Published in final edited form as:

Shock. 2015 April ; 43(4): 327–333. doi:10.1097/SHK.0000000000000297.

Protection by enteral glutamine is mediated by intestinal epithelial cell peroxisome proliferator-activated receptor gamma during intestinal ischemia/reperfusion:

PPAR γ mediates protection by glutamine

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Abstract

We have demonstrated that enteral glutamine provides protection to the post ischemic gut and that PPAR γ plays a role in this protection. Using Cre/lox technology to generate an intestinal-epithelial cell (IEC) specific PPAR γ null mouse model, we now investigated the contribution of IEC PPAR γ to glutamine's local and distant organ protective effects. These mice exhibited absence of expression of PPAR γ in the intestine but normal PPAR γ expression in other tissues. Following one hour of intestinal ischemia under isoflurane anesthesia, wild type and null mice received enteral glutamine (60 mM) or vehicle followed by 6 hours of reperfusion or 7 days in survival experiments and compared to shams. Small intestine, liver, and lungs were analyzed for injury and inflammatory parameters. Glutamine provided significant protection against gut injury and inflammation with similar protection in the lung and liver. Changes in systemic TNF α reflected those seen in the injured organs. Importantly, mice lacking IEC PPAR γ had worsened injury and inflammation and glutamine lost its protective effects in the gut and lung. The survival benefit found in glutamine treated wild type mice was not observed in null mice. Using an IEC-targeted loss-of-function approach, these studies provide the first in vivo confirmation in native small intestine and lung that PPAR γ is responsible for the protective effects of enteral glutamine in reducing intestinal and lung injury and inflammation and improving survival. These data suggest that early enteral glutamine may be a potential therapeutic modality to reduce shock-induced gut dysfunction and subsequent distant organ injury.

Keywords

intestinal-cell (IEC) specific PPAR γ null mouse; cre/lox; neutrophil infiltration; multiple organ dysfunction syndrome

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DISCLOSURES

No conflicts of interest, financial, or otherwise, are declared by the authors.

INTRODUCTION

Multiple organ dysfunction syndrome (MODS) remains the most common cause of death in severely injured patients in shock who do not die from early uncontrolled hemorrhage or brain injury.(1) The institution of early enteral nutrition decreases septic morbidity and therefore lessens the incidence of post-injury MODS.(2,3) However, standard practice is to begin enteral nutrition once resuscitation is complete, well after the localized (gut) and systemic production and release of multiple pro-inflammatory mediators has occurred. The separation of nutritional support from the provision of key nutrients such as glutamine has led to a further appreciation of the immunomodulatory and anti-inflammatory benefits of isolated nutrients.(4) The mortality of MODs remains high, in part due to the lack of early therapeutic interventions to prevent shock-induced gut dysfunction.(5) The purpose of the current study was to determine if glutamine may represent such an intervention.

Glutamine is a conditionally essential amino acid after critical illness or injury.(6) We have been interested in glutamine's gut protective effects and have shown that as a metabolizable nutrient, it can protect against gut injury and impaired absorption by supplying the post ischemic gut with needed ATP.(7) Additionally, these protective effects are mediated in vitro and in vivo by peroxisome proliferator-activated receptor gamma (PPAR γ). (8-11) PPAR γ is a member of the nuclear receptor superfamily of transcription factors and is highly expressed in adipocytes, intestinal epithelium, immune cells, skeletal muscle cells, and hepatocytes.(12) Its most important natural ligand is 15-deoxy PG J2 while the glitazones are well studied synthetic ligands that have been shown to reduce intestinal inflammation in models of colitis.(13)

In the current study we assessed the role of intestinal epithelial PPAR γ in glutamine's protection from intestinal ischemia/reperfusion-induced intestinal and distant organ (lung) injury. We generated conditional null mice with an intestinal epithelial cell specific deletion of PPAR γ and tested the hypothesis that enteral glutamine protects the gut and mitigates distant organ (lung) injury via intestinal PPAR γ .

MATERIALS AND METHODS

All animal procedures performed were protocols approved by the University of Texas Houston Medical School Animal Welfare Committee. The experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals. All animals were housed at constant room temperature with a 12:12-h light-dark cycle with access to food and water ad libitum. Male C57BL/6J mice, 8-10 weeks of age were used for all experiments.

