Effect of ghrelin on inflammatory response in lung contusion

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Received 12 October 2011; accepted 5 December 2011
Available online 18 October 2012

KEYWORDS
Adenosine deaminase; Ghrelin; Lung contusion; MMP-2; TGF-β1

Abstract The purpose of this study was to investigate the effects of ghrelin on inflammatory response and tissue damage following trauma-induced acute lung injury. Thirty male wistar albino rats (300–400 g) were randomly assigned into three groups: control group (n = 6), lung contusion plus saline (saline-treated, n = 12), and lung contusion plus ghrelin (ghrelin-treated, n = 12). Saline- or ghrelin-treated traumatic rats were sacrificed at two time points (24 and 72 hours) after lung contusion. Blood was collected for the analysis of serum adenosine deaminase (ADA). Tissue transforming growth factor-beta 1 (TGF-β1) and matrix metalloproteinase-2 (MMP-2) levels were measured by enzyme-linked immunosorbent assay and histopathological examination was performed on the lung tissue samples. Our results indicated that ghrelin significantly reduced morphologic damages. Serum ADA activities were significantly decreased after lung contusion and this decline started early with ghrelin treatment. TGF-β1 and MMP-2 levels in lung tissue were elevated at 72 hours after lung contusion and treatment with ghrelin significantly increased TGF-β1 level and reduced MMP-2 level. In conclusion, our study demonstrates that acute lung injury initiated proinflammatory responses and ghrelin administration showed an anti-inflammatory effect in lung contusion.

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Introduction

Lung contusion is a significant concern in blunt chest trauma and an independent risk factor for the development of pneumonia, acute lung injury and acute respiratory distress syndrome (ARDS) [1]. Lung contusion displays pathophysiological alterations and is characterised by an inflammatory response in the pulmonary parenchyma. Lung injury causes activation of cells such as blood leukocytes and tissue macrophages, which release different mediators such as cytokines [2]. Transforming growth factor-beta 1 (TGF-β1) is a cytokine that regulates the expression of the extracellular matrix proteins. During tissue repair, the rate of extracellular matrix protein synthesis was balanced with protein degradation, which was catalysed by matrix metalloproteinases (MMP). During acute tissue injury, extracellular nucleotide levels are significantly increased due to the release of ATP or ADP from the intracellular space into the extracellular space. Extracellular nucleotides are rapidly hydrolyzed to adenosine by adenosine deaminase (ADA) in the extracellular space [3]. Recent studies demonstrate that endogenous adenosine plays an important role in modulating inflammatory response in acute lung injury [4,5].

Ghrelin is a gastrointestinal hormone that is primarily synthesised and secreted by the stomach. It is an endogenous ligand for the growth hormone secretagogue receptor and has a strong stimulatory effect on growth hormone release [6,7]. Administration of ghrelin has been implicated in the regulation of food intake, body weight, gastrointestinal motility, cardiovascular functions, enzyme release, cell proliferation, and reproduction in vitro or in vivo [6–8]. Ghrelin inhibits the production of inflammatory cytokines [9,10]. Wu et al. [11] demonstrated that ghrelin administration improved pulmonary blood flow, decreased pro-inflammatory cytokines and reduced acute lung injury. In this preliminary study, the authors offer ghrelin as a novel treatment for severe sepsis-induced acute lung injury. In the literature, there is no study evaluating the effect of ghrelin in trauma-induced acute lung injury. Therefore, we aimed to investigate the effect of ghrelin on ADA, TGF-β1, and MMP-2 levels during remodelling of extracellular matrix in trauma-induced acute lung injury.

Materials and methods

Thirty male wistar albino rats, weighing 300–400 g, were obtained from the Experimental Research Centre of Zonguldak Karaelmas University, Faculty of Medicine. All rats were on a 12-hour light, 12-hour dark cycle at 20–21°C and provided with food and water ad libitum. All experimental protocols were approved by the Zonguldak Karaelmas University Animal Care and Use Committee.

Blunt chest trauma and experimental design

Animals were randomly divided into three groups: control (n = 6), lung contusion plus saline (saline-treated, n = 12), and lung contusion plus ghrelin (ghrelin-treated, n = 12). Rats (except those of the control group) were anaesthetised with ketamine/xylazine (60/10 mg/kg, intramuscularly). Blunt chest trauma was performed using the model for isolated bilateral lung contusion described by Raghavendran et al. [12]. This model involves dropping a cylindrical weight (400 g) from a height of 50 cm onto a mobile lexon platform positioned over the site of thorax trauma. Rats were received either saline or ghrelin (rat ghrelin 150 ug/kg, Sigma Chemical, St. Louis, MO, USA) after trauma was performed. Ghrelin was injected (0.3 mL) on the hind limb and repeated once a day, during the entire study period, according to the method published by Schwenke et al. [13]. In both the saline- and ghrelin-treated groups, rats were decapitated at 24 and 72 hours following trauma injury. After decapitation, blood was collected by cardiac puncture and serum was stored at −80°C for the determination of total ADA activity. Lung tissue was excised for determining the levels of TGF-β1 and MMP-2 as well as for histopathologic examination.

