JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

JPET #92122

Title page:

Metformin prevents endotoxin-induced liver injury after partial hepatectomy

Ina Bergheim, James P. Luyendyk, Chad Steele, Gilandra K. Russell, Luping Guo, Robert A. Roth and Gavin E. Arteel

Department of Pharmacology and Toxicology and the James Graham Brown Cancer Center, University of Louisville Health Sciences Center, Louisville, KY, USA (IB, GKR, LG, GEA)

Department of Pharmacology and Toxicology, National Food Safety and Toxicology Center, Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA (JPL, RAR)

Department of Pediatrics, Division of Pulmonology Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA. (CS)

Running Title Page: Metformin and liver failure

Corresponding author:

Gavin E. Arteel

Department of Pharmacology and Toxicology

University of Louisville Health Sciences Center

Louisville, KY, 40292

Phone#: (502) 852-5157

FAX#: (502) 852-3242

e-mail: gavin.arteel@louisville.edu

Text pages: 30

Number of Figures: 8

Number of Tables: 3

Number of references: 40

Abstract: 214 words, **Introduction:** 500 words, **Discussion:** 1049 words

Recommended section assignment: Gastrointestinal, Hepatic, Pulmonary, & Renal

Abbreviations*:* ALP, alkaline phosphatase; AST, aspartate aminotransferase; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-monocyte colony stimulating factor; IFNγ, interferon γ; IL-1α, interleukin 1 α ; IL-1β, interleukin 1 β; IL-2, interleukin 2 ; IL-4, interleukin 4; IL-6, interleukin 6; IL-10, interleukin 10; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; Metformin, 2-(N,N-dimethylcarbamimidoyl)guanidine; NASH, non-alcoholic steatohepatitis; PAI-1, plasminogen activator inhibitor 1; PEPCK, phosphenolpyruvate carboxykinase; PH, partial hepatectomy; TNFα, tumor necrosis factor α; tPA, tissue-type plasminogen activator; uPA, urokinase plasminogen activator.

Abstract

Metformin [2-(N,N-dimethylcarbamimidoyl)guanidine] is a drug used in the treatment of type 2 diabetes. Recent studies have suggested that metformin may have effects in addition to lowering serum glucose concentrations (e.g., anti-inflammatory). The aim of the present study was to determine whether metformin prevents the inflammatory reaction and liver damage in a model of post-surgical sepsis. Accordingly, rats underwent 2/3 partial hepatectomy (PH; or sham surgery); 48h after surgery, animals were administered endotoxin (LPS; 1.5 mg/kg i.v.). Both PH and LPS alone caused some minor liver damage. However, their combined effect (PH/LPS) was synergistic, leading to robust hepatic damage, as indicated by plasma enzymes and histological assessment. Although metformin treatment did not alter changes caused by PH alone, it almost completely blunted the effects of LPS in the PH/LPS group. Increases in biomarkers of inflammation (e.g. IL-6, INFγ, and neutrophil number) were also blunted by metformin treatment. Furthermore, PH/LPS caused a >200× increase in hepatic PAI-1 mRNA expression and plasma PAI-1 protein. These increases were associated with inhibition of hepatic uPA activity and an increase in fibrin deposition, indicative of local thrombosis. These effects were markedly reduced by metformin treatment. In conclusion, these data demonstrate that metformin prevents liver damage in a model of post-surgical sepsis in rats by decreasing proinflammatory and hemostatic responses.

Introduction

Post surgical sepsis is a major cause of morbidity and mortality after extensive liver resection, with risk increasing with the magnitude of the resection (D'Amico and Cillo, 1999). Studies in experimental models have suggested that, in addition to the normal risks associated with GI tract surgery, patients undergoing surgical resection for the treatment of cancer or in living-related donor liver transplantation may be exquisitely prone to serious complications of sepsis after surgery. For example, the clearance function of the reticuloendothelial system is impaired by partial hepatectomy in rats (Arii et al., 1985). Furthermore, injection of a sublethal dose of LPS into partial hepatectomized rats causes high mortality (Mochida et al., 1990; Tsuchiya et al., 2004). Although Gram-negative bacteria can directly damage tissue, it is hypothesized that the major part of injury during sepsis is mediated by the host response to the infection.

Two host responses proposed to play significant roles in tissue damage after sepsis are the inflammatory (Strassheim et al., 2002) and the hemostatic responses (Jagneaux et al., 2004). Sepsis is associated with a proinflammatory response characterized by an increase of proinflammatory cytokines such as interleukin (IL)-1β, interleukin (IL)-6, or tumor necrosis factor (TNF)-α. Additionally, a sustained by elevation of the acute phase protein type-1 plasminogen activator inhibitor (PAI-1) involved in the inhibition of fibrinolysis and local pro-coagulant state occurs with sepsis (Pralong et al., 1989). It has been shown that sepsis and several other pathophysiological conditions such as trauma and hemorrhage often induce hyperglycemia and insulin resistance (Carter, 1998; Ma et al., 2004). In critically ill patients, intensive insulin therapy leads to a decrease of the incidence of sepsis and mortality thus improving clinical outcome of patients (Van Den Berghe et al., 2001). Insulin treatment in animal

models of endotoxemia and thermal injury was found to alter the hepatic inflammatory response (i.e. decrease levels of proinflammatory cytokines) and to improve liver morphology and function (Jeschke et al., 2002; 2004). Taken together, these data suggest that therapies to overcome insulin resistance associated with critical illness (e.g. sepsis) are important for the clinical outcome.

Metformin (dimethylbiguanide) is a drug used in the treatment of type 2 diabetes. Recent studies have suggested that metformin has effects in addition to lowering serum glucose concentrations. For example, metformin reduces hepatic inflammation in animal models of non-alcoholic steatohepatitis (NASH), as well as in humans with NASH (Lin et al., 2000; Marchesini et al., 2001; Nair et al., 2004). Indeed, the protective effect metformin under these conditions was associated with attenuating the proinflammatory response to TNFα in the liver (Lin et al., 2000). In addition, metformin is a potent inhibitor of the expression of PAI-1 in vitro and in vivo (Standeven et al., 2002; He et al., 2003). Therefore, metformin might elicit protective effects in post-surgical sepsis by blocking activation of two critical pathways (i.e., inflammation and coagulation). This hypothesis was tested by determining the effect of metformin treatment on injury in a model of post-surgical sepsis based on partial hepatectomy and subsequent LPS administration.

Methods

Animals

Rats were housed in a pathogen-free barrier facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) and procedures were approved by the local Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (225-250g) were obtained from Harlan Laboratories (Bar Harbor, ME). Food and tap water were allowed ad libitum during all experimental periods. The timeline of the experimental design is shown schematically in Figure 1. Animals underwent a 2/3 partial hepatectomy (or sham surgery) under isoflurane anesthesia (Waynforth and Flecknell, 1992). Body temperature was maintained by a heating pad during the surgeries. Animals were allowed to recover for 48 h. Animals were subsequently administered LPS ("PH/LPS"; 1.5 mg/kg i.v.; Sigma, St. Louis, MO) or vehicle ("PH"; saline) as described by Mochida *et al.*(1990). Animals also received metformin (200 mg/kg i.p.; Sigma, St. Louis, MO) or vehicle (saline) 18 and 2.5 h prior to LPS injection. Six h after LPS injection, animals were anesthetized with sodium pentobarbital (150 mg/kg i.p.) for sacrifice and tissue harvesting. Heparinized blood was collected from the vena cava just prior to sacrifice by exsanguination and plasma stored at –80°C for further analysis. Portions of liver tissue were frozen immediately in liquid nitrogen, while others were fixed in neutral buffered formalin (10%) or frozen-fixed in OCT mounting media (Tissue Tek, Hatfield, PA) for later sectioning and mounting on microscope slides.

Clinical chemistry and pathologic evaluation

Plasma levels of aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, and lactate dehydrogenase (LDH) were analyzed using kits purchased from Fisher

Scientific (Pittsburgh, PA). The concentration of functionally active PAI-1 in plasma was assessed using an ELISA kit purchased from Molecular Innovations Inc (Southfield, MI). The concentration of plasma insulin was determined using an ELISA kit purchased from ALPCO diagnostics (Windham, NH). The concentration of plasma glucagon was determined using an ELISA kit purchased from Wako Chemicals USA (Richmond VA). The concentration of plasma resistin was determined using an ELISA kit purchased from Biovendor LLC. (Candler, NC). Paraffin sections of liver (5 μm) were stained for hematoxylin and eosin to assess liver necrosis. Neutrophil infiltration was evaluated by staining using AS-D chloroacetate esterase (Sigma Chemical Co., St. Louis, MO). Neutrophil numbers in liver were counted in 10 randomly selected fields (20×), and data from each tissue section were pooled to determine means.