Generation of intestinal-specific PPAR γ deficient mice

Cre/lox technology was used to generate intestinal-specific PPAR γ deficient mice as previously described. (14) Homozygous floxed PPAR γ (f/f) mice with VFB background were generously provided by Dr. F Gonzalez (NIH). Floxed mice were bred with C57BL/6J for 7 generations to get PPAR γ ^{f/f} mice with C57BL/6J background. The targeted allele, containing loxP sites flanking exon 2 of the PPAR γ gene, was crossed into a transgenic

mouse expressing Cre recombinase under control of the villin1 to yield mice with a targeted deletion of PPAR γ in intestinal villi and crypt cells.

Quantitative real time polymerase chain reaction (PCR)

Total RNA was extracted from small and large bowel, heart, liver, lung and kidney using TRI reagent (Sigma, St. Louis). One μ g of RNA was used to reverse cDNA using Bio-Rad iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) then 50ng of cDNA was used to perform quantitative real-time PCR to check PPAR γ mRNA expressions using TaqMan® Gene Expression Assays with a PPAR γ probe (Mm01184321, Applied Biosystems) as a target and an actin probe (Mm00607939_s1, Applied Biosystems) as the internal reference. The relative expression level of target genes was normalized to that of the internal reference gene and to a calibrator sample that was run on the same plate. The normalized relative expression levels were automatically calculated using commercial software (StepOne Software V2.1, Applied Biosystems).

Western Blot

Target tissues were homogenized and lysates were prepared with RIPA buffer (Sigma, Milwaukee, WI) containing protease inhibitor cocktail (Sigma, Milwaukee, WI). The lysates were denatured, loaded on 4% to 12% linear gradient NuPAGE gel, and transferred to Immobilon PVDF blotting membrane (Millipore, Billerica, Massachusetts). The membranes were blocked for 1 hour at room temperature Odyssey blocking buffer (Li-cor, cat: 927-40000), incubated with rabbit anti-PPAR γ antibody (1:1000, Cell Signaling, cat:5468) overnight at 4°C, followed by washing and incubation with IRDye 800CW conjugated goat anti-rabbit IgG (1:5000, Li-cor, cat: 926-32211) for 1 hour at room temperature, then the membrane was washed and scanned with Odyssey CLx Infrared Imaging System (Li-cor, Lincoln, Nebraska).

Gut ischemia/reperfusion

Animals were anesthetized under inhalation of isoflurane. For gut ischemia/reperfusion (I/R), a midline laparotomy was performed and the superior mesenteric artery was clamped for 60 minutes. Reperfusion was established one hour later by removal of the clamp. Animals were sacrificed after six hours and the full thickness jejunum, liver, and lung harvested and bronchial alveolar fluid obtained. Sham animals underwent an identical procedure without superior mesenteric artery occlusion. Animals were randomly divided into 6 groups (8 animals/group): WT sham, KO sham, WT subjected to I/R + vehicle, KO subjected to I/R + vehicle, WT subjected to I/R plus glutamine, and KO subjected to I/R plus glutamine. For animals in the glutamine group, 60 mM glutamine was instilled into the intestine at the onset of reperfusion and compared to vehicle treated animals as we have described (11). For survival experiments, the superior mesenteric artery was occluded for 75 minutes then the animals were observed for 7 days (15-18 animals/group).(15)

Intestinal studies

To evaluate intestinal histopathology, 5- μ m sections were stained with hematoxylin and eosin. For quantitative estimation of damage, sections were scored with a light microscope

in a blinded manner. The morphological criteria were used as described by Chiu with a scoring range from 0 to 5, where 5 is the most severely injured. (16)

Neutrophil infiltration was assessed in intestinal sections by staining with Naphthol AS-D Chloroacetate Esterase Cytochemical Staining kit (Sigma Diagnostics, Milwaukee, WI), which identifies specific leukocyte esterases. To quantitate assessment of neutrophil infiltration into the intestinal mucosa, a total of 50 high-power fields ($\times 400$) under a light microscope were counted for positively stained cells in a blinded fashion.

Myeloperoxidase (MPO) activity was measured using the MPO Colorimetric Activity Assay Kit (BioVision Research Products, Mountain View, CA, USA). Activity was normalized according to milligrams of total protein in tissue lysates. Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc. Hercules, CA, USA) was used to determine protein concentration.

Apoptotic cells were stained in sections using an In Situ Cell Death Detection Kit according to the manufacturer's instructions (Roche, Indianapolis, IN). The apoptotic index was calculated as a ratio of the positively stained cell number to the total cell number in each field. At least of 400 cells were counted.

