pH-dependent changes of nitric oxide, peroxynitrite, and reactive oxygen species in hepatocellular damage

SHU, Zhijun, Martin Jung, Hans-G. Beger, Michael Marzinzig, Fulì Han, Urwe Butzer, Uwe B. Bruckner, and Andreas K. Nussler. pH-dependent changes of nitric oxide, peroxynitrite, and reactive oxygen species in hepatocellular damage. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1118–G1126, 1997.—Low arterial blood pH and sustained nitric oxide (NO) production are critical parameters in inflammatory events such as sepsis, and appropriate treatment is still under debate. Because the stability of nitrogen and oxygen intermediates is dependent on the surrounding pH, we investigated whether the relationship among NO, peroxynitrite (ONOO⁻), and reactive oxygen species production also depends on the pH value, particularly with respect to their effects on hepatocellular damage. Our studies demonstrate that the extracellular pH influences NO and hydroxyl radical (OH) production in hepatocytes. Acidification (pH 7.0) of the medium revealed a significant increase (P < 0.05) of OH-like radicals, enhanced hepatocellular damage, and a sharp drop in cellular glutathione (GSH) content compared with levels measured at physiological or alkaline pH conditions. Furthermore, inhibition of NO synthesis at all pH conditions resulted in decreased NO production and cellular GSH levels but a simultaneous increase of OH-like radicals and hepatocellular damage with a maximum seen at pH 7.0. Our results suggest that hepatocellular damage is in part regulated by the surrounding pH and that inhibition of NO synthesis at acidic conditions (e.g., in sepsis) leads to increased reactive oxygen-mediated cell injury.

hepatocytes; inducible nitric oxide synthase; oxygen radicals; sepsis

SEVERE INFECTIONS IN SEPTIC patients can lead to hepatic dysfunction, which can be life threatening to the critically ill (9). Low arterial blood pressure, due in part to increased nitric oxide (NO) formation, along with a decrease of blood pH and sustained acidosis, is frequently observed in severe inflammatory events (6). During sepsis the optimal treatment of metabolic dysfunction and hypotension is still a matter of debate, whereas low arterial blood pH and the resulting acidosis can be partially corrected by infusing NaHCO₃.

Under septic conditions the liver plays a pivotal role, resulting in numerous metabolic changes. NO is a molecule that is abundantly produced in liver cells during infections and has been linked with several “pathophysiological” changes within the liver, such as hepatic artery blood flow, glucogenolysis, prostaglandin synthesis, or hepatocellular damage (20, 22, 35, 41). On the other hand, recent publications suggest that the inhibition of NO synthase (NOS) can overcome pathophysiological effects, such as hypotension or certain forms of multiple organ dysfunction, caused by sustained NO overproduction (32, 40).

NO is produced by at least three separately identified NOS isoforms (19). NOS1 and NOS3 are constitutively expressed, whereas NOS2 is synthesized in cells and tissues after exposure to cytokines and/or various pathogens. In the liver the overproduction of NO can have profound cytostatic actions on target cells or the cells that produce NO. We have shown that NOS2 is regulated in rat, human, baboon, and mouse hepatocytes by inflammatory cytokines, endotoxin (lipopolysaccharide (LPS)), and growth factors (24, 26, 30). Increased NOS2 synthesis in hepatocytes can lead to the suppression of DNA synthesis (24, 29), profound reduction in protein synthesis (8) and in mitochondrial aconitase (36), the inhibition of GAPDH (29), and cytochrome P-450 activity (37), as well as apoptosis (24). On the other hand, NO has been suggested to protect against hepatic thrombosis and oxygen radical-mediated injury during endotoxemia (5, 15, 20, 22, 35).

