Metformin Prevents Endotoxin-Induced Liver Injury after Partial Hepatectomy

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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 postsurgical sepsis. Accordingly, rats underwent 2/3 partial hepatectomy (PH; or sham surgery); 48 h 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., interleukin 6, interferon γ, and neutrophil number) were also blunted by metformin treatment. Furthermore, PH/LPS caused a >200 × increase in hepatic plasminogen activator inhibitor 1 (PAI-1) mRNA expression and plasma PAI-1 protein. These increases were associated with inhibition of hepatic urokinase plasminogen activator 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 postsurgical sepsis in rats by decreasing proinflammatory and hemostatic responses.

Postsurgical 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 gastrointestinal 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, IL-6, or tumor necrosis factor (TNF)-α. Additionally, a sustained elevation of the acute phase protein type 1 plasminogen activator inhibitor (PAI-1)
involved in the inhibition of fibrinolysis and local procoagulant 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 the 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 nonalcoholic steatohepatitis, as well as in humans with nonalcoholic steatohepatitis (Lin et al., 2000; Marchesini et al., 2001; Nair et al., 2004). Indeed, the protective effect of 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 postsurgical 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 postsurgical sepsis based on partial hepatectomy and subsequent LPS administration.

Materials and Methods

Animals. Rats were housed in a pathogen-free barrier facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the local Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (225–250 g) 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 Fig. 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 hours 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, whereas others were fixed in neutral buffered formalin (10%) or frozen-fixed in ortihne carbyl transferase-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). Neutrophil numbers in liver were counted in 10 randomly selected fields (20x’), 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-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, and 0.1% SDS) containing protease (20 μM AEBSF, 10 μM EDTA, 1 μg/ml bestatin, 1 μg/ml E64, 1 μg/ml leupeptin, and 1 μg/ml phenylmethylsulfonyl fluoride), tyrosine phosphatase (1 mM Na2VO4, 1.2 mM NaN3, 28 μM C6H12O6Na2, and 2 mM imidazole), and serum/threonine phosphatase (4.6 μM cantharidin, 20 μM bromotetramisole oxalate, and 0.1 μg/ml microcin) inhibitors (Sigma). Anticytokine/chemokine antibody-conjugated beads were added to individual wells of a 96-well filter plate and attached using vacuum filtration. After washing, 50 μl of prediluted standards (range between 32,000 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 prediluted multiplex detection antibody was added for 30 min. After washing, 50 μl of prediluted 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 are expressed as picograms of cytokine per microgram 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) was 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% [w/v] Nonidet P-40, 8.5% [w/v] sodium deoxycholate, 0.1% [w/v] SDS) in PBS (pH 7.4) containing protease, tyrosine phospha-

<table>
<thead>
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<th>−48h</th>
<th>−18h</th>
<th>−2.5h</th>
<th>0h</th>
<th>+6h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial Hepatectomy or Sham Surgery</td>
<td>Metformin 200mg/kg BW, i.p.</td>
<td>Vehicle</td>
<td>LPS 1.5 mg/kg BW, i.v. or Saline</td>
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<td>Sacrifice</td>
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tase, and serine/threonine phosphatase inhibitors (see above). Re-
spective lysates (100 μg of protein/well) were placed in sample buffer 
[2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.002% bron-
 phenol blue] and were separated on 12% SDS-polyacrylamide gels 
containing 2% nonfat dry milk powder (Bio-Rad) and 75 mM/ml 
plasminogen (Sigma). Plasminogen-free gels run in parallel were 
used to confirm that the activity detected was plasminogen-depen-
dent. Gels were incubated twice for 30 min in 2.5% (v/v) Triton X-100 
solution and washed three times for 30 min in developing buffer (50 mM Tris, 0.1 M glycine, and 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, and 10% acetic acid for 2 h and destain-
ing in 45% methanol and 10% acetic acid for 30 min. Densitometric 
analysis was performed using Image Quant software (Amersham 

