The presence of oxidized low-density lipoprotein and inducible nitric oxide synthase expression in renal damage after intestinal ischemia reperfusion

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Abstract
Intestinal ischemia/reperfusion (I/R) is a complex phenomenon that causes destruction of both local and remote tissues. The objective of this study was to investigate the possible participation of oxidized low-density lipoproteins (oxLDLs) and inducible nitric oxide synthase (iNOS) expression in renal tissue damage after intestinal I/R. The superior mesenteric artery was blocked for 30 minutes, followed by 24 hours of reperfusion. At the end of the reperfusion period, renal tissues were removed; the presence of oxLDL, superoxide dismutase enzyme activity, malondialdehyde levels, and iNOS expression were evaluated. I/R resulted in positive oxLDL staining in renal tissue. Compared with control rats, tissue from the I/R group showed significantly higher malondialdehyde levels and lower superoxide dismutase enzyme activity. Strong and diffuse iNOS expression was present in the I/R group. Our findings support the hypothesis that I/R of intestinal tissue results in oxidative and nitrosative stress and enhances lipid peroxidation in the end organ. These data show that oxLDL accumulates in rat renal tissue after intestinal I/R. Antioxidant strategies may provide organ protection in patients with reperfusion injury, at least by affecting interactions with free radicals, nitric oxide, and oxLDL. This study demonstrates for the first time that oxLDL may play a role in renal tissue damage after intestinal I/R. Antioxidant strategies may be beneficial for protection from reperfusion injury.

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Introduction

Intestinal ischemia/reperfusion (I/R) is a complex phenomenon that causes local and remote tissue damage and even multiple-organ failure. Multiple-organ failure after serious illness or injury is a major cause of death in the surgical intensive care unit, and the exact pathophysiology of this syndrome is still unclear. Intensive care unit mortality directly correlates with the number of failed organ systems, and associated mortality rates are 60–80% depending on the source of obstruction [1]. It was recently suggested that remote organ injuries are not directly because of exogenous factors, such as bacteria or toxins, but are largely a consequence of the host’s own endogenously produced mediators. Cytokines, reactive oxygen species (ROS), nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cell adhesion molecules are some mediators that both interact with each other and are involved in multiple-organ failure syndrome [2–4].

During reperfusion of the intestine, there is an increase in superoxide production. The absence of effective dismutation of the superoxide anion allows for its rapid reaction with NO to form the potent reactive nitrogen species peroxynitrite, which can react with cellular proteins and lipids and induce general organ dysfunction. Membrane-associated polyunsaturated fatty acids are readily attached by ROS in a process that results in the peroxidation of lipids. Peroxidation of membrane lipids disrupts membrane fluidity and cell compartmentation, which can result in cell lysis [2].

Free radicals not only induce oxidative stress in the body but also initiate lipid peroxidation and maintain oxidation of low-density lipoproteins (LDLs) [5]. One of the major and early lipid peroxidation products is oxidized LDL (oxLDL). Cell-derived reactive oxygen intermediates can affect lipid metabolism, creating an oxLDL particle that is more atherogenic than the native LDL. OxLDL leads to foam cell formation, inflammatory reactions, and cellular hyperplasia [6–8]. We recently showed for the first time that oxLDL may play a role in pathologies, including terminal ileum, liver, and lung damage after intestinal I/R [9]: lung edema and pulmonary effusion [10]; obstructive jaundice in rats [11]; and psoriatic skin in psoriatic patients [12]. We also showed that oxLDL accumulation might play a role in renal tissue injury after renal I/R [13].

Previous studies have suggested that severe intestinal I/R injury is accompanied by systemic inflammatory changes and that the kidney is one of the target organs for effector cells and molecules generated from distant organ damage [2]. The effect of intestinal I/R on lipid peroxidation in rat kidney tissue has not been thoroughly investigated. Accumulating evidence suggests that renal dysfunction may occur after intestinal I/R [14–19]. In the present study, we investigated (1) the presence of oxLDL in renal tissue after intestinal I/R and (2) the association between oxLDL and iNOS expression and superoxide dismutase (SOD) activity in renal damage.

