Protection against acute kidney injury via A₁ adenosine receptor-mediated Akt activation reduces liver injury after liver ischemia and reperfusion in mice

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A1 adenosine receptors reduce renal injury after hepatic IR

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Text pages: 39
Number of tables: 1
Number of figures: 12
Number of references: 47
Number of words in the Abstract: 230
Number of words in the Introduction: 543
Number of words in the Discussion: 1365

Recommended section assignment
Gastrointestinal, Hepatic, Pulmonary, and Renal

List of non-standard abbreviations
A1AR = A1 adenosine receptor, AKI = acute kidney injury, Akt = protein kinase B, CCPA = 2-chloro-N6-cyclopentyladenosine, DMSO = dimethylsulfoxide, DPCPX = 8-cyclopentyl-1,3-dipropylxanthine, EGFP = enhanced green fluorescent protein, IR = ischemia reperfusion, HIF = hypoxia inducible factor, KW-3902 = 8-(noradaman-3-yl)-1,3-dipropylxanthine, ERK MAPK = extracellular signal-regulated kinase mitogen activated protein kinase, PD98059 = 2′-amino-3′-methoxyflavone
Abstract

Hepatic ischemia reperfusion (IR) injury causes acute kidney injury (AKI). However, the contribution of AKI to the pathogenesis of liver IR injury is unclear. Furthermore, controversy still exists regarding the role of A1 adenosine receptors (A1AR) in AKI. In this study, we determined whether exogenous and endogenous A1AR activation protects against AKI with subsequent liver protection after hepatic IR in mice. We found that after hepatic IR, A1 knockout (KO) mice and A1AR antagonist-treated A1 wild type (WT) mice developed worse AKI and liver injury compared to vehicle-treated A1WT mice. Moreover, a selective A1AR agonist protected against hepatic IR induced AKI and liver injury in A1WT mice. Renal A1AR-mediated kidney protection plays a crucial role in protecting the liver after IR as: 1) selective unilateral renal lentiviral overexpression of human A1ARs (EGFP-huA1AR ) in A1KO mice protected against both kidney and liver injury sustained after liver IR, 2) removing the EGFP-huA1AR lentivirus injected kidney from A1KO mice abolished both renal and hepatic protection after liver IR and 3) bilateral nephrectomy before hepatic ischemia abolished the protective effects of A1AR activation in A1WT mice. Finally, inhibition of Akt but not ERK MAPK prevented the kidney and liver protection afforded by A1AR agonist treatment. Taken together, we show that endogenous and exogenous activation of renal A1ARs protect against liver and kidney injury following liver IR in vivo via pathways involving Akt activation.
Introduction

Hepatic ischemia and reperfusion (IR) is a frequent cause of acute liver failure during the perioperative period and occurs frequently after major liver resection or liver transplantation (Lee, et al., 2009, Davis, et al., 2002). Acute kidney injury (AKI) is common in patients who sustain hepatic IR injury and the development of AKI in addition to liver injury greatly increases mortality and morbidity during the perioperative period (Davis, et al., 2002). We recently developed a murine model of liver IR induced AKI characterized by early renal endothelial cell death and severe renal vascular impairment with subsequent renal inflammation due to cytokine and neutrophil infiltration, filamentous (F)-actin degradation and proximal tubular necrosis (Lee, et al., 2009).

Our laboratory also previously demonstrated that exogenous and endogenous A1 adenosine receptor (AR) activation protected against direct renal IR injury in vivo (Joo, et al., 2007, Lee, et al., 2004, Lee and Emala, 2001, Lee and Emala, 2000). We also demonstrated that selective renal expression of human A1ARs (huA1AR) via lentiviral gene delivery attenuated renal IR injury in mice lacking A1ARs (Kim, et al., 2009). Furthermore, we showed that A1AR activation can modulate liver IR injury in mice (Kim, et al., 2008). However, other investigators have reported that a nonselective AR antagonist (theophylline) or selective A1AR antagonists (DPCPX, KW-3902) improved renal function, urine output and renal hemodynamics against direct renal injury induced by insults such as cisplatin, gentamicin or glycerol (Yao, et al., 1994, Heidemann, et al., 1989, Bowmer, et al., 1986, Kellett, et al., 1989). Therefore, the role of A1ARs in renal injury remains controversial and furthermore, it is in unknown whether A1ARs protect
against renal injury induced by remote liver injury following liver IR. In addition, it is unclear whether the renal protective effect of renal A₁AR activation directly contributes to the reduction of liver injury after hepatic IR.

Activation of A₁ARs in renal proximal tubule cells and vascular endothelial cells initiates several cytoprotective kinase signaling cascades including ERK mitogen activated protein kinase (MAPK) and Akt (Joo, et al., 2007). Since ERK MAPK and Akt signaling pathways are known to protect against endothelial cell apoptosis (Buckley, et al., 1999, Kennedy, et al., 1999) and since hepatic IR induced AKI directly causes renal endothelial cell apoptosis with subsequent vascular dysfunction and neutrophil infiltration (Lee, et al., 2009), we hypothesized that the A₁AR-mediated activation of ERK MAPK and Akt signaling pathways may protect against renal endothelial cell apoptosis and reduce AKI after liver IR.

In this study, we sought to further elucidate the role of the renal A₁AR activation in attenuating renal and hepatic injury due to hepatic IR. We used A₁AR knockout (A₁KO) mice in addition to the pharmacological manipulation of A₁ARs with a selective agonist and a selective antagonist in A₁AR wild type (A₁WT) mice. We also achieved selective renal expression of human A₁ARs (huA₁ARs) in the kidneys of A₁KO mice. We tested the following hypotheses: 1) Genetic deletion or pharmacologic blockade of A₁ARs in mice would exacerbate AKI after hepatic IR, 2) pre-ischemic activation of A₁ARs would protect against AKI after hepatic IR in A₁WT mice and 3) A₁AR-mediated protection against hepatic IR-induced AKI is via activation of pre-existing cytoprotective kinases including ERK MAPK and Akt. We also tested the hypothesis that renal protection with A₁AR activation is directly responsible for the hepatic protection after
liver IR via 2 approaches: 1) We bilaterally nephrectomized mice to determine whether the hepatic protection with A1AR agonist treatment is attenuated or eliminated in these cohorts of mice and 2) We determined whether selective renal expression of huA1ARs in A1KO mice would reduce both kidney and liver injury after liver IR.
Methods

Detailed methods describing mice, surgery and anesthesia protocols, immunohistochemistry and RNA isolation are available as On Line Supplementary Information.

