N-acetylcysteine and atorvastatin alleviates lung injury due to ischemia-reperfusion injury in rats

Da-Wei Guo, Chien-Ying Wang, Hsin-Chin Shi

1. INTRODUCTION

Ischemia-reperfusion (IR) injury is the most common and serious sequela of resuscitation from major surgery, severe injury, and hemorrhagic shock. Restoration of mesenteric blood flow, frequently forgone early in shock, may contribute to IR injury pathogenesis. Reactive oxygen species (ROS) formation, lipid peroxidation, and inflammatory responses mainly mediated IR injury, resulting in systemic inflammatory response syndrome (SIRS). SIRS can induce secondary injury to distant organs, causing remote organ failure, multiple organ dysfunction, or even death. These injuries manifest as inflammatory cytokine release, oxygen free-radical formation, and neutrophil infiltration in injured tissue. Acute lung injury is the most severe remote organ injury and one of the primary causes of death after IR injury. ROS formation is the primary pathogenesis of lung injuries due to mesenteric IR injury. ROS, generated in damaged tissues and cells, trigger the activation of various signaling pathways, promote the inflammatory reaction, and damage the intestinal mucosal barrier function in the process of intestinal IR injury. IR allows ROS to escape gut microvascular into systemic circulation and generate a cascade of various biological events, including acute lung injury. Various anti-inflammatory drugs including antioxidant and antiapoptotic drugs are used for preventing tissue injury. N-acetylcysteine (NAC) and atorvastatin (ATOR) have pleiotropic effects and are strong antioxidants, which reduce oxidative stress and prevent IR injury. NAC, a sulphydryl group transmitter commonly used in clinical practice, is a strong antioxidant, which directly scavenges ROS through its thiol groups. ATOR is an antidyslipidemic for lowering cholesterol and primary and secondary prevention of cardiovascular diseases. ATOR are supposed to act as anti-inflammatory drugs through various pathways. In present study, we evaluated the therapeutic efficacy of pretreatment with NAC and ATOR in a mesenteric IR rat model.

2. METHODS

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of National Yang-Ming University.

Abstract

Background: Acute lung injury is a major cause of death following severe injury and ischemia-reperfusion (IR). We investigated the protective effect of pretreatment with N-acetylcysteine (NAC) and atorvastatin (ATOR) in a mesenteric IR rat model.

Methods: Male rats were randomly divided into five experimental groups: sham; mesenteric IR; and ATOR, NAC, ATOR + NAC (A + N) pretreatment followed by IR. Blood gas and cytokine levels, biochemistry, and cell count were analyzed. Lung injury was evaluated through histopathology and by using the wet-to-dry lung weight (W/D) ratio.

Results: Following IR, significant changes were noted in biochemistry, cytokine, and lung injury. Compared with those in the IR group, neutrophil-to-lymphocyte ratio, lactate and alanine aminotransferase (ALT) levels were lower in all pretreatment groups, and creatinine and alkaline phosphatase (ALKP) levels were lower only in the A + N group. Blood pH and base excess (BE) were higher, and partial pressure of carbon dioxide in venous blood (PvCO2) lowered significantly in the ATOR and A + N groups than those in the IR group, and bicarbonate (HCO3−) levels increased only in the A + N group. Lung injury scores and W/D indicated significant attenuation in the A + N group. Compared with those in the IR group, tissue tumor necrosis factor-α levels were significantly lower in all the pretreatment groups and interleukin-1β levels were lower in the A + N group.

Conclusion: NAC and ATOR decreased inflammation and lung injury following mesenteric IR in rats. NAC and ATOR may alleviate lung injury more efficiently in combination than individually.

Keywords: Atorvastatin; Mesenteric ischemia-reperfusion; N-acetylcysteine; Pretreatment; Rats

1. INTRODUCTION

Ischemia-reperfusion (IR) injury is the most common and serious sequela of resuscitation from major surgery, severe injury, and hemorrhagic shock. Restoration of mesenteric blood flow, frequently forgone early in shock, may contribute to IR injury pathogenesis. Reactive oxygen species (ROS) formation, lipid peroxidation, and inflammatory responses mainly mediated IR injury, resulting in systemic inflammatory response syndrome (SIRS). SIRS can induce secondary injury to distant organs, causing remote organ failure, multiple organ dysfunction, or even death. These injuries manifest as inflammatory cytokine release, oxygen free-radical formation, and neutrophil infiltration in injured tissue. Acute lung injury is the most severe remote organ injury and one of the primary causes of death after IR injury. ROS formation is the primary pathogenesis of lung injuries due to mesenteric IR injury. ROS, generated in damaged tissues and cells, trigger the activation of various signaling pathways, promote the inflammatory reaction, and damage the intestinal mucosal barrier function in the process of intestinal IR injury. IR allows ROS to escape gut microvasculature into systemic circulation and generate a cascade of various biological events, including acute lung injury.

