Adrenomedullin Prevents Lung Injury after Hepatic Ischemia-Reperfusion Damage

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ABSTRACT

Objective: Acute lung injury is a complication after hepatic ischemia/reperfusion (I/R) and might be responsible for high mortality rate. The aim of this study was to demonstrate that adrenomedulline administration may prevent lung injury after hepatic I/R by downregulation of proinflammatory cytokines.

Materials and Methods: This study was performed by using 54 male Wistar rats. The rats were randomly allocated into 3 groups and in groups were randomly allocated into 1st, 2nd, and 4th hour subgroups. After I/R, AM (12μg/kg) was infused for 30 minute via portal vein. Blood and tissue samples were collected 1, 2 and 4 hour after reperfusion. Hepatic I/R induced lung injury, as characterized by lung edema, histopathologic changes and proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were evaluated.

Results: The TNF-α enzyme activity in the first hour was found to be significantly higher in the I/R group than in the Sham and AM group (p=0.027 and p=0.041, respectively). The levels of TNF-α in the sham-operated group were similar in the AM group (p=0.310). Significant differences were found only in the second hour IL-6 measurements (p=0.038). Similar differences in caspase-9 enzyme activity in the sham-operated and AM group (p=0.291). Treatment with AM decreased lung injury after hepatic I/R injury.

Conclusions: Acute lung injury was decreased by AM treatment after hepatic I/R injury.

Key Words: Adrenomedullin, hepatic ischemia/reperfusion, lung injury.

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ÖZET

Amaç: Akut akciğer hasarı, hepatik iskemi/reperfüzyon (I/R) sonrası görülen bir komplikasyondur ve yüksek mortalite oranından sorumludur. Bu çalışmamız amacı, adrenomedüllin uygulamasının hepatik I/R sonrası akciğer hasarını proinflamatuar sitokinlerin downregüle edilmiş yolu ile önleyebileceğini göstermektir.

Yöntemler: Bu çalışma 54 erkek Wistar rat kullanılarak gerçekleştirildi. Ratlar randomize olacak şekilde 3 gruba ayrıldı. Bu grupta tedavi veya I/R'dan sonra 1. ve 4. saat alt gruplarına ayrıldı. İskemi sonlandırılduktan sonra, AM (12μg / kg) portal ven yoluyla 30 dakika verildi. Kan ve doku örnekleri reperfüzyondan 1, 2 ve 4 saat sonra toplandı. Akciğer ödemi, histopatolojik değişiklikler ve plazma tümör nekroz faktör-α (TNF-α) ve interlökin-6 (IL-6) gibi proinflamatuar sitokin düzeyleri değerlendirildi.

Bulgular: Birinci saatteki TNF-α enzim aktivitesi I/R grubunda, Sham ve AM grubundan anlamlı olarak daha yüksek bulundu (sırasyla, p=0.027 ve p=0.041, sırasıyla). Sham grubunda TNF-α seviyeleri, AM grubunda de benzer bulundu (p=0.310). İkinci saatde caspase-9 enzim aktivitesi, AM grubunda de benzer bulundu (p=0.291). Adrenomedüllin uygulamasının akciğer hasarı hepatik I/R grubuna göre daha az olduğu gösterildi.

Sonuç: Hepatik I/R hasarı sonrası oluşan akut akciğer hasarı adrenomedüllin uygulanması ile azaltılabilir.

Anahtar Sözcüklər: Adrenomedullin, hepatic ischemia/reperfusion, akciğer hasarı.
INTRODUCTION

One of the difficulties arising in the post-operative period after liver transplantation and liver resection is lung injury. Lung injury after liver surgery is a serious complication affecting the prognosis of patients. Meanwhile, lung injury cause leads to death (1,2). It was observed that pulmonary edema developed within hours especially after liver transplantation. It was reported in experiments with animals that there were increased flow of neutrophil and pulmonary vascular structuring and temporary hepatic artery clamping (3). This injury occurring in the lung is attributed to hepatic I/R injury. This may cause lung injury and related complications (4,5). Pro-inflammatory and anti-inflammatory cytokines are released in consequence of the activation in hepatic Kupffer cells forming during I/R, and oxygen radicals are generated (6). Many cytokines such as TNF-α, IL-1β, IL-6, prostaglandins and reactive oxygen products released during blood circulation first come to the capillary layer of the lung after circulation (7,8). Many different methods such as ischaemic preconditioning, remote pre-conditioning and various agents pharmacologically were tried so as to prevent hepatic I/R injury and they are still being tried (9). Of these precautions, surgical techniques are restricted or impossible to implement in some cases. Therefore, trying pharmacological agents in reducing hepatic I/R is considered as a more acceptable method. However, no molecules preventing distant organ damage such as liver occurring after hepatic I/R.