Pulmonary histopathology

The lungs were harvested and fixed in 10% buffered formalin for 24 hours. The tissue were embedded in paraffin and then stained with hematoxylin eosin (HE). A semi-quantitative histopathologic assessment using a lung injury score was performed based on the following histologic features; edema, congestion, intra-alveolar haemorrhage and inflammation, neutrophil margination in blood vessels, neutrophil accumulation in interstitial tissues, and cellular hyperplasia. Each feature was graded as absent (0), mild (1), moderate (2), or severe (3), with a score of 0–3 [14]. The lung injury score was calculated by adding the individual scores for each category.

Biochemical analysis

ADA activity (U/L) was assayed with a commercial kit (BEN, Milano, Italy) that is based on the enzymatic colorimetric method using Shimadzu UV 1601 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Adenosine is deaminated by ADA and the ammonia obtained from this reaction reacts with α-ketoglutarate. The latter reaction is catalysed by glutamate dehydrogenase. The enzyme activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>ADA (IU/L)</th>
<th>TGF-β1 (pg/mg protein)</th>
<th>MMP-2 (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>32.6 ± 2.55</td>
<td>39.11 ± 17.5</td>
<td>10.65 ± 0.68</td>
</tr>
<tr>
<td>Saline-24 hr (n = 6)</td>
<td>28.9 ± 3.65</td>
<td>36.78 ± 16.4</td>
<td>11.27 ± 1.47</td>
</tr>
<tr>
<td>Saline-72 hr (n = 6)</td>
<td>22.9 ± 5.27</td>
<td>54.20 ± 34.3</td>
<td>16.24 ± 5.44</td>
</tr>
<tr>
<td>Ghrelin-24 hr (n = 6)</td>
<td>21.9 ± 5.32</td>
<td>29.00 ± 6.12</td>
<td>11.57 ± 3.73</td>
</tr>
<tr>
<td>Ghrelin-72 hr (n = 6)</td>
<td>20.5 ± 4.82</td>
<td>75.84 ± 19.34</td>
<td>10.84 ± 1.84</td>
</tr>
</tbody>
</table>

All data are mean ± standard deviation.
ADA = adenosine deaminase; MMP-2 = matrix metalloproteinase-2; TGF-β1 = tissue transforming growth factor-beta 1.
was proportional to the decrease in absorbance of NADH and was measured at 340 nm. The detection limit of the assay was 3–150 IU/L with intra- and inter-assay coefficients of variable less than 5%.

Tissue specimens were homogenised in Tris/Tween-80 buffer, and protein concentrations were determined as described previously [15,16]. The concentrations of TGF-β1 (Invitrogen, Camarillo, USA) and MMP-2 (Boster Biosciences, Wuhan, China) were determined using sandwich enzyme-linked immunoassay technique according to the manufacturer’s recommendations. Tissue TGF-β1 and MMP-2 results are expressed per milligram of tissue protein.

Statistical analyses
All data were analysed using SPSS 13 software (SPSS Inc., Chicago, IL) and presented as mean ± standard deviation. Differences between the groups were evaluated by one-way analysis of variance test and Mann–Whitney U test. Differences in values were considered significant at \( p < 0.05 \).

Results
The biochemical characteristics of the control, saline-, and ghrelin-treated groups are shown in Table 1.

Serum ADA activities were decreased in traumatic rats. There was a significant difference in serum ADA levels at 24 hours between the saline- and ghrelin-treated groups (\( p < 0.05 \)), but there was no difference at 72 hours (\( p > 0.05 \); Fig. 1).

There was no difference in tissue TGF-β1 and MMP-2 levels at 24 hours (\( p > 0.05 \)). Tissue TGF-β1 level was increased at 72 hours in both groups. This enhancement was higher in the ghrelin-treated group (\( p < 0.05 \)). Tissue MMP-2 level was increased only in the saline-treated group (\( p < 0.05 \), Fig. 2).