It is well established that hepatocytes produce various nitrooxide and reactive oxygen intermediates (ROI) in response to cytokines, pathogens, or other forms of cell injury (1, 14, 21). Among these oxygen species (e.g., superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (OH)), the formation of OH is considered to be one of the major toxic radical forms (1, 14, 38). OH or OH-like radicals can be formed when O₂⁻ reacts with H₂O₂ in the presence of iron or when NO reacts with superoxide to form peroxynitrite (ONOO⁻). The latter can either spontaneously rearrange to form nitrate (NO₃⁻) or undergo cleavage to generate OH-like radicals and nitrite (NO₂⁻) (4). Interestingly, the formation and stability of OH-like radicals either by the iron-catalyzed Fenton and Haber-Weiss chemistry or via ONOO⁻ is favored in the presence of an acidic pH (2, 7). In the present study, we hypothesized that pH changes in experimental sepsis interfere with the production of reactive oxygen species and NO intermediates, hence directly affecting the hepatocellular damage. In addition, we investigated the consequences of NOS inhibition at different pH values on reactive radical formation and hepatocellular damage.

EXPERIMENTAL PROCEDURES

Materials. William's medium E, penicillin-streptomycin, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and calf serum were purchased from GIBCO BRL (Paisley, Scotland). Percoll was obtained from Pharmacia (Uppsala, Sweden). Western blot kit was supplied by Amersham [enhanced chemiluminescence (ECL), Buckinghamshire, UK]. Corynebacterium parvum (C. parvum) was purchased from...
Immunohem Research (Hamilton, MT). Cytokines used in this study included rat recombinant interferon-γ (IFN-γ; Genzyme, Cambridge, MA), human recombinant interleukin-1β (IL-1β; Cistron, Pine Brook, NJ), and murine tumor necrosis factor-α (TNF-α; Genzyme). Collagenase H was obtained from Boehringer Mannheim (Mannheim, Germany). NaHCO₃, HCl, gelatin, and LPS (Escherichia coli 0111:B4) were purchased from Sigma (Deisenhofen, Germany). All other reagents were also obtained from Sigma unless otherwise indicated.

Animals. Adult male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 250 ± 50 g were used in accordance with the institutional animal welfare guidelines of the University of Ulm and the Government of the Land Baden-Württemberg. C. parvum (20 mg/kg body wt) diluted in physiological saline was injected intravenously 4 days before liver perfusion. Control animals were injected with 0.9% saline (5). Animals were kept at constant temperature (22–24°C) and humidity, under a 12:12-h dark-light cycle, and 75 µl Dapsone (4,4'-diaminodiphenylsulfone; Cistron, Pine Brook, NJ) incubated according to the experimental protocol for 12 h. Cells were then scraped off the culture dishes, pelleted, washed three times with PBS, and homogenized in buffer containing 20 mM N-tris(hydroxymethyl)methyl-2-aminoeth-
aminoethylglycine, DTT, and glycerol, respectively, with 1 M HCl or NaHCO₃. Cell homogenates were then subjected to three rapid freeze-thaw cycles and centrifuged at 100,000 g for 60 min at 4°C. The cytosolic fractions (crude cytosol) were stored at −80°C until further processing. Protein concentrations were measured using a commercially available test based on the Lowry reaction (Sigma). Equal amounts of total protein (50 µg) obtained from rat hepatocyte cytosol were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrohoresitically transferred to nitrocellulose membranes in the presence of 20% methanol, 25 mM tris(hydroxymethyl)aminomethane, and 192 mM glycine, at pH 8.3. Nonspecific binding to the membrane was blocked by 5% nonfat dry milk in PBS-Tween 20 at 4°C overnight. The blots were washed twice in PBS-Tween 20 and then incubated with an affinity-purified immunoconjugated secondary antibody (goat-anti-rabbit IgG, conjugated with horseradish peroxidase (Amersham), dilution 1:1,500) at room temperature for 1 h. After incubation, membranes were subjected to three washing procedures and incubated with the primary antibody (goat-anti-rabbit IgG, conjugated with horseradish peroxidase (Amersham), dilution 1:1,500) at room temperature for 1 h. After incubation, membranes were washed three times with PBS-Tween 20 and developed with 10 ml of a 1:1 mixture of solution 1 and solution 2 of the ECL detection system (Amersham) for 1 min, dried immediately, and exposed to film for 0.5–15 min.