Bio-Plex analysis of hepatic cytokine and chemokine levels

To quantify cytokine and chemokine levels in liver, a multiplex suspension protein array was performed using the Bio-Plex Protein Array System and a Rat Cytokine 9-plex Panel (Bio-Rad, Hercules, CA). This method of analysis is based on Luminex™ technology and simultaneously measures IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, and TNF-α at the protein level. Briefly, total hepatic protein was extracted from snap-frozen liver samples using a lysis buffer (20 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1 % Nonidet P-40, 1 % sodium deoxycholate, 0.1 % SDS) contained protease (20 μM AEBST, 10 μM EDTA, 1 μg/ml bestatin, 1 μg/ml E64, 1 μg/ml leupeptin, and 1 μg/ml PMSF), tyrosine phosphatase (1 mM Na_3VO_4, 1.2 mM Na_2MoO_4, 4.8 mM $C_4H_4O_6Na_2$, and 2 mM imidazole) and serine/threonine phosphatase (4.6 μM cantharidin, 20 μM bromotetramisole oxalate, and 0.1 μg/ml microcystin) inhibitors (Sigma, St. Louis, MO). Anti-cytokine/chemokine antibody conjugated beads were added to individual

wells of a 96-well filter plate and adhered using vacuum filtration. After washing, 50 µl of pre-diluted standards (range between 32,000 pg/ml and 1.95 pg/ml) or liver lysate was added and the filter plate was shaken at 300 rpm for 30 min at room temperature. Thereafter, the filter plate was washed and 25 µl of pre-diluted multiplex detection antibody was added for 30 min. After washing, 50 µl pre-diluted streptavidin-conjugated phycoerythrin was added for 10 min followed by an additional wash and the addition of 125 µl of Bio-Plex assay buffer to each well. The filter plate was analyzed using the Bio-Plex Protein Array System and concentrations of each cytokine and chemokine were determined using Bio-Plex Manager Version 3.0 software. Data is expressed as pg cytokine/µg of total liver protein.

Zymographic analysis of hepatic plasminogen activator activity

The activity of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) were determined in liver samples as described by Bezerra *et al*. (2001) Briefly, total protein was extracted from frozen liver tissue samples using lysis buffer [1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS in PBS (pH 7.4)] containing protease, tyrosine phosphatase, and serine/threonine phosphatase inhibitors (see above). Respective lysates (100 µg protein/well) were placed in sample buffer (2% SDS, 80 mM Tris-HCl (ph 6.8), 10% glycerol, and 0.002% bromophenol blue) and were separated on 12% SDS-polyacrylamide gels containing 2% non-fat dry milk powder (Biorad, Hercules, CA) and 75 mU/ml plasminogen (Sigma, St. Louis, MO). Plasminogen-free gels run in parallel were used to confirm that the activity detected was plasminogen dependent. Gels were incubated twice for 30 min in 2.5% v/v Triton-X-100 solution and washed three times 30 min in developing solution (50 mM Tris, 0.1 M glycine, 0.1 M sodium chloride, ph 8.0) followed by a 16 h incubation in developing buffer at

37°C. The caseinolytic activity was detected by staining the gel for 2 h in 0.1% amido black, 45% methanol, 10% acetic acid for 2 h and destaining in 45% methanol, 10% acetic acid for 30 min. Densitometric analysis was performed using Image Quant software (Amersahm Biosciences Corp, Piscataway, NJ).

Immunohistochemical detection of fibrin deposition

Sections of frozen liver (8 μm-thick) were fixed in 10% buffered formalin containing 2% acetic acid for 30 minutes at room temperature. Sections were blocked with PBS containing 10% horse serum (i.e., blocking solution; Vector Laboratories, Burlingame, CA) for 30 min, followed by incubation overnight at 4°C with goat anti-rat fibrinogen antibody diluted (1:1000, ICN Pharmaceuticals, Aurora, OH) in blocking solution. Sections were washed three times, 5 minutes each, with PBS and incubated for 3 h with donkey anti-goat secondary antibody conjugated to Alexa 594 (1:1000, Molecular Probes, Eugene, OR). Sections were washed with PBS and visualized using a fluorescent microscope. No staining was observed in controls for which the primary or secondary antibody was eliminated from the staining protocol. Liver sections from all treatment groups were stained at the same time.

RNA isolation and real-time rt-PCR

Total RNA was extracted from liver tissue samples by a guanidium thiocyanate-based method (RNA STAT 60 Tel-Test, Ambion, Austin, TX). RNA concentrations were determined spectrophotometrically, and 1 μg total RNA was reverse transcribed using an AMV reverse transcriptase kit (Promega, Madison, WI) and random primers. Polymerase chain reaction (PCR) primers and probes for PAI-1 and β-actin were designed using Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA). Primers were designed to cross introns to ensure that only cDNA and not DNA was amplified (see Table 1). Premade primers and probes for the

catalytic component of phosphenolpyruvate carboxykinase (PEPCK; pck1) were purchased from Applied Biosystems (Foster City, CA). The fluorogenic MGB probe was labeled with the reporter dye FAM (6-carboxyfluorescein). TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used to prepare the PCR mix. The 2× mixture was optimized for TaqMan reactions and contains AmpliTaq gold DNA polymerase, AmpErase, dNTPs with UTP and a passive reference. Primers and probe were added to a final concentration of 300 nM and 100 nM, respectively. The amplification reactions were carried out in the ABI Prism 7700 sequence detection system (Applied Biosystems) with initial hold steps (50°C for 2 min, followed by 95°C for 10 min) and 40 cycles of a two-step PCR (92°C for 15 sec, 60°C for 1 min). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. The comparative C_T method was used to determine fold differences between samples. The comparative C_T method determines the amount of target, normalized to an endogenous reference (β-actin) and relative to a calibrator ($2^{-\Delta\Delta Ct}$). The purity of PCR products was verified by gel electrophoresis.

Statistical analysis

Results are reported as means ± SEM (n = 6-10). ANOVA with Bonferroni's post-hoc test was used for the determination of statistical significance among treatment groups. A *p* value less than 0.05 was selected before the study as the level of significance.

Results

Effect of metformin on liver damage caused by partial hepatectomy and LPS

Figure 2 summarizes the effect of partial hepatectomy (PH) and LPS (alone and combined) on plasma markers of hepatic and tissue damage (AST, ALP, LDH and bilirubin). In animals given sham surgery, LPS alone caused a moderate increase in some of these indices (i.e. AST and LDH) but not others (i.e., ALP and bilirubin). PH alone caused a moderate increase in AST and ALP but not in LDH activity or bilirubin levels. LPS administration after PH caused an increase in all of these plasma parameters. Some of these increases appeared additive compared to the individual effects of LPS and PH (e.g., AST and ALP), whereas others appeared synergistic. For example, neither LPS nor PH alone significantly changed plasma total bilirubin; however, the combination increased this parameter ~6-fold compared to control values. A similar result was observed for LDH activity. Metformin did not blunt any changes in plasma enzymes caused by PH alone (Figure 2). In contrast, metformin treatment prior to LPS injection almost completely blocked the increase of these plasma parameters.

Figure 3 comprises representative photomicrographs depicting hepatic histology as assessed by hematoxylin and eosin staining. No gross pathologic changes were observed in sham or PH-treated animals injected with saline; samples from the latter group are shown to represent both (Figure 3, upper left). Pathology in sham-treated animals administered LPS was similar to the 2 groups described above, with the exception that an increase in inflammatory cell infiltration was observed (not shown). As has been observed by others (Mochida et al., 1990; Tsuchiya et al., 2004), injection of LPS after partial hepatectomy increased inflammatory cell infiltration with areas of coagulative necrosis (Figure 3; lower left). Analogous to results with plasma chemistries (Figure 2), metformin treatment had no noticeable effect on hepatic pathology in the absence of

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

LPS administration (Figure 3; upper right). However treatment of animals with metformin prior to LPS administration significantly blunted changes in liver pathology (Figure 3; lower right). LPS administration 48 h after partial hepatectomy caused a decrease in PCNA-positive cells by ~ 3-fold and an increase in TUNEL-positive cells by ~ 4-fold, markers of proliferation and apoptosis, respectively. Metformin did not alter these effects (data not shown).

To elucidate further the effect of metformin on hepatic inflammation under these conditions, hepatic neutrophil accumulation was assessed by AS-D chloroacetate esterase staining. Figures 4A and B depict representative photomicrographs and quantitative analysis of this staining, respectively. The numbers of hepatic neutrophils in sham or PH-treated animals injected with saline (Figure 4, upper left panel) were within normal ranges and not different from each other; samples from the latter group are shown to represent both (Figure 4; upper left panel). Six h after LPS administration, the number of neutrophils in animals treated with LPS alone was moderately increased by ~5-fold (data not shown). Livers of animals administered LPS after partial hepatectomy displayed a robust ~12-fold increase in the number of neutrophils in liver compared to PH alone. This effect was significantly blunted by metformin treatment by about 30%.

