EXPERIMENTAL STUDY

Antioxidative effects of adrenomedullin and vascular endothelial growth factor on lung injury induced by skeletal muscle ischemia-reperfusion

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Abstract: *Purpose:* The aim of this study was to investigate the effects of adrenomedullin (AM) and vascular endothelial growth factor (VEGF) on lung injury as a remote organ following skeletal muscle ischemia-reperfusion injury in a rat model.
Materials and methods: Thirty-six Wistar rats were randomized into six groups (n=6). Laparotomy was performed in all groups under general anesthesia. Nothing else was done in Group S (Sham). Ischemia reperfusion group (Group I/R) underwent ischemia and reperfusion performed by clamping and declamping of the infrarenal abdominal aorta for 120 minutes, respectively. Group VEGF and Group AM received intravenous infusion of VEGF (0.8 µg/kg) or AM (12 µg /kg) respectively, without ischemia and reperfusion. Group IR+VEGF and Group IR+AM received intravenous infusion of VEGF (0.8 µg/kg) or AM (12 µg /kg) respectively immediately after 2 hours period of ischemia. At the end of reperfusion period. Lung tissue samples were taken for biochemical examination. Total oxidant status (TOS) and total antioxidant status (TAS) levels in lung tissue were determined by using a novel automated method. $p<0.05$ was considered as statistically significant.
Results: TOS levels were significantly higher in Group I/R, when compared with groups S, AM and VEGF (p=0.004, p=0.011, p=0.017, respectively) and significantly lower in groups I/R+AM and I/R+VEGF, when compared with Group I/R (p=0.018, p=0.006, respectively). TAS levels were significantly higher in Group I/R, when compared with groups S, AM and VEGF (p=0.006 p=0.016, p=0.016, respectively) and significantly lower in Group I/R+AM, when compared with Group I/R (p=0.016).
Conclusion: These findings indicate that AM and VEGF acted effectively on the prevention of lung injury induced by skeletal muscle ischemia-reperfusion injury in a rat model *(Fig. 2, Ref. 30)*. Full Text in PDF *www.elis.sk*.
Key words: ischemia–reperfusion, total oxidant status, total antioxidant status, adrenomedullin, vascular endothelial growth factor, rat.

Ischemia / reperfusion (I/R) induced tissue injury is one of the most important problems in a number of surgical procedures including extremity revascularization, abdominal aortic surgery, replantation, transplantation and muscular flap reconstruction (1, 2). Reperfusion of ischemic tissues results in both a local and a systemic inflammatory response that, in turn, may result in widespread microvascular dysfunction and altered tissue barrier function (3). Skeletal muscle I/R elicits oxidative stress and causes inflammation in lung tissues that may lead to lung injury.

Adrenomedullin (AM) is a novel hypotensive peptide was discovered in 1993, in human pheochromocytoma by monitoring the elevating activity of platelet cAMP (4). AM has a range of biological actions including vasodilatation, bronchodilatation, inhibition of apoptosis, cell growth, regulation of hormone secretion, natriuresis, antimicrobial and anti-inflammatory effects (5, 6).

Vascular endothelial growth factor (VEGF) is a potent angiogenic mitogen acting exclusively on endothelial cells (7). Widespread distribution of VEGF and its specific receptors in the vasculature implies an important role for VEGF in maintenance of normal vascular function and development (8). It has been demonstrated that VEGF stimulates nitric oxide release from vascular endothelial cells (9, 10).

In this experimental study, we aimed to evaluate the oxidative effects of AM and VEGF on lung injury induced by skeletal muscle I/R.

Materials and methods

Experimental groups

Thirty-six adult Wistar-albino rats, weighing 200 to 250 g were used in this study. Rats were housed in cages at an average temperature of 22 °C in a light dark cycle-controlled environment with free access to food and tap water. The protocols of this experi-

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mental study were approved by the Animal Ethics Committee of Gazi University. All animals received human care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and the Use of Laboratory Animals" prepared by the National Academy of Science and published by the National Institutes of Health (NIH publication no. 85–23, revised in 1985).

Study design

Rats were randomized into six groups (n=6/group). The sham group (Group S) underwent midline laparotomy and dissection of the infrarenal abdominal aorta (IAA) without cross-clamping; ischemia reperfusion group (Group I/R) underwent laparotomy and cross-clamping of the IAA for 120 minutes and then 120 minutes of reperfusion; Group VEGF and Group AM underwent laparotomy and received intravenous infusion of VEGF (0,8 µg/kg) or AM (12 µg /kg) respectively, without ischemia and reperfusion; Group I/R+VEGF and Group I/R+AM received intravenous infusion of VEGF (0,8 µg/kg) or AM (12 µg /kg), respectively, immediately after 120 minutes of ischemia.

