Multiple Organ Diseases

TNF-α and plasma D(-)-lactate levels in rats after intestinal ischemia and reperfusion

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Objective To study the potential role of tumor necrosis factor-α (TNF-α) induction in the development of mucosal barrier dysfunction in rats caused by acute intestinal ischemia-reperfusion injury, and to examine whether pretreatment with monoclonal antibody against TNF-α (TNF-α MoAb) would affect the release of D(-)-lactate after local gut ischemia followed by reperfusion. Methods Anesthetized Sprague-Dawley rats underwent superior mesenteric artery occlusion for 75 min followed by reperfusion for 6 hr. The rats were treated intravenously with either TNF-α MoAb (20 mg/kg) or albumin (20 mg/kg) 30 min prior to the onset of ischemia. Plasma D(-)-lactate levels were measured in both the portal and systemic blood by an enzymatic spectrophotometric assay. Intestinal TNF-α mRNA expression as well as protein levels were also measured at various intervals. In addition, a postmortem examination was performed together with a macro pathological evaluation based on a four-grade scoring system.

Results Intestinal ischemia resulted in a significant elevation in D(-)-lactate levels in the portal vein blood in both the control and treatment groups (P<0.05). However, animals pretreated with TNF-α MoAb at 6 hr after reperfusion showed significant attenuation of an increase in both portal and systemic D(-)-lactate levels when compared with those only receiving albumin (P<0.05). In the control animals, a remarkable rise in intestinal TNF-α level was measured at 0.5 hr after clamp release (P<0.01); however, prophylactic treatment with TNF-α MoAb completely nullified the increase of local TNF-α levels seen in the control animals. Similarly, after anti-TNF-α MoAb administration, intestinal TNF-α mRNA expression was markedly inhibited, which showed significant differences when compared with the control group at 0.5 hr, 2 hr and 6 hr after the release of occlusion (P<0.05-0.01). In addition, the pathological examination showed marked intestinal lesions that formed during ischemia, which were much worse upon reperfusion, particularly at the 6 hr time point. These acute injuries were obviously attenuated in animals receiving TNF-α MoAb.

Conclusions It appeared that acute intestinal ischemia was associated with failure of the mucosal barrier, resulting in increased plasma D(-)-lactate levels in both portal and systemic blood. These results suggest that TNF-α appears to be involved in the development of local damage associated with intestinal ischemic injury. Moreover, prophylactic treatment with TNF-α MoAb exerts preventive effects on ischemia/ reperfusion-induced circulating D(-)-lactate elevation and gut injury. ( J Geriatr Cardiol 2004;1(2):119-124.)

Key Words D(-)-lactate; ischemia/reperfusion injury, intestinal; tumor necrosis factor-α; monoclonal antibody; intestinal mucosal barrier

Introduction

Multiple organ dysfunction syndrome is the leading cause of mortality in patients surviving initial resuscitation and surgical intervention after major trauma and hemorrhage. From both experimental and clinical data, there is increasing evidence suggesting that one of the important initiating events in sepsis complications that follows ischemia and reperfusion injury might be the access of bacteria and endotoxin into the circulation. Intestinal ischemia and subsequent reperfusion, which may potentiate the development of sepsis and remote organ failure, occur commonly in critically ill patients. This local intestinal inflammatory process is associated with activation of systemic mediators including oxygen derived free radicals, arachidonic metabolites, complement products, and cytokines. Recently, there has been increasing evidence implicating tumor necrosis factor-α (TNF-α) in particular as
an important mediator in the pathogenesis of multiple organ
dysfunction secondary to intestinal ischemia/reperfusion.\(^1\) In
this respect, Catty et al\(^2\) reported that the intestinal injury
could lead to prompt access of endotoxin into the portal
venous circulation, followed by release of TNF-\(\alpha\) into the
systemic circulation. Similarly a recent study of ours
demonstrated that overproduction of TNF-\(\alpha\) may be one of
the most significant factors responsible for the pathophysiological
alterations following acute intestinal injury.\(^3,4\) Moreover, shock-induced intestinal injury might
cause the gut to become a cytokine (TNF-\(\alpha\) and interleukin
6) generating organ in case of hemorrhage.\(^5\) It therefore
seems very conceivable that TNF-\(\alpha\) is an important
pathogenic mediator for both local intestinal and remote
organ dysfunction, and that inhibition of TNF-\(\alpha\) bioactivity
might be helpful in preventing the development of gut injury
in response to ischemia/reperfusion.

