Effects of Rofecoxib, a Selective Cyclooxygenase-2 Inhibitor, on Endothelial Dysfunction, Lipid Peroxidation, and Hepatocyte Morphology in Rats with Sepsis-Induced Liver Damage

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ABSTRACT

Background: Sepsis remains a difficult problem for clinicians, with its systemic effects and high morbidity and mortality rates. The roles of oxidative stress, endothelial dysfunction, and lipid peroxidation in sepsis-induced organ damage are being investigated.

Objective: The aim of this study was to investigate the effects of selective cyclooxygenase (COX)-2 inhibition on tissue lipid peroxidation, endothelial dysfunction, and hepatic cell morphology in a rat model of sepsis.

Methods: Thirty rats with sepsis induced by cecal ligation and puncture were divided equally into 3 groups: treatment group (rofecoxib 1 mg/kg PO), control group (saline 1 mL PO), and sham group (sham surgery only). All the rats were sacrificed 1 day after sepsis induction. The livers were removed using a median laparotomy for histopathologic and biochemical analysis.

Results: Histomorphologic hepatic damage and lipid peroxidation were significantly reduced in the rofecoxib treatment group compared with the control group ($P < 0.05$ and $P = 0.001$, respectively). Endothelial nitric oxide synthase and inducible nitric oxide synthase staining of liver samples was statistically significantly reduced in the treatment group compared with the control group (both, $P < 0.001$). The hepatic nitric oxide level and malonyldialdehyde activity decreased significantly ($P < 0.001$ and $P = 0.001$, respectively) in the rofecoxib group compared with the control group. Hepatic myeloperoxidase activity was similar between the treatment and control groups.

Conclusion: Further investigation of selective COX-2 inhibition as an alternate therapeutic choice for sepsis-induced hepatic damage should be considered. (*Curr Ther Res Clin Exp.* 2004;65:278–291) Copyright © 2004 Excerpta Medica, Inc.

Key words: lipid peroxidation, nitric oxide, selective COX-2 inhibitor, experimental sepsis.

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INTRODUCTION

Sepsis is a major problem in intensive care units despite advances in the understanding of the pathophysiologic mechanisms of this condition and the development of many therapeutic strategies for its management. Sepsis, a clinical syndrome resulting from an exaggerated systemic response by the host to infection, may lead to multiple organ failure and death.¹ Acute peritonitis is a common source of sepsis or systemic inflammation induced by multiple organ failure.²

The liver is thought to be one of the organs most affected by sepsis because it plays a pivotal role in the immunologic defense mechanisms of the organism.^{1,3-5} The liver has 2 opposing roles in sepsis: it serves as (1) a source of inflammatory mediators and (2) a target organ for the effects of the inflammatory mediators. The liver is pivotal in modulating the systemic response to severe infection because it contains the largest mass of macrophages (Kupffer cells) in the body.^{1,4,5}

In vitro and in vivo experiments⁶⁻⁸ have indicated that the hemodynamic and metabolic changes that occur during sepsis involve marked increases in the production of nitric oxide (NO), cytokines, and eicosanoids. The benefits of NO include maintenance of tissue perfusion, inhibition of platelet aggregation, and improvement of splanchnic circulation.⁹ In sepsis, NO has a complex role: it regulates blood pressure and antimicrobial defense mechanisms and modulates the inflammatory response. The hyperdynamic response and increased cytokine levels in sepsis cause the induction of inducible NO synthase (iNOS) in the endothelial cells of numerous tissues. Although iNOS expression seems to be beneficial in acute sepsis, its overexpression in sepsis can be detrimental.^{10,11} It can cause the overproduction of NO and causes NO to induce vasodilatation¹² and refractory hypotension, which may contribute to organ damage.¹³⁻¹⁵ Increases in iNOS and NO can be detected systemically and in the liver, kidneys, and small intestine.¹¹

One treatment for sepsis involves inhibiting the consequences of iNOS activation. Interactions of transcription factors, inducers, cofactors, and regulators of iNOS are important in understanding the development of iNOS inhibitors.¹² Inhibition through the administration of L-arginine analogues, depletion of arginine, inhibition of cofactors, modulation of gene transcription, and scavenging of NO have been studied.¹² Human studies have been conducted only with nonselective L-arginine analogues.¹² Reduction of mortality from sepsis as a result of these treatments has not been reported, however. It is anticipated that iNOS-specific compounds will be clinically useful, and future human trials will focus on these agents. In addition, although ideal therapy for treating the vasodilatation in sepsis is not available, research into the pathophysiology of NOS in sepsis has clarified the complexities surrounding this therapeutic dilemma.¹²