Various anti-inflammatory drugs including antioxidant and antiapoptotic drugs are used for preventing tissue injury. N-acetylcysteine (NAC) and atorvastatin (ATOR) have pleiotropic effects and are strong antioxidants, which reduce oxidative stress and prevent IR injury. NAC, a sulphydryl group transmitter commonly used in clinical practice, is a strong antioxidant, which directly scavenges ROS through its thiol groups, increasing intracellular glutathione levels. ATOR is an antidyslipidemic for lowering cholesterol and primary and secondary prevention of cardiovascular diseases. ATOR are supposed to act as anti-inflammatory drugs through various pathways. In present study, we evaluated the therapeutic efficacy of pretreatment with NAC alone, ATOR alone, and NAC combined with ATOR in preventing lungs injury following IR of the superior mesenteric artery (SMA) in rats.

2. METHODS

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of National Yang-Ming University.
2.1. Experimental groups

In total, 30 male Sprague Dawley rats, weighing 250-300g and aged 7-8 weeks, were obtained from BioLasco Taiwan Co. Ltd (Taipei City, Taiwan). All rats were fed standard rat chow and filtered water and kept under constant standard conditions: room temperature (23°C ± 2°C), 55% ± 5% humidity, and 12-h light-dark cycle. The following drugs were administrated as follows: NAC (Nang Kuang Pharmaceutical Co. Ltd., Tainan, Taiwan), ATOR (Pfizer Inc., New York, NY, USA), NAC + ATOR, or placebo (0.9% NaCl) for 3 days before surgery. The final dose of drugs was administered 12 hours before surgical manipulation. Animals were randomly allocated to five experimental groups (n = 6 for each group) as follows: (1) sham group (no pretreatment and sham for mesenteric IR); (2) IR group (placebo through oral gavage and intraperitoneal injection, followed by mesenteric IR); (3) ATOR group (ATOR, 40 mg/kg body weight/d, through oral gavage, followed by mesenteric IR); (4) NAC group (NAC, 300 mg/kg body weight/d, through intraperitoneal injection, followed by mesenteric IR); and (5) ATOR + NAC group (A + N; both drugs followed by mesenteric IR).

2.2. Mesenteric IR

Mesenteric IR was performed according to our previous study. Animals were exposed to 3% isoflurane gas for inducing anesthesia. The animals were placed in the supine position and the abdomen was opened by a 2 cm midline laparotomy incision. The bowel was then returned to the abdominal cavity, which was reopened, and the vascular clamp was removed. Mesenteric ischemia was confirmed when mesenteric pulsation was lost and the intestinal segment became pale. The bowel was returned to the abdominal cavity, and the incision was closed using interrupted 4-0 nylon sutures. After 60 minutes of ischemia, the abdomen was reopened, and the vascular clamp was removed. Mesenteric reperfusion was confirmed by the return of pulsation and color, and the bowel was then returned to the abdominal cavity, which was closed again. After 60 minutes of reperfusion, the animals were exsanguinated through the inferior vena cava and sacrificed. The subsequent sampling and analysis were performed using numbers tags rather than groups to ensure blindness in the experiments.

2.3. Blood analysis

Blood samples were collected in an Eppendorf tubes (Scientific Specialties, Lodi, CA, USA). The serum was separated by centrifugation at 3000 g for 10 min at 4°C). Blood gas analysis was performed on VetStat Electrolyte and Blood Gas Analyzer (IDEXX Laboratories, Inc., Westbrook, ME, USA). Chemical analysis of serum was performed on VetTest Chemistry Analyzer (IDEXX Laboratories, Inc.). Whole-blood analysis was performed on Sysmex XT-1800iv (Sysmex Europe GmbH, Co., Norderstedt, Germany).