Murine model of hepatic IR.

After Columbia University IACUC approval, male A1WT or A1KO mice (20-25g) were subjected to partial 60 min liver IR as described previously (Lee, et al., 2009, Kim, et al., 2008). To determine the role of exogenous manipulations of A1ARs in hepatic IR injury, some mice were treated with a single dose of a selective A1AR agonist 2-chloro-N6-cyclopentyladenosine (CCPA, 0.1 mg/kg ip.) or a selective A1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.4 mg/kg ip.), 15 min. before hepatic ischemia. CCPA and DPCPX were dissolved first in DMSO and then further diluted in saline for the final DMSO concentration of ~0.5%. Sham operated mice were treated with vehicle, CCPA or DPCPX and were subjected to laparotomy and identical liver manipulations without vascular occlusion. Five and 24 hrs after reperfusion, plasma was collected for the measurement of creatinine and ALT. In separate cohorts of mice, kidneys were collected at 5 hrs after reperfusion to measure the expression of pro-inflammatory mRNA induction and vascular permeability and at 24 hrs after reperfusion to measure vascular permeability, neutrophil infiltration, apoptosis and histological evaluation of renal tubular injury as described below (and On Line Supplementary Information).
In a separate cohort of A1WT mice, we removed both kidneys before liver ischemia to determine whether the renal A1AR activation is directly responsible for reducing liver and kidney injury after liver IR. Preliminary studies demonstrated that mice subjected to bilateral nephrectomy and 60 min. of liver ischemia had significantly worse liver injury with high mortality. These findings support the hypothesis that impaired or lack of renal function increases hepatic injury in mice. Therefore, in binphrectomized mice, we reduced the hepatic ischemia time to 45 min.

**Intra-renal lentivirus delivery in vivo in A1KO mice**

Generation of lentivirus encoding EGFP or EGFP-huA1AR and in vivo transduction was described previously (Kim, et al., 2009) ([Online Supplementary Information](#)). We utilized 3 techniques to detect the expression of EGFP or EGFP-huA1AR in the kidney and liver after intra-renal injection of lentivirus; 1) direct visualization of EGFP in frozen sections, 2) immunohistochemistry for huA1ARs and 3) RTPCR for the EGFP-A1AR transgene in the liver and kidney tissues as described previously (Kim, et al., 2009). Two days after intrarenal injection of lentivirus encoding EGFP (100 μl) or EGFP-huA1AR (20 μl or 100 μl) into the left kidney of A1KO mice, we induced liver IR injury. In some mice, we removed the EGFP or EGFP-huA1AR lentivirus injected left kidney before liver ischemia to determine whether the EGFP-huA1AR overexpressing kidneys are directly responsible for reducing liver and kidney injury after liver IRI.

**Plasma ALT activity and creatinine level**
The plasma ALT activities were measured using the Infinity™ ALT assay kit according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA). Plasma creatinine was measured by an enzymatic creatinine reagent kit according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA). This method of creatinine measurement largely eliminates the interferences from mouse plasma chromagens well known to the Jaffe method (SLOT, 1965).

Enzyme Linked ImmunoSorbent Assay for plasma TNF-α and IL-6 after liver IR

Twenty-four hrs after liver reperfusion, the plasma TNF-α and IL-6 levels were measured with mouse specific ELISA kits according to the manufacturer’s instructions (eBiosciences, San Diego, CA).

Histological analysis of renal injury

For histological preparations, kidney tissues were fixed in 10% formalin solution overnight. After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin-eosin (H&E). Renal H&E sections were evaluated for the severity of renal proximal tubule injury in the cortico-medullary junction by counting the number of hypereosinophilic (necrotic) cells in 100X fields by an experienced pathologist (VDD) who was blinded to the treatment each animal had received as described previously (Lee, et al., 2009).

Assessment of kidney inflammation
Kidney inflammation was determined by the detection of neutrophil infiltration by immunohistochemistry 24 hrs after hepatic IR and by measuring mRNA encoding markers of inflammation, including keratinocyte derived cytokine (KC), intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractive protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor-alpha (TNF-α) 5 hrs after liver IR as described previously (Lee, et al., 2009) (On Line Supplementary Information).

Assessment of kidney and liver vascular permeability

Changes in kidney and liver vascular permeability were assessed by quantitating extravasation of Evans blue dye (EBD) into the tissue as described by Awad et al. (Awad, et al., 2006) with minor modifications (Lee, et al., 2009) (On Line Supplementary Information).

Detection of kidney apoptosis

We utilized in situ Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End-Labeling (TUNEL) assay and DNA laddering assay to detect renal apoptosis after liver IR as described previously (Chen, et al., 2008, Chen, et al., 2009) (On Line Supplementary Information).

F-actin staining of kidney sections
As breakdown of F-actin occurs early after IR, we visualized the F-actin cytoskeleton by staining with phalloidin as an early index of renal injury (Molitoris, 1997) (On Line Supplementary Information).

Potential roles of ERK MAPK and Akt in A1AR-mediated renal protection after liver IR

Inhibitors of ERK MAPK (PD98059) and Akt (wortmannin) signaling intermediates were used in this protocol. The doses of inhibitors of PD98059 and wortmannin were selected based on previous in vivo studies (Joo, et al., 2006, Joo, et al., 2007). In addition, we performed preliminary experiments to demonstrate that the dosage and method of administration of PD98059 and wortmannin we used effectively blocked the phosphorylation of ERK and Akt in vivo, respectively (Joo, et al., 2007). To test the hypothesis that ERK MAPK and/or Akt participate in A1AR-mediated protection against liver IR induced AKI, we pretreated the mice with PD98059 (an inhibitor of MEK1 to inhibit ERK phosphorylation, 1 mg/kg, i.p.) or wortmannin (an inhibitor of PI3K to inhibit Akt phosphorylation, 1 mg/kg, i.p.) 15 min. before CCPA injection.

