Protective Effects of N-acetylcysteine and a Prostaglandin E1 Analog, Alprostadil, Against Hepatic Ischemia: Reperfusion Injury in Rats

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ABSTRACT

Ischemia–reperfusion (I/R) injury has a complex pathophysiology resulting from a number of contributing factors. Therefore, it is difficult to achieve effective treatment or protection by individually targeting the mediators or mechanisms. Our aim was to analyze the individual and combined effects of N-acetylcysteine (NAC) and the prostaglandin E1 (PGE1) analog alprostadil on hepatic I/R injury in rats. Thirty male Sprague-Dawley rats were randomly divided into five groups (six rats per group) as follows: Control group, I/R group, I/R + NAC group, I/R + alprostadil group, and I/R + NAC + alprostadil group. The rats received injections of NAC (150 mg/kg) and/or alprostadil (0.05 μg/kg) over a period of 30 min prior to ischemia. These rats were then subjected to 60 min of hepatic ischemia followed by a 60-min reperfusion period. Hepatic superoxide dismutase (SOD), catalase, and glutathione levels were significantly decreased as a result of I/R injury, but they were increased in groups treated with NAC. Hepatic malondialdehyde (MDA), myeloperoxidase (MPO), and nitric oxide (NO) activities were significantly increased after I/R injury, but they were decreased in the groups with NAC treatment. Alprostadil decreased NO production, but had no effect on MDA and MPO. Histological results showed that both NAC and alprostadil were effective in improving liver tissue morphology during I/R injury. Although NAC and alprostadil did not have a synergistic effect, our findings suggest that treatment with either NAC or alprostadil has benefits for ameliorating hepatic I/R injury.

Key words: Alprostadil, Ischemia–reperfusion, N-acetylcysteine, Prostaglandin E1 analog

INTRODUCTION

Hepatic ischemia–reperfusion (I/R) is a key clinical problem associated with numerous adverse events such as hypovolemic shock,1 disseminated intravascular coagulation,2 liver transplantation and surgery,3 cardiac failure and arrest, alcohol toxicity,4 and several other pathological conditions. The observed pathophysiology of hepatic I/R injury is as follows. The liver is a highly
They were treated according to the regulations in the
examined by measuring different biochemical parameters. These
alprostadil against hepatic I/R effects against hepatic I/R injury remain unknown.
Although alprostadil has been used for several years in patients
blood with a concomitant increase in oxygen delivery to tissues.
platelet aggregation, and ameliorates the rheological properties of
product of purine metabolism that causes vasodilation, decreases
the adenosine levels in plasma. Adenosine is an endogenous by
function of the smooth muscles of peripheral blood vessels. It functions
vasodilator actions. Numerous experiments
are mainly due to its physiological role as an ROS scavenger.
NAC has been clinically used for
more than 30 years, primarily as a mucolytic agent. It has also been
used in states of decreased glutathione (GSH) and oxidative stress
in HIV infections, cancer, heart diseases, and other diseases. Due to
its hepatoprotective activity, intravenous and oral administrations
of NAC have been used extensively for the treatment of acetamino
phen poisoning. The diverse pharmacological applications of NAC
are mainly due to its physiological role as an ROS scavenger.[12,13]
Prostaglandin E1 (PGE1) is a naturally occurring prostaglan
d. It was originally introduced as a therapeutic agent because of
its potent direct vasodilator actions. Numerous experiments indicate a beneficial effect of PGE1 when intravenously admini
stered to patients with chronic vascular disease due to its pos
ible direct vasodilatory effect causing an increase in local tissue perfusion.[18] Alprostadil is a synthetic form of PGE1 that can
function as a PGE1 analog by producing a strong relaxant effect
on the smooth muscles of peripheral blood vessels. It functions as a peripheral vasodilator and platelet aggregation inhibitor by interacting with specific G-protein coupled receptors to increase the adenosine levels in plasma. Adenosine is an endogenous by
product of purine metabolism that causes vasodilation, decreases
leukocyte activities, provides for endothelial protection, inhibits platelet aggregation, and ameliorates the rheological properties of
blood with a concomitant increase in oxygen delivery to tissues.[17] Although alprostadil has been used for several years in patients
with liver transplants,[16] the mechanisms related to its protective
effects against hepatic I/R injury remain unknown.
In the present study, the protective effects of NAC and/or
alprostadil against hepatic I/R-induced tissue damage in rats were
examined by measuring different biochemical parameters. These
included the levels of the following parameters: (1) an end prod
uct of lipid peroxidation; (2) liver malondialdehyde (MDA); (3) antioxidant enzymes, including catalase (CAT), superoxide
dismutase (SOD), and GSH; and (4) indicators of inflammation,
including myeloperoxidase (MPO) activity and nitric oxide (NO).

MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (250-300 g) were obtained from the
animal center of the National Science Council, Taiwan, Republic
of China. They were fed a standard diet and were given water
ad libitum. They were treated according to the regulations in the
“Guide for the Care and Use of Laboratory Animals” (National Academy Press, 1996). The use of animals for our experiments
was approved by the ethics committee of the National Yang-Ming
University, Taipei, Taiwan.
Thirty rats were randomly divided into five groups (six rats in
each group) as follows: Control, I/R, I/R + NAC, I/R + alprostadil,
and I/R + NAC + alprostadil groups. Rats in the control group were
neither subjected to I/R injury nor any treatment. In the I/R groups
with treatments, the rats were subjected to 60 min of hepatic ischemia
followed by a 60-min reperfusion period. For the treatment
groups, an infusion pump (KDS220; KD Scientific Co., Holliston,
MA, USA) was used to infuse 150 mg/kg of NAC and/or 0.05 µg/ kg of alprostadil into the femoral vein continuously for 30 min prior
to 60 min of hepatic ischemia followed by 60 min of reperfusion.

Animal model and parameters for hepatic I/R injury
The rat model with I/R injury was generated as previously de
scribed.[17,18] Briefly, each male Sprague-Dawley rat was anesthetized
with urethane (1.25 g/kg, intraperitoneally) and its trachea was
cannulated for artificial respiration with a ventilator. A polyethyl
ene (PE-50) catheter was inserted into the femoral artery to monitor
blood pressure using a polygraph (TA240S; Gould Co., USA) and
for drug administration. The liver was exposed through an upper
midline incision and two pieces of fine silk thread were looped
into a snare with a piece of PE-90 tubing provided for the occlu
sion of the blood supply to the median and left lobes (left branch).
To study I/R injury, ischemia and reperfusion period (each main
tained for 60 min) of the median/left lobes with immediate
oclusion of the right lobe vasculature was carried out. One hour
after completing the reperfusion procedure, the initial ischemic–re
perfused median/left lobes were resected and determination of the
levels of MPO, MDA, GSH, CAT, SOD, and NO was made. Blood samples for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurements were collected immediately after
femoral catheterization and completion of the reperfusion procedure.

Biochemical analyses

MPO assay
MPO activity in the liver tissue was determined using a pro
cedure similar to that of Hillegas et al.[19] Harvested liver samples

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were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0) and centrifuged at 41,400 × g for 10 min. The pellets were then suspended in 50 mM PB containing 0.5% hexadecyl trimethyl ammonium bromide. After three cycles of freezing and thawing, with sonication between each cycle, the samples were centrifuged at 41,400 × g for 10 min. An aliquot (0.3 ml) was added to 2.3 ml of a reaction mixture containing 50 mM PB, 0.19 mg/ml o-dianisidine, and 20 mM H₂O₂ solution. Enzyme activity was defined as the amount of MPO that caused a change in the absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

**MDA assay**

A liver sample (0.5 g) was homogenized in 4.5 ml of thiobarbituric acid (TBA) reagent containing 0.375% TBA, 15% trichloroacetic acid, and 0.25 N HCl. Samples were boiled for 15 min, cooled, and centrifuged. Absorbance of the supernatants was spectrophotometrically measured at 532 nm.[20]