(D(-))-lactate is produced by endogenous bacteria found
in the gastrointestinal tract, and mammals do not possess
the enzyme systems to rapidly metabolize it.\(^6\) An increase in
plasma D(-)-lactate might thus reflect an efflux of bacteria and/or its products into circulation due to mucosal injury. It
has been reported that serum D(-)-lactate is increased in
animal models of acute intestinal ischemia and simple
obstruction.\(^7\) Moreover, our previous study suggested that
plasma D(-)-lactate might be a useful marker for intestinal
injury following both ischemia and reperfusion injuries.\(^8\) However, it remains unclear whether elevation of plasma
D(-)-lactate, which reflects a significant deficit in mucosal
function, is associated with proinflammatory cytokine
formation following acute intestinal injury.

The present research was designed to study the
potential role of TNF-\(\alpha\) in the pathogenesis of mucosal
barrier dysfunction in rats caused by acute intestinal ischemia-reperfusion injury. In addition, we attempted to
examine whether pretreatment with monoclonal antibody
against TNF-\(\alpha\) (TNF-\(\alpha\) MoAb) would affect the release of
D(-)-lactate after local gut ischemia followed by
reperfusion.

Materials and methods

Intestinal ischemia and reperfusion injury

Adult male Sprague-Dawley rats, weighing 340-360 g,
were obtained from the Academy of Military Medical
Science, Beijing. The animals were allowed to rest for at
least one week after arrival before being used in this
experiment. The rats were kept on a temperature controlled
surgical board (38 ± 1°C) and allowed to breathe spontaneously and were anesthetized by intramuscular
injection of a mixture of ketamine/xylazine (112/15 mg/kg
body weight). Following the induction of anesthesia, the
right femoral artery was cannulated under aseptic conditions
with a polyethylene catheter connected to a blood pressure
monitor. Another cannula in the right jugular vein was used to
administer agents and fluid. The present experiment was
carried out without systemic heparinization being given
during the ischemic episode and the subsequent reperfusion.
Animals had only the cannula filled with heparinized Ringer’
s lactate (8 units/ml), followed by back-washing of the line
with a small amount of heparinized blood. All heparin
batches were checked before use to ensure they were free of
endotoxin.

After performing a midline laparotomy, the superior
mesenteric artery (SMA) was isolated from the surrounding
connective tissue near its aortic origin. Following a 30 min
stabilization period, SMA was occluded with an atraumatic
clip applied to the root of the mesentery. The intestine was
dehydrated during the occlusion, and there was no venous stasis or
congestion. The abdomen was then covered with sterile
saline-moist gauze. After 75 min of ischemia, the arterial
clip was removed to allow reperfusion. Reperfusion was
confirmed by the return of pulsations to the mesenteric
vascular arcade. The laparotomy incision was closed, and
the anesthetized animals underwent monitoring for an
additional 6 hr during reperfusion.

Experimental design

Before the induction of anesthesia, the rats were
randomized to receive treatment with either TNF-\(\alpha\) MoAb
(kindly provided by Celltech, UK) or the control protein
(Albumin, Immuno AG, Austria), TNF-\(\alpha\) MoAb (20 mg/kg
body weight, treatment group) or a protein control
preparation (control group) of the same dosage was
administered intravenously 30 min prior to the onset of
ischemia. In each group, six animals were sacrificed at each
of the following time points; before occlusion (0 min), end
of ischemia (75 min), and 0.5 hr (105 min), 2 hr (195
min), and 6 hr (435 min) after release of the clamp. Portal
blood samples were obtained at each time point by portal
vein puncture before the animals were sacrificed, and
limited to once per animal to avoid potential contamination.
Plasma was separated by centrifugation and stored at -70°C
until analyzed.