Photomicrographs showed edema, haemorrhage in the alveolus, and infiltration of inflammatory cells into the lung interstitium and alveolar spaces in traumatic rats (Fig. 3A). Histopathologic analysis of the lung was performed according to scoring system (Fig. 3B). After lung contusion, there was no significant difference in histologic scores between the two groups at 24 hours (\( p > 0.05 \)). On comparing the saline- and ghrelin-treated groups at 72 hours, the ghrelin-treated group showed obvious reduction in inflammatory cell infiltration and significant improvement in lung tissue (\( p < 0.05 \)).

Discussion
Lung contusion is an important concern in trauma patients and is characterized by increased alveolar edema, hemorrhage, vasocongestion, and inflammatory cell infiltration [1]. Epithelial cell death, inflammatory response, and fibrosis are seen after lung contusion. Recent studies indicate that ghrelin has protective effects in acute lung injury models. The biologic effects of ghrelin are mediated through the ghrelin receptors that have been demonstrated in human lung parenchyma and pulmonary artery wall [17,18]. Zhou et al. [19] reported decreased serum proinflammatory cytokine levels, improved morphologic damage, and pulmonary parameters by treatment with ghrelin in pancreatitis-induced acute lung injury model. Furthermore, the ghrelin-treated group demonstrated significant improvement in lung architecture in sepsis-induced acute lung injury while administration of a specific ghrelin receptor antagonist worsened the survival rate [11]. In our study, we found significantly reduced edema, congestion, hemorrhage, inflammation, neutrophil...
infiltration, and cellular hyperplasia in the ghrelin-treated group. This is the first study demonstrating significant improvement in histopathologic findings following ghrelin treatment in lung contusion.

MMP play an important role in inflammation and extracellular matrix degradation. MMP-2 levels were increased in broncho-alveolar-lavage (BAL) fluid after pulmonary ischemia–reperfusion injury [20]. Fligiel et al. [21] investigated 28 ARDS patients and reported elevated MMP-2 in BAL fluid within 48 hours from the time of onset of ARDS. Ghrelin treatment has an anti-inflammatory effect in postinfarct myocardial remodelling and inhibits MMP-2 expression [22]. In our study, we observed a significant decrease in MMP-2 levels following ghrelin treatment.

TGF-β1 stimulates fibroblast proliferation and increases the synthesis of extracellular matrix proteins including collagens. TGF-β signalling plays an important role in the development of fibrosis in late phases after acute lung injury [23]. Kaminski et al. [24] showed increase in TGF-β1 expression as early as 2 days after bleomycin-induced acute lung injury. Fahy et al. [25] investigated BAL fluid samples of ARDS patients and found increased TGF-β1 levels in early

**Figure 3.** Effect of ghrelin on lung histology in lung contusion. (A) Histopathologic evaluation is shown 24 hours (×40) and 72 hours (×400) after lung contusion; (B) histopathologic scoring of lung injury in traumatized rats treated with saline and ghrelin. Data are mean ± standard deviation. *p < 0.05 versus saline in 24-hour group. **p < 0.05 versus ghrelin in the 24-hour group. †p < 0.05 versus saline in the 72-hour group.
phases (24 hours). Kolb et al. [26] found increased TGF-β1 levels by Day 7 but no change on Day 2 in acute lung injury initiated by IL-1β. We also found a significant increase in TGF-β1 levels at 72 hours after blunt trauma. In concordance with previous studies, our findings support that TGF-β1 is an essential modulator in the early stages of acute lung injury repair.

TGF-β1 plays an important role in down-regulating the inflammatory process by inhibiting the functions of activated cells and even promoting apoptosis [27,28]. Moreover, TGF-β1 plays a role in regulating the extracellular matrix by decreasing degradation of matrix proteins through a reduction in protease synthesis and an increase in the synthesis of protease inhibitors [29]. In our study, we found a significant increase in TGF-β1 levels at 72 h after ghrelin administration. Thus, we suggest that TGF-β1 may contribute to the anti-inflammatory effect of ghrelin.

Adenosine deaminase is an indicator of cellular immunity and responsible for adenosine degradation. Adenosine is a signalling molecule produced as a result of cell stress or injury. It plays a direct role in the regulation of inflammation and responsible for adenosine degradation. Ghrelin administration. Thus, we suggest that TGF-β1 may contribute to the anti-inflammatory effect of ghrelin.

In conclusion, our study demonstrates that lung contusion initiated pro-inflammatory responses and ghrelin administration showed an anti-inflammatory effect during this process. Further studies with detailed physiological investigation must be undertaken to elucidate the exact mechanism of ghrelin in lung injury.

References