Materials and methods

Protocols (rat intestinal I/R model)

Experiments were performed on adult Sprague-Dawley rats of both sexes weighing 200–230 g (Zonguldak Karaelmas University Research Laboratory). Approval for all experiments was obtained from the Zonguldak Karaelmas University Animal Experiments Local Ethics Committee. Rats were housed under standard laboratory conditions with a 12-hour light/dark cycle and were allowed free access to food and water. During the experimental procedure, the animals were placed in separate cages and kept at room temperature (22 °C). The procedures and protocols of the study were in accord with our institutional guidelines, which are parallel to the “Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, revised 1985).”

Fourteen animals were divided into two groups of 7 rats [control group and superior mesenteric artery (SMA) occlusion group]. The procedure used for inducing SMA occlusion was performed as previously described [20]. Animals were anesthetized by the administration of intramuscular ketamine (90 mg/kg) and xylazine (9 mg/kg). The abdomen was shaved, and following a midline laparotomy, the SMA was occluded for 30 minutes with an Aesculap Yasargil clip (Aesculap Inc., PA 18034, USA) (catalog no: FD 725). Thereafter, flow was restored for 24 hours. Between surgical interventions, the incision was sutured and covered with plastic wrap to minimize fluid losses. In the control group, the SMA was exposed, but not occluded, and animals were followed for 30 minutes to simulate the ischemic interval. The reperfusion interval was simulated respectively, and the animals were then subjected to the same procedures. Animals received normal saline (1 mL) subcutaneously immediately after the surgery and were housed individually and given free access to water.

In the SMA occlusion group, renal tissues were harvested following reperfusion and used for determining LDL oxidation levels, iNOS expression, malondialdehyde (MDA) levels, and SOD activity, and for histological examination. The same analysis procedure was performed in the sham-operated control group.

Histological examination

For the histopathological examination, tissues were immersed in 10% formalin and allowed to fix for 2–3 days. Cross-sections (10 μm) were processed for standard hematoxylin and eosin staining. These sections were then examined via a light microscope and photographed.

MDA measurement

Tissue MDA levels were assessed according to the method described by Mihara and Uchiyama [21]. Tissues were homogenized with cold 1.15% KCl to make a 10% homogenate. Phosphoric acid (1%, 3 mL) and thiobarbituric acid aqueous solution (0.6%, 1 mL) were added to 0.5 mL of the 10% homogenate. The mixture was heated for 45 minutes on a boiling water bath. After cooling, 4 mL of n-butanol was added and mixed vigorously. The butanol phase was separated by centrifugation, and absorbance was measured at 535 nm and 520 nm on a Shimadzu UV 1601 spectrophotometer (Shimadzu, MD 21046, USA). The difference was used as the thiobarbituric acid value and 1,1,3,3-tetraethoxypropane was used as a standard. The MDA
The protein concentration in the supernatant fraction was determined using the method of Lowry et al. [22] [Reagent A, 2% Na₂CO₃ in 0.1 N NaOH; Reagent B, 0.5% CuSO₄ · 5H₂O in 1% sodium or potassium tartrate; Reagent C, alkaline copper solution; mix 50 mL of Reagent A with 1 mL of Reagent B; Reagent E, Folin reagent (Folin Ciocalteau reagent, Sigma Co. (St. Louis, MD 63103, USA)). Sample (0.2 mL) and Reagent C (1 mL) were mixed and left standing for 10 minutes at room temperature. Reagent E (0.10 mL) was added and the mixture was left standing for 30 minutes. Absorbance was measured at 750 nm using a Shimadzu UV 1601 spectrophotometer. Bovine serum albumin (0.2 mL) and Reagent E (0.10 mL) were added and the mixture was left standing for 10 minutes at room temperature. Absorbance was measured at 560 nm on a Shimadzu UV 1601 spectrophotometer. Percent of 0.8 mmol/L CuCl₂ reagent to each tube at 30-seconds intervals and incubated for 20 minutes. Xanthine oxidase reagent was added to each tube at 30-seconds intervals and incubated for 20 minutes. Xanthine oxidase reagent was freshly prepared with ice-cold 2 mol/L NH₄SO₄, and the final concentration of xanthine oxidase was 167 U/L. The reaction was terminated by adding 1 mL of 0.8 mmol/L CuCl₂ reagent to each tube at 30-seconds intervals. The absorbance of each sample was measured at 560 nm on a Shimadzu UV 1601 spectrophotometer. Percent inhibition was calculated by the following formula:

\[
\text{A blank} - \text{A sample} \times 100%
\]

One unit of SOD was defined as the amount of protein that inhibits the rate of NBT reduction by 50%. SOD activity was calculated per milligram tissue protein (unit/milligram protein).