Plasma D(-)-lactate measurement

Plasma D (-)-lactate levels were measured by an
enzymatic spectrophotometric assay with slight modification
using a centrifugal analyzer.\(^9\) In brief, heparin-plasma was
deproteinized with 4 mol/L perchorlic acid and neutralized
with 2 mol/L K\(_2\)CO\(_3\). D (-)-lactate concentration was
determined by coupled enzymatic reactions using D (-)-
lactate dehydrogenase (D-LDH; Sigma Chemical Co.,
USA), alanine aminotransferase, and diaphorase in the
presence of NAD$^+$ and 2-p-iodophenyl-3-p-nitrophenyl-5-nitrobenzenetrazolium chloride. This resulted in the reduction of the formation of colored formazan, which was measured at 500 nm after 0.5 and 15 min.

**Intestinal TNF-α levels and semi-quantitative analysis of TNF-α mRNA expression**

Animals were killed and intestinal tissues were collected. Tissue TNF-α level was measured using a commercially available enzyme-linked immunosorbent assay kit according to the manufacturer’s directions (Genzyme, USA). To investigate the mRNA expression of TNF-α, we isolated RNA from the small intestine at different intervals after acute injury and performed reverse transcription coupled polymerase chain reaction (RT-PCR). The densities of both TNF-α mRNA as target and β-actin mRNA expression as an internal standard were scanned to assess the ratio of RT-PCR product for TNF-α to that for β-actin. The TNF-α/β-actin ratios were normalized by comparing with the values obtained from sham controls.

**Myeloperoxidase (MPO) assay**

Neutrophil infiltration in the small intestine was determined by measuring myeloperoxidase (MPO) activity. Briefly, at the time of sacrifice, the proximal intestinal tissue was excised, frozen in liquid nitrogen, and pulverized. The thawed tissue was placed in 50 mmol/L potassium phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethyl ammonium bromide. Then, the intestinal tissue was homogenized, sonicated, and centrifuged at 4°C. The resultant supernatant was assayed for MPO activity using a spectrophotometric reaction with o-dianisidine hydrochloride (Sigma Chemical Co., USA) at 460 nm. MPO units were calculated as the change in absorbance over time. On the average duplicated samples were used for analysis.

**Morphological evaluation**

Postmortem examination was performed blindly together with a macropathological evaluation based on a pre-established four-grade scoring system. For histological examination, small intestinal tissue samples were collected and fixed in 10% buffered formalin for light microscopic sections. The sections were stained with hematoxylin-eosin.

**Statistical analysis**

Data were expressed as mean ± SEM. Differences between groups were determined with an unpaired Student t test, and differences within each group were analyzed by repeated-measures ANOVA. Means were considered significantly different if the probability of error was less than 0.05.

**Results**

The animals in both groups were similar with respect to body weight (control vs TNF-α MoAb: 355 ± 2 vs 350 ± 4 g) and baseline measurements of systemic hemodynamics (control vs TNF-α MoAb; mean arterial pressure, 105 ± 2 vs 104 ± 3 mmHg; cardiac output, 181 ± 3 vs 182 ± 4 ml/min). During 75 min of SMA occlusion the mean arterial pressure tended to fall in all animals but no significant difference between control and treatment groups was found. Subsequently, blood pressure declined rapidly upon release of the clamps and remained at a low level (less than 65 mm Hg) for the 6 hr reperfusion period in the control group, but this decrease in blood pressure was significantly blunted by pretreatment with TNF-α MoAb (P < 0.01). All animals survived throughout the time course of the experiment. The degree of intestinal ischemia-reperfusion injury was consistently associated with intestinal, hepatic, and pulmonary damages. The mortality was about 75% at 24 hr in untreated animals.

As shown in Table 1, 75 min intestinal ischemia resulted in a significant elevation in D(-)-lactate levels in the portal vein blood compared to baseline values in both the control and treatment groups (P < 0.05). Plasma D(-)-lactate levels had a tendency to further increase after reperfusion up to 6 hr. However, the increase in D(-)-lactate at the end of the observation period was significantly attenuated in animals pretreated with TNF-α MoAb as compared with those only receiving albumin (P < 0.05). Similar alterations in D(-)-lactate were also found in the systemic circulation. There were no significant differences between the portal and the systemic circulation of either group at any point of time.