**GSH assay**

Hepatic GSH levels were determined using a commercial GSH assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). This assay uses a carefully optimized enzymatic recycling method in which glutathione reductase is used to quantify GSH. Liver tissue was first homogenized in 5-10 ml of cold buffer [either 50 mM MES or phosphate, pH 6-7, containing 1 mM ethylenediaminetetraacetic acid (EDTA)] per gram of liver tissue and centrifuged at 10,000 × g for 15 min at 4°C, followed by deproteinization with metaphosphoric acid. After adding triethanolamine solution, an assay cocktail [a mixture of MES buffer (11.25 ml), reconstituted cofactor mixture (0.45 ml), reconstituted enzyme mixture (2.1 ml), water (2.3 ml), and reconstituted 5,5’dithiobis (2-nitrobenzoic acid) (0.45 ml)] was prepared and total GSH in each of the deproteinated samples was determined spectrophotometrically at 405 or 414 nm.

**CAT assay**

CAT activity was determined using a commercial chemical CAT assay kit (Cayman Chemical Co.). This assay utilizes the peroxidative function of CAT to determine the enzyme activity. Liver tissue was homogenized in 5-10 ml of cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) per gram of liver tissue and centrifuged at 10,000 × g for 15 min at 4°C. The sample was then mixed sequentially with hydrogen peroxide, potassium hydroxide, Purpald, and potassium periodate, and then read at 540 nm.

**SOD assay**

Hepatic SOD activity was determined using a commercial SOD assay kit (Cayman Chemical Co.). This assay utilizes a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. Liver tissue was homogenized in 5-10 ml of cold buffer (20 mM HEPES buffer, pH 7.2, containing 1 mM EDTA, 210 mM mannitol, and 70 mM sucrose) per gram of liver tissue and centrifuged at 1500 × g for 5 min at 4°C. The reaction was initiated by adding xanthine oxidase and incubated at room temperature for 20 min, after which the absorbance was read at 450 nm.

**NO assay**

Hepatic NO activity was determined using a commercial nitrate/nitrite colorimetric assay kit (Cayman Chemical Co.). This assay measures the total nitrate/nitrite concentrations in a simple two-step method. The first step involves the conversion of nitrate to nitrite by nitrate reductase. The second step uses Griess reagent that converts nitrites into deep purple azo compounds which can be measured photometrically to determine the concentration of NO₃⁻.

**Histological analysis**

Rats were sacrificed by decapitation and the livers were harvested. Small pieces of liver tissue were placed in a 10% (v/v) formalin solution and processed routinely by embedding them in paraffin. Tissue sections (4-5 μm) were stained with hematoxylin and eosin and examined under a light microscope. Results were interpreted by pathologists.

**Statistical analysis**

Results are expressed as means ± standard errors of the mean (SEMs). The concentrations of MPO, MDA, GSH, CAT, SOD, NO, ALT, and AST in different experimental groups were compared using the Wilcoxon rank sum test. A P value of < 0.05 indicated statistical significance.

**RESULTS**

**Hepatic I/R induction and effects of NAC and/or alprostadil**

During the experimental procedure, blood pressure was continuously monitored using a polygraph. The blood pressure of all experimental rats remained stable and none of them died during I/R induction. To evaluate the level of liver damage after the reperfusion procedure, blood samples from different groups were collected immediately via the femoral catheters after completing the reperfusion procedure. As shown in Table 1, the ALT and AST levels increased significantly in the I/R group as compared with the control group, while treatment with either NAC or alprostadil ameliorated these increases in ALT and AST. These results indicated that although the liver damage had been attenuated, there was no significant difference between combined treatment (NAC + alprostadil) and each treatment administered separately.