Lung studies

Lungs were harvested and sections fixed with 10% neutral buffered formaldehyde solution then embedded in paraffin and sectioned. At least two sections from each animal were stained with hematoxylin and eosin then scored using a 4-point scale for six separate criteria. (17) The overall lung injury score was calculated by averaging the six indices of injury.

Lung sections were stained using a Naphthol AS-D Chloroacetate Esterase Cytochemical Staining kit (Sigma Diagnostics, Milwaukee, WI), which identifies specific leukocyte esterases. Two random images were taken from each lung section with a light microscope (Nikon Eclipse Ti, Melville, NY) at 200X and quantified using ImageJ software (NIH). The neutrophil infiltration is presented as the percentage of neutrophil positive staining area / total lung tissue area.

Infiltration of neutrophils was assessed by MPO immunofluorescence staining and MPO activity. For immunofluorescence, paraffin embedded tissue was cut into 5 μm -thick sections then incubated with MPO primary antibody (1:100 MPO mouse monoclonal antibody, Abcam, Cambridge, MA) followed by incubation with secondary antibody (goat anti-mouse, Alexa Fluor 568, Invitrogen, Carlsbad, CA). Two random images were taken from each lung section with a fluorescent microscope (Nikon Eclipse Ti, Melville, NY) at 200X and quantified using ImageJ software (NIH). MPO activity was measured using a commercially available kit per manufacturer's instructions (Enzo Life Sciences, ADI-907-029).

Liver studies

Liver tissue was harvested and sections fixed with 10% neutral buffered formaldehyde solution then embedded in paraffin and sectioned. At least two sections from each animal were stained with hematoxylin and eosin then scored on a scale from zero to three. (18)

Systemic parameters

Blood was obtained at the time of sacrifice and TNF- α was determined using the mouse tumor necrosis factor alpha (TNF α) ELISA kit from MyBioSource (San Diego, CA) according to manufacturer's instructions. Three liver function assays were also performed, aspartate aminotransferase (AST), alanine aminotransferase (ALST) and alkaline phosphatase (alk phos) according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO)

Statistical analysis

All values are expressed as mean $\pm$ SEM. Data were analyzed by one way analysis of variance with Bonferroni correction. $P < 0.05$ was considered significant.

RESULTS

Intestinal-specific PPAR γ deficiency

To investigate the contribution of IEC PPAR γ to glutamine's gut protective effects, conditional null mice with intestinal epithelial cell specific deletion of PPAR γ was generated using a floxed PPAR allele and Cre recombinase under the control of the villin gene promoter. The intestinal specific deletion in PPAR γ is shown in Figure 1. MRNA and protein expression is present in lung, heart, liver, and kidney, but is not detectable in the small or large intestine (Fig 1A and C). WT mice have approximately 40 fold more PPAR γ mRNA expression in the small intestine compared to KO mice (Fig. 1B), which display normal growth and development.

Luminal glutamine loses its gut protective effects in intestinal-specific PPAR γ null mice

Intestinal morphology in sham null mice was similar to WT sham mice (0.12 ± 0.13 vs 0.14 ± 0.13) (Fig. 2A). Severe damage occurred after I/R in the WT intestine (2.90 ± 0.55) which was significantly worsened in null mice (4.00 ± 0.35). As expected, luminal glutamine protected against mucosal damage in the WT mice (1.90 ± 0.54) but this effect was abolished in the intestine of null mice (3.40 ± 0.65).

Intestinal inflammation was first evaluated by specific leukocyte esterase staining (Fig. 2B). There were rare neutrophils detected in the sham WT (3.6 ± 2.4) and KO (5.6 ± 3.6) intestines. Following intestinal I/R, there was a significant increase in neutrophil infiltration in the KO intestine (148.4 ± 23.9) compared to the WT (95.0 ± 23.3). Luminal glutamine administration attenuated neutrophil infiltration in the WT (59.0 ± 16.3) but not in the KO intestine (133.6 ± 21.2). Consistent with neutrophil infiltration, MPO activity was higher in the KO intestine (87.0 ± 12.5) than in the WT controls (63.1 ± 7.5 ; $p < 0.01$) and significantly lessened by glutamine in the WT (44.4 ± 10.3) ($p < 0.05$) but not in the KO intestine (75.8 ± 16.5). MPO activity was minimal in both the WT (6.6 ± 2.5) and KO (8.6 ± 2.4) sham intestines.

