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STAT3 Does Not Regulate Acute Liver Injury After Ischemia/Reperfusion

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Abstract

Background—Hepatic ischemia/reperfusion (I/R) injury is an important complication of liver surgery and transplantation. Regulation of this injury response occurs at the cellular and molecular levels. Previous studies have shown that interleukin-6 (IL-6) is a negative regulator of the acute inflammatory injury occurring as a result of hepatic I/R. The Signal Transducer and Activator of Transcription-3 (STAT3) is a key target of receptor signaling for IL-6. Both IL-6 and STAT3 have been implicated in the protective effects of ischemic preconditioning of the liver. However, there have been no studies that have directly addressed the potential role of STAT3 in regulating acute inflammatory liver injury induced by I/R. In the current study, we investigated whether blockade of STAT3 phosphorylation altered the injury response to hepatic I/R injury.

Methods—Male Balb/c mice were subjected to 90 minutes of partial hepatic ischemia followed by reperfusion with or without treatment with specific inhibitors of STAT3 activation, AG490 (selective JAK2 inhibitor) or STATTIC (direct inhibitor of STAT3 phosphorylation). Mice were sacrificed at 8 and 24 hours after reperfusion.

Results—STAT3 activation was induced by I/R. This activation was partially inhibited by administration of AG490 and almost completely abrogated by treatment with STATTIC. Despite the blockade of STAT3, neither AG490 nor STATTIC had any effect on acute liver injury induced by I/R. Treatment with STATTIC did reduce hepatic neutrophil accumulation.

Conclusion—The data suggest that STAT3 is not a central regulator of acute liver injury induced by I/R.

Keywords

STAT3; hepatic ischemia/reperfusion; neutrophil accumulation

Introduction

Hepatic ischemia/reperfusion (I/R) injury is common complication of liver resection and transplantation, and trauma surgery (1-3). A primary component of this injury is the induction of an acute inflammatory response that leads to significant tissue damage and

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organ dysfunction both locally and remotely (3-5). The mechanisms of acute inflammation occurring as a result of hepatic I/R injury have been widely studied and have led to the identification of numerous regulatory mediators that attempt to prevent or temper uncontrolled inflammatory responses (6).

One such regulatory mediator is interleukin-6 (IL-6), which has been shown to be protective against warm hepatic I/R injury. Administration of recombinant IL-6 prior to hepatic I/R reduced liver injury and increased proliferation promoting faster recovery (7). Knockout of IL-6 resulted in increased hepatocellular injury after I/R and exogenous treatment of knockout mice with IL-6 reduced injury (7). IL-6 is believed to mediate its hepatoprotective effects on hepatic I/R through down regulation of TNFα, thereby blunting the resulting inflammatory cascade (7,8). More recently, IL-6 has also been demonstrated to be protective in ischemic preconditioning of liver (8). Ischemic preconditioning confers hepatoprotection as evidence by improved survival and decrease hepatocellular injury in wild type mice undergoing hepatic I/R. However, this survival benefit is negated in IL-6-knockout mice (8).

The primary signaling mechanism utilized by IL-6 is the Janus kinase (Jak)-Signal Transducer and Activated by Transcription (STAT) pathway. In response to IL-6 binding to its receptor, Jak2 is activated and in turn phosphorylates STAT3 monomers(9). Phosphorylation of STAT3 allows dimerization and translocation to the nucleus(10). Ischemic preconditioning of liver has been shown to induce STAT3 phosphorylation, which is associated with reduced I/R injury and improved recovery (8). While the roles of both endogenous and exogenous IL-6 on hepatic I/R have been investigated, the role of STAT3 has not. In the present study, we sought to determine if STAT3 was activated during hepatic I/R injury and whether this activation was required for regulation of the acute inflammatory response to hepatic I/R.