Effect of metformin on hepatic cytokine protein concentration

To further assess the inflammatory response after PH and LPS, hepatic protein levels of the proinflammatory cytokines IL-1α, IL-1β, IL-6, INFγ, and TNFα were quantitated by using the Bio-Plex Protein Array System (see Materials). Results are summarized in Table 2. In livers of animals with sham surgery, treatment with LPS alone caused an increase of most proinflammatory cytokines. PH alone had no effect on protein levels of proinflammatory cytokines in the liver. However, LPS administration after PH resulted in a moderate (e.g. TNFα

and INFγ) to robust (e.g. IL-1α, IL-1β, and IL-6) increase of all proinflammatory cytokines determined. Specifically, levels of IL-1α, IL-1β, and IL-6 increased by ~10-, ~29-, and ~23-fold, respectively under these conditions, whereas levels of TNFα and INFγ were only moderately increased by ~2.8- and ~7–fold over animals undergoing PH only. Although metformin had no significant effect on the levels of these cytokines/chemokines in the absence of LPS, it significantly blunted the effect of LPS on IL-6 and INFγ by ~53%. Additionally, hepatic protein levels of the anti-inflammatory mediators IL-2, IL-4, IL-10, and GM-CSF were determined (see Table 2). In sham-treated animals administration of LPS lead to a 3.8- fold increase of IL-10 over controls. PH alone had no effect on IL-10 levels but levels of IL-2 were decreased by ~77%. Administration of LPS after PH led to a ~7-fold increase of IL-2 but had no apparent effect on hepatic IL-10 levels. Concomitant treatment with metformin had no effect on hepatic IL-2 and IL-10 in the presence or absence of LPS. No differences were found in hepatic levels of IL-4 and GM-CSF between the groups.

Robust induction of PAI-1 by PH/LPS: prevention by metformin

As mentioned above, PAI-1 is a major regulator of fibrinolysis that is known to be induced by LPS.(Kruithof, 1988; Sawdey and Loskutoff, 1991) Since metformin can prevent PAI-1 induction (Anfosso et al., 1993), the effect of PH/LPS and metformin on PAI-1 expression and protein were determined. Expression of PAI-1 mRNA was analyzed in whole liver tissue by real-time rt-PCR, and PAI-1 levels in plasma were determined by ELISA (Figure 5). Hepatic expression of PAI-1 after PH in the absence of LPS was low (Figure 5, upper panel) and did not differ from sham-treated animals (data not shown). Injection of LPS caused a ~15-fold increase in the expression of PAI-1 in livers of sham-treated animals. In contrast, hepatic expression of PAI-1 mRNA levels was increased by ~300-fold in liver of animals with PH. A similar pattern

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

was observed in plasma PAI-1 protein levels, determined by ELISA (Figure 5, lower panel). Again, in the absence of LPS-treatment PAI-1 levels did not differ between shams and PH, however LPS-treatment increased plasma PAI-1 levels ~87-fold. Plasma PAI-1 levels of PH/LPS animals were ~150-fold greater in compared to PH alone. Metformin treatment did not alter hepatic message or plasma PAI-1 protein levels in the absence of LPS; however, it significantly blocked the increase in these parameters caused by LPS. For example, the increase in hepatic PAI-1 expression caused by LPS under these conditions was blunted by >80% by metformin treatment (Figure 5, upper panel). A similar pattern was observed in the plasma protein levels (Figure 5, lower panel).

Metformin restores uPA activity after LPS injection

The effect of PH/LPS and metformin on the activity of uPA and tPA in livers after partial hepatectomy was determined by zymography. Figure 6 depicts a representative zymogram demonstrating plasmin-mediated casein lysis induced by uPA in whole liver tissue. Bands indicating the activity of both enzymes (uPA and tPA) were detected in all samples. The band corresponding to tPA activity was very faint and did not appear to be altered with any treatments. Conversely, the band corresponding to uPA gave a strong signal in liver tissue (Figure 6A). In the absence and presence of LPS, the activity of uPA in livers of sham-treated animals was ~2 fold higher than in livers after PH (data of shams not shown). In contrast, PH/LPS caused a ~50% decrease in the activity of uPA (Figures 6A and B) compared to PH alone. Although having no effect on uPA activity in the PH group, metformin treatment completely prevented the decrease in activity observed in the PH/LPS group (Figure 6).

To further assess the effects of metformin on the hemostatic system, deposition of fibrin was determined in liver samples after PH and LPS. Figure 8A comprises representative

photomicrographs depicting immunofluorescent detection of fibrin deposition. In accordance with uPA activity and PAI-1 expression, 6h hours after LPS administration, fibrin deposition was significantly increased by ~3-fold compared to PH alone. Although having no effects on basal fibrin levels in the absence of LPS (Figure 7A upper right), metformin treatment completely abolished the increase in fibrin deposition caused by LPS under these condition.

Effect of partial hepatectomy and LPS on indices of hepatic carbohydrate metabolism: effect of metformin

A potential mechanism by which metformin may mediate protective effects in liver is via alteration of insulin-responsiveness of the organ. Therefore, plasma levels of key regulators of hepatic carbohydrate metabolism (insulin and glucacon) and insulin signaling (resistin) were determined (Table III). In sham surgery animals, injection of LPS caused a significant increase in the plasma levels of all 3 peptide hormones; specifically insulin, glucagon and resistin were elevated 1.5-, 6.9-, and 1.3-fold, respectively, by LPS in these animals. Interestingly, PH alone caused a significant increase in plasma levels of insulin and glucagon, with values 2.0- and 2.9-fold higher, respectively, than animals that underwent sham surgery; in the absence of LPS, metformin significantly blunted the increase caused by PH, with values similar to those of animals that underwent sham surgery.

In contrast to the results in animals that underwent sham surgery, the injection of LPS after PH caused a significant decrease in circulating insulin levels by ~50%. The combination of PH and LPS caused a synergistic increase in plasma glucagon levels, with values ~19-fold higher in comparison with animals that underwent sham surgery without LPS. Resistin levels were also more robustly increased in by the combination of PH and LPS with values 2-fold higher than in animals that underwent sham surgery without LPS. Figure 8 shows the mRNA expression of the

catalytic component of PEPCK (pck1) after PH and LPS. The combination of PH and LPS caused a signicant 3-fold increase in the mRNA levels of this gene. While having no effect on the expression in the absence of LPS, metformin significantly attenuated the increase caused by LPS, with values similar to those of control (Figure 9). Metformin did not significantly alter the expression of other 2 other genes involved in hepatic gluconeogensis (glucose 6 phosphatase) or glycolysis (glucokinase) under these conditions (not shown).

Discussion

Metformin protects from endotoxin induced inflammation and liver damage in a model of bacterial sepsis

Animal models resembling conditions of surgical trauma, hemorrhage, burns and sepsis have been found to be useful tools to investigate mechanisms underlying the protective effects of drugs in these devastating clinical conditions. Here, a rodent model that mimics the early events of post-surgical sepsis after partial liver resection was employed, as first described by Mochida *et al*. (1990). This model represents a synergistic effect between PH and LPS; specifically, partial hepatectomy under these conditions sensitizes the liver to a 2nd "hit" caused by LPS (see Figure 2). Work by other groups have employed this model to investigate underlying mechanisms of liver and tissue damage under these conditions as well as to identify potential new drug therapies to reduce or prevent morbidity/mortality associated with sepsis after hepatic resection (e.g.,Tsuchiya et al., 2004). As discussed above, recent studies have suggested that metformin may protect against inflammatory liver damage (Lin et al., 2000; Marchesini et al., 2001; Nair et al., 2004). This model was therefore employed to test the hypothesis that metformin will protect from the early events of post-surgical sepsis.

Treatment of rats with metformin prior to LPS injection in this model significantly blunted subsequent liver damage as assessed by plasma indices of damage (Figure 2) and histologic assessment (Figures 3 and 4). The protective effect of metformin treatment correlated with a blunting of the proinflammatory response (i.e. IL-6 and INFγ), without any apparent effect on the hepatic anti-inflammatory response (i.e. IL-2 and IL-10) (Table II). While the effects of metformin on survival were not performed in the current study, the blunting of serum enzymes

(Figure 2) and cytokine production (Table II) observed here correlates with similar protective effects in work by others in which survival was determined (e.g., Tsuchiya et al., 2004).

Robust induction of PAI-1 after PH: protection by metformin

As mentioned above, in addition to inflammation, sepsis caused by Gram-negative bacteria is frequently associated with activation of the hemostatic system (Morrison and Ryan, 1987; Cybulsky et al., 1988). Furthermore, elevated PAI-1 levels are associated with a predisposition to thrombosis in a number of clinical conditions, including sepsis. In addition, it has been shown that PAI-1 levels correlate well with the severity of the infection during sepsis (Pralong et al., 1989; Mesters et al., 1996; Mavrommatis et al., 2001). PAI-1 is also induced in rodent models of sepsis/endotoxemia (Quax et al., 1990; Sawdey and Loskutoff, 1991; Yamamoto and Loskutoff, 1996). Here, hepatic PAI-1 mRNA levels were increased 300-fold along with a concomitant 150-fold increase in PAI-1 protein levels in plasma (Figure 5); this increase of PAI-1 was significantly blunted by metformin pretreatment. While the mechanism(s) by which PAI-1 is elevated by Gram-negative bacterial products is not fully understood, previous work in rodents indicates that proinflammatory cytokines such as TNFα, IL-1β, and IL-6 might be critically involved (Healy and Gelehrter, 1994; Seki and Gelehrter, 1996; Fearns and Loskutoff, 1997). For example, Fearns and Loskutoff (1997) demonstrated that the induction of PAI-1 caused by bacterial endotoxin is mediated by TNFα. However, the specific mechanism(s) by which metformin prevented the induction of PAI-1 under the current conditions is unclear and the focus of future studies.