In addition to its effects on the vascular system, NO mediates some cellular events and damage in the liver during sepsis. In the inflammatory tissues, NO rapidly reacts with superoxide radicals to form peroxynitrite, a highly reactive

oxidant species. Oxygen free radicals (OFRs) are intermediates produced from the partial reduction of molecular oxygen in biologic systems. The toxic effect of OFRs has been demonstrated in several forms of cellular damage and a variety of disease states.¹⁶ Common conditions in which an increased production of OFRs may be largely responsible for host cell damage are inflammatory disorders, oxygen toxicity, circulatory shock, and postischemic reperfusion damage.¹⁷⁻¹⁹ OFRs, as measured by the lipid peroxide byproduct malonyldialdehyde (MDA), are generated during endotoxic shock and sepsis,²⁰ and have been shown to be elevated in sepsis.^{21,22} The mechanism of OFR generation in sepsis is most likely leukocytes primed by activated complement fragments (polymorphonuclear membrane-bound oxidase), which is activated by the C5a component of the complement system²³ and is a consequence of exposure to endotoxin.²⁴ OFRs generated during sepsis not only damage cellular membranes by direct lipid peroxidation but also cause an increase in arachidonic acid turnover,² which leads to increased levels of leukotrienes and prostaglandins, which have immunologic and hemodynamic implications in the pathophysiology of sepsis.²⁰

As a result, treatments have also focused on the inadequate inflammatory response and the antioxidant status that characterize the excessive systemic inflammatory response in the early phase of sepsis. Because direct detection of OFRs is difficult *in vivo*, many studies of tissue damage due to inflammation have relied on a beneficial response to treatment with antioxidant agents or on elevated products of the action of OFRs, including lipid peroxides, as indirect evidence that OFRs were involved.^{21,22,25}

Finally, in patients with sepsis, production of arachidonic acid metabolites by cyclooxygenase (COX) increases, but the pathophysiologic role of these prostaglandins is unclear. In animal models, inhibition of COX by treatment with ibuprofen before the onset of sepsis reduced the physiologic abnormalities and improved survival.²⁶ In patients with sepsis, treatment with ibuprofen reduces levels of prostacyclin and thromboxane and decreases fever, tachycardia, oxygen consumption, and lactic acidosis, but it does not prevent the development of shock or acute respiratory distress syndrome and it does not improve survival.²⁶

The optimal therapy is debatable, and improved treatment is necessary to increase the host response and host monitoring. The aim of this study was to investigate the effects of a selective COX-2 inhibitor (ie, rofecoxib) on tissue lipid peroxidation, endothelial dysfunction, and hepatic cell morphology in a rat model of sepsis.

MATERIALS AND METHODS

Study Design

This animal study was conducted at the Departments of General Surgery, Biochemistry, and Histology and Embryology, University of Celal Bayar (Manisa,

Turkey). All study procedures were performed in strict accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals. Approval for the study protocol was obtained from the university ethics committee.

Thirty Sprague-Dawley rats weighing between 170 and 190 g were used. All animals were given a laboratory diet and tap water ad libitum for 10 days prior to the experiment. The animals were starved for 24 hours before the experiment. Study drugs were administered during the 24-hour period prior to the induction of sepsis.

The 30 animals were divided equally into 3 groups. The treatment group received rofecoxib 1 mg/kg orogastric tube over 24 hours. The control group received saline 1 mL PO over 24 hours. The sham group underwent median laparotomy with handling of the cecum without any drug treatment. In the treatment and control groups, a median laparotomy was performed to induce sepsis by ligation and puncture of the cecum. After fecal contamination of the visceral peritoneum was observed, the abdomen was closed with 3-0 silk sutures. All rats were sacrificed using decapitation on the first day of abdominal sepsis.

Biochemical Analysis

After decapitation of the rats, the livers were immediately removed and washed with ice-cold (4°C) saline, placed in glass bottles, labeled, and stored at -80°C until homogenization. Immediately prior to homogenization, the livers were cut into small pieces using scissors. They were then homogenized in 4 volumes of ice-cold Tris hydrochloride buffer (50 mmol/L; pH, 7.5), using a basic homogenizer (Ultra-Turrax T25, IKA Werke GmbH & Co. KG, Staufen, Germany) for 2 minutes at 10,000 rpm. Clear upper supernatant fluid was taken and myeloperoxidase (MPO) activity, considered a marker of the inflammatory response to sepsis, was determined. All procedures were performed at 4°C.