Intestinal I/R leads to epithelial cell apoptosis (19) and PPAR γ has been shown to possess anti-apoptotic properties.(20) We therefore examined the effect of PPAR γ deficiency on intestinal epithelial cell apoptosis (Fig. 2C). There were very few apoptotic cells in both the WT and KO sham intestines (1.0 ± 1.2 and 2.0 ± 1.9 , respectively). However, apoptosis was

dramatically increased by I/R in the WT (40.6 ± 11.6) and further increased in the KO intestine (59.0 ± 11.4). Similar to histopathology and inflammation, glutamine treatment protected against cell apoptosis in the WT intestine (24.0 ± 6.5), but this protective effect was lost in the KO intestine (51.0 ± 12.9).

Luminal glutamine mitigates lung injury in wild type but not intestinal-specific PPAR γ null mice

Lung histopathology was similar in WT and null mice (1.14 ± 0.40 vs 1.28 ± 0.42) but was increased by I/R in WT (5.0 ± 0.48) and in null mice (7.0 ± 0.75) (Fig. 3A). Luminal glutamine was protective in WT (3.14 ± 0.34) but not null mice lungs (5.4 ± 0.72).

Lung inflammation was evaluated by leukocyte esterase staining as well as by myeloperoxidase activity and immunostaining (Fig. 3). There was no difference in neutrophil staining between sham WT and null mice (0.38 ± 0.03 vs $0.44 \pm 0.04\%$) (Fig. 3B). Intestinal I/R significantly increased neutrophil staining in WT mice lungs ($1.6 \pm 0.20\%$) and in null mice ($2.2 \pm 0.20\%$). Glutamine was protective in WT ($1.0 \pm 0.1\%$) but not in null mice lungs ($1.9 \pm 0.1\%$). MPO activity showed similar findings with no differences between shams (2.67 ± 0.62 WT vs 3.07 ± 0.49 ng/mg protein KO), an increase after I/R in WT (8.73 ± 1.49 ng/mg protein) and null mice lungs (9.76 ± 1.24 ng/mg protein). Again, no protection was seen by glutamine in null (9.06 ± 0.96 ng/mg) mice, only WT mice lungs (6.78 ± 1.04 ng/mg protein). MPO immunostaining is shown in Fig. 3C and findings were consistent with MPO activity.

Liver histopathologic injury reduced by luminal glutamine in wild type but not intestinal-specific PPAR γ null mice

Liver injury was assessed as the liver receives blood flow directly from the intestine. Although liver histopathology was the same magnitude as gut injury, the pattern of changes was similar. There were no differences between sham WT and null mice (0.03 ± 0.02 vs 0.06 ± 0.02) but there was a significant increase by I/R in WT mice (0.89 ± 0.08) with a further increase in null mice (1.32 ± 0.06) (Fig. 4). Luminal glutamine was protective in WT (0.43 ± 0.06) but not in the null mice liver (1.09 ± 0.09). Results of liver parameters are shown in Table 1. Although there was an increase in AST, ALT, and alkaline phosphatase in all groups compared to shams, there were not significant differences between I/R groups.

Changes in systemic TNF α reflect changes in injured organs

Systemic TNF α levels were measured, as this cytokine is known to be elevated in injured patients and after gut ischemia. Changes in TNF α levels were similar to those found in the gut, lung, and liver. Levels were similar between sham WT and KO mice (17.0 ± 2.8 pg/ml) but dramatically increased after gut I/R in WT mice (526.0 ± 30.3 pg/ml) with a further significant increase in KO mice (839.3 ± 54.6). Luminal glutamine lessened TNF α in WT (203.5 ± 12.9 pg/ml) but not KO mice (746.3 ± 50.0 pg/ml).

Intestinal specific PPAR γ deficiency increases mortality in glutamine treated mice after gut I/R

All sham WT and PPAR γ null mice survived (Fig. 5). Luminal glutamine significantly improved survival in WT mice after gut I/R. However, the improvements in survival seen after glutamine in WT mice were not observed in KO mice.

DISCUSSION

We used an intestinal epithelial cell (IEC)-targeted loss-of-function approach to determine the role of IEC PPAR γ in both gut and lung protection by luminal glutamine in the post ischemic gut. We demonstrated that glutamine provided significant protection against gut injury and inflammation with similar protection in the lung. Importantly, in mice lacking IEC PPAR γ glutamine lost its protective effects. Additionally, the survival benefit found in the glutamine treated wild type mice were not observed in null mice.