Immunohistochemical Detection of Fibrin Deposition. Sec-
tions of frozen liver (8-μm thick) were fixed in 10% buffered formalin 
containing 2% acetic acid for 30 min 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 solu-
tion. Sections were washed three times, 5 min each, with PBS and 
incubated for 3 h with donkey anti-goat secondary antibody conju-
gated to Alexa 594 (1:1000; Molecular Probes, Eugene, OR). Sections 
were washed with PBS and visualized using a fluorescent micro-
scope. 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 ex-
tracted from liver tissue samples by a guanidium thiocyanate-based 
method (RNA STAT 60 Tel-Test; Ambion, Austin, TX). RNA concen-
trations were determined spectrophotometrically, and 1 μg of total RNA 
was reverse transcribed using an avian myeloblastosis virus 
reverse transcriptase kit (Promega, Madison, WI) and random prim-
ers. 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 compo-
nent of phosphoenolpyruvate carboxykinase (PEPCK; pck1) were 
purchased from Applied Biosystems (Foster City, CA). The fluoro-
genic MGB probe was labeled with the reporter dye FAM (6-carboxy-
fuchsia). TaqMan Universal PCR Master Mix (Applied Biosys-
tems, Foster City, CA) was used to prepare the PCR mix. The 2× 
mixture was optimized for TaqMan reactions and contains AmpTaq 
gold DNA polymerase, AmpErase, deoxy nucleoside-5′-triphosphates 
with UTP, and a passive reference. Primers and probe were added to 
a final concentration of 300 and 100 nM, respectively. The amplifica-
tion 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 s, 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 compara-
tive C_{T} method determines the amount of target, normalized to an 
endogenous reference (β-actin) and relative to a calibrator (2^{-ΔΔC_{T}}). 
The purity of PCR products was verified by gel electrophoresis.

Statistical Analysis. Results are reported as means ± S.E.M. 
(n = 6–10). Analysis of variance with Bonferroni’s post hoc test was 
used for the determination of statistical significance among treat-
ment 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 Par-
tial Hepatectomy and LPS. Figure 2 summarizes the ef-
fect of partial hepatectomy (PH) and LPS (alone and com-
bined) 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 seemed additive 
compared with the individual effects of LPS and PH (e.g., 
AST and ALP), whereas others seemed synergistic. For 
example, neither LPS nor PH alone significantly changed 
plasma total bilirubin; however, the combination increased this 
parameter ~6-fold compared with control values. A similar 
result was observed for LDH activity. Metformin did not 
blunt any changes in plasma enzymes caused by PH alone 
(Fig. 2). In contrast, metformin treatment prior to LPS injec-
tion almost completely blocked the increase of these plasma 
parameters.

Figure 3 comprises representative photomicrographs de-
picting 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 (Fig. 3, top 
left). Pathology in sham-treated animals administered LPS 
was similar to the two groups described above, with the 
exception that an increase in inflammatory cell infiltration 
was observed (not shown). As others have observed (Mochida 
et al., 1990; Tsuchiya et al., 2004), injection of LPS after 
partial hepatectomy increased inflammatory cell infiltration 
with areas of coagulative necrosis (Fig. 3; bottom left).

TABLE 1

<table>
<thead>
<tr>
<th>PCR primer and probe sequences for the detection of PAI-1 and β-actin in rat liver</th>
<th>Sense (5’-3’)</th>
<th>Antisense (5’-3’)</th>
<th>Probe (5’-3’)</th>
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<tr>
<td>PAI-1</td>
<td>CACCCAGCTTTTGGACCTGA</td>
<td>TCAGCTCAGCCACGGTCTCC</td>
<td>CCAGGCTGCCCGCTCCTCC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CGCTCCACGACACAGA</td>
<td>AGCCACGATCCACACAGA</td>
<td>AAATCTTGCTCCTTCCAGGSCAAATA</td>
</tr>
</tbody>
</table>