Malonyldialdehyde Analysis

Tissue MDA level, a marker of lipid peroxidation, was determined using a spectrophotometer (UV-120IV, Shimadzu Corporation, Tokyo, Japan) according to the method described by Ohkawa et al.²⁴ An external standard curve was prepared using 1,1,3,3-tetraethoxypropane. MDA levels are expressed as nmol/g protein.

Nitric Oxide Analysis

NO was not measured directly, but the surrogate markers of NO were analyzed. NO level was determined in the homogenate, and the homogenate was then centrifuged at 5000g for 1 hour to remove debris. Because nitrite (NO_2^-) and nitrate (NO_3^-) levels can be used to estimate NO production,²⁷ we measured the concentrations of these stable NO oxidative metabolites in homogenized hepatic tissue. Determination of NO_2^- and NO_3^- was based on the Griess reac-

tion,²⁷ in which a chromophore with a strong absorbance at 545 nmol/L is formed when NO_2^- reacts with a mixture of naphthylethylenediamine and sulfanilamide.²⁷ A standard curve was established with a set of serial dilutions (100 to 5 $\mu\text{mol/L}$) of sodium nitrite. The resulting standard curve was then used to calculate the unknown sample concentrations. NO data are expressed as $\mu\text{mol/g}$ protein.

Myeloperoxidase Activity

The method described by Wei and Frenkel²¹ was used for the tissue MPO assay. In this method, 1.3 mL of 25 mmol/L 4-aminoantipyrine 2% phenol solution and 1.5 mL of 1.7 mmol/L hydrogen peroxide (H_2O_2) were added and equilibrated for 3 to 4 minutes. After establishing the basal rate, a 0.2-mL sample of filtered supernatant was added to the cuvettes and quickly mixed. Increases in absorbance at 510 nmol/L-min were recorded. One unit of MPO activity is defined as that which degrades 1 μmol of H_2O_2 /min at 25°C. MPO data are expressed as U/g protein.

Histologic Assessment

All specimens were fixed in 10% formalin for 24 hours. Specimens were washed and dehydrated in a graded series of ethanol (50%–100%) and were then embedded in paraffin. Sections 5-mm thick were cut and prepared for histochemical and immunohistochemical staining. Hematoxylin–eosin staining was used for histologic diagnosis. The liver specimens were assessed for sinusoidal dilatation, periportal edema, necrosis, hemorrhage, perisinusoidal cell infiltration, and abnormal hepatocyte morphology. All specimens were assessed under light microscopy by the same histologist who was blinded to treatment assignment. For each liver slide, a minimum of 10 fields were examined and assigned for severity of changes using scores of none (–), mild (+), moderate (++), and severe (+++).

Immunohistochemical Assessment

For immunohistochemical staining, sections were first incubated at 60°C overnight and then incubated in xylene for 30 minutes. After washing with a decreasing series of ethanol (100%–50%), sections were washed with distilled water and phosphate-buffered saline (PBS) for 10 minutes. Sections were then treated with 2% trypsin in Tris buffer (50 mmol/L Tris base and 150 mmol/L sodium chloride dissolved in deionized water) at 37°C for 15 minutes and washed with PBS. Sections were delineated with a Dako pen (DakoCytomation Denmark A/S, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 minutes to inhibit endogenous peroxidase activity. Sections were then washed with PBS and incubated for 18 hours at 4°C with the following primary antibodies: anti-endothelial nitric oxide synthase (eNOS) in a 1/200 dilution (SA-258, BIOMOL International LP, Hamburg, Germany), anti-iNOS in a 1/100 dilution (61-770, Zymed Laboratories, Inc., South San Francisco, Califor-

nia), and Ki67 in a 1/100 dilution (RB-081-A1, NeoMarkers, Fremont, California). Sections were then washed 3 times for 5 minutes each with PBS, followed by incubation with biotinylated immunoglobulin G and then with streptavidin-peroxidase conjugate (DakoCytomation Denmark A/S). All incubation steps were separated by 3 washing steps. After washing 3 times for 5 minutes with PBS, sections were incubated with diaminobenzidine substrate containing diaminobenzidine (DakoCytomation Denmark A/S) for 5 minutes to stain immunolabeling and then with Mayer's hematoxylin. Sections were covered with mounting medium and were analyzed using light microscopy with a BX 40 microscope (Olympus Diagnostica, Tokyo, Japan). Control samples were processed in a similar manner, but the primary antibody step was omitted. Two observers blinded to treatment assignment assessed the staining scores independently. Staining intensity was graded as 1 = mild, 2 = moderate, and 3 = strong.