Protein determination and reagents

Protein contents were determined with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as a standard. Unless otherwise specified, all chemicals were obtained from Sigma (St. Louis, MO).

Statistical analysis
The data were analyzed with t-tests when means between two groups were compared or with one-way (e.g., plasma creatinine or ALT) ANOVA plus Tukey post hoc multiple comparison test to compare mean values across multiple treatment groups. The ordinal values of the kidney injury scores were analyzed by the Kruskal-Wallis nonparametric test with Dunn posttest comparison between groups. In all cases, $P < 0.05$ was taken to indicate significance. All data are expressed as mean ± SEM.
Results

A1AR modulation affects renal and hepatic function after liver IR injury

A1WT and A1KO mice that underwent sham operations had similar baseline renal and hepatic function (Fig. 1). Our model of hepatic IR resulted in severe kidney dysfunction 24 hrs after reperfusion indicated by significant increases in plasma creatinine levels as described previously (Kim, et al., 2008, Lee, et al., 2009). However, 24 hrs after hepatic IR injury the A1KO mice had significantly higher plasma Cr and ALT compared to the A1WT mice (Fig. 1). A1WT mice pretreated with DPCPX before hepatic ischemia also had significantly higher Cr and ALT at 24 hrs compared to vehicle-treated A1WT mice subjected to liver IR (Fig. 1). Exogenous A1AR activation with CCPA treatment protected the A1WT mice against AKI and liver injury after hepatic IR injury (Fig. 1). We determined that DPCPX- or CCPA-treated A1KO mice subjected to IR had similar Cr and ALT compared to the A1KO mice subjected to liver IR alone confirming the in vivo selectivity of these drugs for the A1AR (Fig. 1). Injection of CCPA or DPCPX alone without hepatic IR (CCPA-Sham or DPCPX-Sham) had no effect on renal or hepatic function (Fig. 1).

Bilateral nephrectomy before hepatic ischemia abolishes CCPA-mediated hepatic protection after liver IR

Pilot studies demonstrated that mice subjected to bilateral nephrectomy and 60 min. of liver ischemia had significantly worse liver injury (ALT=29587±4252 U/L, N=6) with high mortality (50%). These findings support the hypothesis that the kidneys modulate liver injury after hepatic IR in mice. Therefore, in mice subjected to bilateral
nephrectomy, we reduced the hepatic ischemia time to 45 min. Bilateral nephrectomy caused significant rises in plasma Cr in both vehicle- (Cr=3.1+0.2 mg/dL, N=5) and CCPA-treated A\textsubscript{1}WT mice (Cr=3.1+0.1 mg/dL, N=5) in 24 hrs. In addition, bilateral nephrectomy before 45 min. of hepatic ischemia caused significant liver injury (ALT=21086±1594 U/L, N=5) 24 hr after reperfusion. Furthermore, bilateral nephrectomy before 45 min. of hepatic ischemia abolished the hepatic protective effects of CCPA (ALT=26212±1802 U/L, N=5, P=0.073 vs. bilateral nephrectomy and 45 min. hepatic IR) demonstrating that the renal activation of A\textsubscript{1}AR is critical in producing both renal as well as hepatic protection after liver IR.

**Unilateral renal injection of EGFP-huA\textsubscript{1}AR lentivirus in A\textsubscript{1}KO mice protects against hepatic and renal injury after liver IR**

We demonstrated previously that selective in vivo renal expression of EGFP or EGFP-huA\textsubscript{1}AR after intra-renal lentiviral gene delivery in mice is possible without major expression in the contralateral kidney or in the liver (Kim, et al., 2009). We confirmed selective left renal expression of EGFP-huA1AR via 1) direct visualization of EGFP in frozen sections of the injected kidney, contralateral kidney or liver, 2) immunohistochemistry for huA\textsubscript{1}ARs and 3) RTPCR for the EGFP-A\textsubscript{1}AR transgene in the liver and kidney tissues (data not shown) as we described previously (Kim, et al., 2009).

The average plasma level of ALT and Cr in the sham-operated A\textsubscript{1}KO mice renally-injected with lentivirus encoding EGFP or EGFP-huA\textsubscript{1}AR was similar to the levels obtained from A\textsubscript{1}KO sham-operated mice not renally injected with lentivirus (Fig. 2). In A\textsubscript{1}KO mice renally injected with EGFP lentivirus, the plasma level of Cr and ALT
significantly increased at 24 hrs after liver IR. The increases in ALT and Cr were significantly suppressed in A1KO mice renally injected (48 hrs prior to liver IR) with EGFP-huA1AR lentivirus in a dose-dependent manner at 24 hrs after liver IR.

Removing the EGFP-huA1AR lentivirus injected kidney before hepatic ischemia abolishes both renal and hepatic protection after liver IR in A1KO mice.

Unilateral nephrectomy of the EGFP-huA1AR lentivirus (100 μl) injected kidney before hepatic ischemia completely abolished the hepatic (ALT=22447±2544 U/L, N=4) and renal (Cr=1.26±0.07 mg/dL, N=4) protective effects of renal huA1AR overexpression in A1KO mice (previously shown in Figure 2) demonstrating that the overexpression of huA1AR in the injected kidney plays a crucial role in protecting the kidney and liver after liver IR. Unilateral nephrectomy of the EGFP lentivirus injected kidney in A1KO mice before hepatic ischemia did not significantly change the hepatic (ALT=19356±1032 U/L, N=4) and renal injury (Cr=1.37±0.07 mg/dL, N=4) compared to the A1KO mice with 2 intact kidneys 24 hr after reperfusion (Figure 3).

A1AR modulation affects plasma TNF-α and IL-6 after liver IR

In sham-operated animals, the plasma TNF-α (N=4) and plasma IL-6 (N=4) levels were very low (Fig. 3). The plasma TNF-α (N=6, P<0.01) and IL-6 (N=6, P<0.01) levels increased markedly in A1WT mice 24 hrs after liver IR (Fig. 3). The A1KO mice and DPCPX-treated A1WT mice showed significantly higher plasma TNF-α and plasma IL-6 levels when compared to the A1WT mice 24 hrs after liver IR. In contrast, the A1WT
mice treated with CCPA showed significantly reduced plasma levels of TNF-α (N=5, and IL-6 levels when compared to the A₁WT mice 24 hrs after liver IR.