**The antioxidant activity of NAC and/or alprostadil in the liver**

Because antioxidant enzymes are important for maintaining an optimal chemical reducing environment to protect liver from ROS damage, we analyzed the activities of several of these liver enzymes. As shown in Figure 1a, CAT levels were decreased after I/R was induced, and the decrease was significantly lower than that in the control group. Treatment with NAC and NAC + alprostadil improved the CAT activity, while alprostadil alone had no effect on the CAT activity, compared with the I/R group. The level of hepatic SOD in the liver tissue also decreased after I/R induction, which was significantly lower in the control group. Compared with the I/R group, separate treatments with NAC and alprostadil and their combined administration clearly...
increased the SOD levels. However, there were no synergistic effects of the combined treatment as compared with the results of separate treatments [Figure 1b]. We also measured the levels of GSH, which participates in many metabolic processes that protect cells against the actions of free radicals. As shown in Figure 1c, hepatic GSH levels in the liver tissue decreased after I/R induction, which was significantly lower than in the control group. Compared with the I/R group, GSH levels were significantly increased in the NAC group (226.9 ± 11.0 μM) and in the NAC + alprostadil group (202.1 ± 12.9 μM). The mean liver MDA level, an indicator of lipid peroxidation, was also estimated. The MDA value was significantly higher in the I/R group than in the control group. Compared with the I/R group, the mean MDA level was significantly decreased in the I/R + NAC group. However, there was no significant difference in the MDA levels in either the alprostadil group or the NAC + alprostadil group compared with the I/R group [Figure 1d].

The anti-inflammatory effect of NAC and/or alprostadil in the liver

Because the inflammatory response after I/R plays a critical role in cell damage, we examined the hepatic MPO levels. This enzyme is reflective of tissue infiltration by neutrophils. As shown in Figure 2a, the mean hepatic MPO level in the I/R group was significantly increased in comparison to the control group. Interestingly, the MPO level in the I/R group that received NAC treatment was lower compared with the I/R group ($P < 0.05$), whereas there were no significant changes in the MPO levels in the I/R group treated with either alprostadil or NAC + alprostadil.

We also evaluated the levels of another indicator of inflammation, NO. As shown in Figure 2b, the NO levels in the collected liver tissue increased after I/R, and the increase was significantly higher than that in the control group. Compared with the I/R group, the liver NO levels were significantly lower in the I/R + NAC, I/R + alprostadil, and I/R + NAC + alprostadil groups.

Figure 1. Effects of ischemia–reperfusion (I/R) and pretreatment with NAC and/or alprostadil on (a) catalase, (b) superoxide dismutase, (c) glutathione, and (d) malondialdehyde levels in liver tissues. Procedures are described in Materials and Methods. *$P < 0.05$ as compared with the I/R group.
HISTOLOGICAL RESULTS

In order to assess the physiological effects of these treatments in terms of liver protection, we examined liver histology after I/R induction. As shown in Figure 3, in the control group, normal liver parenchyma cells appeared with both hepatocytes and sinusoids around the central vein with a regular morphology. In the I/R group, hepatocytes were prominently swollen with marked vacuolization. Congestion was noted in enlarged sinusoids. The liver parenchyma cells accompanying both hepatocytes and sinusoids around the central vein showed abnormal morphology. In the I/R + NAC group, the I/R + alprostadil group, and the I/R + NAC + alprostadil group, hepatocytes and sinusoids exhibited normal morphology which indicated well-preserved liver parenchyma cells.

DISCUSSION

Hepatic I/R injury occurs in many clinical scenarios such as transplantation, trauma, and hepatectomy, and extensive research has been done to improve its clinical results. The aim of some studies was to restore the blood supply to the ischemic liver; however, this could lead to further damage.

This paradox has led to an uncertainty regarding the effectiveness of these treatments. Because I/R injury has a complex pathophysiology that results from a number of contributing factors, it is difficult to achieve effective treatment or protection by targeting only individual mediators or mechanisms. During recent years, the most promising protective strategy against I/R injury to be explored is preconditioning. Preconditioned fasting, ischemia, pharmaceuticals, hyperosmolar solutions, and local somatothermal stimulation (LSTS) have appeared to increase the resistance of cells to ischemia and reperfusion events.[21-26]

The goal of this study was to analyze the combined effects of chemicals in preventing liver damage during hepatic I/R injury. For this study, we adopted a rat model that included the simultaneous occlusion of the right lobe vasculature along with reperfusion of the median/left lobes while making sure that the ischemic lobes were well reperfused. This was suggested to be a good model for...
studying hepatic I/R injury.\textsuperscript{17,18,27}

