunal sections were H&E stained and scored for mucosal injury (75–150 villi per section); (B) Total peroxidase levels were determined by measuring oxidation of 3,3',5,5'-tetramethylbenzidine (TMB); Intestinal sections were also used to analyze ex vivo (C) LTB₄ and (D) PGE₂ production. Peroxidase, LTB₄ and PGE₂ were normalized to mg protein within the tissue. *, Significant difference between sexes within the same treatment (P 0.05), as determined by unpaired t-test; **, Significant difference between untreated I/R and NS-398 treated mice within the same sex (P 0.05). Each bar is representative of 5–10 animals per group.

Table 1

Real Time PCR Primer Sequences

Gene	T _m (°C) ^a	Sequence
HIF-1α	51	FWD: ATGAGAGAAATGCTTACACAC
		REV: TGAGGTTGGTTACTGTTGG
COX-2	54	FWD: ATCCTGCCAGCTCCACCG
		REV: TGGTCAAATCCTGTGCTCATACAT
A5-LOX	52	FWD: ATCTGAGCGAGTCAAGAACC
		REV: TGAAAATGTTCCCTTCCTGT
Ribosomal 18s ^b	56	FWD: GGTGATCCTGCCAGTAGC
		REV: GCGACCAAAGGAACCATAAC

^a Annealing temperature^b House-Keeping gene to which genes of interest were normalized