A₁AR modulation affects renal tubular necrosis after liver IR

Representative kidney histological slides (cortico-medullary junction) from A₁WT mice, A₁KO mice, DPCPX-treated A₁WT mice and CCPA-treated A₁WT mice subjected to 60 min. ischemia and 24 hrs reperfusion and A₁WT mice or A₁KO mice subjected to the sham-operation are shown in Figure 4 (A-F, magnification, 400X). In kidneys from the A₁WT mice subjected to liver IR, we observed multifocal acute tubular injury including S3 segment proximal tubule necrosis indicated by hypereosinophilia (arrow showing a single, hypereosinophilic cell, C). Correlating with significantly worsened renal function, significantly increased numbers of hypereosinophilic necrotic proximal tubules were observed in A₁KO mice and DPCPX-pretreated A₁WT mice subjected to hepatic IR injury compared to the A₁WT mice (D and E). Furthermore, A₁WT mice pretreated with the A₁AR agonist CCPA showed significantly less number of hypereosinophilic necrotic proximal tubule cells (F). The quantifications of hypereosinophilic necrotic proximal tubule cells (400X field) in S3 segment of the kidney were performed. We failed to detect hypereosinophilic necrotic cells from sham-operated mice (A₁WT sham, N=4; A₁KO sham, N=4). The A₁WT mice subjected to hepatic IR resulted in a significant number of hypereosinophilic necrotic renal proximal tubule cells 24 hrs after hepatic IR (8.4±1.1 hypereosinophilic necrotic cells/400X field, N=5, p<0.01 vs. sham). A significantly higher percent of proximal tubule necrosis developed in the A₁KO mice (15.7±2.9 hypereosinophilic necrotic cells/400X field, N=5, p<0.05 vs.
A1WT mice subjected to liver IR) or in DPCPX-pretreated A1WT mice (14.3±2.0 hypereosinophilic cells/400X field, N=5, p<0.05 vs. A1WT mice subjected to liver IR) compared to A1WT mice subjected to IR. A1WT mice pretreated with the A1AR agonist (CCPA) before IR injury showed reduced proximal tubule hypereosinophilic necrotic cells (5.3±0.8 hypereosinophilic cells/400X field, P<0.05 vs A1WT mice subjected to liver IR). Injection of CCPA or DPCPX alone (without hepatic IR) had no effect on kidney histology.

A1AR modulation affects renal pro-inflammatory mRNA expression after liver IR injury

Hepatic IR injury was associated with significantly increased renal pro-inflammatory mRNA expression (ICAM-1, TNF-α, KC, MCP-1 and MIP-2) 5 hrs after hepatic IR. Kidneys from A1KO or A1WT mice treated with DPCPX showed increased expression of all pro-inflammatory mRNAs studied after liver IR (Fig. 5). In contrast, CCPA pretreatment significantly suppressed the increases in pro-inflammatory mRNA expression 5 hrs after hepatic IR. Injection of CCPA or DPCPX alone (without hepatic IR) had no effect on pro-inflammatory gene expression in the kidney.

A1AR modulation affects renal neutrophil infiltration 24 hrs after IR injury

Figures 6 shows representative images (400X magnification) of neutrophil immunohistochemistry of kidney sections from sham-operated A1WT (A) or A1KO mice (B), A1WT or A1KO mice subjected to liver IR (C and D) or A1WT subjected to IR after DPCPX or CCPA pretreatment (E and F). In sham-operated mice, we were unable to detect neutrophils in the kidney (N=4 each). Sixty min. of hepatic ischemia and 24 hrs of
reperfusion resulted in a significant neutrophil recruitment into the kidneys of A1WT mice. In the A1WT mice subjected to liver IR, we detected 9.25±2 neutrophils/field (100X magnification, N=6). The A1KO mice subjected to liver IR injury had significantly higher neutrophil counts (30.3±3.3 neutrophils/field, N=5) compared with the A1WT mice 24 hrs after IR (P<0.05). The A1WT mice pretreated with DPCPX before hepatic IR injury also had increased neutrophil infiltration (31.3±6.7 neutrophils/field, N=5) compared to the A1WT mice 24 hrs after IR (P<0.05). In contrast, the A1WT mice pretreated with an A1AR agonist (CCPA) and subjected to hepatic IR injury had significantly reduced neutrophil infiltration (1.1±1 neutrophils/field, N=5). Injection of CCPA or DPCPX alone (without hepatic IR) had no effect on neutrophil infiltration into the kidney.

A1AR modulation affects the severity of renal apoptosis after liver IR injury

Sham-operated A1WT or A1KO mice did not exhibit DNA laddering in kidneys (representative of 4 experiments, Fig. 7). However, 60 min. of hepatic ischemia and 24 hrs of reperfusion resulted in DNA fragmentation in the kidneys of A1WT mice (Fig. 7). The A1KO mice subjected to liver IR showed increased renal DNA laddering in kidneys compared to the A1WT mice subjected to liver IR. Moreover, DNA from mice pretreated with the A1AR antagonist (DPCPX) or agonist (CCPA) displayed increased or decreased fragmentation in the kidneys, respectively compared to the A1WT mice subjected to liver IR. Sham-operated mice demonstrated few TUNEL-positive cells in the kidneys 24 hrs after hepatic ischemia (Fig. 8A and 8B). The TUNEL staining (representative of 4 experiments) showed that endothelial cell apoptosis was predominant in the kidney after
60 min hepatic ischemia and 24 hrs of reperfusion (Fig. 8C-F). However, the degree of endothelial apoptosis in A1KO mice was significantly greater at 24 hrs after reperfusion (Fig. 8D). Moreover, DPCPX-pretreated A1WT mice showed increased number of TUNEL positive cells compared to A1WT mice (Fig. 8E) whereas the A1WT mice pretreated with the A1AR agonist (CCPA) and subjected to IR injury resulted in reduced TUNEL positive endothelial cells (Fig. 8F). Injection of CCPA or DPCPX alone (without hepatic IR) had no effect on renal endothelial apoptosis.