**SOD assay**

Assay for SOD activity involves the inhibition of nitroblue tetrazolium (NBT) reduction with xanthine and xanthine oxidase, which was used as a superoxide generator [23]. Tissues were weighed, and 1.15% KCl was added to make a 10% homogenate. A total of 2.45 mL SOD measurement reactive solution (40 mL 0.3 mmol/L xanthine, 20 mL 0.6 mmol/L EDTA, 20 mL 150 μmol/L NBT, 12 mL 400 mmol/L Na₂CO₃, and 6 mL 1 g/L bovine serum albumin) was added to 0.5 mL of the 10% homogenate. At 25°C, 0.05 mL xanthine oxidase reagent was added to each tube at 30-seconds intervals and incubated for 20 minutes. Xanthine oxidase reagent was freshly prepared with ice-cold 2 mol/L NH₄SO₄, and the final concentration of xanthine oxidase was 167 U/L. The reaction was terminated by adding 1 mL of 0.8 mmol/L CuCl₂ reagent to each tube at 30-seconds intervals. The absorbance of each sample was measured at 560 nm on a Shimadzu UV 1601 spectrophotometer. Percent inhibition was calculated by the following formula:

\[
\frac{A \text{ blank} - A \text{ sample}}{A \text{ blank}} \times 100\%
\]

**Immunofluorescent staining method for rat tissue**

The presence of oxLDL in the tissue sections of I/R-induced and sham-operated rats was evaluated using an immunofluorescent staining method. Rat tissues were obtained and stored at −85°C in a deep freeze. Slides were prepared from frozen tissue biopsy sections, which were cut at 7-μm thickness. Slides were further divided into two pieces: one was used for the test and the other for the negative control. Around 30 μL of human polyclonal anti-oxLDL IgG solution (Immco Diagnostics, New York, NY, USA) was added to only the test slides as the primary antibody, and the control slides were treated with the same amount of phosphate-buffered saline (PBS) solution. After 30 minutes of incubation in a humid chamber at room temperature, both the control and test slides were washed with PBS, and 30 μL of fluorescent isothiocyanate-labeled anti-human IgG was administered as a conjugate substance. For an additional 30 minutes, the slides were kept and incubated at room temperature and then washed with the standard PBS solution. After open-air drying, slides were examined under fluorescent microscopy at 200× and 400× magnification (Leica DMRX, Wetzlar, Germany).

**Statistical analysis**

Results were expressed as means ± standard deviation. Comparisons between groups were made using unpaired Student t tests. Values of p less than 0.05 were considered to be statistically significant.

**Results**

**Histological damage following I/R**

There was no tissue damage in the control renal (Fig. 1A) and terminal ileum (Fig. 1B) tissues. Mononuclear cell infiltration at the tubulus was detected in the I/R group kidneys (Fig. 1C). Mononuclear cell infiltration was observed in the villus tips of the lamina propria of the terminal ileum in the I/R group (Fig. 1D).

**The effect of intestinal I/R on renal tissue oxLDL accumulation**

Significant positive immunofluorescent staining was observed in the I/R group renal tissue (Fig. 2B). Positive immunofluorescent staining was not present in the control group (Fig. 2A).

**The effect of intestinal I/R on renal tissue MDA levels and SOD activity**

The mean MDA levels and SOD activity of all groups are shown in Table 1. The tissue MDA level was significantly higher, and tissue SOD activity was significantly lower, in the I/R group (p < 0.05).

**Concentration was calculated per tissue weight (nanomole MDA/gram tissue weight).**

**Tissue protein assay**

The protein concentration in the supernatant fraction was calculated from Sigma was used as the protein standard. The protein concentration was calculated per milligram tissue protein (unit/milligram protein).

**iNOS immunohistochemical staining method in tissue**

Biopsy materials were fixed in 10% buffered formaldehyde and 3–5-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, tissue sections were boiled in 10 nm citrate buffer at pH 6.0 for 10–20 minutes followed by cooling at room temperature for 20 minutes. These sections were then incubated with primary antibodies (NOS, iNOS, Ab-1 Rabbit P-Ab Neo-markers from Biogen Medical (NeoMarkers Inc. Fremont, CA, USA)). All of the samples were immunohistochemically analyzed using the avidin-biotin complex method.
The effect of I/R on renal tissue iNOS expression

In the control rat kidney, we observed a mild iNOS immunochemical staining reaction only in the proximal tubulus epithelial cell cytoplasm, whereas staining was not observed in the glomerular basement membrane (Fig. 3A). On the other hand, strong iNOS staining reactions were observed in both the proximal tubulus epithelial cell cytoplasm and glomerular basement membrane in the I/R group kidneys (Fig. 3B). Large magnification of glomerular staining is shown in Fig. 3B.