The fact that metformin can prevent hemostasis has received recent attention in the treatment of type II diabetes (Grant, 2003). Here, a similar mechanism of action of metformin is proposed. Specifically, it is hypothesized that by preventing the induction of PAI-1, that the

decreased fibrinolysis and increased hemostasis caused by LPS after partial hepatectomy is prevented. PAI-1 is a potent inhibitor of both uPA and tPA and likely impairs fibrinolysis by blocking these enzymes (Kruithof, 1988). Recent studies suggest that plasminogen activators can also protect against tissue damage by promoting clearance of matrix and cellular debris from the field of injury (Bezerra et al., 2001). Studies with knockout mice indicate that uPA is more critical for fibrinolysis than is tPA (Carmeliet et al., 1994). Furthermore, Yamamoto and Loskutoff (1996) reported that fibrin accumulation in tissue after LPS injection associated more strongly with impaired uPA activity rather than tPA. Similar to the findings of others in mouse (Bezerra et al., 2001), an association between uPA activity and fibrin deposition was observed under the current conditions. Specifically, PH/LPS decreased uPA activity and increased fibrin deposition; both changes were completely prevented by concomitant metformin administration.

An additional possible mechanism by which metformin is protective in the current model is via insulin sensitization. Specifically, acute insulin resistance occurs after various stresses, such as sepsis, hemorrhagic shock and surgical trauma (Chaudry et al., 1974; Carter, 1998; Ma et al., 2004). Furthermore, Jeschke et al. (2004) have shown that insulin prevents the systemic inflammatory response and liver damage due to endotoxemia in rats. Recent work by Ma et al. (2004) demonstrated that hepatic insulin resistance during hemmorhagic shock is mediated by TNFα. Here, we observed a synergistic effect of PH+LPS on circulating levels of glucagon (Table III). Further, resistin, a recently identified peptide hormone known to regulate hepatic insulin resistance (Muse et al., 2004; Satoh et al., 2004) was elevated by PH+LPS (Table III). Lastly, the expression of PEPCK, a surrogate marker of hepatic insulin resistance (see Postic et al., 2004, for review) was elevated by PH+LPS in this study and was attenuated by metformin administration (Figure 8). Taken together, these data support the hypothesis that insulin

resistance (and protection by metformin) may contribute to the results observed here. Interestingly, PAI-1 knockout mice were recently demonstrated to be protected against liver damage due to hemorrhagic shock (Lagoa et al., 2005). Furthermore, PAI-1 induction correlates with the extent of insulin resistance during septic shock (Pandey et al., 2005). Inflammation, insulin resistance and the induction of PAI-1 may therefore be linked events in liver; a similar mechanism has been proposed for PAI-1 induction and the increased risk of atherosclerosis in diabetes (e.g., Bastard et al., 2000).

Taken together, these data demonstrate that metformin attenuates post-surgical sepsis by suppressing proinflammatory cytokines, PAI-1 expression and maintaining hepatic uPA activity. In addition, the results of the present study also support the hypothesis that the therapeutic effects of metformin are not limited solely to type-2 diabetes per se, but may also have beneficial effects in other conditions associated with hepatic inflammation, such as sepsis.

References

Anfosso F, Chomiki N, Alessi MC, Vague P, and Juhan-Vague I (1993) Plasminogen activator inhibitor-1 synthesis in the human hepatoma cell line Hep G2. Metformin inhibits the stimulating effect of insulin. *J Clin Invest* 91:2185-2193.

Arii S, Shibagaki M, Takahashi S, Taki Y, Yokoo N, Jikko A, Tanaka J, Kamiyama Y, Takasan H, Ozawa K, and . (1985) Changes in the reticuloendothelial phagocytic function after partial hepatectomy. *J Lab Clin Med* 105:668-672.

Bastard JP, Pieroni L, and Hainque B (2000) Relationship between plasma plasminogen activator inhibitor 1 and insulin resistance. *Diabetes Metab Res Rev* 16:192-201.

Bezerra JA, Currier AR, Melin-Aldana H, Sabla G, Bugge TH, Kombrinck KW, and Degen JL (2001) Plasminogen activators direct reorganization of the liver lobule after acute injury. *Am J Pathol* 158:921-929.

Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, and Mulligan RC (1994) Physiological consequences of loss of plasminogen activator gene function in mice. *Nature* 368:419-424.

Carter EA (1998) Insulin resistance in burns and trauma. *Nutr Rev* 56:S170-S176.

Chaudry IH, Sayeed MM, and Baue AE (1974) Insulin Resistance in Experimental Shock. *Arch Surg* 109:412-415.

Cybulsky MI, Chan MK, and Movat HZ (1988) Acute inflammation and microthrombosis induced by endotoxin, interleukin-1, and tumor necrosis factor and their implication in Gram-negative infection. *Lab Invest* 58:365-378.

D'Amico D and Cillo U (1999) Impact of severe infections on the outcome of major liver surgery: a pathophysiologic and clinical analysis. *J Chemother* 11:513-517.

Fearns C and Loskutoff DJ (1997) Induction of plasminogen activator inhibitor 1 gene expression in murine liver by lipopolysaccharide. Cellular localization and role of endogenous tumor necrosis factor-alpha. *Am J Pathol* 150:579-590.

Grant PJ (2003) Beneficial effects of metformin on haemostasis and vascular function in man. *Diabetes Metab* 29:6S44-6S52.

He G, Pedersen SB, Bruun JM, Lihn AS, and Richelsen B (2003) Metformin, but not thiazolidinediones, inhibits plasminogen activator inhibitor-1 production in human adipose tissue in vitro. *Horm Metab Res* 35:18-23.

Healy AM and Gelehrter TD (1994) Induction of Plasminogen-Activator Inhibitor-1 in Hepg2 Human Hepatoma-Cells by Mediators of the Acute-Phase Response. *Journal of Biological Chemistry* 269:19095-19100.

Jagneaux T, Taylor DE, and Kantrow SP (2004) Coagulation in sepsis. *Am J Med Sci* 328:196-204.

Jeschke MG, Einspanier R, Klein D, and Jauch KW (2002) Insulin attenuates the systemic inflammatory response to thermal trauma. *Molecular Medicine* 8:443-450.

Jeschke MG, Klein D, Bolder U, and Einspanier R (2004) Insulin attenuates the systemic inflammatory response in endotoxemic rats. *Endocrinol* 145:4084-4093.

Kruithof EK (1988) Plasminogen activator inhibitors--a review. *Enzyme* 40:113-121.

Lagoa CE, Vodovotz Y, Stolz DB, Lhuillier F, McCloskey C, Gallo D, Yang R, Ustinova E, Fink MP, Billiar TR, and Mars WM (2005) The role of hepatic type 1 plasminogen activator inhibitor (PAI-1) during murine hemorrhagic shock. *Hepatology* 42:390-399.

Lin HZ, Yang SQ, Chuckaree C, Kuhajda F, Ronnet G, and Diehl AM (2000) Metformin reverses fatty liver disease in obese, leptin-deficient mice. *Nat Med* 6:998-1003.

Ma Y, Toth B, Keeton AB, Holland LT, Chaudry IH, and Messina JL (2004) Mechanisms of hemorrhage-induced hepatic insulin resistance: role of tumor necrosis factor-alpha. *Endocrinol* 145:5168-5176.

Marchesini G, Brizi M, Bianchi G, Tomassetti S, Zoli M, and Melchionda N (2001) Metformin in non-alcoholic steatohepatitis. *Lancet* 358:893-894.

Mavrommatis AC, Theodoridis T, Economou M, Kotanidou A, El Ali M, Christopoulou-Kokkinou V, and Zakynthinos SG (2001) Activation of the fibrinolytic system and utilization of the coagulation inhibitors in sepsis: comparison with severe sepsis and septic shock. *Intensive Care Med* 27:1853-1859.

Mesters RM, Florke N, Ostermann H, and Kienast J (1996) Increase of plasminogen activator inhibitor levels predicts outcome of leukocytopenic patients with sepsis. *Thromb Haemost* 75:902-907.

Mochida S, Ogata I, Hirata K, Ohta Y, Yamada S, and Fujiwara K (1990) Provocation of massive hepatic necrosis by endotoxin after partial hepatectomy in rats. *Gastroenterology* 99:771-777.

Morrison DC and Ryan JL (1987) Endotoxins and disease mechanisms. *Annu Rev Med* 38:417-432.

Muse ED, Obici S, Bhanot S, Monia BP, McKay RA, Rajala MW, Scherer PE, and Rossetti L (2004) Role of resistin in diet-induced hepatic insulin resistance. *J Clin Invest* 114:232-239.

Nair S, Diehl AM, Wiseman M, Farr GH, Jr., and Perrillo RP (2004) Metformin in the treatment of non-alcoholic steatohepatitis: a pilot open label trial. *Aliment Pharmacol Ther* 20:23-28.

Pandey M, Loskutoff DJ, and Samad F (2005) Molecular mechanisms of tumor necrosis factor-alpha-mediated plasminogen activator inhibitor-1 expression in adipocytes. *FASEB J* 19.