**A1AR modulation affects renal and hepatic vascular permeability 5 hrs after liver IR**

Analyses of EBD extravasations in mice subjected to sham-operation or liver IR are shown in Figure 9. The increase in EBD contents were significantly higher for A1KO mice and A1WT mice treated with DPCPX and subjected to liver IR. In contrast, the A1WT mice treated with CCPA before liver IR had significantly reduced EBD extravasation 5 hrs after liver IR. Injection of CCPA or DPCPX alone (without hepatic IR) had no effect on renal and hepatic vascular permeability.

**A1AR modulation affects the degradation of renal F-actin after liver IR**

In Figure 10, 24 hrs post-hepatic IR induced disruptions of the F-actin cytoskeleton in renal tubular epithelial cells are shown. A1WT or A1KO mice subjected to sham surgery showed intense staining in tubular epithelial and in the basal plasma membrane (Fig. 10A and 10B). In contrast, kidneys from A1WT mice subjected to liver IR showed loss of F-actin staining in the tubular epithelial cells (Fig. 10C). In addition, the A1KO mice and the A1WT mice treated with DPCPX and subjected to liver IR
showed even more loss of F-actin integrity (Fig. 10D and 10F). In contrast, the A1WT mice treated with CCPA before liver IR show significantly better preserved F-actin structure after liver IR as the staining is quite similar to that of sham-operated mice (Fig. 10E). Mean fluorescent intensity analysis for proximal tubule F-actin is shown in Figure 11. Injection of CCPA or DPCPX alone (without hepatic IR) had no effect on renal F-actin integrity.

Signaling pathways of A1AR-mediated renal protection: critical role for the Akt pathway

We probed the renal protective signaling pathways activated by acute A1AR activation in mice subjected to liver IR. We have demonstrated previously (Joo, et al., 2007) that acute A1AR activation resulted in rapid phosphorylation of ERK MAPK and Akt in A1WT but not in A1KO mice. To determine whether ERK and/or Akt phosphorylation mediate the cytoprotective signaling of acute A1AR activation mediated renal protection after hepatic IR, A1WT mice were pretreated with PD98059 (a MEK1 inhibitor) or wortmannin (a PI3K inhibitor) prior to CCPA treatment. We have demonstrated previously that the doses of PD98059 and wortmannin used effectively blocked phosphorylation of ERK and Akt, respectively, in mice in vivo (Joo, et al., 2007, Joo, et al., 2006). We found that the inhibition of PI3K but not MEK1 prevented the renal as well as hepatic protection with acute A1AR activation after hepatic IR (Fig. 12). Although the dose of PD98059 was effective in inhibiting MEK1 (Joo, et al., 2006), inhibition of ERK phosphorylation did not prevent the renal protection with acute A1AR activation. Inhibitors alone had no effect on renal or hepatic function (Fig. 12) after IR injury.
Discussion

We demonstrate in this study that renal A1AR activation is directly responsible for both hepatic and renal protection after liver IR in mice as 1) removing both kidneys prior to liver IR completely abolished the hepatic and renal protective effects observed in A1WT mice, 2) selective renal expression of huA1ARs in A1KO mice reduced both kidney and liver injury after liver IR and 3) removing the EGFP-huA1AR lentivirus injected kidney prior to liver IR in A1KO mice abolished the hepatic and renal protective effects of huA1AR expression. In addition, 1) mice deletionally lacking the A1ARs (A1KO mice) or A1WT mice pre-treated with an A1AR antagonist (DPCPX) demonstrate greater renal injury, 2) kidneys from A1KO or DPCPX-pretreated A1WT mice showed increased tubular necrosis (hypereosinophilia), apoptosis (DNA laddering and TUNEL staining) and inflammation (neutrophil infiltration and pro-inflammatory mRNA and cytokine expression), 4) exogenous activation of A1ARs with CCPA before hepatic ischemia attenuated renal injury in A1WT mice and 5) blocking Akt prevented the A1AR-mediated renal protection after hepatic IR.

AKI due to hepatic IR injury results in rapid, immediate apoptotic destruction of renal endothelial cells with subsequent individual cell necrosis of proximal tubules and interstitial neutrophil infiltration and inflammation (Lee, et al., 2009). This is clinically significant as hepatic IR is a frequent cause of AKI during the perioperative period and the incidence of renal dysfunction after major liver surgery or liver transplantation approaches 40-80% (Betrosian, et al., 2007, Davis, et al., 2002). Furthermore, our model of hepatic IR induced AKI appears to resemble clinically observed AKI in humans manifesting as combination of apoptosis, necrosis and inflammation. Hepatic IR induced
murine AKI model may lead to development of pharmacological therapies capable of attenuating endothelial cell apoptosis, renal tubular necrosis/inflammation and cytokine-mediated attack observed in human AKI.

Release of adenosine after stress (e.g., hypoxia, IR) with subsequent activation of ARs has been proposed to auto-protect against cell death in several cell types (Dinour and Brezis, 1991, Walsh, et al., 1995). Indeed, recent elegant studies combining genetic deletion as well as pharmacological inhibition have demonstrated that ecto-5'-nucleotidase (CD73), the enzyme involved in extracellular adenosine production by converting AMP to adenosine, is critical for hepatic (Hart, et al., 2008) and renal protection (Grenz, et al., 2007) by ischemic preconditioning in mice. Furthermore, Eltzschig, et al. have demonstrated that diminished adenosine uptake via HIF-1 dependent repression of equilibrative nucleoside transporter types 1 and 2 greatly enhances the extracellular adenosine levels (Eltzschig, et al., 2005, Morote-Garcia, et al., 2009). These studies imply that endogenous adenosine production is critical in protecting against hypoxia or ischemia induced organ injury. Previous studies also have demonstrated that activation of cell surface A1ARs, in particular, produces cytoprotective effects against IR injury in many organ systems including the heart, kidney and brain (Kim, et al., 2009, Lankford, et al., 2005, Lee, et al., 2004, Heurteaux, et al., 1995). Mechanistically, A1AR activation produces several cellular effects that are suited to attenuate the multifaceted pathophysiology of AKI (endothelial and renal tubular cell apoptosis, inflammation and necrosis).