Statistical analysis. Values are expressed as means ± SE. Significance of differences was determined with the use of the analysis of variance test (Statview statistics program; Abacus Concepts). Statistical significance was established at P < 0.05.

RESULTS

pH-dependent NO₂⁻-NO₃⁻ formation. Figure 1A depicts NO₂⁻ plus NO₃⁻ levels measured in normal rat hepatocytes after stimulation with CM. Incubation with CM at a physiological pH (7.4 ± 0.1) led to a potent increase of NO formation that was not significantly changed when the pH in the medium was adjusted to pH 7.0 ± 0.1. In contrast, incubation of nonprimed hepatocytes with CM at pH 7.8 ± 0.1 showed a significant increase (P < 0.05) in NO levels compared with values detected at pH 7.4 ± 0.1. Severe infection, as seen in septic shock syndrome for example, is characterized by two phases, a priming phase to trigger metabolic changes followed by a second insult such as LPS (9, 41). Therefore, C. parvum-primed hepatocytes were exposed only to LPS to mimic a second insult. Isolated hepatocytes from in vivo primed rats showed a "constitutively" high level of NO production (96.2 ± 10 µM measured as NO₂⁻ plus NO₃⁻) at a physiological pH. This generation was unchanged when the pH in the medium was either decreased to pH 7.0 or increased to pH 7.8 (Fig. 1B). However, the NO production was increased to 239.3 ± 18 µM when C. parvum-primed hepatocytes were exposed to LPS at pH 7.4 ± 0.1. Even though an increased NO formation (96.2 ± 10 vs. 163.2 ± 5.7 µM) was detected in response to LPS at an acidic pH, this increase was significantly (P < 0.01) lower than NO levels measured at a physiological or alkaline pH.

pH-dependent hepatic OH-like radical formation. The formation and stability of OH or OH-like radicals, either through the metal-catalyzed Fenton and Haber-Weiss chemistry or via the formation of ONOO⁻, is dependent on the surrounding pH levels (2, 7, 16) and has been shown to damage cells (33, 43), including hepatocytes (38). Therefore, we investigated whether the generation of OH or OH-like radicals also relies on the pH in primed and normal hepatocytes when exposed to LPS and CM, respectively. Figure 2 shows that acidification of the medium (pH 7.0) results in a significant (P < 0.01) OH-like radical production (measured as 2,3-DHB plus 2,5-DHB) compared with levels measured at physiological or alkaline medium conditions. Incubation of normal or C. parvum-primed hepatocytes with CM or LPS resulted in a further OH-like radical production (2,3-DHB plus 2,5-DHB) compared with levels measured at physiological or alkaline medium conditions. The increase was, however, only significant (P < 0.05) at pH 7.0. It is interesting to note that the total amount of OH-like radical formation decreased with the alkalinization of the surrounding pH (7.0 > 7.4 > 7.8) in stimulated and unstimulated hepatocyte cultures (Fig. 2, A and B).
PH-dependent ONOO⁻ formation. Recent evidence suggests that the formation of ONOO⁻ and its stability are more likely under acidic rather than physiological or alkaline pH conditions (2, 7, 16). Because septic patients frequently suffer from acidosis with a low blood pH in addition to increased NO levels, we performed studies to investigate whether we would observe an increased ONOO⁻ formation under acidic conditions. In supernatants of normal and C. parvum-primed hepatocytes we detected a significant (P < 0.05) increase of ONOO⁻ after the exposure to CM or LPS (Table 1). The highest increase was observed at pH 7.0. It is interesting to note that at pH 7.0 normal hepatocytes showed a high background level of oxidized dihydrodihromazine 123 that was not completely abolished after L-NMMA addition. Although we added catalase to the cultures, one possible explanation for this background might be the presence of increased H₂O₂ formation that can cause the oxidation of dihydrodihromazine 123 in the presence of cytochrome-c or endogenous peroxidases.