Adrenomedullin was introduced into the world of science for the first time in 1923 as a molecule having the structure of a peptide composed of 52 amino acids (10). It has been used in many diseases such as sepsis, shock, rheumatoid arthritis and inflammatory intestinal diseases in whose pathophysiology inflammation took on important responsibilities since that date (11). Later, it was found that AM had many biological activities such as vasodilatation, bronchodilatation, diuretic effects, aldosterone secretion inhibition, neurotransmission and anti-microbial effects (12). The effects of AM on inflammation have been the focus especially in recent years. Many clinical and animal experiments indicating that AM expression increase in acute liver damage (13,14). It was demonstrated that it was expressed especially in bronchial epithelium, bronchial smooth muscle, pulmonary vascular structures and macrophage (13). Besides, its anti-inflammatory effects were also shown in some liver disease models (14). The aim of the present study was to evaluate the effect of AM administration after hepatic I/R induced lung injury in rats.

MATERIALS AND METHODS

This experiment were reviewed and approved by the Institutional Animal Care and Use Committee at Gazi University (Protocol No: G.U.E.T-05052). All animal procedures in this study were conducted in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources.

Animals and Study Design

This study was performed by using 54 male Wistar rats, weighting 220–270 gram. The animals were housed for at least 5 day before surgery, with free access to a standard diet and water on 12 hours of light and 12 hours of dark in individual cages. All experiments were performed with rats that had fasted for 12 hours before surgery and all procedures were performed using sterile surgical technique. Following randomization, the rats were divided into three groups. Rats were subjected to 1 hour of 70% hepatic ischemia following clamping of the portal vein and hepatic artery, which supply the left lateral and median lobes of the liver (segments II–IV), using an atraumatic vascular clamp (Harvard Apparatus Inc.; Holliston, MA, USA) for 1 hour, followed by different periods of reperfusion.

Rats were anesthetized with intraperitoneal ketamine [100 mg/kg body weight (BW)] and xylazine [20 mg/kg] and prepared for aseptic surgery. A midline incision extending from the xiphoid to the pubis was made. A polyethylene catheter (PE-50, ID 0.28, OD 0.61; Portex, Hythe, UK) was inserted into the ileocecal vein to the portal vein for infusion of the drugs. The liver was exposed with retractors placed in the flank, and a clamp was attached to the xiphoid and elevated. The ligamentous attachments from the liver to the diaphragm were freed. In order to avoid splanchnic congestion, we used a model of partial liver ischemia. Partial liver ischemia was induced by selective clamping of the portal vein and hepatic artery, which supply the left lateral and median lobes of the liver (segments II–IV), using an atraumatic vascular clamp (Harvard Apparatus Inc.; Holliston, MA, USA) for 1 hour, followed by different periods of reperfusion.

Blood Analysis

Blood samples were collected from the vena cava in Sham groups (1st, 2nd and 4th hours). To separate plasma the samples were centrifuged at 3000 revolutions for 10 minutes and blood samples were stored at -80 °C until assayed. To determine the levels of TNF-α and IL-6, a commercial solid phase sandwich enzyme-linked immunosorbent assay (ELISA) from Biosource International (Camarillo, CA, USA) was used. TNF-α levels were determined from a standard curve for recombinant TNF-α and; concentrations were expressed as pg/mL. The ELISA detection limit for TNF-α was 3 pg/mL.