2.4. Histopathology

Lung samples from the most superior region of the right middle lobe (nondependent site) and the most inferior region of the superior-posterior segment of the right lower lobe (dependent site) were obtained with a constant positive end-expiratory pressure (5 cm H2O). These samples were immersed in buffered 4% formaldehyde, embedded in paraffin, and cut into sections before staining with hematoxylin and eosin. The lung histology slides were examined and scored by a blinded pathologist. The lung tissues of the rats were harvested by one of the authors. Lung histology slides were prepared by a specialist and marked using sequential numbers. The pathologist who examined and scored the observations was blinded to this study.

Injury was scored according to the presence of abnormal observations, namely alveolar hemorrhage, interstitial inflammatory cells, edema, and necrosis. On the histology slides, injury severity was graded using a 5-point scale: grade 0, no abnormal observations; grade 1, <1/4; grade 2, 1/4-1/2; grade 3, 1/2-3/4; and grade 4, ≥3/4 of the microscopic field included the abnormal observations. For each slide, five or more fields were examined to minimize regional variations.

2.5. Wet-to-dry lung weight ratio

An index of pulmonary edema was determined using the wet-to-dry lung weight (W/D) ratio. The left lower lung was excised and weighed immediately (wet weight). The lung tissue was then dried in an oven for 5 days at 60°C and reweighed (dry weight). The W/D was calculated by dividing the wet weight by the dry weight, as described previously.

2.6. Cytokine levels

The left upper lung was excised and rinsed with phosphate-buffered saline to remove excess blood. A 0.5-g piece of lung tissue was homogenized in 1 mL of tissue protein extraction reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) and total protein concentrations were determined using Pierce BCA protein assay kit (Thermo Fisher Scientific Inc.). The concentration of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Duoset rat; R&D Systems, Minneapolis, MN, USA) according to manufacturer’s instructions. The optical density was read at 450 nm, with a reference wavelength of 540 nm, on the TECAN Infini 200 (Tecan Group Ltd., Männedorf, Switzerland) ELISA reader. All assays used recommended buffers, diluents, and substrates. The linear graph for cytokine levels was obtained using a series of eight 2-fold dilutions of rat IL-1β (4000-62.5 pg/mL), IL-6 (8000-125 pg/mL), and TNF-α (4000-62.5 pg/mL) standard; to quantitate tissue cytokine levels, readings

Table

<table>
<thead>
<tr>
<th>Group</th>
<th>N/L, %</th>
<th>Lactate, mmol/L</th>
<th>BUN, mg/dL</th>
<th>CREA, mg/dL</th>
<th>AST, U/L</th>
<th>ALT, U/L</th>
<th>ALKP, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>0.18 (0.25-0.13)a</td>
<td>2.69 (4.32-1.53)a</td>
<td>18.5 (23-13)</td>
<td>0 (0)a</td>
<td>62 (78-16)a</td>
<td>82.5 (108-54)a</td>
<td>200 (350-141)a</td>
</tr>
<tr>
<td>IR</td>
<td>2.13 (5.24-1.11)</td>
<td>5.68 (11.32-2.81)</td>
<td>31 (40-24)</td>
<td>0.25 (0.4-0)</td>
<td>230 (415-90)</td>
<td>308 (969-172)</td>
<td>279.5 (375-95)</td>
</tr>
<tr>
<td>ATOR</td>
<td>0.98 (1.45-0.69)a</td>
<td>3.67 (5.25-1.68)</td>
<td>31 (32-24)</td>
<td>0.1 (0.2-0)</td>
<td>190 (476-126)</td>
<td>208 (281-157)</td>
<td>161.5 (226-115)</td>
</tr>
<tr>
<td>NAC</td>
<td>1.49 (2.12-0.96)b</td>
<td>3.63 (3.91-1.94)b</td>
<td>34 (38-31)</td>
<td>0.1 (0.2-0)</td>
<td>105 (160-61)</td>
<td>171 (196-114)</td>
<td>155 (260-129)</td>
</tr>
<tr>
<td>A + N</td>
<td>1.06 (1.68-0.82)b</td>
<td>3.23 (4.12-2.17)b</td>
<td>26.5 (34-20)</td>
<td>0 (0)a</td>
<td>122 (190-90)</td>
<td>192.5 (220-125)</td>
<td>119 (219-70)b</td>
</tr>
</tbody>
</table>

ALT = alanine Aminotransferase; ALKP = alkaline phosphatase; A + N = atorvastatin + N-acetylcysteine; AST = aspartate aminotransferase; ATOR = atorvastatin; BUN = blood urea nitrogen; CREA = creatinine; IR = ischemia-reperfusion; NAC = N-acetylcysteine; N/L = neutrophil-to-lymphocyte ratio; SHAM = sham treatment.
a<0.05 vs IR group.