Postic C, Dentin R, and Girard J (2004) Role of the liver in the control of carbohydrate and lipid homeostasis. *Diabetes Metab* 30:398-408.

Pralong G, Calandra T, Glauser MP, Schellekens J, Verhoef J, Bachmann F, and Kruithof EK (1989) Plasminogen activator inhibitor 1: a new prognostic marker in septic shock. *Thromb Haemost* 61:459-462.

Quax PH, van den Hoogen CM, Verheijen JH, Padro T, Zeheb R, Gelehrter TD, van Berkel TJ, Kuiper J, and Emeis JJ (1990) Endotoxin induction of plasminogen activator and plasminogen activator inhibitor type 1 mRNA in rat tissues in vivo. *J Biol Chem* 265:15560-15563.

Satoh H, Nguyen MT, Miles PD, Imamura T, Usui I, and Olefsky JM (2004) Adenovirus-mediated chronic "hyper-resistinemia" leads to in vivo insulin resistance in normal rats. *J Clin Invest* 114:224-231.

Sawdey MS and Loskutoff DJ (1991) Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-alpha, and transforming growth factor-beta. *J Clin Invest* 88:1346-1353.

Seki T and Gelehrter TD (1996) Interleukin-1 induction of type-1 plasminogen activator inhibitor (PAI-1) gene expression in the mouse hepatocyte line, AML 12. *J Cell Physiol* 168:648-656.

Standeven KF, Ariens RA, Whitaker P, Ashcroft AE, Weisel JW, and Grant PJ (2002) The effect of dimethylbiguanide on thrombin activity, FXIII activation, fibrin polymerization, and fibrin clot formation. *Diabetes* 51:189-197.

Strassheim D, Park JS, and Abraham E (2002) Sepsis: current concepts in intracellular signaling. *Int J Biochem Cell Biol* 34:1527-1533.

Tsuchiya H, Kaibori M, Yanagida H, Yokoigawa N, Kwon AH, Okumura T, and Kamiyama Y (2004) Pirfenidone prevents endotoxin-induced liver injury after partial hepatectomy in rats. *J Hepatol* 40:94-101.

Van Den Berghe G, Wouters P, Weekers F, Verwaest C, Bruyninckx F, Schetz M, Vlasselaers D, Ferdinande P, Lauwers P, and Bouillon R (2001) Intensive insulin therapy in critically ill patients. *N Engl J Med* 345:1359-1367.

Waynforth HB and Flecknell PA (1992) *Experimental and surgical technique in the rat*. Academic Press, San Diego, CA.

Yamamoto K and Loskutoff DJ (1996) Fibrin deposition in tissues from endotoxin-treated mice correlates with decreases in the expression of urokinase-type but not tissue-type plasminogen activator. *J Clin Invest* 97:2440-2451.

Footnotes:

This work was supported, in part, by grants from the National Institute of Alcohol Abuse and Alcoholism (NIAAA) and National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

Legends for Figures

Figure 1 Schematic depiction of the experimental protocol. Male rats were treated with endotoxin (1.5 mg/kg i.v.) 48 h after 70% hepatectomy or sham surgery. Metformin (200 mg/kg) was administered intraperitoneally (i.p.) 18 and 2.5 h before endotoxin injection. Animals were sacrificed 6 h after LPS injection.

Figure 2 Effect of metformin on plasma indices of tissue damage. Animals and treatments are described in Methods. Plasma levels of aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and bilirubin were determined spectrophotometrically. Data are means ± SEM (n = 6-10). $^{a}P<0.05$ compared with animals injected with saline instead of LPS; $^{b}P<0.05$ compared to animals that underwent a sham operation; $^{c}P<0.05$ compared to the absence of metformin. PH = partial hepatectomy

Figure 3 Effect of LPS and metformin on liver histology after partial hepatecomy. Animals and treatments are as described in Methods. Representative photomicrographs (200×) of livers from animals that underwent PH alone (upper left), PH + metformin (upper right), PH + LPS (lower left) and PH + LPS + metformin (lower right). Arrows indicating a region of coagulative oncotic necrosis.

Figure 4 Effect of LPS and metformin on neutrophil accumulation after partial hepatectomy. Animals and treatments are as described in Methods. (A) Representative photomicrographs (200×) depicting neutrophils (pink; arrows) against a hematoxylin counterstain

(blue) from animals after partial hepatectomy. (B) Quantitative analysis of neutrophil infiltration (original magnification, 200×). Data are expressed as % of controls (means ± SEM, n = 4-6) ; [a]P<0.05 compared with animals injected with saline instead of LPS; [b]P<0.05 compared to the absence of metformin.

Figure 5 Effect of LPS and metformin on hepatic PAI-1 expression and plasma levels after partial hepatectomy. Expression of PAI-1 mRNA (A) and plasma concentrations (B) were determined by real-time rt-PCR and ELISA, respectively (see Methods). Data are expressed as % control (means ± SEM, n = 4-6). Figure insets show data expressed on a log scale. [a]P<0.05 compared with animals injected with saline instead of LPS; [b]P<0.05 compared to the absence of metformin.

Figure 6 Effect of LPS and metformin on hepatic uPA after partial hepatectomy in rats. (A) Representative zymographs demonstrating plasmin mediated casein lysis by uPA in liver tissue. (B) Quantitative image analysis of uPA activity in liver. Data are expressed as means ± SEM (n = 4-6). [a]P<0.05 compared with animals injected with saline instead of LPS; [b]P<0.05 compared to the absence of metformin.

Figure 7 Effect of metformin on fibrin deposition 6 h after endotoxin injection in rats with partial hepatectomy . Representative photomicrographs (original magnification, 200×) depicting immunohistochemistry of hepatic fibrin. Light grey indicating fibrin deposition. PP = periportal, CL = centrilobular.

Figure 8 Effect of metformin on the expression of PEPCK after endotoxin injection in rats with partial hepatectomy. Expression of the catalytic subunit of PEPCK (pck1) mRNA was determined by real-time rt-PCR (see Methods). Data are expressed as % control (means ± SEM, n = 4-6). [a]$P<0.05$ compared with animals injected with saline instead of LPS; [b]$P<0.05$ compared to the absence of metformin.

Table I PCR primer and probe sequences for the detection of PAI-1 and β-actin in rat liver.

	Sense (5'-3')	Antisense (5'-3')	Probe (5'-3')
PAI-1	CACCAACATTTTGGACGCTGA	TCAGTCATGCCCAGCTTCTCC	CCAGGCTGCCCCGCCTCCTC
β-actin	GGCTCCCAGCACCATGAA	AGCCACCGATCCACACAGA	AAGATCATTGCTCCTCCTGAGCGCAAGTA

Table II Hepatic protein levels of proinflammatory and anti- inflammatory cytokines.

Cytokine	Sham Surgery		Partial Hepatectomy			
	- LPS	+ LPS	- LPS		+ LPS	
				+ Metformin		+ Metformin
IL-1α	20 ± 3	183 ± 51[a]	31 ± 7	35 ± 5	322 ± 35[a,b]	238 ± 55[a]
IL-1β	23 ± 5	238 ± 61[a]	20 ± 4	17 ± 3	585 ± 188[a,b]	689 ± 69[a]
IL-6	7 ± 0.8	48 ± 13[a]	7± 1.3	11 ± 1.6	169 ± 38[a,b]	79 ± 21[a,c]
TNFα	0.6 ± 0.1	8.7 ± 2.2[a]	1.5 ± 0.3	1.8 ± 0.2	4.2 ± 0.9	3.9 ± 0.8
INFγ	0.3 ± 0.1	2.3 ± 0.1[a]	0.7 ± 0.1	0.9 ± 0.2	4.6 ± 0.8[a,b]	2.2 ± 0.3[a,c]
IL-2	5.9 ± 1.3	9.1 ± 2.1	1.3 ± 0.2[b]	2.3 ± 0.8[b]	9.0 ± 0.5	9.5 ± 1.3
IL-10	0.7 ± 0.1	2.8 ± 0.6[a]	1.3 ± 0.2	2.2 ± 0.7	3.2 ± 0.2[a]	3.5 ± 0.3[a]

Animal treatments and Bio-Plex Protein Array are as described in Methods. Results normalized to total protein. Cytokines/ chemokines that were not significantly altered by treatment are not displayed (i.e. IL-4 and GM-CFS). Data (pg cytokine/μg total protein) are means ± SEM (n = 5-6). [a]$P<0.05$ compared with animals injected with saline instead of LPS; [b]$P<0.05$ compared to animals that underwent a sham operation; [c]$P<0.05$ compared to the absence of metformin.

Table III Plasma levels of insulin, glucagon and resistin.

Peptide	Sham Surgery		Partial Hepatectomy			
	- LPS	+ LPS	- LPS		+ LPS	
				+ Metformin		+ Metformin
Insulin	528±72	811±87[a]	1130±57[b]	510±68[c]	637±113[a]	628±102
Glucagon	271±59	1862±254[a]	796±129[b]	297±123[c]	5251±532[a,b]	2322±439[a,c]
Resistin	13.8±1.5	18.6±0.8[a]	13.7±1.2	11.1±0.9	28.0±2.7[a,b]	18.4±1.8[a,c]

Animal treatments and ELISAs for plasma insulin, glucagon and resistin are as described in Methods. Data (ng/ml) are means ± SEM (n = 5-6). [a]$P<0.05$ compared with animals injected with saline instead of LPS; [b]$P<0.05$ compared to animals that underwent a sham operation; [c]$P<0.05$ compared to the absence of metformin.