Discussion

Intestinal I/R induces multiple-organ dysfunction associated with high rates of mortality, especially in aged patients [1]. The outcome of intestinal ischemia may be either acute as a consequence of sudden occlusion of the mesenteric arterial supply or chronic as a result of progressive atherosclerosis. Reperfusion injury may develop in conditions, such as shock and resuscitation, as well as in many abdominal surgical interventions in which decreased blood supply is further maintained. Regardless of whether I/R injury is the patient’s primary pathophysiological issue or is secondary to other disease states, progression to systemic inflammatory response syndrome often ensues [24], and acute renal failure is a common sequela of the malignant progression of this syndrome in critically ill patients.

Mesenteric I/R induces remote organ injury and has been used as a model for multiple-organ failure syndrome. Several researchers have shown that hepatic and pulmonary dysfunction may occur after mesenteric I/R [1, 9]. However, the effect of mesenteric I/R on the kidney has received relatively little attention [14–19]. LaNoue et al. [25] showed that I/R is associated with a profound reduction in renal blood flow, and this renal hypoperfusion is temporally related to decreased renal tissue ATP levels and acute tubular dysfunction, as manifested by enhanced sodium loss from the kidney. Our results indicate that I/R in the mesenteric artery is associated with histopathological damage in renal tissue. Accumulation of oxLDL, increases in iNOS expression and MDA levels, and decreases in SOD activity participated in this damage. These data support the hypothesis that intestinal I/R results in oxidative and nitrosative stress and enhances lipid peroxidation in renal tissue of the rat.

Generation of ROS after reperfusion can result in oxidative damage of lipids, proteins, and nucleic acids. Our results demonstrated that the MDA level, which acts as an indicator of lipid peroxidation, was significantly increased in renal tissue after intestinal I/R. Lipid peroxidation is a crucial factor in the propagation of cellular damage from I/R injury and leads to increased permeability of the plasma membrane, as well as mitochondrial and lysosomal membranes.

This is the first study to directly show accumulation of oxLDL molecules in the renal tissue by fluorescent immunostaining methods after intestinal I/R in rats. Because our control rat kidneys showed no signs of oxLDL accumulation, we can conclude that oxLDL might have played an important role in I/R-induced renal damage. We know that free radicals initiate and maintain oxidation of LDL. When oxidized, unsaturated fatty acids undergo molecular rearrangement leading to the generation of peroxo fatty acids.
They readily decompose, particularly in the presence of redox metals, such as iron and copper, and generate aldehydes, such as MDA, 4-hydroxynonenal, and others. We recently showed that oxLDL accumulates in renal tissue after renal I/R [13] and in intestinal, lung, and liver tissues after intestinal I/R [9]. Rothenbach et al. [26] demonstrated that severe intestinal I/R contributes to down-regulation of renal function. This decrease in renal function is caused in part by toxic oxygen metabolites, which occur in the milieu of altered renal eicosanoid release, reflecting a decrease in vasodilator and an increase in vasoconstrictor eicosanoids. Kosaka et al. [27] found that I/R in the kidney triggers iNOS and lectin-like oxLDL receptor-1 induction. Lectin-like oxLDL receptor-1 is a newly identified cell surface major receptor in endothelial cells and is specific for oxLDL. Reperfusion after organ ischemia accompanies superoxide generation that accelerates lipid peroxidation.

Table 1  Mean renal malondialdehyde (MDA) concentration and superoxide dismutase (SOD) activity in the control and ischemia/reperfusion (I/R) group rat tissues

<table>
<thead>
<tr>
<th>Group (n = 7)</th>
<th>MDA (nmol/g tissue)</th>
<th>SOD (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.9 ± 8.2</td>
<td>0.69 ± 0.08</td>
</tr>
<tr>
<td>I/R</td>
<td>272.6 ± 89.8</td>
<td>0.40 ± 0.03</td>
</tr>
</tbody>
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Values are the mean ± SD.
Intestinal I/R resulted in significantly decreased renal SOD levels, whereas renal MDA levels were significantly increased.

a $p < 0.05$ using Student t test

Figure 2.  (A) Immunofluorescent staining in renal tissue of the control group. (B) Significant positive immunofluorescent staining, which indicates accumulation areas of oxidized low-density lipoprotein, was observed in the ischemia/reperfusion group (indicated by white arrows) (×400).