Complicating the issues however, is that several investigators have reported that a nonselective AR antagonist (theophylline) or selective A1AR antagonists (DPCPX, KW-
3902) improved renal function, urine output and renal hemodynamics in several models of nephrotoxin AKI induced by cisplatin, gentamicin or glycerol. (Yao, et al., 1994, Bidani and Churchill, 1983). In addition, Lin et al. have demonstrated that theophylline increased renal plasma flow and glomerular filtration rate after ischemic renal injury (Lin, et al., 1988). These studies were performed based on the observation that A1AR antagonism increases urine output, solute transport and renal blood flow. Indeed A1AR antagonists reversed these indices of renal injury in toxin- and ischemic-models of ARF (Yao, et al., 1994, Yao, et al., 2000).

We have previously demonstrated that the degree of renal injury is directly proportional with the degree of hepatic injury after liver IR (Lee, et al., 2009). We have also previously demonstrated that A1KO mice as well as A1WT mice treated with a selective A1AR antagonist (DPCPX) developed significantly worse liver injury (alanine aminotransferase, liver necrosis, neutrophil infiltration, and apoptosis) compared to the A1WT mice 24 hrs after liver IR injury (Kim, et al., 2008). Taken together, our previous as well as current findings imply that endogenous as well as exogenous A1AR plays an important role in hepatic and renal protection. Furthermore, we now show in this study that renal A1AR activation is directly responsible for both hepatic and renal protection after liver IR in mice by 3 direct experimental data: 1) Removing the kidneys prior to liver IR completely abolished the hepatic and renal protective effects CCPA in A1WT mice, 2) Selective expression of huA1ARs in A1KO mice decreases AKI as well as liver injury and 3) Removing the EGFP-huA1AR lentivirus injected kidney prior to liver IR in A1KO mice abolished the hepatic and renal protective effects of huA1AR expression. We confirmed that systemic spill over of EGFP-huA1AR lentivirus (to the contralateral
kinder and/or to the liver) cannot explain the hepatic and renal protective effects with EGFP-huA₁AR lentivirus injection. Instead, we propose that the direct EGFP-huA₁AR-mediated reduction in AKI provided hepatic protection after IR. Taken together, our findings imply that liver IR-mediated AKI potentiates the liver injury further and protecting the kidney reduces liver damage after IR.

Acute renal protection after hepatic IR with A₁AR activation is mediated by Akt activation as inhibition of phosphoinositide 3-kinases with wortmannin prevented CCPA-mediated renal protection after hepatic IR. The serine/threonine kinase Akt is an important component of cell survival pathways in many cell types (Hausenloy, et al., 2004, Hausenloy, et al., 2005). In particular, Akt has diverse functions to counteract apoptosis including inhibition of mitochondrial cytochrome c and phosphorylation of several pro-apoptotic factors (e.g., bad, caspase 9, glycogen synthase kinase 3) (Cross, et al., 2000). Akt can also increase the activity of HSP27 in certain cell types (Rane, et al., 2001, Rane, et al., 2003, Konishi, et al., 1997) promoting filamentous (F) actin stability. Better preserved F-actin cytoskeleton in the kidneys of mice subjected to liver IR after CCPA treatment may have contributed to reduced renal tubular necrosis and apoptosis observed in these mice.

Our results show that bilateral nephrectomy exacerbates hepatic IR injury in mice. Bilateral nephrectomy induced extra-renal organ dysfunction has been described (Paladino, et al., 2009). Reduced renal cytokine elimination after nephrectomy may contribute to increased plasma and hepatic pro-inflammatory cytokine levels and exacerbate hepatic IR injury. Indeed, increased plasma IL-6 after bilateral nephrectomy contributes to lung injury in mice (Klein, et al., 2008). Taken together, it is possible that
loss of renal cytokine clearance may induce extra-renal organ (e.g., intestine, lung) injury that can potentiate cytokine release further exacerbating systemic inflammatory response.

One of the limitations of this study is that the cell types(s) targeted in the kidney via A1AR activation was not directly elucidated. A1AR signaling has been extensively characterized in various non-immune cells and includes modulation of adenylyl cyclase, protein kinase C, phosphoinositide 3 kinase (PI3K) and ERK MAPKs (Hasko, et al., 2008). Hepatic IR induced AKI is characterized by early renal endothelial cell apoptosis with subsequent proximal tubule necrosis (Lee, et al., 2009). Furthermore, we have previously showed direct in vitro renal tubular protective effects of A1ARs against both anoxic and oxidant induced necrosis (Lee and Emala, 2002a, Lee and Emala, 2002b). Therefore, we propose that both renal endothelial as well as renal tubule cells are targeted by the A1ARs. It remains to be determined in future in vitro studies whether hepatocytes are also directed targeted via A1AR activation.

In summary, we demonstrate in this study that endogenous A1AR activation provides protection against hepatic IR induced AKI injury by reducing endothelial apoptosis, renal proximal tubular necrosis and inflammatory changes. We propose that renal endothelial and/or tubular A1ARs serve to protect against renal insults that occur after hepatic IR via Akt-dependent mechanisms. Given the protective benefit of endogenous and exogenous A1AR activation against hepatic IR induced AKI and that hepatic IR is common in patients after liver surgery, liver transplantation or sepsis, our findings may have important future therapeutic implications. The finding that loss of renal function (after bilateral nephrectomy) potentiated liver injury after IR is an interesting finding that requires further investigation.
References


Foot Notes

This work was supported by National Institute of Health Grant [DK-58547].
Legends for Figures

Figure 1. Comparison of mean plasma creatinine (A) and ALT activity (B) measured from sham-operated and vehicle-treated A1WT (N=4) and A1KO mice (N=4), sham-operated A1WT mice given 0.1 mg/kg CCPA (N=4) or 0.4 mg/kg DPCPX (N=4) injection, A1WT mice (N=6-10) or A1KO mice (N=6-10) pretreated with vehicle, A1WT (N=6-9) or A1KO (N=6) mice pretreated with DPCPX or CCPA and subjected to liver IR. Plasma creatinine and ALT activity were measured at 24 hrs after reperfusion for each mouse. *P<0.05 vs. respective A1WT or A1KO sham group. #P<0.05 vs. A1WT+Vehicle hepatic IR group. Error bars represent 1 SEM.