Immunohistochemistry

Lung tissue samples were taken from the rats in the Sham operated group 1st, 2nd and 4th hours after laparotomy or lung tissue samples were taken only from the rats in hepatic I/R+AM sub-groups in the 1st, 2nd and 4th hours following the 30 minute infusion. Resected lung tissue was fixed in 10% buffered formalin for 7 days and embedded in paraffin. Sections of 3-μm thickness were obtained from the paraffin blocks and stained with H&E for histological examination. The sections were placed onto polylysine plates and immunohistochemically dyed with Caspase-9 primer antibody (Caspase 9/ LAP 6, Ab-6, Ca++ + RB- 1570- PO Labvision/ Neo Markers Corporation, Fremont, CA, 94539 USA). The degree of immunoactivity with Caspase-9 was evaluated as follows: (-): nil, (+): mild, (++): moderate, (+++): strong.

Light Microscopy Evaluation

Morphologic alterations in the lungs were examined at 1st, 2nd and 4th hours after completion of treatment by light microscopy. The samples of the lung tissue obtained from the sham operated, control I/R and AM treatment groups were fixed in 10 % for 72 hours. Then, they were washed in alcohols of various degrees and embedded in paraffin. Sections of 3 μm thicknesses were obtained from the paraffin blocks and were stained with H&E. Morphologic examinations were performed in a blinded fashion using a Leica DM 4000B microscope and documented by photographs.

Statistical Analysis

SPSS 20.0 programme (IBM, Co., Armonk, NY, USA) was employed in statistical analyses. Sample size was predetermined using a power analysis: α=0.05 and power of 0.8 (SD: 1.22, mean difference: 2.28, normal two sided test). The analysis showed that 6 rats per group would be sufficient. The data analysis were performed by Leica DM 4000B microscope and documented by photographs.

RESULTS

Effects of adrenomedullin treatment on serum levels of TNF-α and IL-6

Serum concentrations of TNF-α and IL-6 were measured with the use of ELISA. On comparing the groups in terms of TNF-α enzyme activity, significant changes were found between groups in the 1st and 4th hour measurements (p=0.039 and p<0.0001, respectively). Accordingly, the TNF-α enzyme activity in the first hour was found to be significantly higher in I/R group than in Sham and AM treatment group (p=0.027 and p=0.041, respectively) (Table 1). The values of TNF-α levels for the sham and AM treatment group, on the other hand, were found to be similar (p=0.832). In a similar way, the fourth hour TNF-α enzyme activity in I/R group was found to be higher than in the sham and AM treatment group (p<0.0001 and p<0.0001, respectively) (Table 1). The levels of TNF-α in sham group were similar to those in the AM treatment group (p=0.310).
On comparing the groups in terms of IL-6 enzyme activity, significant differences were found only in the second hour measurements \((p=0.038)\). The second hour IL-6 enzyme activity in I/R group was found to be significantly higher than in the Sham and AM treatment group \((p=0.025\) and \(p=0.028\), respectively). There were no significant differences in the levels of IL-6 for AM treatment group as compared with sham-operated animals \((p=0.864)\).

### Table 1. The results of both in-groups and sub-groups

|                         | TNF-α (pg/m) | IL-6 (pg/m) | Caspasee-9 |
|-------------------------|--------------|-------------|------------|
| **Sham group**          |              |             |            |
| 1st hour                | 155±88’      | 6528±4044   | 1.8±0.4'   |
| 2nd hour                | 204±27       | 9770±2693   | 1.6±0.5’   |
| 4th hour                | 107±42’      | 960±2682    | 1.8±0.4’   |
| **Control (I/R) group** |              |             |            |
| 1st hour                | 228±124      | 8007±2667   | 2.3±0.5    |
| 2nd hour                | 489±166      | 6713±1077   | 2.5±0.5    |
| 4th hour                | 285±96       | 856±506     | 2.8±0.4    |
| **AM group**            |              |             |            |
| 1st hour                | 133±63’      | 5990±1928   | 1.5±0.5    |
| 2nd hour                | 220±79       | 6916±2407   | 1.3±0.5’   |
| 4th hour                | 165±93’      | 6975±3272   | 1.1±0.4’   |

*; Compared with control (I/R) group \(p<0.05\)
#: Compared with sham group \(p<0.05\)

**Effects of adrenomedullin on lung morphology**

As shown in Figure 1A, 1B and 1C, lungs of sham-operated animals had a normal microscopic appearance. Comparing the groups according to lung tissue caspase-9 enzyme activity, significant differences were found between the groups at all measurement times \((p=0.033\) and \(p=0.05, p<0.0001\), respectively). The first hour caspase-9 enzyme activity was found to be significantly higher in I/R group than in sham group \((p=0.011)\). The caspase-9 enzyme activity in AM treatment group were similar to those in the sham group \((p<0.05)\). The caspase-9 enzyme activity in the second hour was found to be significantly higher in I/R group than in sham and AM treatment group \((p=0.002\) and \(p=0.015\), respectively).