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

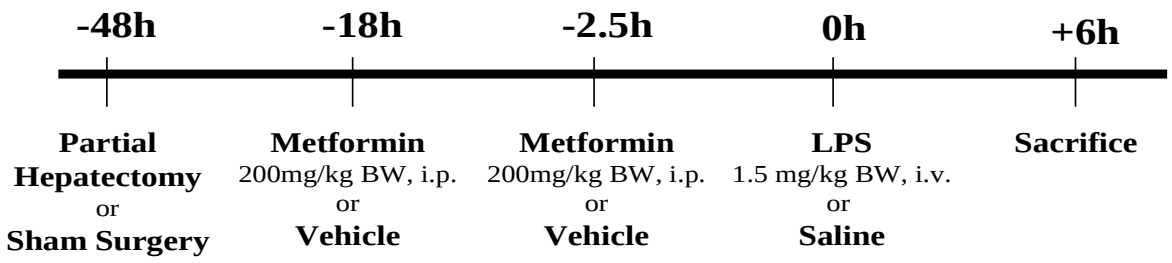


Figure 1

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

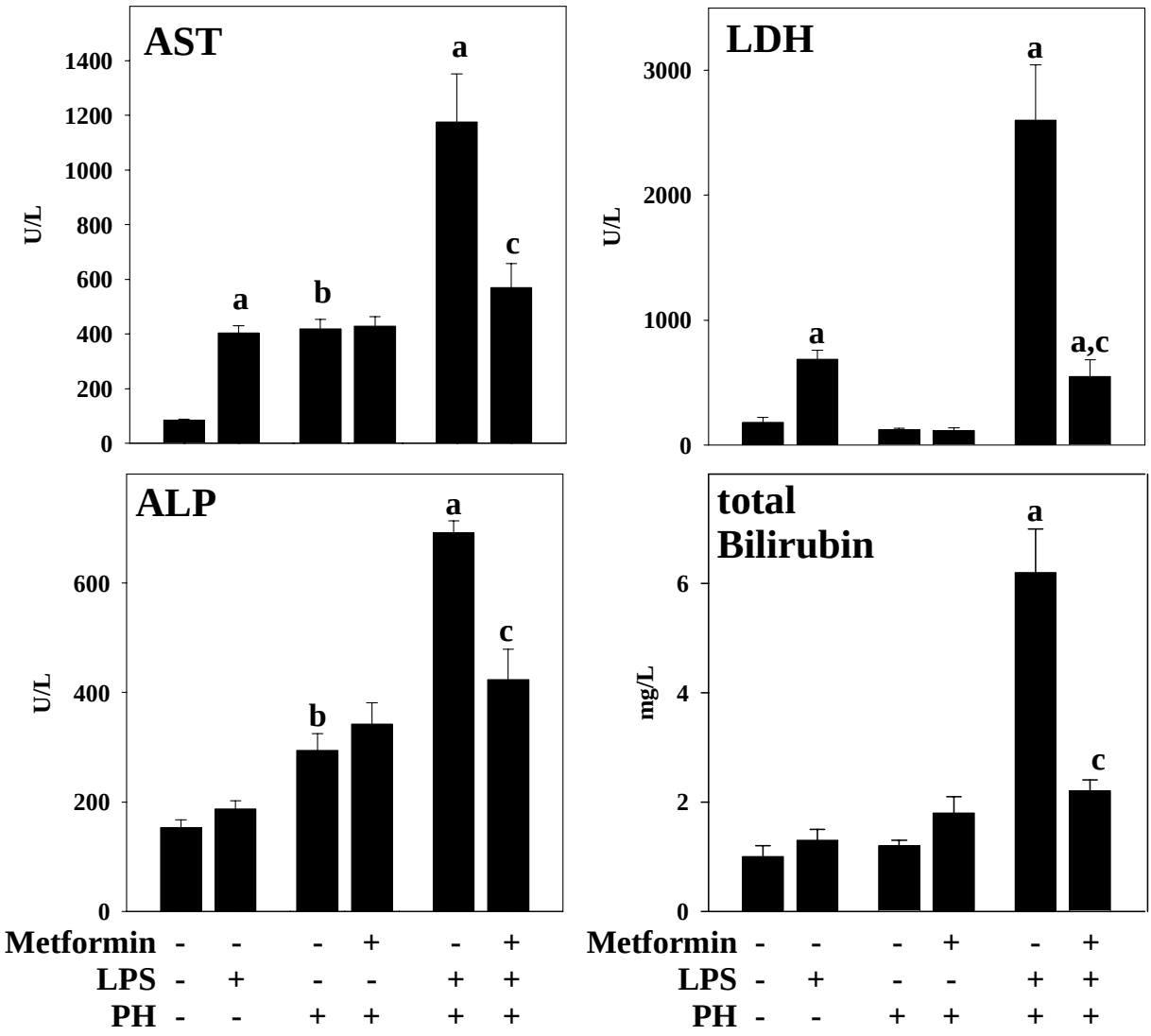


Figure 2

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

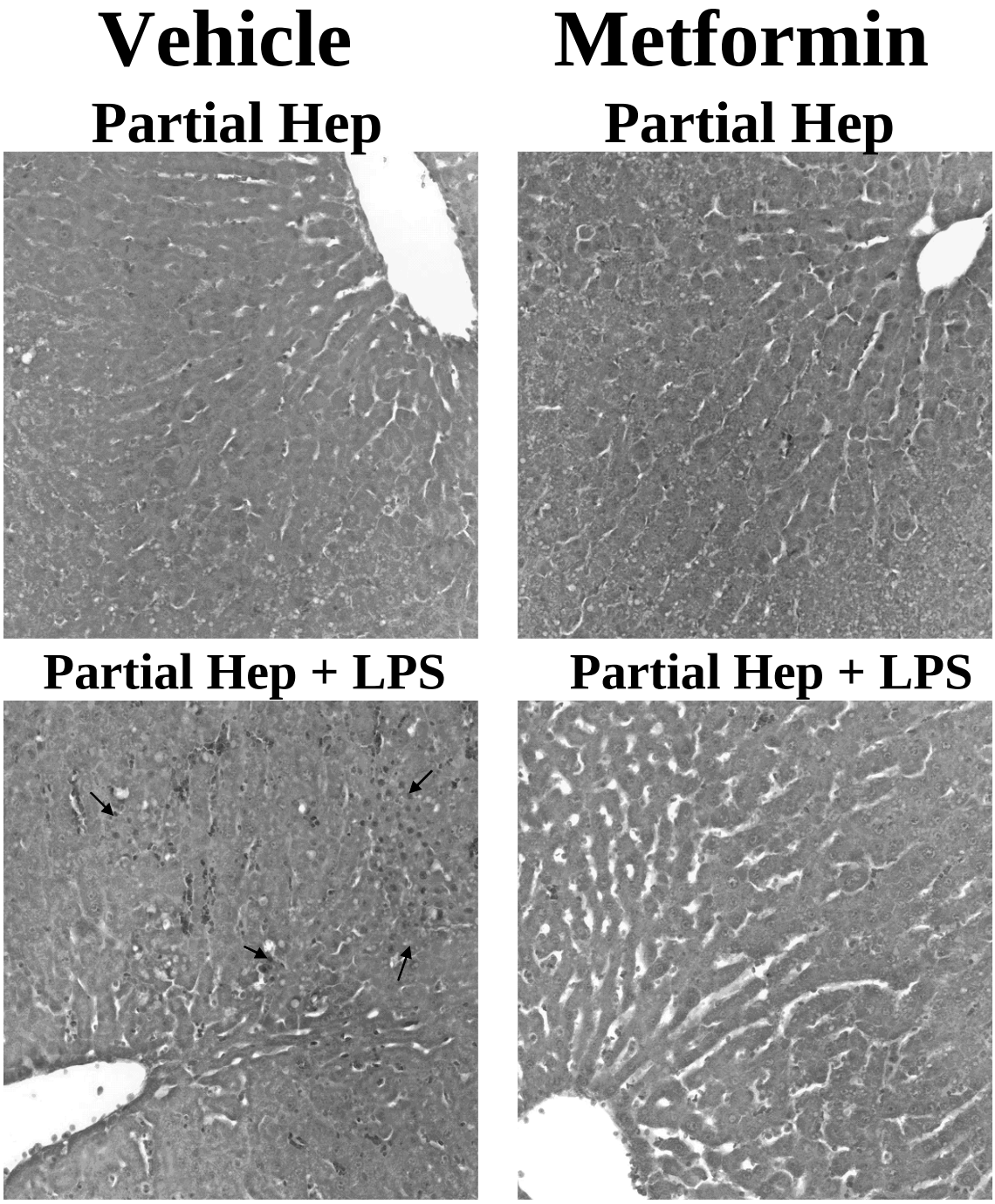


Figure 3