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were compared with a standard concentration curve created using MS Excel (Microsoft Corporation, Redmond, WA, USA).

2.7. Statistical analysis
Statistical analysis was performed using SigmaPlot 13 for Windows (Systat Software, Inc., Chicago, IL, USA). All data are presented as medians with a range. Differences between groups were assessed through Kruskal-Wallis one-way analysis of variance by ranks and Dunn’s post hoc test. Differences within groups were assessed using paired t test to compare lung injury score data between nondependent and dependent sites. A p value of <0.05 was considered statistically significant.

3. RESULTS
3.1. Blood analysis
3.1.1. Cell counts
Total leukocyte counts did not differ among the groups. Percentage of neutrophils/lymphocytes (N/L) was significantly lower in the pretreatment groups than in the IR group; there was no significant difference among the pretreatment groups (Table).

3.1.2. Biochemistry
Serum lactate, blood urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase (ALT), and alkaline phosphatase (ALKP) were assayed. Compared with those in the IR group, lactate and ALT levels were significantly lower in all pretreatment groups, and creatinine and ALKP levels were significantly lower only in the A + N group (Table).

3.1.3. Blood gas
Vein gas exchange levels were presented. Compared with those in the sham group, pH was lower and bicarbonate (HCO₃⁻) levels, and base excess (BE) were lower and partial pressure of carbon dioxide (PvCO₂) was higher in the IR group. Compared with those in the IR group, pH and BE were significantly higher and PvCO₂ significantly lower in the ATOR and A + N groups (Fig. 1A, B, D), significantly higher HCO₃⁻ was noted only in the A + N group (Fig. 1C).

3.2. Lung injury evaluation
3.2.1. Histopathology
The histopathological findings of lung injury in the experimental groups are presented in Figure 2. Increased leukocyte infiltration, edema, and hemorrhage were noted after IR. The

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**Fig. 1** Vein gas exchange levels at the end of the experimental period. Data are presented as medians with total range. Sham (n = 6), no injury; IR (n = 6), no pretreatment; ATOR (n = 6), atorvastatin administered through gavage; NAC (n = 6), N-acetylcysteine administered intraperitoneally; and A + N (n = 6), both drugs administered. *p < 0.05 vs IR group. Vein gas pH (A); vein gas BE (B); vein gas PvCO₂ (D); vein gas HCO₃⁻ (C).
l lung injury score analysis showed decreased injury in all pretreatment groups compared with the IR group; however, significant alleviation was noted only in the A + N group (Fig. 2).

3.2.2. Weight to dry ratio
A higher W/D was noted after IR. Although the pretreatment groups had lower W/D; however, a significant decrease was noted only in the A + N group (Fig. 3).

3.3. Cytokine levels
Proinflammatory cytokines, IL-1β, IL-6, and TNF-α, were analyzed as the biomarkers of acute inflammation in the lung tissue. Compared with the sham group, cytokine levels increased after IR. Compared with those in the IR group, TNF-α levels were significantly lower in all pretreatment groups, whereas IL-1β levels were significantly lower only in the A + N group (Fig. 4).

4. DISCUSSION
Here, in a rat model, NAC, ATOR, and NAC + ATOR protected against remote organ injury induced through mesenteric IR. However, compared with their individual use, the combined use of NAC and ATOR may result in superior biochemical outcomes, gas exchange activity, and lung function preservation. In the A + N group, the improvements in lung condition manifested as high HCO₃⁻ levels and low lung injury scores, W/Ds, and tissue IL-1 levels. Thus, the combination NAC + ATOR has
anti-inflammatory or antioxidant properties and protect against mesenteric IR-induced acute lung damage.