The caspase-9 enzyme activity in sham and AM treatment group, however, were found to be similar differences \((p=0.291)\). Similarly, the caspase-9 enzyme activity in the fourth hour was found to be significantly higher in I/R group than in sham and AM treatment group \((p<0.0001\) and \(p=0.001\), respectively). For each group, repeated measures of TNF-α, IL-6 and caspase-9 (Freidman test) and binary (Wilcoxon test) repeated measures were compared, and no analysis was statistically significant.

![Figure 1A](image1.jpg)

Figure 1A. In sham group 1st hour; the walls of the lung tissue are covered with smooth epithelium; the tissue is formed of alveoli and has a normal lung parenchyma. No pathologic changes are observed in the ductus alveolaris to which the alveoli open. 1B. In sham group 2nd hour; the lung tissue has a normal structure with alveoli containing a terminal bronchiole of small diameter in the middle and ductus alveolaris. 1C. In sham group 4th hour; the lung tissue has a normal structure with oval or round alveoli; the walls are covered with smooth epithelium. 1D. In hepatic I/R group 1st hour; the lung tissue with sporadic formation of minimal vacuolar structures in the parenchyma of the intraalveolar area where hemorrhagic infiltration has started is striking. 1E. In hepatic I/R group 2nd hour; increased hemorrhagic infiltration, vacuolar, and edematous areas are seen in lung tissue. 1F. In hepatic I/R group 4th hour; the lung tissue has a normal structure with alveoli and ductus alveolaris; the walls are covered with smooth epithelium. 1G. In hepatic I/R group 1st hour; the lung tissue with hemorrhagic infiltration is reduced to that of 1st hour group. 1H. In AM group 2nd hour; the alveoli and ductus alveolaris have a similar appearance as that of the lung tissue, and the degree of hemorrhagic infiltration is reduced to that of 1st hour group. 1I. In AM group 4th hour; the characteristics of the lung parenchymal tissue are similar to those of the control group; the alveoli have a normal structure and appearance; the ductus alveolaris have normal width. Hematoxylin and eosin (H&E x 10).
As shown in Figure 2A, 2B and 2C, lungs of 1st, 2nd and 4th hour sham-operated animals had a normal microscopic structure. In hepatic I/R group, the findings of ischemia in lung tissue increased gradually between the 1st hour and 4th hour (Figure 2D and 2F), while in AM treatment group that decreased gradually within the same period (Figure 2G and 2I). In the 4th hour of after hepatic I/R, lung apoptosis was seen to be decreased in AM treatment group. The normal level of apoptosis in the sham group with Caspase-9 increased in severity in I/R group in time starting from the 1st hour (Figure 2D, 2E and 2F), whereas in AM treatment group, its severity was similar to that of the sham group (Figure 2G, 2H and 2I). In AM treatment group of 1st, 2nd and 4th hour; the epithelial cells that form the alveolar wall showed mild to moderate immunoreactivity in the epithelial cells that form the alveoli wall, in the cells of intraalveolar area with Caspase-9 (Figure 2G, 2H and 2I). In I/R group, strong immunoreactivity was observed in the alveolar epithelial cells, cells of the interepithelial area, and endothelial cells with Caspase-9 starting from the 1st to 4th hour (Figure 2D, 2E and 2F). In AM treatment group; however, the degree of immunoreactivity ranged from mild to moderate (Figure 2G, 2H and 2I).

DISCUSSION

Hepatic I/R injury is a mechanism, which may be held responsible for liver failure that may occur after liver transplantation or liver surgery in haemorrhagic or septic shocks and after severe traumas. Organ reperfusion along with the damage caused by ischemia on its own leads to more severe tissue damages. Moreover, liver damage induced by I/R does not only remain in the liver but it also causes non-ischemic remote organ damage. The organs most significantly injured by I/R induced damage are primarily lungs. Several studies have been conducted to explain the mechanisms underlying the damage. While the studies are based on inflammatory mechanisms, they aim to be able to minimise the damage caused by I/R (15). Thus, the focus of systemic proinflammatory cytokine response is on mechanisms so as to explain the pathophysiology of hepatic I/R injury.