Effect of Metformin on inflammation under these conditions, hepatic neutrophil ac-
cumulation was assessed by AS-D chloroacetate esterase staining. Figure 4, A and B, depicts representative photomicrographs and quantitative analysis of this staining, respectively. The numbers of hepatic neutrophils in sham- or PH-treated animals injected with saline (Fig. 4, top left panel) were within normal ranges and not different from each other; samples from the latter group are shown to represent both (Fig. 4; top left panel). Six hours 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 with PH alone. This effect was significantly blunted by metformin treatment by approximately 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 and Methods). 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. 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 led 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). Because 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 (Fig. 5). Hepatic expression of PAI-1 after PH in the absence of LPS was low (Fig. 5, top 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 was observed in plasma PAI-1 protein levels, determined by ELISA (Fig. 5, bottom 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 compared with 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 (Fig. 5, top panel). A similar pattern was observed in the plasma protein levels (Fig. 5, bottom 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 (Fig. 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 (Fig. 6, A and B) compared with 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 (Fig. 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 7 comprises representative photomicrographs depicting immunofluorescent detection of fibrin deposition. In accordance with uPA activity and PAI-1 expression, 6 h after LPS administration fibrin deposition was significantly increased by ~3-fold compared with PH alone. Although metformin treatment had no effects on basal fibrin levels in the absence of LPS (Fig. 7, top right), it abolished the increase in fibrin deposition caused by LPS under these conditions.

### 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 glucagon) and insulin signaling (resistin) were determined (Table 3). In sham surgery animals, injection of LPS caused a significant increase in the plasma levels of all three 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 by the combination of PH and LPS with values 2-fold higher than in animals that underwent sham surgery.
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 significant 3-fold increase in the mRNA levels of this gene. Although 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 (Fig. 8). Metformin did not significantly alter the expression of two other genes involved in hepatic gluconeogenesis (glucose 6 phosphatase) or glycolysis (glucokinase) under these conditions (not shown).

**TABLE 2**

Hepatic protein levels of proinflammatory and anti-inflammatory cytokines

Animal treatments and Bio-Plex Protein Array are as described under Materials and Methods. Results are normalized to total protein. Cytokines/chemokines that were not significantly altered by treatment are not displayed (i.e., IL-4 and GM-CSF). Data (picograms of cytokine per micrograms of total protein) are means ± S.E.M. (n = 5–6).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sham Surgery</th>
<th>Partial Hepatectomy</th>
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<tr>
<td></td>
<td>− LPS</td>
<td>+ LPS</td>
</tr>
<tr>
<td>IL-1α</td>
<td>20 ± 3</td>
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<tr>
<td>IL-1β</td>
<td>23 ± 5</td>
<td>238 ± 61</td>
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<tr>
<td>TNFa</td>
<td>7 ± 0.8</td>
<td>48 ± 12</td>
</tr>
<tr>
<td>INFγ</td>
<td>0.6 ± 0.1</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>IL-2</td>
<td>5.9 ± 1.3</td>
<td>9.1 ± 2.1</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.7 ± 0.1</td>
<td>2.8 ± 0.6</td>
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*a P < 0.05 compared with animals injected with saline instead of LPS.

*b P < 0.05 compared with animals that underwent a sham operation.

*c P < 0.05 compared with the absence of metformin.

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**Fig. 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 Materials and Methods). Data are expressed as percentage of control (means ± S.E.M., n = 4–6). 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 with the absence of metformin.

**Fig. 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 ± S.E.M. (n = 4–6). a, P < 0.05 compared with animals injected with saline instead of LPS; b, P < 0.05 compared with the absence of metformin.
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 postsurgical sepsis after partial liver resection was used 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 second "hit" caused by LPS (see Fig. 2). Other groups have used 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., Tsuji 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 used to test the hypothesis that metformin will protect against early damage caused by postsurgical 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 (Fig. 2) and histologic assessment (Figs. 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 2). Although the effects of metformin on survival were not performed in the current study, the blunting of serum enzymes (Fig. 2) and cytokine production (Table 2) observed here correlates with similar protective effects in work by others in which survival was determined (e.g., Tsuji 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 (Morris 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 (Fig. 5); this increase of PAI-1 was significantly blunted by metformin pretreatment. Although the mechanism(s) by which PAI-1 is elevated by Gram-negative bacterial products are 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 are unclear and the focus of future studies.
The fact that metformin can prevent hemostasis has received recent attention in the treatment of type 2 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, 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 probably 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 is 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 endotoxia in rats. Recent work by Ma et al. (2004) demonstrated that hepatic insulin resistance during hemorrhagic shock is mediated by TNFα. Here, we observed a synergistic effect of PH+LPS on circulating levels of glucagon (Table 3). Furthermore, 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 3). Finally, the expression of PEPCK, a surrogate marker of hepatic insulin resistance (for review, see Postic et al., 2004), was elevated by PH+LPS in this study and was attenuated by metformin administration (Fig. 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 postsurgical 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


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