In the control animals, a remarkable rise in intestinal TNF-α level was measured at 0.5 hr (P < 0.01) after clamp release, gradually decreasing up to the end of the observation period (P < 0.05-0.01, Table 2). Prophylactic treatment with TNF-α MoAb, however, markedly annulled the local TNF-α levels seen in the control animals. As shown in Table 2, intestinal TNF-α mRNA was significantly expressed in the controls at 0.5 h, peaking at 2 hr following reperfusion (P < 0.01). After anti-TNF-α MoAb administration, intestinal TNF-α mRNA expression was markedly inhibited, which showed significant difference when compared with control group at 0.5, 2 and 6 hr after the release of occlusion (P < 0.05-0.01).

Intestinal MPO activity was significantly increased at 0.5 and 6 hr after reperfusion in control animals (Table 3). However, anti-TNF-α administration prevented the increase in intestinal MPO and significantly reduced the
MPO activity compared to those in the controls (P < 0.05-0.01). Before the induction of intestinal ischemia the animals were noted to have no abnormal alteration of the bowels. SMA occlusion (75 min) resulted in marked intestinal damage, which was much worse upon reperfusion in untreated animals. The macrophathological evaluation scores were similar between animals treated with albumin and TNF-α MoAb within 0.5 hr after reperfusion, while they were much lower in the treatment group than in the controls at 2 and 6 hr (Table 3).

On histologic examination of the intestine (Fig. 1), most of the control animals showed evidence of remarkable mucosal injury including edema, hemorrhage and local necrosis at the end of occlusion. Reperfusion elicited a progressive destruction of the lamina propria and transmural necrosis together with inflammatory cell infiltration, particularly at the 6 hr point (Fig. 1B). These acute injuries were obviously less pronounced in animals receiving TNF-α MoAb 0.5 hr prior to the induction of ischemia (Fig. 1C). In addition, translocating bacteria were commonly found in the intestinal wall at various time points following reperfusion, yet there was no difference in the degree of their translocation between those treated with albumin or anti-TNF-α MoAb.

**Discussion**

Intestinal permeability, as a measure of gut mucosal function, has been reported to be increased following endotoxin administration, hypovolemic shock, trauma, and thermal injury, although changes in intestinal permeability have not been consistently correlated with the development of subsequent infectious complications. Recently, our group provided further supporting evidence for the above conclusions. By employing anti-endotoxin treatment, it was found that neutralization of endotoxin could attenuate the intestinal injury and reduce the translocation rate of bacteria after prolonged hemorrhage.

### Table 1. The effect of TNF-α MoAb on plasma D(-)-lactate levels in portal and systemic circulations (mg/ml, M ± SEM)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Portal blood</th>
<th>Systemic blood</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TNF-α MoAb</td>
</tr>
<tr>
<td>0</td>
<td>3.25 ± 0.30</td>
<td>3.10 ± 0.29</td>
</tr>
<tr>
<td>75</td>
<td>7.15 ± 0.98</td>
<td>6.73 ± 1.10</td>
</tr>
<tr>
<td>105</td>
<td>7.65 ± 1.04</td>
<td>6.09 ± 0.27</td>
</tr>
<tr>
<td>195</td>
<td>10.02 ± 1.45</td>
<td>7.49 ± 0.68</td>
</tr>
<tr>
<td>435</td>
<td>14.87 ± 3.56</td>
<td>7.00 ± 0.74</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01, compared to baseline within group; # P < 0.05, compared to control group

### Table 2. Intestinal TNF-α levels and TNF-α mRNA expression in both groups after ischemic and reperfusion injury (M ± SEM)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>TNF-α level (µg/mg protein)</th>
<th>TNF-α mRNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TNF-α MoAb</td>
</tr>
<tr>
<td>0</td>
<td>37.3 ± 3.2</td>
<td>34.9 ± 4.1</td>
</tr>
<tr>
<td>75</td>
<td>45.5 ± 4.9</td>
<td>31.6 ± 5.2</td>
</tr>
<tr>
<td>105</td>
<td>182.8 ± 24.3 **</td>
<td>26.1 ± 5.3 # #</td>
</tr>
<tr>
<td>195</td>
<td>160.6 ± 18.4 **</td>
<td>20.5 ± 4.4 # #</td>
</tr>
<tr>
<td>435</td>
<td>74.3 ± 15.9 **</td>
<td>30.1 ± 6.7</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01, compared to baseline within group; # P < 0.05, # # P < 0.01, compared to control group