AM - a peptide obtained for the first time by Kitamura et al in 1993 from human phaeochromocytoma cells- are also available in such cells as adrenocortical cells, neurons, glial cells, fibroblasts, macrophages, epithelial cells and various neoplastic cells (16). A study conducted found that there were differences between the AM levels in the pulmonary artery and in the left ventricle, and the study attributed the difference to clearance of AM from the blood by lungs (17). Besides, it was also shown in some other studies that the amount of AM per tissue after AM injection was the highest in the lungs (18). Because the highest amount of AM was in the lung tissues, it was thought that the greatest effect was in the lungs. Therefore, this study aimed to describe the diminishing effects of AM on hepatic I/R induced lung injury.

Following hepatic I/R, TNF-α and IL-6 values began to rise, and both reached the maximum value in the second hour. In the fourth hour, however, reduction was observed in both cytokines. The values of TNF-α and IL-6 in rats especially in groups to which AM was administered were found to be lower than in rats to which hepatic I/R was administered.

Liver Kupffer cells are activated in the second hour, which is regarded as the early phase of I/R, and this causes the production of proinflammatory cytokines such as TNF-α and IL-6. This study is in parallel the data presented in the literature. Yet, the data in this study is insufficient to assess the long-term effects of reperfusion.

Excessive release of multiple cytokines was the main reason of the organ inflammatory injury. The release of pro-inflammatory cytokines, such as the activation and migration of TNF-α, IL-6, IL-8, can induce neutrophils and cause systemic inflammatory damage of various organs (19-21). TNF-α was the one of the highlight cytokine. TNF-α, a pleiotropic cytokine, may mediate direct toxicity to mitochondria, and induce apoptotic or necrotic cell death. This secreted TNF-α had local, systemic and remote organ effects, notably on the lung (22). Dwivedi et al. have shown that intestinal I/R induced considerable lung injury, as characterized by lung edema, histopathologic changes, increased proinflammatory cytokines [TNF-α and IL-6] levels in the lungs (18). Colletti et al. have shown that lung and liver injury following hepatic I/R in the rat is increased by exogenous lipopolysaccharide, which also increases hepatic TNF production in vivo and in vitro (23). Administration of AM/ adrenomedullin-binding protein-1 (AMBP-1) after ischemia mitigated lung injury and dramatically downregulated proinflammatory cytokines. Lung injury was also ameliorated by delayed AM/AMBP-1 treatment as evidenced by improvement in lung histology (18). Carrizo et al. demonstrated that administration of AM and AMBP-1 reduced TNF-α, IL-1β, IL-6, transaminases, lactate and creatinine levels, attenuated tissue injury, and improved survival (24). Kerem et al. have shown that the levels of these cytokines TNF-α and IL-1β were found to be significantly high following hepatic I/R, however it was seen that the levels of proinflammatory cytokines, apoptosis and necrosis, were significantly decreased by AM treatment (25).
Many studies making efforts to explain the pathophysiology of the anti-inflammatory property of AM are available, and one of those studies demonstrated that AM stimulated the release of TNF-α and IL-6 from murine macrophage-like RA264.7 cells which were stimulated by endotoxin (26,27). It was demonstrated that the IL-6 levels in circulation were influential in the prediction of ARDS severity (28). IL-6 levels in this study were also observed to be in the highest level in the hepatic I/R group in the AM group that decreased gradually within the same period. In the AM group, its severity was similar to that of the sham group. Meanwhile, consistent results were obtained from the morphological changes and immunohistochemistry. Setting out from these findings, this study may be said to be significant in that it demonstrates that AM mitigates the apoptosis of pulmonary endothelial cells beginning after the second hour after AM administration in particular, and it has similarities with other studies (30).

In conclusion, we have shown that pulmonary damage associated with hepatic I/R as characterized by lung edema and histopathologic changes is mediated by the release of inflammatory mediators such as TNF-α and IL-6. These events lead to histopathological consequences such as ischemia, immunopathologies and cytokine disturbances in lung tissue. Therefore our results showed that administration of AM had a beneficial therapeutic effect on hepatic I/R induced lung injury.

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