Statistical Analysis

All data are expressed as mean (SEM). Groups of data were compared using the Student *t* test for unpaired data or analysis of variance followed by the Tukey multiple-comparison test. The assessment of nonparametric variables was made by Kruskal-Wallis and chi-square tests. Values of $P < 0.05$ were regarded as significant. For all statistical analyses, we used SPSS software version 11.0 (SPSS Inc., Chicago, Illinois).

RESULTS

Cecum ligation and puncture-induced experimental sepsis resulted in histomorphologic changes in the livers of the rats.

Biochemistry

After treatment, the MDA level was significantly lower in the rofecoxib group compared with the control group (mean [SEM], 0.44 [0.11] vs 0.74 [0.15] nmol/g protein, respectively; $P = 0.001$). The NO level was significantly lower in the rofecoxib-treated rats compared with the control group (mean [SEM], 2.92 [0.32] vs 4.34 [0.38] $\mu\text{mol/g}$ protein, respectively; $P < 0.001$). NO levels were also significantly reduced in the rofecoxib-treated group compared with the sham group ($P = 0.013$). MPO activity was similar between the treatment and control groups (mean [SEM], 0.18 [0.11] vs 0.33 [0.22] U/g protein, respectively) (Table I, Figure 1).

Histology

Sinusoidal dilatation was significantly decreased in the treatment group compared with the control group (mean [SEM], 2.10 [0.23] and 2.80 [0.33], respectively; $P < 0.01$). Among rofecoxib-treated rats, sinusoidal dilatation was severe in 2 rats (20%), moderate in 4 (40%), and mild in 4 (40%). In the control group,

Table I. Mean (SEM) biochemical data after treatment (N = 30 rats).

Variable	Rofecoxib (n = 10)	Control (n = 10)	Sham (n = 10)	P (Rofecoxib vs Control)	P (Rofecoxib vs Sham)	P (Control vs Sham)
MDA, nmol/g protein	0.44 (0.11)	0.74 (0.15)	0.60 (0.21)	<0.001	NS	NS
NO, μ mol/g protein	2.92 (0.32)	4.34 (0.38)	1.82 (0.23)	<0.001	0.013	0.011
MPO, U/g protein	0.18 (0.11)	0.33 (0.22)	0.10 (0.08)	NS	NS	NS

MDA = malonyldialdehyde; NO = nitric oxide; MPO = myeloperoxidase.

Figure 1. Means (SEM) and ranges of nitric oxide (NO) and malonyldialdehyde (MDA) levels after treatment, by group. * $P < 0.001$ versus control group; † $P = 0.013$ versus sham group; ‡ $P = 0.001$ versus control group; § $P = 0.011$ versus control group.

8 rats (80%) had mild or moderate sinusoidal dilatation, and 2 (20%) had no dilatation (**Table II**).

In the hepatic samples, edema was similar between the treatment and control groups (mean [SEM], 0.50 [0.17] and 0.60 [0.13], respectively). Necrosis was significantly lower in the rofecoxib-treated group compared with the control group (0.30 [0.15] vs 0.70 [0.15], respectively; $P = 0.04$). Hemorrhage was similar in the treatment and control groups (mean [SEM], 0.60 [0.16] vs 0.80 [0.13], respectively). Sepsis-induced abnormal perisinusoidal inflammatory cell infiltration and abnormal hepatocyte morphology were significantly lower in the treatment group compared with the control group ($P = 0.023$ and $P < 0.001$, respectively) (**Table II**). Liver specimens from both groups are shown in **Figure 2**.

Immunohistochemistry

The eNOS and iNOS staining of the hepatic samples in the treatment and control groups showed statistical significance ($P < 0.001$ and $P < 0.001$, respectively) (**Table II**). eNOS and iNOS levels were similar between the treatment and sham

Table II. Mean (SEM) histomorphologic data after treatment (N = 30 rats).