Materials and Methods

Hepatic I/R Injury Model

This project was approved by the University of Cincinnati Animal Care and Use Committee and was in compliance with the National Institutes of Health guidelines. Male wild-type mice on a BALB/c background (Jackson Laboratory, Bar Harbor, ME) weighing 22-28 g were subjected to either sham surgery or hepatic I/R as previously described (11). The mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). A midline laparotomy was performed and an atraumatic clip was used to interrupt blood supply to the left lateral and median lobes of the liver. The caudal lobes retained intact portal and arterial inflow and venous outflow, preventing intestinal venous congestion. After 90 minutes of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion. Sham operated mice underwent the same protocol without vascular occlusion. I/R control mice had 100μl of saline intraperitoneally at the time of reperfusion. To evaluate the effect of STAT3 blockade on hepatic I/R, dose-response experiments were performed using AG490 (Calbiochem San Diego, CA) a selective JAK2 inhibitor in 50μl of 2.5% DMSO and STATTIC (Calbiochem San Diegao, CA) a direct inhibitor of STAT3 phosphorylation in 100μl of 2.5% DMSO. The agents were administered intaperitoneally at the time of reperfusion. Test doses of AG490 ranged from 6ug to 12.5ug while doses of STATTIC range from 12μg to 25μg. Effective STAT3 blockade was achieved and confirmed by Western blot analysis. The effective doses of AG490 and STATTIC were found to be 12.5 μg and 25μg respectively. Study mice were injected intraperitoneally with 25μg of STATTIC or 12.5μg AG 490 in 2.5% DMSO just after reperfusion. An additional group of mice received only vehicle (100μl of 2.5% DMSO). Control mice received saline at reperfusion. Mice were sacrificed after the indicated periods of reperfusion, and blood and samples of ischemic lobes and non-ischemic lobes of the liver were taken for analysis.

Western Blot Analyses

Liver samples were homogenized in lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCL, 0.1% NP-40, 0.6 mM PMSF, 0.5 mM DTT, Protase Inhibitor Cocktail). Cellular debris was removed by centrifugation at 5,000 rpm and the pellet resuspended in buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 25% Glycerol, 0.6 mM PMSF, 0.5 mM DTT, Protase Inhibitor Cocktail) and incubated on ice for 30 minutes with periodic vortexing. Cellular debris was again removed using centrifugation at 14,000 rpm and the supernatant nuclear extract was collected. Protein concentrations of each sample were determined. Nuclear extracts containing equal amounts of protein in equal volumes of sample buffer were separated in a denaturing 10% polyacrylimide gel and transferred to a 0.1 μm pore nitrocellulose membrane. Nonspecific binding sites were blocked with tris-buffered saline (TBS; 40 mM Tris, pH 7.6, 300 mM NaCl) containing 5% non-fat dry milk for 1 hour at room temperature. Membranes were then incubated with antibodies to phosphorylated-STAT3 or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS with 0.1% Tween 20 (TBST). Membranes were washed and incubated with goat anti-mouse IgG-HRP secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Blood and Tissue Analysis

Blood was obtained by cardiac puncture for analysis of serum alanine amino transferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made using a diagnosis kit by bioassay (Wiener Laboratories, Rosario, Argentina). Liver tissues were fixed in 10% neutral-buffered formalin, processed and then embedded in paraffin for light microscopy. Sections were stained with hematoxylin and eosin for histological examination. Neutrophil accumulation in liver sections was determined by counting the number of neutrophils in a high power field (HPF; 40X). For each section, five HPF were examined. Neutrophil data is expressed as mean number of PMN per HPF. Severity of liver injury was also assessed using the Suzuki classification of sinusoidal congestions, necrosis and ballooning. Tissue levels of TNF-α and MIP-2 were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed with a one-way analysis of variance with subsequent Student-Newman-Keuls test. Differences were considered significant when $P<0.05$.

Results

Hepatic STAT3 activation is increased by I/R and can be effectively inhibited in vivo

To determine whether STAT3 was activated in the liver acutely after I/R, liver nuclear extracts were immunoblotted for phosphorylated STAT3. Livers from sham-operated controls showed no evidence of phospho-STAT3 (Figure 1). However, after 90 minutes of ischemia and 8 or 24 hours of reperfusion, there was marked activation of STAT3 (Figure 1). To evaluate if STAT3 activation could be effectively blocked in vivo, we tested two inhibitors of STAT3 phosphorylation, AG 490 and STATTIC. Treatment with AG490 resulted in partial inhibition of STAT3 activation, whereas STATTIC was much more effective in blocking STAT3 after ischemia and 24 hours of reperfusion (Figure 2).