Figure 2. Comparison of mean plasma creatinine (A, mg/dL) and ALT activity (B, U/L) measured from sham-operated A1WT (N=4) and A1KO mice (N=4), sham-operated A1KO renally injected with EGFP encoding lentivirus (100 μl, N=5), A1WT mice (N=6) or A1KO mice (N=6) subjected to hepatic IR, A1KO mice renally injected with EGFP (N=5) or EGFP-huA1AR encoding lentivirus (20 μl or 100 μl, N=5 each) and subjected hepatic IR. Mice were renally injected with lentivirus 48 hrs prior to sham-surgery or hepatic IR. Plasma ALT and creatinine was measured at 24 hrs after reperfusion. *p<0.05 vs. appropriate sham-operated mice. # p<0.05 vs. A1WT mice subjected to liver IR. +P<0.05 vs. A1KO mice injected with EGFP lentivirus. Error bars represent 1 SEM.

Figure 3. Plasma TNF-α and IL-6 levels (in pg/ml) in sham-operated A1WT (A1WT sham, N=4) and A1KO mice (A1KO sham, N=4), A1WT (A1WT hepatic IR, N=6) or A1KO mice (A1KO hepatic IR, N=5-8) subjected to 60 min. hepatic ischemia and 24 hrs
of reperfusion, A1WT mice pretreated with 0.4 mg/kg DPCPX (A1WT hepatic IR+DPCPX, N=5-8) or with 0.1 mg/kg CCPA (A1WT hepatic IR+CCPA, N=5) and subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion. Data are presented as means ± SEM. *P<0.05 vs. respective A1WT or A1KO sham group. #P<0.01 vs. A1WT hepatic IR group.

Figure 4. Representative (of 4-5 slides) hematoxylin and eosin staining photomicrographs in kidney sections from sham-operated A1WT (A1WT sham, A) and A1KO mice (A1KO sham, B), A1WT (A1WT hepatic IR, C) or A1KO mice (A1KO hepatic IR, D) subjected to 60 min. liver ischemia and 24 hrs of reperfusion, A1WT mice pretreated with 0.4 mg/kg DPCPX (A1WT hepatic IR+DPCPX, E) or 0.1 mg/kg CCPA (A1WT hepatic IR+CCPA, F) and subjected to 60 min. liver ischemia and 24 hrs of reperfusion (magnification 400X, cortico-medullary junction). Hypereosinophilic proximal tubules (arrows) visible in A1WT mice subjected to liver IR are increased in A1KO mice or DPCPX-treated A1WT mice subjected to liver IR.

Figure 5. (A) Representative gel images of semi-quantitative RT-PCR results for GAPDH, murine A1AR, TNF-α, ICAM-1, KC, MCP-1 and MIP-2 mRNAs of kidney tissues from sham-operated A1WT and A1KO mice (A1WT sham, N=3; A1KO sham, N=3), A1WT or A1KO subjected to 60 min. hepatic ischemia and 5 hrs of reperfusion (A1WT hepatic IR, N=6; A1KO hepatic IR, N=5), A1WT mice pretreated with 0.4 mg/kg DPCPX (A1WT hepatic IR+DPCPX, N=5) or 0.1 mg/kg CCPA (A1WT hepatic IR+CCPA, N=5) and subjected to 60 min. hepatic ischemia and 5 hrs of reperfusion. (B)
Densitometric quantification of relative pro-inflammatory mRNA band intensities normalized to GAPDH from RT-PCR reactions. Data are presented as means ± SEM. *P<0.05 vs. respective A1WT or A1KO sham group. #P<0.05 vs. A1WT hepatic IR group.

Figure 6. Representative photomicrographs (of 3-5 experiments) of immunohistochemistry for neutrophils (arrows indicating brown granules) in kidney (400X) sections from sham-operated A1WT (A1WT sham, A) and A1KO mice (A1KO sham, B), A1WT (A1WT hepatic IR, C) or A1KO (A1KO hepatic IR, D) subjected to 60 min. hepatic ischemia and 24 hrs reperfusion, A1WT mice pretreated with 0.4 mg/kg DPCPX (A1WT hepatic IR+DPCPX, E) or 0.1 mg/kg CCPA (A1WT hepatic IR+CCPA, F) and subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion.

Figure 7. Representative gel images (of 4 experiments) demonstrating DNA laddering as an index of DNA fragmentation in the kidney tissues from sham-operated A1WT (A1WT sham) and A1KO mice (A1KO sham), A1WT (A1WT hepatic IR) or A1KO mice (A1KO hepatic IR) subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion, A1WT mice pretreated with 0.4 mg/kg DPCPX (A1WT hepatic IR+DPCPX) or with 0.1 mg/kg CCPA (A1WT hepatic IR+CCPA) and subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion. Apoptotic DNA fragments were extracted according to the methods of Herrmann et al. (Herrmann, et al., 1994). This method of DNA extraction selectively isolates apoptotic, fragmented DNA and leaves behind the intact chromatin.
Figure 8. Representative fluorescent photomicrographs (of 4 experiments) illustrate apoptotic nuclei (TUNEL fluorescent stain, magnification 400X) in kidney sections from sham-operated A1WT (A1WT sham, A) and A1KO mice (A1KO sham, B), A1WT (A1WT hepatic IR, C) or A1KO (A1KO hepatic IR, D) subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion, A1WT mice pretreated with 0.4 mg/kg DPCPX (A1WT hepatic IR+DPCPX, E) or 0.1 mg/kg CCPA (A1WT hepatic IR+CCPA, F) and subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion. In the kidney, endothelial cells predominantly underwent apoptotic death (short, thick arrows) with sparing of renal proximal tubule cells (long, thin arrows) as illustrated in Figure 6D.