### Table 3. The effect of TNF-α MoAb on intestinal MPO activities and macropathological evaluation scores (M ± SEM)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>MPO (mU/mg protein)</th>
<th>Evaluation score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TNF-α MoAb</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>TNF-α MoAb</td>
</tr>
<tr>
<td>0</td>
<td>184 ± 51</td>
<td>207 ± 60</td>
</tr>
<tr>
<td>75</td>
<td>214 ± 42</td>
<td>193 ± 35</td>
</tr>
<tr>
<td>105</td>
<td>309 ± 31 *</td>
<td>190 ± 30 #</td>
</tr>
<tr>
<td>195</td>
<td>266 ± 67</td>
<td>157 ± 45</td>
</tr>
<tr>
<td>435</td>
<td>447 ± 72 **</td>
<td>153 ± 43 # #</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01, compared to baseline within group; # P < 0.05, # # P < 0.01, compared to control group
Intestinal ischemia/reperfusion may be an initial event leading to the spread of endotoxin and to bacterial translocation, which in turn will induce an excessive activation of endogenous inflammatory mediators including TNF-α and interleukins. In this respect, it is of interest to note that a significant increase in portal endotoxin concentration was already found during the ischemia phase, while intestinal TNF-α mRNA expression and peak tissue TNF-α levels occurred relatively late after reperfusion. To further define the potential role of TNF-α in the pathogenesis of mucosal barrier dysfunction, we studied whether administration of anti-TNF-α MoAb could protect against local damage resulting from intestinal ischemia and reperfusion. In the present study, pretreatment with TNF-α antibody significantly blunted the elevation in tissue TNF-α mRNA expression and intestinal TNF-α protein levels as seen in the controls, and markedly attenuated intestinal neutrophil infiltration as well as mucosal injury. The data reported here and shown in previous studies provide further evidence that TNF-α not only appears to be the early cytokine of systemic inflammation in this model but also is involved in the etiology of local damage associated with intestinal ischemic injury.

The mechanisms of preventive effects on ischemia/reperfusion-induced circulating D(-)-lactate release and gut injury by TNF-α MoAb treatment cannot be determined from the current study, but several factors may contribute to such beneficial actions. Firstly, the infusion of recombinant human TNF-α in animals has been shown to cause ischemia and hemorrhagic lesions of the gastrointestinal tract. In addition, the effects of TNF-α on the permeability of the epithelial barrier have been found to be dose-dependent on one hand and rapidly

Fig. 1. Histologic changes in the jejunum of the small intestine (original magnification × 200).
A: Sham-operated animal showing normal villi; B: Six hr after reperfusion in an animal receiving control protein. Denuded villi with lamina propria and hemorrhage are observed, and there is increased infiltration of the lamina propria by inflammatory cells; C: Six hr after reperfusion in an animal receiving TNF-α MoAb. Moderate subepithelial edema and epithelial lifting at the tips of the villi are found. No other significant abnormalities are present in the mucosa of this treatment group.
reversible or inhibited by a monoclonal antibody to TNF-α on the other. Thus, a blockade of intestinal TNF-α activity and inhibition of TNF-α mRNA expression by prophylactic treatment with TNF-α MoAb would exert direct protection to the gut. Secondly, TNF-α has the ability to activate neutrophils, including increased neutrophil aggregation and adherence to endothelial cells, which appears to be implicated in the process of neutrophil-mediated gut injury. The data presented in this experiment show that pretreatment of animals undergoing intestinal ischemia/reperfusion with an anti-TNF-α agent completely inhibited neutrophil sequestration in the bowel as measured by MPO activity. Thirdly, TNF-α has been reported to be a pivotal cytokine in the development of hypotension and remote organ dysfunction. In this experiment, attenuation of the systemic shock state associated with acute ischemia/reperfusion injury by TNF-α MoAb administration might improve the perfusion pressure of hyperperfused organs including the intestine, which is likely to contribute to a decrease in mucosal permeability and amelioration of intestinal barrier damage.

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References