Figure 3.  Immunohistochemical localization of inducible nitric oxide synthase (iNOS) expression in renal tissue of the (A) control group (indicated by red arrows) (×20) and (B) ischemia/reperfusion (I/R) group (×40). (A) Mild positive staining was obtained in the proximal tubulus epithelial cell cytoplasm in the control rats (brown staining indicated by arrows). The glomerular basement membrane of control rats displayed negative staining for iNOS (indicated by white arrows). (B) A strong iNOS staining reaction was obtained in the proximal tubulus epithelial cell cytoplasm and glomerular basement membrane in the I/R group (brown staining indicated by arrows). Large magnification of glomerular staining is shown.
and oxLDL generation. The absence of effective dismutation of the superoxide anion allows for its rapid reaction with NO to form the potent reactive nitrogen species peroxynitrite, which can react with cellular proteins and lipids (LDL oxidation) and induce general organ dysfunction [28–30]. Noiri et al. [31] suggested that the peroxynitrite scavenger ebselen reduces nitrotyrosine formation in ischemic kidneys and improves the functional outcome. These observations corroborate our results and support the hypothesis that reactive nitrogen intermediates derived from NO may contribute to LDL oxidation in injured tissues, and that iNOS expression and LDL oxidation are important participants of I/R injury of end organs, including the kidney. On the other hand, accumulating evidence suggests that endothelial activation, dysfunction, and injury by oxLDL are an early key step in the development of atherosclerosis and distant organ injury [27,32].

Growing evidence indicates that NO has potent effects on renal function, including modulation of renal and glomerular hemodynamics, renin secretion, the tubuloglomerular feedback response, and sodium excretion [33]. Despite growing evidence suggesting important roles for NO in renal function, relatively little is known regarding the expression and function of iNOS along the normal nephron. A number of studies have reported that the iNOS protein is not seen in the kidney under basal conditions [34]. However, recently, the presence of "constitutive" iNOS mRNA has been demonstrated in a number of nephron components [35,36]. Morrissey et al. [35] suggested that there may be tonic influences in the outer medulla of the normal rat kidney, resulting in the "steady-state" presence of an iNOS mRNA. Tojo et al. found that a polyclonal antibody to an induced form of NO immunolabeled the terminal afferent arteriole and thick ascending limb of Henle of the normal rat kidney but not glomerular mesangial cells [37]. In control rats, we observed a mild iNOS staining reaction only in the proximal tubulus epithelial cell cytoplasm but not in the glomerular basement membrane. In our study, detection of iNOS expression in control rats supports this concept that iNOS may be "constitutively" and differentially expressed in specific renal cell types and participates in the regulation of renal function as previously proposed [35–39]. The presence of iNOS under basal conditions may be important in modulating renal functions, such as regulation of blood flow and salt and water excretion [38]. In our I/R group rats, there was a striking increase in iNOS expression in proximal tubulus epithelial cell cytoplasm when compared with the controls, and the strong iNOS staining reaction in the glomerular basement membrane differed from control group rats. These results suggest that intestinal I/R leads to augmentation of iNOS expression in various regions of the kidney. The major question remaining concerns the physiologic function of iNOS in the normal kidney, particularly in proximal tubulus epithelial cells.

In conclusion, our study confirms the previously documented finding that the kidney is one of the target organs for effector cells and molecules generated from distant organ injury. This study is the first description of oxLDL accumulation in renal tissue after 30 minutes ischemia/24 hours reperfusion of SMA in rats. These results support that I/R injury may extend beyond the ischemic area at risk to include injury of remote, nonischemic organs, and that oxidative and nitrosative stress may play a major role in tissue damage. Moreover, we believe that accumulation of oxLDL in tissue has an important role in the events that result in progressive tissue damage. Augmentation of iNOS expression may play a role in the progression of inflammation and LDL oxidation. On the other hand, this study suggests that cellular oxidative stress is a critical step in reperfusion-mediated injury in renal tissue, for example, end-organ damage, and that antioxidant strategies may provide organ protection in patients with reperfusion damage, at least by affecting interaction with free radicals, NO, and oxLDL.

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References