Figure 9. Quantification of EBD extravasations as indices of vascular permeability of kidney (A) and liver (B) tissues from sham-operated A1WT (A1WT sham, N=4) and A1KO mice (A1KO sham, N=4), A1WT (A1WT hepatic IR, N=7) or A1KO mice (A1KO hepatic IR, N=7) subjected to 60 min. hepatic ischemia and 5 hrs of reperfusion, A1WT mice pretreated with 0.4 mg/kg DPCPX (A1WT hepatic IR+DPCPX, N=6) or with 0.1 mg/kg CCPA (A1WT hepatic IR+CCPA, N=6) and subjected to 60 min. hepatic ischemia and 5 hrs of reperfusion. Data are presented as means ± SEM. *P<0.05 vs. respective A1WT or A1KO sham group. #P<0.05 vs. A1WT hepatic IR group.

Figure 10. Representative fluorescent photomicrographs (of 4 experiments) of phalloidin staining of the kidney tissues (magnification 400X) from sham-operated A1WT (A1WT sham, A) and A1KO mice (A1KO sham, B), A1WT (A1WT hepatic IR, C) or A1KO mice (A1KO hepatic IR, D) subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion,
A1WT mice pretreated with 0.4 mg/kg DPCPX (A1WT hepatic IR+DPCPX, E) or 0.1 mg/kg CCPA (A1WT hepatic IR+CCPA, F) and subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion. In the kidney, F-actin stains of proximal tubular epithelial cells are prominent in the brush border from sham-operated mice (*) which is severely degraded in the kidneys of mice subjected to liver IR (#).

Figure 11. Quantification of mean renal proximal tubule F-actin intensity in kidney tissues from sham-operated A1WT (A1WT sham) and A1KO mice (A1KO sham), A1WT (A1WT hepatic IR) or A1KO mice (A1KO hepatic IR) subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion, A1WT mice pretreated with 0.4 mg/kg DPCPX (A1WT hepatic IR+DPCPX) or 0.1 mg/kg CCPA (A1WT hepatic IR+CCPA) and subjected to 60 min. hepatic ischemia and 24 hrs of reperfusion.. *P<0.05 vs. respective A1WT or A1KO sham group. #P<0.05 vs. A1WT hepatic IR group.

Figure 12. Plasma creatinine (A, mg/dL) and ALT activity (B, U/L) in A1WT mice after injection with vehicle (A1WT Vehicle hepatic IR) or with 0.1 mg/kg CCPA 15 min. (A1WT CCPA hepatic IR) before 60 min. hepatic ischemia and 24 hrs of reperfusion. Some A1WT mice were pretreated with PD98059 (PD, an inhibitor of MEK1 to inhibit ERK phosphorylation, 1 mg/kg, i.p.) or with wortmannin (an inhibitor of PI3K to inhibit Akt phosphorylation, 1 mg/kg, i.p.) 15 min. before vehicle (A1WT Wort hepatic IR (N=5) or A1WT PD hepatic IR (N=5)) or CCPA treatment (A1WT CCPA+Wort hepatic IR (N=5) or A1WT CCPA+PD hepatic IR (N=5)). Data are presented as means ± SEM. Inhibition of PI3K→Akt pathway but not MEK→ERK MAPK prevents acute A1AR
activation induced renal protection after hepatic IR. *P<0.05 vs. A\textsubscript{1}WT Vehicle hepatic IR group.
Table 1. RT-PCR primers used in this study. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; A1AR, A1 adenosine receptor, ICAM-1, intercellular adhesion molecule-1; KC, keratinocyte derived chemokine, MIP-2, macrophage inflammatory protein 2, MCP-1, monocyte chemoattractant protein 1, TNFα = tumor necrosis factor alpha.

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**Figure 1**

60 min. liver ischemia and 24 hr Reperfusion

**A)**

Creatinine (mg/dL)

- $A_1$ WT+Vehicle
- $A_1$ WT+DPCPX
- $A_1$ WT+CCPA
- $A_1$ KO+Vehicle
- $A_1$ KO+DPCPX
- $A_1$ KO+CCPA

**B)**

ALT (U/L)

- $A_1$ WT+Vehicle
- $A_1$ WT+DPCPX
- $A_1$ WT+CCPA
- $A_1$ KO+Vehicle
- $A_1$ KO+DPCPX
- $A_1$ KO+CCPA
Figure 2

60 min. liver ischemia and Reperfusion

A) Creatinine (mg/dL)

B) ALT (U/L)

A1 WT sham
A1 KO sham
A1 KO EGFP Sham
A1 WT Hepatic IR
A1 KO EGFP Hepatic IR
A1 KO 10 μl huA1 AR EGFP hep IR
A1 KO 20 μl huA1 AR EGFP hep IR
Figure 3  
60 min. liver ischemia and 24 hrs of Reperfusion

[Graph showing plasma TNF-α and IL-6 levels across different treatment groups]
Figure 4

A) A<sub>1</sub>WT Sham

B) A<sub>1</sub>KO Sham

C) A<sub>1</sub>WT hepatic IR

D) A<sub>1</sub>KO hepatic IR

E) A<sub>1</sub>WT hepatic IR + DPCPX

F) A<sub>1</sub>WT hepatic IR + CCPA
Figure 5A

60 min. liver ischemia and 5 hrs of Reperfusion
Figure 5B: 60 min. liver ischemia and 5 hrs of Reperfusion

- **TNF-α mRNA/GAPDH over sham**
- **ICAM-1 mRNA/GAPDH over sham**
- **MCP-1 mRNA/GAPDH over sham**
- **KC mRNA/GAPDH over sham**
- **MIP-2 mRNA/GAPDH over sham**
Figure 6

A) $A_1$ WT Sham

B) $A_1$ KO Sham

C) $A_1$ WT hepatic IR

D) $A_1$ KO hepatic IR

E) $A_1$ WT hepatic IR + DPCPX

F) $A_1$ WT hepatic IR + CCPA
Figure 7

60 min. liver ischemia and 24 hrs of Reperfusion
Figure 9

(A) EBD extravasation (μg EBD/g dry kidney tissue)

(B) EBD extravasation (μg EBD/g dry liver tissue)
Figure 10

A) $A_1$WT Sham  

B) $A_1$KO Sham

C) $A_1$WT hepatic IR  

D) $A_1$KO hepatic IR

E) $A_1$WT hepatic IR + DPCPX  

F) $A_1$WT hepatic IR + CCPA
Figure 11

60 min. liver ischemia and 24 hrs of Reperfusion
Figure 12

60 min. liver ischemia and 24 hrs of Reperfusion