Blockade of STAT3 activation has no effect on I/R-induced liver injury

In order to determine if the increased STAT3 activation we observed in the liver after I/R was involved in regulation of the injury response, we next examined the effects of blockade

of STAT3 on hepatic I/R injury. Neither treatment with AG 490 nor STATTIC had any effect on liver injury as measured by serum ALT (Figure 3). These findings were confirmed by histological examination. Mice undergoing sham surgery had normal liver architecture (Figure 4A), whereas mice undergoing 90 minutes of ischemia and 8 hours of reperfusion had large areas of necrosis (Figure 4B). Mice undergoing 90 minutes of ischemia and 8 hours of reperfusion plus vehicle, AG 490, or STATTIC, all had similar degrees of hepatocellular necrosis (Figure 4C, D, and E, respectively). Similarly, hepatocellular necrosis after 24 hours of reperfusion was similar amongst the treatment groups (data not shown). There was no difference in injury severity scores as assessed by the Sukuki Classification (Table 1). However, when neutrophil accumulation was evaluated by counting the number of neutrophils per high powered field (HPF), we found no differences between treatment groups after 8 hours of reperfusion (Figure 5). In contrast, when we examined livers after 24 hours of reperfusion, we found that treatment with vehicle (2.5% DMSO) significantly reduced liver neutrophil accumulation compared to untreated controls (Figure 5). Treatment with AG 490 was similar to vehicle treatment, whereas treatment with STATTIC significantly reduced neutrophil accumulation compared to vehicle controls (Figure 5). There was no difference in expression of the inflammatory mediators, TNF-α and MIP-2, between groups to account for this difference in neutrophil accumulation.

Discussion

To the best of our knowledge, this is the first study that has evaluated the function of STAT3 during acute liver injury induced by I/R. STAT3 has been implicated as a key protective mediator of the protective effects resulting from ischemic preconditioning in the liver (8,12,13). That study demonstrated that STAT3 and IL-6 were increased after ischemic preconditioning and protected the liver from hepatic I/R injury and that the hepatoprotection and activation of STAT3 was dependent upon IL-6. Earlier studies have shown that IL-6 is protective against acute I/R injury and promote hepatocyte proliferation (7,8). Based on these previous findings, we hypothesized that STAT3 represented an endogenous protective mechanism that would prevent uncontrolled inflammation and regulate the injury response.

Surprisingly, we found no evidence that STAT3 is protective during the acute hepatic injury caused by I/R. These results are important as they contrast with the earlier studies of IL-6 (7,8), and therefore suggest that the protective effects of IL-6 on acute I/R injury are unrelated to STAT3 activation. While our study did not directly address the mediator(s) responsible for induction of STAT3 activation, and therefore cannot conclude whether or not it was due to IL-6, what our study does demonstrate is that STAT3 activation does not regulate the acute injury response after I/R. This suggests that the protective effects of IL-6 may be mediated by a different signaling pathway. IL-6 is known to activate other signaling pathways in hepatocytes that are independent of STAT3, including mitogen activated protein kinases, protein kinase C, and peroxisome proliferator activated receptor-δ (14,15). Interrogation of the roles of these pathways in acute I/R injury is warranted.

Another interesting finding of this study was that blockade of STAT3 with STATTIC resulted in reduced neutrophil accumulation after 24 hours of reperfusion. These findings are similar to those in models of renal I/R and glomerular nephrology in which blockade of STAT3 resulted in reduced recruitment of inflammatory cells (16,17). Those studies showed that the reduced inflammation was associated with decreased expression of chemokines and adhesion molecules at the site of injury. Other studies have demonstrated that STAT3 positively regulates chemokine production in macrophages (18). Taken together with our current data, the observed reduction in neutrophil recruitment may be due to effects of STATTIC on hepatic production of neutrophil chemoattractants or perhaps vascular expression of adhesion molecules. Despite this possibility, the reduced neutrophil

recruitment observed in the present study with STATTIC treatment did not alter the injury response and therefore is likely not relevant to the development of I/R injury. It may, however, be more relevant to the recovery and healing response occurring over days after I/R. We have previously shown that this recovery response is associated with activation of STAT3 (19).