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

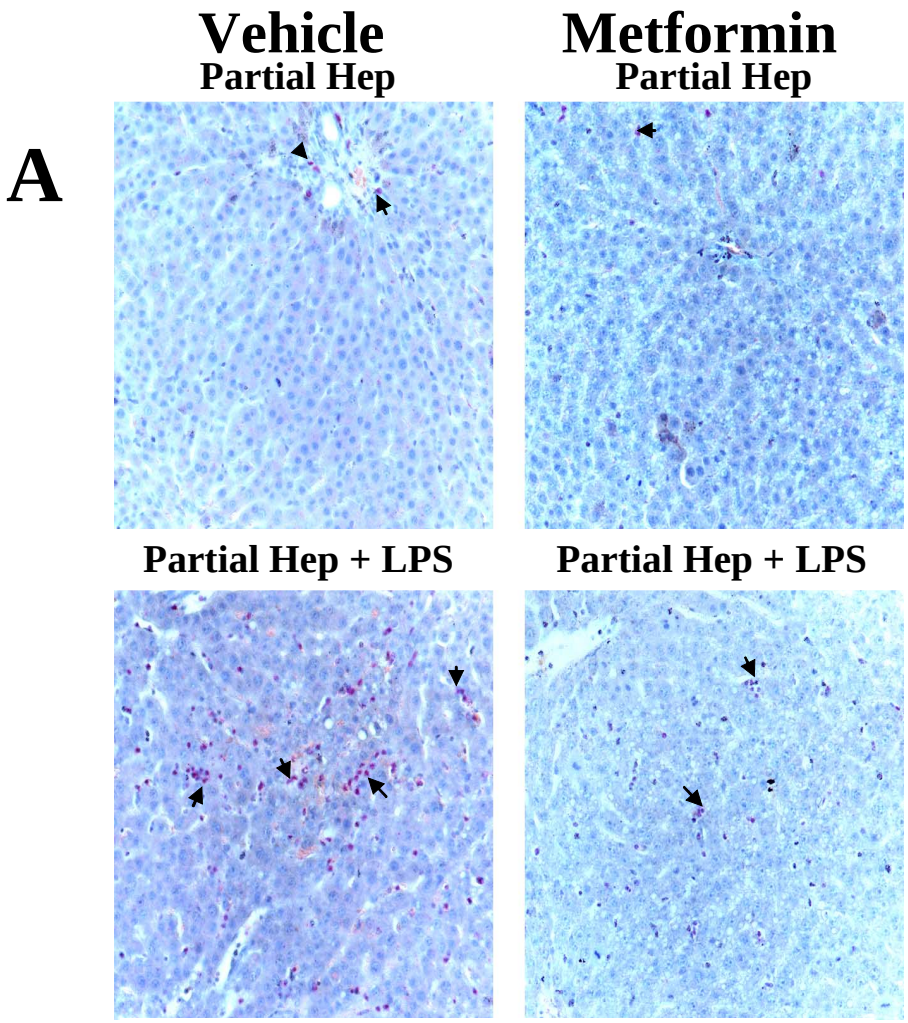


Figure 4

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

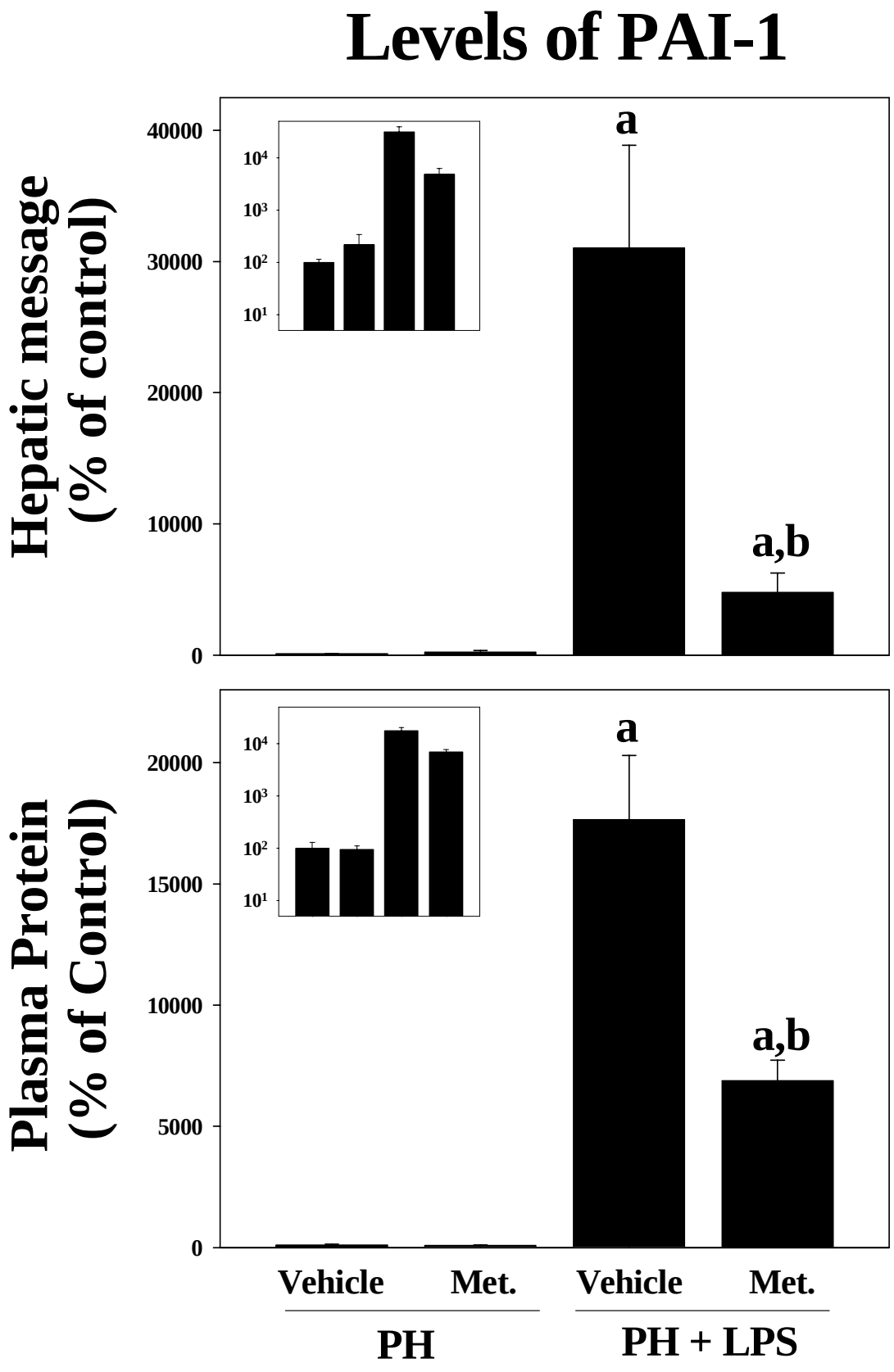
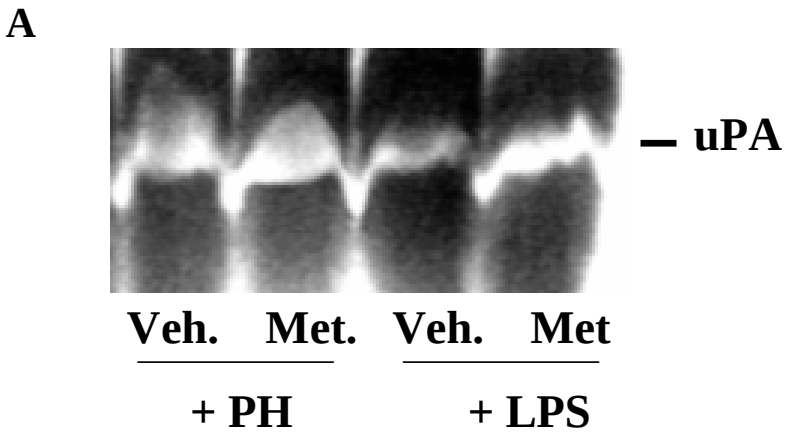


Figure 5

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.