PH-dependent hepatocellular damage. Cell viability was consistently 88 ± 3.5% in all cell cultures after 24 h of treatment, demonstrating that the observed differences were not due to cell death. As shown in Fig. 3, A and B, the transaminase baseline changed in treated and nontreated cultures, depending on the pH in the medium. The highest release of LDH and AST was observed in nonstimulated normal and C. parvum-primed hepatocytes at acidic pH conditions. Furthermore, we observed in cytokines and/or LPS-stimulated normal or C. parvum-primed hepatocytes a significant (P < 0.05) increase of LDH release at acidic (pH 7.0) medium conditions. The lowest AST and LDH levels were measured at pH 7.8 in stimulated or nonstimulated normal and C. parvum-primed hepatocytes (Fig. 3, A and B).

PH-dependent effects of hepatic NOS inhibition by L-NMMA. Several recent studies suggest that the inhibition of NO synthesis in sepsis promotes oxygen radical-mediated hepatic injury (5, 15, 20, 22, 35). Thus we studied OH-like formation and NO formation under acidic, physiological, and alkaline medium conditions in the presence or absence of inflammatory stimuli and/or L-NMMA. In addition, we measured the LDH and AST release under these circumstances to elucidate the hepatic cellular damage caused by both the pH changes in the medium and the inhibition of NO. Inhibition of NOS by L-NMMA in CM-stimulated normal hepatocytes resulted only at pH 7.0 in a significant (P < 0.01) release of OH (measured as 2,5-DHB) and LDH in the supernatants (Figs. 4A and 5A). In con-

Table 1. Superoxide and peroxynitrite formation under different pH conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Superoxide</th>
<th>Peroxynitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.0</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>Control hepatocytes</td>
<td>21.9 ± 2.8</td>
<td>3.7 ± 1.9</td>
</tr>
<tr>
<td>+CM</td>
<td>5.5 ± 1.6</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>+CM + 0.5 mM L-NMMA</td>
<td>16.8 ± 3.4</td>
<td>9.6 ± 2.3</td>
</tr>
<tr>
<td>C. parvum-primed hepatocytes</td>
<td>1.6 ± 1.2</td>
<td>8.6 ± 2.5</td>
</tr>
<tr>
<td>+LPS</td>
<td>3.2 ± 0.4</td>
<td>3.6 ± 0.7</td>
</tr>
<tr>
<td>+LPS + 0.5 mM L-NMMA</td>
<td>6.7 ± 2.1</td>
<td>9.6 ± 1.8</td>
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Values are means of triplicates ± SE of 3 separate experiments in nmol·60 min⁻¹·10⁶ cells⁻¹. pH-dependent superoxide and peroxynitrite formation was measured in normal and C. parvum-primed rat hepatocytes. Hepatocytes were exposed either to medium (control), cytokine mixture [CM; 10 μg/ml lipo polysaccharide (LPS), 5 U/ml interleukin-1β, 100 U/ml interferon-γ, 500 U/ml tumor necrosis factor-α], or LPS (10 μg/ml) in the presence or absence of 0.5 mM N⁵-monomethyl-l-arginine (L-NMMA). The pH value in the culture medium was strictly adjusted to 7.0 ± 0.1, 7.4 ± 0.1, and 7.8 ± 0.1, respectively, with 1 M HCl or NaHCO₃. Superoxide and peroxynitrite levels were measured as described. ND, not detected.
The inhibition of NOS in LPS-treated C. parvum-primed hepatocytes led to a significant increase of OH (measured as 2,5-DHB) and LDH at all pH conditions (Figs. 4B and 5B).

**Effects of pH-dependent changes and NO synthesis inhibition on GSH levels.** It has been demonstrated that the presence of GSH is necessary for a functional NO synthesis of the purified enzyme (39) and in endothelial cells (12). In addition, it was shown that extracellular GSH is a protecting compound against oxidative liver damage (23). Therefore, we investigated whether hepatocellular GSH levels are dependent on the surrounding pH, NO synthesis induction, and inhibition.

Figure 6, A and B, shows that GSH content is higher in normal nonprimed hepatocytes than in C. parvum-primed hepatocytes under all culture conditions. Acidification of the medium (pH 7.0) resulted in a significant decrease of GSH levels in normal and C. parvum-primed hepatocytes compared with levels detected at pH 7.4 and 7.8. The induction of NO synthesis by cytokines and/or LPS in normal or C. parvum-primed hepatocytes resulted in cellular GSH depletion under all pH conditions. Moreover, GSH content was further decreased when NO synthesis was inhibited by L-NMMA.