In this model, the time period of I/R is important. If the ischemia period is either too short (<20 min) or too long (>90 min), it would result in either minimal damage or irreversible structural and functional changes, respectively. Sinusoidal perfusion failure is aggravated when the ischemic time period is prolonged to 60 min.\textsuperscript{28} Using 60 min of median/least lobar ischemia followed by 60 min of reperfusion as a model, our results showed distinct functional alterations [Table 1]. In addition, this model provided a reproducible system for studying the protective effects of NAC and/or alprostadil on hepatocytes resulting from I/R injury.

NAC is known to counteract oxidative stress and replenish GSH levels. This drug has been shown to have antioxidant activity \textit{in vitro}\textsuperscript{29} and \textit{in vivo}\textsuperscript{30,31}. Nagasaki et al.,\textsuperscript{32} showed that in a GSH-depleted liver, NAC prevented hepatic injury and improved its integrity after hepatic I/R injury, by acting not only as a substrate for GSH synthesis but also as a direct scavenger of free radicals. However, the evidence that NAC can reduce I/R injury and prevent complications after major surgery is not conclusive.

For example, in a randomized trial, Khan et al., found no significant difference between the control and NAC-treated group in terms of peak serum transaminase levels and the pathological severity of I/R injury.\textsuperscript{29,33} The present study demonstrated that NAC administration could improve liver functions and significantly decrease I/R-induced elevations of MPO, MDA, and NO activities while maintaining the levels of GSH, SOD, and CAT. Our histological findings also support the protective role of NAC toward the liver.

We determined the levels of several antioxidant indicators because oxygen-derived free radicals are widely known to be produced in many organs with I/R injury, such as the heart and liver. Recent evidence indicates that reactive oxygen metabolites play a fundamental role in the hepatotoxicity due to various xenobiotics and medications.\textsuperscript{14–36} Lipid peroxidation is known to play an essential role in damaging the cell membrane through reactive oxygen radicals. I/R injury has been shown to increase MDA levels, which is an important index of lipid peroxidation.\textsuperscript{37,38}

In addition, NO is synthesized by a family of enzymes designated as nitric oxide synthases (NOSs). Among these, the inducible isoforms release NO in large amounts during inflammatory or immunologic reactions and are involved in host tissue damage responses. Superoxide anion is known to react with NO to produce peroxynitrite (ONOO\textsuperscript{-}) that can readily modify proteins and other molecules.\textsuperscript{39} Thus, apart from being an indicator of inflammation, NO can also interact with free radicals and cause further cell damage. In addition to NO, the enzyme MPO, which is restricted primarily to polymorphonuclear cells, can also serve as an indicator of inflammation and the generation of ROS because an increase in MPO activity reflects tissue neutrophil infiltration. MPO plays a fundamental role in oxidant production by neutrophils. Neutrophils are a potential source of oxygen free radicals\textsuperscript{40} and are considered to be the major effector cells involved in tissue damage that occurs in several inflammatory diseases.\textsuperscript{41,42} Previous studies have reported that the addition of neutrophils to the perfusate accentuated I/R injury in the isolated perfused kidney. Consistent with this, we observed a significant increase in the MPO activity in the liver tissue after hepatic I/R injury.

Mechanisms that have been proposed to explain I/R renal injury include anoxia followed by the release of oxygen-derived free radicals during reperfusion. It is reasonable to hypothesize that an increase in antioxidant enzyme levels, such as SOD and CAT, and the maintenance of GSH as well as the decrease in MDA and MPO levels due to NAC could ameliorate the liver damage due to I/R injury.