In summary, the present study demonstrates that STAT3 activation during acute I/R injury does not regulate the injury response, but may be involved in the recruitment of neutrophils. These findings provide additional information regarding the divergent roles of STAT3 in liver injury and repair/regeneration. It appears that STAT3 is not a significant player in the injury response, but is essential for recovery and regeneration.

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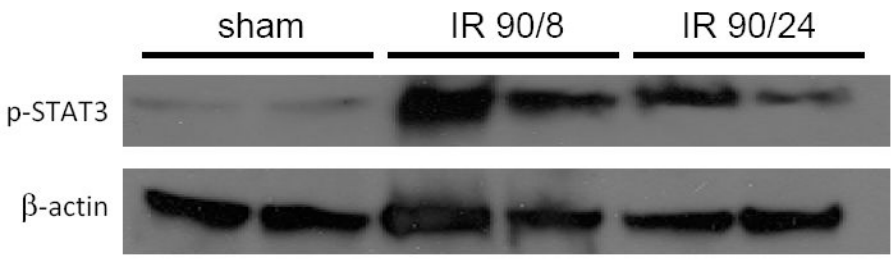

Figure 1.

Hepatic activation of STAT3 after I/R. Liver nuclear extracts from sham-operated mice showed negligible phosphorylated-STAT3. After 90 minutes of partial hepatic ischemia and 8 or 24 hours of reperfusion (IR 90/8 and IR90/24, respectively), abundant amounts of phosphorylated-STAT3 was found in nuclear extracts. β-actin was stained as a loading control.

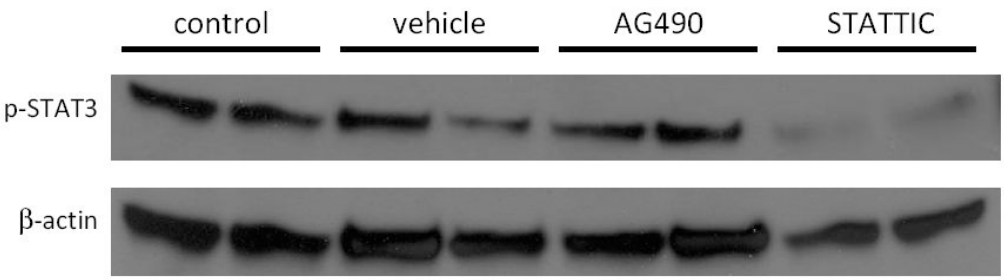

Figure 2.

Pharmacologic blockade of STAT3 activation during I/R injury. Liver nuclear extracts from mice undergoing 90 minutes of partial hepatic ischemia and 24 hours of reperfusion and treated with nothing (control), vehicle, AG490, or STATTIC, were analyzed for STAT3 activation by Western blot. Abundant phosphorylated-STAT3 was observed in control and vehicle groups. Treatment with AG 490 resulted in partial inhibition of STAT3 activation, whereas STATTIC was much more effective in blocking STAT3 activation. β-actin was stained as a loading control.