**pH-dependent superoxide formation.** Once superoxide is formed in a cell it can react in several ways. With NO it can form ONOO" and react to NO₂ and NO₃. To address the issue of whether the dramatic drop of intracellular GSH is the result of a possible increased oxidative damage when L-NMMA is added to hepatocyte cultures, we measured O₂ levels at all culture conditions in the presence and absence of L-NMMA. As shown in Table 1 we found the highest O₂ level in control supernatants, with the highest degree at pH 7.0. Addition of CM or LPS resulted always in a drop of O₂ levels that was partially corrected in the presence of L-NMMA. These results underline the protective role of NO in hepatocytes, but they also show that radicals can act in concert on the antioxidative GSH system.

**pH-dependent NOS2 protein expression.** To determine whether the changes of NO production seen at different pH conditions were associated with different hepatocellular NOS2 protein expression, Western blot analyses were performed on cell lysates from treated and nontreated hepatocyte cultures. Stimulation of normal hepatocytes with CM resulted under all pH conditions in a similar degree of NOS2 protein expression (data not shown). Although NOS2 was constitutively expressed in C. parvum-primed hepatocytes and further increased after LPS stimulation, no significant changes were observed among the different pH conditions (Fig. 7).
DISCUSSION

During severe inflammatory events such as sepsis, several metabolic and pathophysiological changes take place, including the secretion of nitrogen and oxygen intermediates. Parallel to these changes local or systemic acidification is observed, followed by severe acidosis and organ dysfunction (e.g., liver). Although it is established that NO controls liver blood flow and hepatic metabolism, its role in cellular and molecular mechanisms of cell injury and/or protection is far from clear. Under septic conditions, NOS overexpression in the liver can lead to several consequences: the decrease of protein and DNA synthesis, enhanced antimicrobial activity, inhibition of GAPDH, inhibition of enzymes of mitochondrial respiration, inhibition of cytochrome P-450, apoptosis, and intracellular nonheme iron-nitrosyl formation (19). On the other hand, NO has been described to be cytoprotective in the liver by preventing oxygen-mediated cellular injury (5, 15, 20, 22, 35).

Because the cytotoxic potential as well as the stability of oxygen and nitrogen intermediates seem strongly dependent on the surrounding pH (2, 7, 16, 33), the aim of the present study was to investigate the effects of pH changes on both free radical formation and hepatocellular damage.

Our experiments clearly revealed that the formation of OH or OH-like radicals, ONOO⁻, NO production, and hepatocellular damage are strictly dependent on pH conditions in normal and C. parvum-primed hepatocytes. Modest acidification (pH 7.0) of the medium as seen in sepsis resulted in an increased OH-like radical and LDH baseline in normal and C. parvum-primed hepatocytes compared with levels measured at physiological or alkaline medium conditions. The stimulation of normal and C. parvum-primed hepatocytes with CM and LPS, respectively, led to an increased NO production and a further OH-like radical generation. However, significant hepatocellular damage (measured as LDH release) in normal and C. parvum-primed hepatocytes was only found at pH 7.0. It is noteworthy that the NO production in LPS-stimulated C. parvum-primed hepatocytes is lower in acidic than in physiological or alkaline conditions. Our results suggest that the generation of OH-like radicals either by the superoxide-driven Fenton reaction or via ONOO⁻ in septic livers may be faster and more stable under acidic conditions compared with the reaction of NO with superoxide at physiological or alkaline conditions. In keeping with this evidence is our observation that the relative amounts of superoxide decrease in parallel with an increased NO production in stimulated hepatocytes at all culture conditions, which demonstrates the antioxidative effects of NO by scavenging superoxide.