We also examined the protective effects of alprostadil, an analog of PGE1, against liver injury. Prostaglandins are released primarily by activated Kupffer cells during the reperfusion phase.\textsuperscript{44} Several animal studies have shown that prostaglandins are effective for treating ischemic liver injury owing to their ability to increase liver perfusion, inhibit platelet aggregation, and also provide direct cytoprotection in a model of isolated perfused cat liver.\textsuperscript{45} The protective actions of PGE1 may be related to its ability to reduce both the release of proteases and the generation of oxygen free radicals by activated leukocytes. Because of the synergistic role between platelets and leukocytes and the interaction of these cells with liver sinusoidal endothelial cells (LSECs) during the reperfusion phase,\textsuperscript{46} it is conceivable that PGE1’s effects on leukocyte adherence may account for its favorable actions.

Greig et al., found that after reperfusion and progression to primary dysfunction, liver function could be restored by treatment with PGE1.\textsuperscript{47} However, the use of pharmacological doses of natural prostaglandins in clinical settings is limited because of drug-related side effects.\textsuperscript{48} Thus, synthetic prostaglandin analogs have been developed which show milder side effects and longer half-lives.\textsuperscript{49} Several of these analogs have actually improved animal survival and prevented parenchymal injury after prolonged periods of warm hepatic inflow occlusion.\textsuperscript{50} In the current study, we observed that alprostadil could attenuate the infiltration of neutrophils. However, the value of MPO only increased slightly after the treatment of alprostadil, indicating its liver protection effect might come from pathways other than inhibiting the adhesion of neutrophils.

SOD activity and its amounts are decreased after I/R injury presumably via the inactivation of mature, active SOD within mitochondria.\textsuperscript{50} Manson et al., were the first to demonstrate that the administration of SOD could enhance skin flap survival after arterial and venous occlusion.\textsuperscript{51,52} Similarly, cardiopuglic solutions containing SOD could enhance postoperative cardiac contractile

### Table 1. Effects of hepatic I/R injury and pretreatment with NAC and/or alprostadil on alanine aminotransferase and aspartate aminotransferase activities in plasma

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>ALT (U/I)</th>
<th>AST (U/I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54.3±5.8</td>
<td>108.0±17.0</td>
</tr>
<tr>
<td>I/R</td>
<td>829.5±131.9*</td>
<td>1424.3±266.5*</td>
</tr>
<tr>
<td>I/R+NAC</td>
<td>375.3±62.6,*</td>
<td>448.8±109.0,*</td>
</tr>
<tr>
<td>I/R+alprostadil</td>
<td>481.0±42.4,*</td>
<td>832.7±140.2,*</td>
</tr>
<tr>
<td>I/R+NAC+alprostadil</td>
<td>318.2±113.2,*</td>
<td>588.1±171.7,*</td>
</tr>
</tbody>
</table>

*P<0.05 as compared with the control group. P<0.05 as compared with the I/R group; ALT: Alprostadil; AST: Aspartate
function after hypothermic global ischemia in dogs.\textsuperscript{[53]}

In a study by Yang et al.,\textsuperscript{[54]} NO had direct toxic effects on LSECs during hypoxia reoxygenation. First, NO production and the expression of ENOS and iNOS were increased in LSECs during hypoxia reoxygenation. Second, an NO inhibitor, N-o-nitro-l-arginine, protected LSECs against apoptosis, while an NO activator, S-nitroso-N-acetylcysteinate, increased LSEC apoptosis during hypoxia reoxygenation. Our results showing that NAC and alprostadil mediated inhibition of NO production were correlated with better clinical results, indicating a direct toxic role of NO in hepatic I/R injury. However, our results showed that treatment with both NAC and PGE1 was not synergistic. In our study, we have established that alprostadil is a less potent SOD inducer than NAC. Because NAC is a strong antioxidant, the antioxidative effect of alprostadil might not significantly improve the effect of NAC with regard to the reducing activity. Also, it is probable that alprostadil plays a role in downstream of the effect of NAC, such as ROS-induced inflammation signaling; thus, no synergistic effect could be offered by alprostadil in combination of the treatment of NAC.

In summary, preconditioning with NAC and/or alprostadil treatments had a beneficial effect in protecting the rat liver against I/R injury. These protective effects provided by NAC or alprostadil are easily applied and may provide new avenues for the prevention of ischemic liver disease or problems that arise in liver transplantation.

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