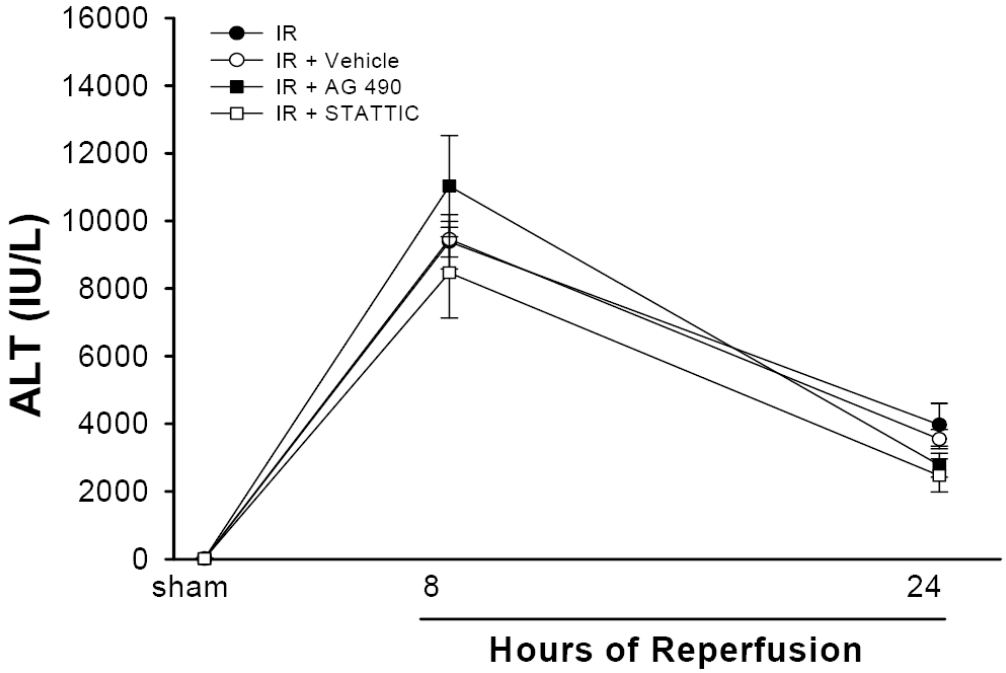

Figure 3.

Effect of STAT3 blockade on acute hepatic I/R injury. Liver injury in mice undergoing 90 minutes of partial hepatic ischemia and 24 hours of reperfusion and treated with nothing (IR), vehicle, AG490, or STATTIC, was evaluated by measuring serum ALT. Data are mean ± SEM with n=4 per group.

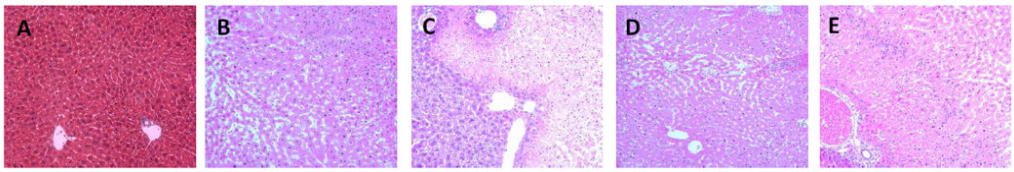

Figure 4.

Effect of STAT3 blockade on liver histolopathology. Liver sections from sham-operated mice showed normal hepatic architecture (A). After 8 hours of reperfusion, livers from control mice had the typical large areas of hepatocellular necrosis with neutrophil accumulation (B). Livers from mice treated vehicle (C), AG 490 (D), or STATTIC (E), all had similar degrees of hepatocellular necrosis. Original magnification was 40X.