**ONOO⁻** has been reported to exert several cytotoxic effects in various cell types (4, 7, 33, 43). In normal and C. parvum-primed hepatocytes, we measured ONOO⁻...
levels at all culture conditions after the stimulation with CM and LPS. Although we were unable to detect large amounts of ONOO\(^-\) at pH 7.4 and pH 7.8, our data are still in agreement with recent published work by Radi et al. (33). These authors reported that cellular damage caused by exogenous addition of ONOO\(^-\) is enhanced under acidic rather than alkaline pH conditions. This finding of pH-dependent cytotoxicity of ONOO\(^-\) was further substantiated by data from Crow et al. (7), who demonstrated that a less toxic ONOO\(^-\) isoform is synthesized at alkaline pH conditions. The same line of evidence stresses our observations of low ONOO\(^-\) formation, which correlates with the lowest percentage of cell damage. However, from our experimental setup we were unable to distinguish between different isoforms of ONOO\(^-\) (7).

The inhibition of NO synthesis in LPS-treated primed hepatocytes caused increased hepatocellular damage at all medium conditions, with a maximum seen at acidic (pH 7.0) conditions. This hepatocellular damage, as observed when NOS is competitively blocked, is most likely due to increased ROI production, as demonstrated by increased superoxide and OH radical production. Under these conditions, increased OH production is generated because NO cannot react with superoxide to form less-toxic intermediates, thus promoting the iron-catalyzed Fenton and Haber-Weiss chemistry. Therefore, we hypothesize that generated radicals and their adducts in hepatocytes are part of a cascade that possesses higher cytotoxic capacity under acidic conditions and may play a minor role under physiological or alkaline pH conditions.

GSH has been shown to protect the liver from ROI toxicity (10, 20, 21, 23). We found that GSH content is decreased in hepatocytes under acidic conditions and declines further when cells are exposed to cytokines and/or LPS. In contrast, at physiological or alkaline medium conditions we observed that reduced OH-like radical and ONOO\(^-\) production was paralleled by increased GSH levels. This increased GSH content at pH 7.4 and pH 7.8 correlated with an enhanced NO production and a reduction of superoxide and hepatocellular damage in LPS-treated C. parvum-primed hepatocytes. Furthermore, increased GSH levels are associated with the necessity of GSH for maximal NOS activity and enzyme stability (39).

Several investigators have shown that the inhibition of NO synthesis in the liver during sepsis promotes oxidative damage (5, 15, 20, 22, 35), resulting in increased ROI production. In addition, recent evidence suggests that NO itself may negatively regulate the antioxidative GSH system by inhibiting GSH reductase via the formation of S-nitroglutathione (3). Therefore, our results suggest that under certain circumstances NO itself can induce or enhance oxidative stress in cells by modifying GSH content, as recently suggested (25). The cytoprotective effects of NO in sepsis are described as follows: scavenging of radical oxygen intermediates, mainly superoxide (43), activation of soluble guanylate cyclase for guanosine 3',5'-cyclic monophosphate synthesis to inhibit platelet adherence (34) or aggregation (28), and neutrophil chemotaxis (17). In addition, there is increasing evidence that NO can antagonize the vasoconstrictive effects of the platelet-activating factor or prostaglandins to maintain hepatic blood flow (42).

On the basis of our data, it seems disadvantageous to reduce NO formation at acidic pH conditions (pH 7.0 ± 0.1) (similar to the acidosis observed in sepsis) because NO could not function as a scavenger for ROI (43). Therefore, we suggest that in sepsis, in which sustained acidosis and hypotension frequently occur, adjusting systemic pH toward the physiological value and then inhibiting NO would be more beneficial for patients than directly blocking sustained NO overproduction. This approach seems to be superior because it reduces both the high OH-like radical baseline and the eventually formed ONOO\(^-\), because either product is more stable at acidic than at physiological or alkaline conditions (2, 7, 16, 33). In addition, our data imply that a reduced OH-like radical baseline as seen under physiological or alkaline pH conditions results in an increased intracellular GSH pool that could additionally serve to detoxify reactive radicals and increase NOS activity and NO production. Finally, our data emphasize the complexity of radical formation and interactions that will need further investigations before any application can be envisaged in vivo.
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