IR injury pathophysiology plays a key role in oxidative stress elevation and mitochondrial dysfunction. The consequent harmful effects result in endoplasmic reticulum stress; the resulting ROS trigger the apoptotic cascade and stimulate an acute inflammatory response. Intestinal IR is expected during clinical stress, including shock and trauma. The lungs become vulnerable particularly after intestinal IR, and the first clinical symptoms precede multiple organ failures, leading to acute respiratory distress syndrome. Although the main mediators of IR-induced damage are inflammation and oxidative stress, the precise underlying mechanisms remain unclear. Similar to our previous study, here, we used an established and validated surgical technique for inducing intestinal IR under complete SMA occlusion.

NAC is a free-radical scavenger and glutathione precursor. We used a high NAC dose (300 mg/kg body weight). The NAC dose and administration pathway were similar to those used by Kalimeris et al, who reported that higher doses (300 mg/kg body weight) of NAC before ischemia improved liver and lung functions more significantly than did lower doses (150 mg/kg body weight). Cerqueira et al administered NAC through continuous infusion (430 mg/kg body weight/h) with effective protection. We noted the advantage of administering NAC after mesenteric IR to protect against remote organ injury, particularly in the lungs. The rats treated with NAC had significantly lower cytokine levels and less neutrophil sequestration in the lungs after IR injury than the untreated rats.

ATOR is a statin that has pleiotropic effects in addition to its cholesterol-lowering activity; the pleiotropic effects include endothelial function modulation, oxidative stress reduction, immunomodulation, and anti-inflammatory action. We administered a high daily dose of ATOR (40 mg/kg body weight) on the basis of a previous study. Relatively high statin concentrations may be necessary in animals, in whom the expression of 3-hydroxy-3-methylglutaryl coenzyme, a reductase, is rapidly upregulated in response to statin administration. A human study reported that a high ATOR dosage (80 mg/d) was associated with significantly reduced high-sensitivity C-reactive protein concentrations, but without serious adverse consequences. Here, ATOR was effective in limiting remote organ damage in intestinal IR models, thus corroborating its protective effects against IR injuries to the lungs, liver, and kidneys reported previously.
We hypothesized that the combination of NAC and ATOR protects against remote organ injury, particularly to the lungs. Both drugs were selected because they are widely used in clinical practice. There are many types of statins, all of which have varied pharmacological properties, such as absorption, affinity, solubility, and excretion, and thus have significantly distinct lung-protective properties. However, in the current study, NAC + ATOR administration before IR injury prevented remote lung injury in rats, thus indicating that the combined use of both drugs limited lung injury more effectively than did their use individually.

To the best of our knowledge, only two studies thus far have investigated the protective effects of NAC and ATOR against lung IR injury. Cusumano et al.22 administered NAC (140 mg/kg) and ATOR (40 mg/kg) to an IR model of rat kidney and found that both drugs modulated the oxidative activity; however, the number of tubular ischemic lesions was relatively low in their NAC + ATOR group. Alexandropoulos et al.27 administered NAC (160 mg/kg) and ATOR (10 mg/kg) to an intestinal IR model and found that NAC and ATOR had relatively superior in kidney- and liver-protective effects, respectively, and that their combined use reduced systemic inflammatory cytokine levels. In our study, after NAC + ATOR use, the creatinine and ALKP levels significantly decreased, but no significant change was noted for other biochemistry markers. Differences in the blood sampling methods and timepoints used might reflect on the final results. Nevertheless, our study confirmed that the combined use of NAC and ATOR reduced the lung injury and modulated the blood gas levels and inflammation in the peripheral blood.

Our study has several limitations. First, we did not procure the blood volume before experiment for baseline measurement to negate the effect of blood loss on our final results; consequently, the lack of basal control values might have obscured the true effect of the drug treatments. Second, for sacrificing fewer rats, we did not add another group with sham treatment without sham IR, which might have clarified the medical sham effect on the final results. Third, we did not compare the molecular mechanisms underlying the protection from either individual or combined drug use; however, the additive effects of the drugs indicated that different pathways existed. Finally, the high drug doses and pre-treatment requirement may restrict the use of these drugs in emergency clinical scenarios, such as acute injury.

In conclusion, pretreatment with NAC, ATOR, or their combination potentially protects against injuries to remote organs, such as the lungs. Moreover, the pretreatment with NAC + ATOR improved gas exchange, attenuated inflammatory cytokine secretion, and reduced lung injury severity in our mesenteric IR rat model. These data are of clinical value, particularly given the common use and relatively safety of NAC and ATOR.

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REFERENCES