Variable	Rofecoxib (n = 10)	Control (n = 10)	Sham (n = 10)	P (Rofecoxib vs Control)	P (Rofecoxib vs Sham)	P (Control vs Sham)
SD	2.10 (0.23)	2.80 (0.33)	1.50 (0.22)	<0.01	0.006	0.007
Edema	0.50 (0.17)	0.60 (0.13)	0.20 (0.13)	NS	NS	0.02
Necrosis	0.30 (0.15)	0.70 (0.15)	0.25 (0.13)	0.04	NS	<0.01
Hemorrhage	0.60 (0.16)	0.80 (0.13)	0.10 (0.01)	NS	0.011	<0.001
PCInf	1.80 (0.29)	2.80 (0.44)	0.90 (0.18)	0.023	0.003	0.005
AbHM	1.80 (0.25)	2.80 (0.29)	1.30 (0.15)	<0.001	<0.001	<0.001
eNOS	1.20 (0.13)	1.70 (0.15)	1.30 (0.15)	<0.001	<0.001	NS
iNOS	1.40 (0.22)	1.60 (0.16)	1.10 (0.01)	<0.001	<0.001	0.022

SD = sinusoidal dilatation; PCInf = perisinusoidal cell infiltration; AbHM = abnormal hepatocyte morphology; eNOS = endothelial nitric oxide synthase; iNOS = inducible nitric oxide synthase.

Figure 2. Liver specimens from (A) the treatment group (rofecoxib 1 mg/kg) and (B) the control group (saline 1 mL). Although perisinusoidal inflammatory cell infiltration, edema, and necrosis were detected in the liver of the control group, healing of liver damage occurred in the treatment group (hematoxylin and eosin; original magnification $\times 400$).

groups. eNOS was similar between the control and sham groups, but iNOS was significantly lower in the control group than in the sham group ($P = 0.022$) (Table II, **Figure 3**).

DISCUSSION

Oxidants released as a result of the inflammatory response were produced in our experimental sepsis and can result in severe cell damage, most notably the peroxidation of cell membrane lipids. Such damage may result from the higher OFR levels, which could actually have stimulated the enzyme activities, as previously reported.²³ Elevated enzyme levels in sepsis might increase tissue damage, possibly by overscavenging superoxide and inhibiting the termination step of lipid peroxidation.²⁵ As we found, hepatic MDA levels were elevated during sepsis in the control group. Rofecoxib-treated animals showed statistically significantly decreased levels of MDA. As previously mentioned, the enhanced lipid peroxidation products in septic rats may indicate cell membrane damage due to OFRs.^{22,25} These OFRs were presumably generated by activated leukocytes as part of the inflammatory response during sepsis.

COX-2 inhibition has been suggested to have different effects on iNOS and eNOS activities and also on NO production, depending on tissue response.²⁶ Our data are in agreement with those of previous studies that reported increased NO production and iNOS activity resulted in increased permeability in sepsis.^{12,28–32} Because overproduction of eicosanoids by COX-2 has been shown to be related to organ damage in animals with sepsis, studies have focused on the potential therapeutic benefits of selective COX-2 inhibitors in preventing sepsis and related pathophysiologic processes.^{29,33–38}

Figure 3. Immunolocalizations of (A, B) endothelial nitric oxide synthase (eNOS) and (C, D) inducible nitric oxide synthase (iNOS) in liver from (A, C) the treatment group (rofecoxib 1 mg/kg) and (B, D) the control group (saline 1 mL). Weak immunoreactivity of eNOS was detected in the treatment group; this immunoreactivity was increased in the control group. Negative iNOS immunoreactivity was observed in the treatment group, and strong and weak immunoreactivities were detected in the control group (immunostaining; original magnification $\times 400$).

In sepsis, hepatic cell damage has been suggested to be one of the most important steps leading to multiple organ failure. Therefore, therapeutic modalities have focused on hepatocytes as target cells in sepsis. In our study, COX-2 inhibition seemed to reverse hepatic damage, as indicated by the histomorphologic scores. Although hepatic MPO activity was decreased in the rofecoxib group, the difference was not statistically significant compared with either the control or the sham group; however, COX-2 inhibition ameliorated hepatic damage. Perisinusoidal inflammatory cell infiltration, edema, necrosis, and hemorrhage were reduced in the rofecoxib group.

In the present study, although histomorphologic and immunohistochemical changes in septic liver were found, mortality and survival of the rats were not

studied. Moreover, the findings of the study may be supported by administration of NO inhibitors, together with selective COX-2 inhibitors, in future clinical and experimental trials.

CONCLUSIONS

Selective COX-2 inhibition prevented lipid peroxidation and tissue damage in the livers of rats during sepsis. The interactions between NOS and the COX pathway in the pathophysiology of sepsis may change the therapeutic efficacy of COX-2 inhibition. Therefore, the effects of a COX-2 inhibitor on NO production and iNOS and eNOS activity, as well as on lipid peroxidation and antioxidant status, may demonstrate efficacy in sepsis or multiple organ failure. As a result, further clinical and experimental study protocols using selective COX-2 inhibitors in the treatment of sepsis may be warranted.

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