The importance of IEC PPAR γ in colonic epithelium has been shown in rodent studies of experimental inflammatory bowel disease. (21,22) Mice with a targeted deletion of PPAR γ in IEC had worsened parameters of colonic disease such as inflammatory lesions and diarrhea. To our knowledge, the current study is the first to examine IEC PPAR γ in the small bowel and the first to demonstrate an increase in mortality with loss of IEC PPAR γ after injury. We chose to examine the small bowel, and the jejunum in particular, as this is the site of glutamine absorption. Mice lacking IEC PPAR γ were generated by crossing the transgenic mouse expressing Cre recombinase under control of the villin1. Because villin 1 is found in epithelial cells of the large and small intestine (23), these mice lack PPAR γ throughout the intestine. We have not investigated other areas of the bowel in our model.

We became interested in the protective role of PPAR γ in the intestine after we demonstrated in vivo that enteral glutamine increased PPAR γ DNA binding activity and was associated with a decrease in mucosal injury and inflammation.(11) In vitro there was a time and concentration-dependent increase in PPAR γ transcriptional activity, but not mRNA or protein, by glutamine.(24) We hypothesized that glutamine functioned through competition for the PPAR γ ligand binding site. However, our data demonstrated that glutamine, via degradation to glutamate, activated PPAR γ via the endogenous PPAR γ intestinal epithelial ligands, 15-S-hydroxyl-eicosatetraenoic acid (15-S-HETE) and dehydrogenated 13-hydroxyoctadecadienoic acid (13-OXO-ODE).

Levy et al have shown that shock, trauma, or sepsis-induced gut injury causes the gut to become a cytokine-generating organ, with toxic gut-derived factors found in the mesenteric lymph that result in distant organ (lung) injury and multiple organ dysfunction syndrome (MODS).(25) As the lung is the first organ to receive mesenteric lymph and MODS remains the most common cause of late deaths in severely injured patients in shock (26), we examined the ability of luminal glutamine to protect against local and distant organ (lung) injury in an established rodent model of MODS.(27) Indeed, gut derived protection by glutamine was similarly demonstrated in the lung. The finding that glutamine lost its protective effects in the lung when intestinal PPAR γ was lacking, highlights the important

role of the intestinal epithelium in MODS. The rather modest changes by glutamine in the liver underscores the important role of the mesenteric lymph.

The early administration of enteral glutamine to patients in shock is feasible, as we have shown in a prospective randomized pilot study of severely injured patients.(28) In fact, glutamine administered during active shock resuscitation after injury was safe and well tolerated. Wischmeyer et al recently published a meta-analysis of 26 randomized controlled studies of intravenous supplementation and also demonstrated benefit. There was a significant decrease in in-hospital mortality and hospital length of stay and a trend towards a reduction in overall mortality, infectious complications, and ICU length of stay.(29) However, a prospective randomized trial by Heyland et al in patients with established MODS, parenteral and enteral glutamine supplementation resulted in a nonsignificant trend towards increased mortality.(30) The reasons for this rather surprising finding are unclear. Unlike in the current laboratory study or in our clinical study in trauma patients, Heyland et al administered glutamine only after the development of MODS and by both the enteral and parenteral routes, thus generating high systemic levels of glutamine. Additionally, many of these patients had underlying renal dysfunction and high serum glutamine levels even prior to supplementation.(31) Though only speculative, correcting low serum levels of glutamine may be maladaptive or glutamine toxicity may have resulted from already high levels of endogenous glutamine.(32) Additionally, prior clinical studies which showed benefit, as well as the current study, all used L-glutamine. In the REDOX trial, glutamine was delivered as alanyl-glutamine. Even though alanyl-glutamine is safe when added to total parenteral nutrition, the dose used in REDOX was high and the alanine contribution may have been detrimental or may have masked the benefit by glutamine. We have shown in our rodent I/R model that there was a significant difference in gut parameters of injury and function between enteral alanine and enteral glutamine, with only glutamine demonstrating protection.(7,9) Although both alanine and glutamine are absorbed across the gut epithelium by sodium-mediated secondary active transport, only glutamine is metabolized by the gut and can maintain energy supplies to the hypoperfused gut.(7) Administering only enteral glutamine and doing so prior to the development of distant organ injury is supported in a more recent article by Heyland et al, and is most applicable to trauma and burn patients.(33) Additionally, enteral supplementation alone does not usually increase systemic levels of glutamine.(34,35) Our findings suggest that the early use of enteral glutamine prior to distant organ injury is safe and may mitigate the development of distant organ failure.