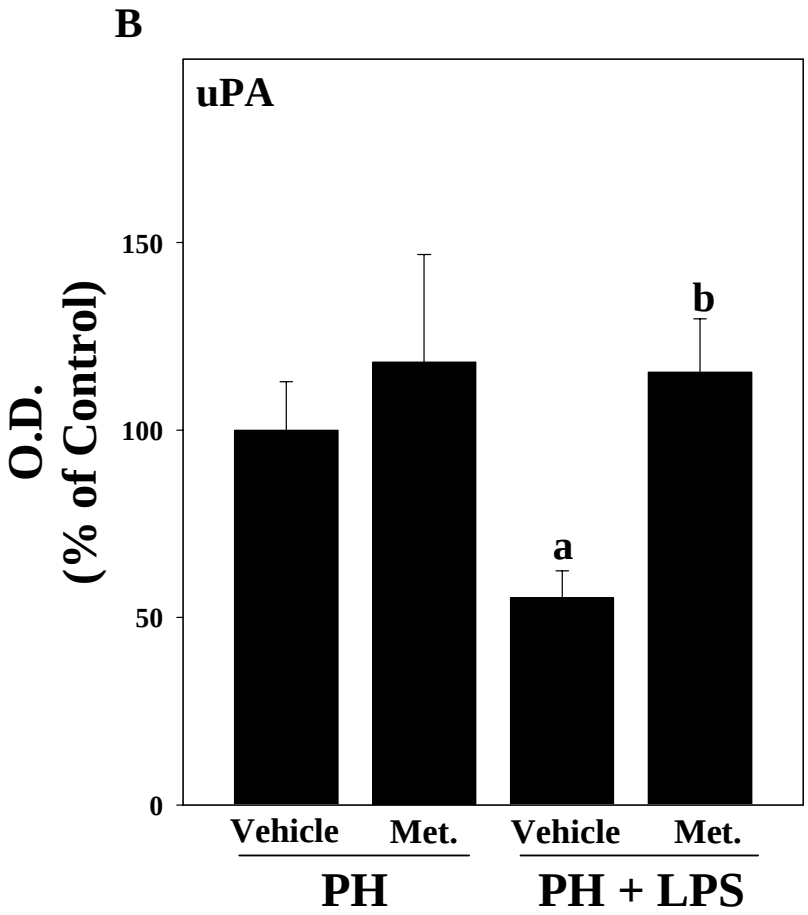


Figure 6

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

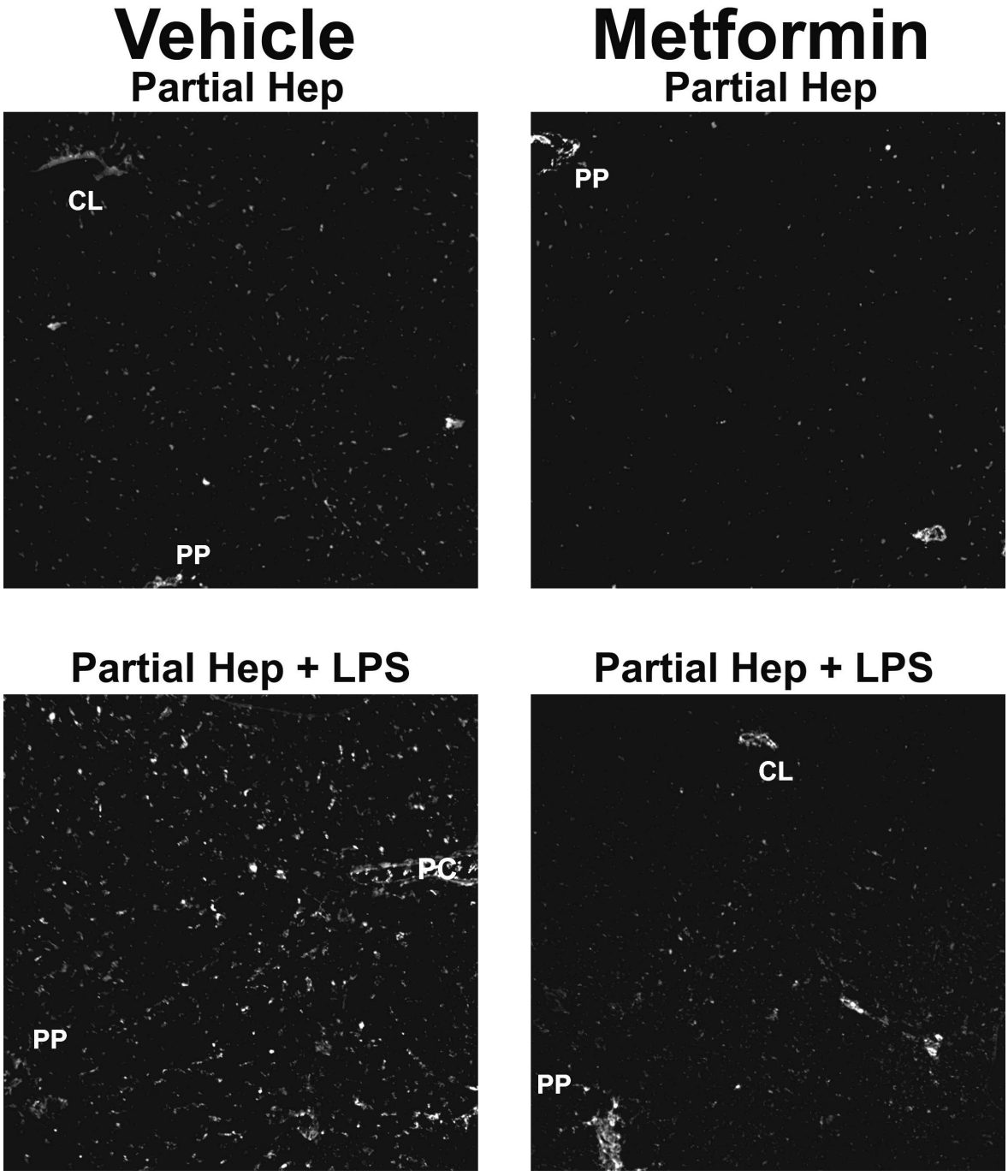


Figure 7

JPET Fast Forward. Published on December 1, 2005 as DOI: 10.1124/jpet.105.092122
This article has not been copyedited and formatted. The final version may differ from this version.

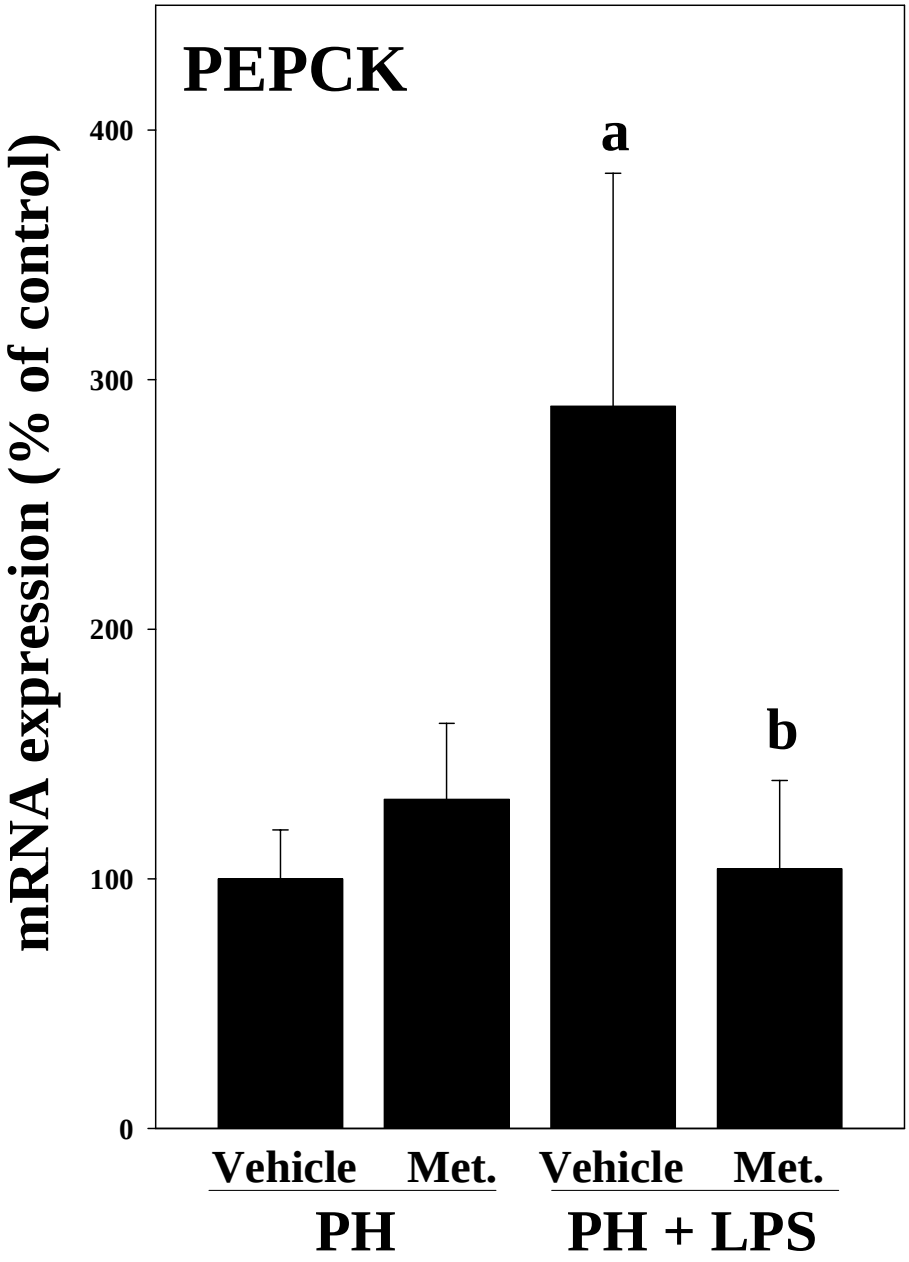


Figure 8