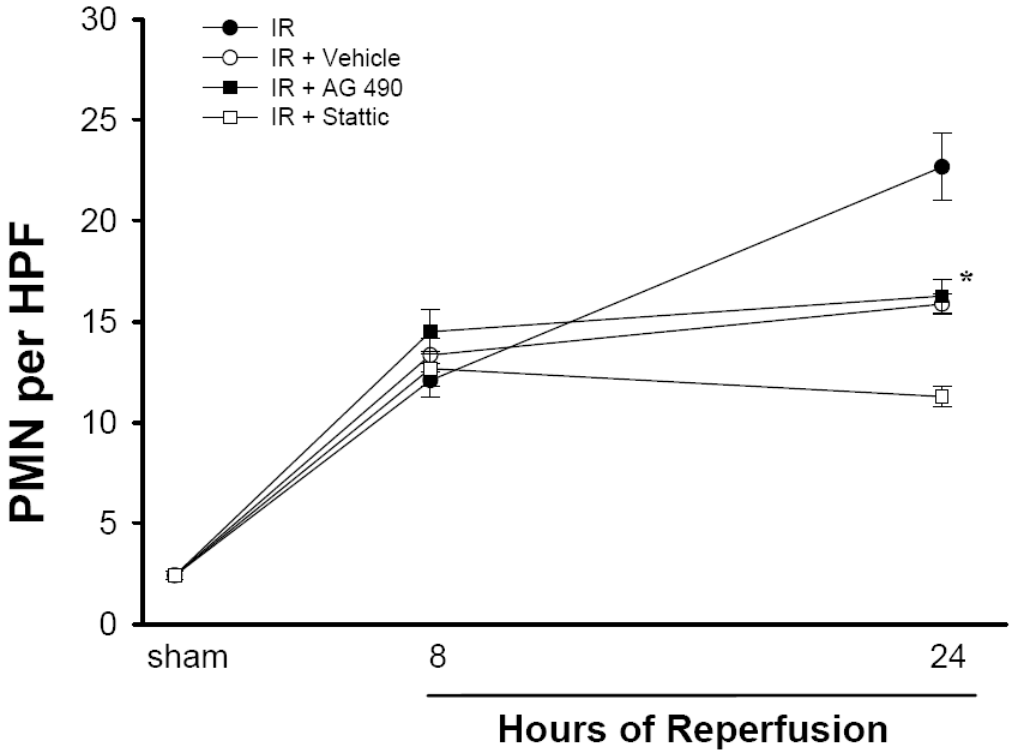

Figure 5.

Effect of STAT3 blockade on neutrophil accumulation during acute I/R injury. Neutrophil accumulation in mice undergoing 90 minutes of partial hepatic ischemia and 24 hours of reperfusion and treated with nothing (IR), vehicle, AG490, or STATTIC, was determined by counting the number of neutrophils per high power field (HPF; 40X). Neutrophil accumulation progressively increased in positive control mice (IR). Treatment with either vehicle or AG490 resulted in a significant decrease in neutrophil accumulation after 24 hours of reperfusion. Treatment with STATTIC resulted in a further reduction in neutrophil accumulation. Data are mean ± SEM with n=4 per group. *P<0.05 compared to control (IR) group. †P<0.05 compared to control (IR) and vehicle (IR+vehicle) groups.

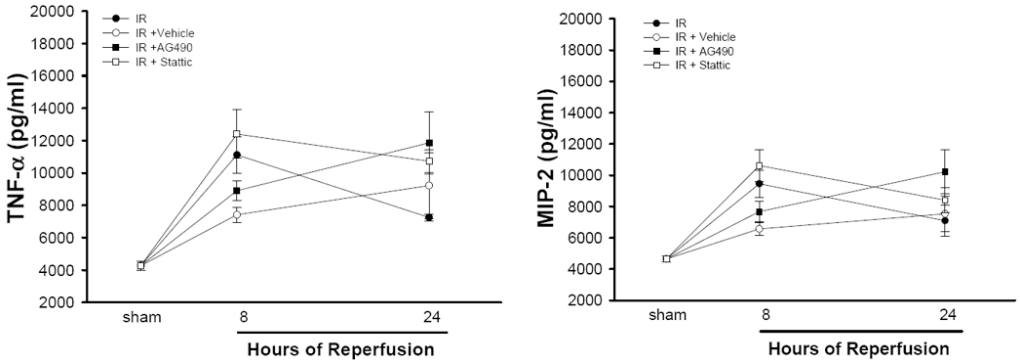

Figure 6.

Effects of STAT3 blockade on liver expression of TNF-α and MIP-2 after hepatic I/R. Liver tissue samples were analyzed by ELISA. Values are mean ± SEM with n=4 per group.

Table 1

Degree of liver injury as assessed by the Suzuki classification system.

	Sinusoidal Congestion (0-4)	Hepatocyte Necrosis (0-4)	Ballooning Degeneration (0-4)
Sham	**0**	**0**	**0**
I/R 8hr	3.5±0.6	3.0±0.8	3.5±0.6
I/R 8hr + vehicle	3.5±1.2	3.8±1.4	2.3±1.1
I/R 8hr + AG490	3.8±0.5	3.3±0.5	2.5±0.6
I/R 8hr + STATTIC	3.8±0.5	3.8±0.5	2.3±0.5
I/R 24hr	2.5±0.7	2.5±0.7	2.0.±0.1
I/R 24hr + vehicle	2.6±0.5	2.8±0.4	2.2±0.4
I/R 24hr + AG490	2.4±0.5	3.2±0.8	2.6±0.5
I/R 24hr + STATTIC	2.2±0.4	3.0±0.9	2.2±0.8

Data are expressed as the mean ± SEM with n=4 per group.