In conclusion, we used an intestinal epithelial cell (IEC)-targeted loss-of-function approach to determine the role of IEC PPAR γ in gut, liver, and lung protection by luminal glutamine in the post ischemic gut. We demonstrated that glutamine provided significant protection against both local (gut) and distant (lung) injury and inflammation, with a lesser degree of protection in the liver. Mice lacking IEC PPAR γ not only demonstrated worsened gut, lung, and liver injury and inflammation but the survival advantage of glutamine was not observed in null mice. These studies demonstrate for the first time that intestinal epithelial cell PPAR γ plays a critical role in enteral glutamine's protection to the post ischemic gut. Additionally, this study supports further investigation of glutamine and other PPAR γ agonists targeting the gut.

Acknowledgments

GRANTS

This work was supported by National Institute of General Medical Sciences Grant (RO1 GM077282)

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Figure 1. Intestinal specific conditional PPAR γ null mice

Distribution of PPAR γ among different organs by mRNA and protein is shown, with specificity of the deletion demonstrated. Homozygous floxed PPAR γ (f/f) mice with VFB background were bred with C57BL/6J for 7 generations to get PPAR $\gamma^{f/f}$ mice with C57BL/6J background. The targeted allele, containing loxP sites flanking exon 2 of the PPAR γ gene, was crossed into a transgenic mouse expressing Cre recombinase under control of the villin1 to yield mice with a targeted deletion of PPAR γ in intestinal villi and crypt cells. (A). Tissue expression of PPAR γ in intestinal specific conditional PPAR γ null mice; (B). Comparison of PPAR γ mRNA between KO and WT small and large intestines; and (C). Protein confirmation showing specificity of PPAR γ deletion.

Figure 2. Effect of intestinal epithelial cell deletion of PPAR γ on gut injury and inflammation
 Wild type and null mice underwent intestinal ischemia following by enteral glutamine or vehicle. After six hours intestine was harvested. Representative images and corresponding quantification of (A) mucosal histologic injury, (B) neutrophil infiltration, and (C) apoptotic index. Mean $\pm$ SEM, ANOVA with Bonferroni correction; n=8/group.

Figure 3. Effect of intestinal epithelial cell deletion of PPAR γ on lung injury and inflammation
Wild type and null mice underwent intestinal ischemia following by enteral glutamine or vehicle. After six hours lung was harvested. Representative images and corresponding quantification of (A) histologic injury, (B) neutrophil infiltration, and (C) myeloperoxidase immunostaining. Mean $\pm$ SEM, ANOVA with Bonferroni correction; n=8/group.

Figure 4. Effect of enteral glutamine on liver injury

Wild type and null mice underwent intestinal ischemia following by enteral glutamine or vehicle. After six hours liver was harvested. Representative images (A) and corresponding quantification of (B) histologic injury. Mean $\pm$ SEM, ANOVA with Bonferroni correction; $n=6$ /group.

Figure 5. Effect of enteral glutamine on survival in wild type and intestinal specific conditional PPAR γ KO mice

Mice were treated with enteral glutamine or vehicle after 75 minutes of intestinal ischemia and survival was determined for seven days. Data were analyzed by the Kaplan-Meier method, $n=15-18/\text{group}$. # $p<0.05$ vs. WT-sham, KO-sham, and WT-IR-Gln; ‡ $p<0.5$ vs WT-IR.

Table 1

Indices of liver function after gut I/IR and luminal glutamine

Groups	AST (U/L)	ALT (U/L)	ALK Phos)(U/L)
WT sham	28.5 ± 3.9	16.6 ± 2.6	62.8 ± 11.0
KO sham	31.5 ± 2.3	17.8 ± 2.2	73.9 ± 11.0
WT IR	94.9 ± 7.6*	61.0 ± 8.4*	184.5 ± 12.2*
KO IR	102.2 ± 10.9*	72.3 ± 7.9*	196.0 ± 9.3*
WT IR gln	71.1 ± 7.2*	44.4 ± 7.9*	137.4 ± 13.8*
KO IR gln	88.3 ± 6.5*	65.8 ± 4.8*	176.1 ± 10.2*

Data are shown as mean ± SEM, n=6/group, ANOVA with Bonferroni correction;

* = p<0.01 vs sham WT and KO.

IR= ischemia/reperfusion, gln-glutamine; AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP phos=alkaline phosphatase