Aortic occlusion and ıschemia reperfusion

Rats were anesthetized with ketamine hydrochloride (Ketalar, 50 mg/kg, intramuscularly, Parke-Davis, Eczacibasi, Istanbul, Turkey) and xylazine hydrochloride (Alfazyne, 2 %, Ege Vet, Izmir, Turkey). Anesthesia was maintained by an additional muscular injection of ketamine hydrochloride and xylazine hydrochloride. The surgical procedures were performed while the rats were placed in a supine position under a heating lamp. The abdomen was shaved, the skin was prepared aseptically and a midline laparotomy was performed. The abdominal aorta was exposed and clamped using an atraumatic microvascular clamp. The aortic occlusion was confirmed by the loss of the distal arterial pulsation. The skin incision was closed and covered with a plastic wrap to maintain the body temperature and fluid balance. After 120 minutes of ischemia, the microvascular clamp was removed and lower extremities were reperfused for 120 minutes. At the end of the reperfusion period, all rats were killed under anesthesia and left lung was harvested for biochemical analyses.

Biochemical examination

Blood samples were obtained after induction of anesthesia and at the end of the surgery. The samples centrifugated at 3000 revolutions per minute for 10 minutes to separate plasma and then blood samples were stored at −80 °C until analysis.

Measurement of serum total oxidant status (TOS)

Plasma total oxidant status (TOS) levels were determined using a commercially available kit, developed by Erel (11) (REL assay diagnostics, Mega Tip, Gaziantep, Turkey). In this method, the oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion produces a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed as µmol H_2O_2 equivalent/L. Hydrogen peroxide and other derivatives of peroxides, produced physiologically in organisms and occurring in higher concentrations under some pathologic conditions, diffuse into plasma. The level of total peroxide was measured and expressed as TOS in this study.

Measurement of serum total antioxidant status (TAS)

Plasma total antioxidant status (TAS) levels were determined using a commercially available kit developed by Erel (REL assay diagnostics, Mega Tip, Gaziantep, Turkey) (12). In this method, hydroxyl radical, which is the most potent radical, is produced via Fenton reaction. In the classical Fenton reaction, the hydroxyl radical is produced by mixing of ferrous ion solution and hydrogen peroxide solution. In the most recently developed assay by Erel, same reaction is used. In the assay, ferrous ion solution, which is present in the Reagent 1, is mixed by hydrogen peroxide, which is present in the Reagent 2. The sequentially produced radicals such as brown-colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. In this assay, antioxidative effect of the sample against the potent free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay has got excellent precision values, which are lower than 3%. The results are expressed as mmol Trolox equivalent.

Statistical analyses

Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) 17.0 program was used for statistical analysis. Kolmogorov–Smirnov test was used for the comparisons to determine the distribution of all variable groups. Variations in TOS and TAS levels were assessed by using Kruskal–Wallis test. Bonferroni adjusted Mann-Whitney U test was used after significant Kruskal–Wallis to determine which group differs from the other. Results were expressed as mean± standard deviation (mean ± SD). Statistical significance was set at a p value <0.05.

Results

There was a statistically significant difference among the groups, when they were compared among themselves by means of TOS levels in lung tissue (p=0.017). TOS levels were significantly higher in Group I/R, when compared with groups S, AM and VEGF (p=0.004, p=0.011, p=0.017, respectively). TOS levels were found to be significantly lower in groups I/R+AM and I/R+VEGF, when compared with Group I/R (p=0.018, p=0.006, respectively) (Fig. 1).

A statistically significant difference was found among the groups, when they were compared among themselves for TAS levels in lung tissue (p=0.012). TAS levels were significantly higher in Group I/R, when compared with groups S, AM and VEGF (p=0.006 p=0.016, p=0.016, respectively). TAS levels were found to be significantly lower in Group I/R+AM, when compared with Group I/R (p=0.016). Figure 2 shows the levels of TAS in study groups.
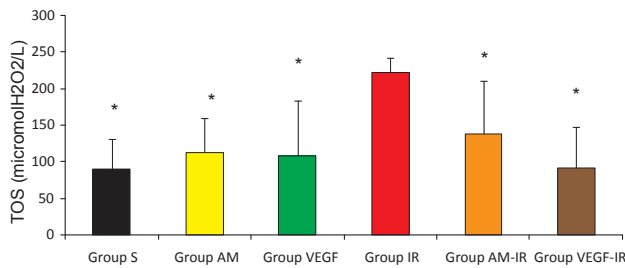
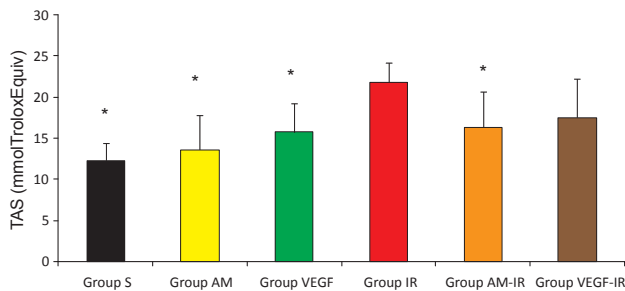**Fig. 1. TOS parameters of the study groups. Each bar represents the mean ± SD. * p<0.05 compared to the Group I/R.**

Fig. 2. TAS values of the groups. Each bar represents the mean ± SD. * p<0.05 compared to the Group I/R.

Discussion

Temporary aortic cross-clamping is accompanied by I/R injury and a systemic inflammatory response syndrome that can lead to non-cardiogenic pulmonary oedema, or acute respiratory distress syndrome in abdominal aortic surgery (13, 14). In the development of lung injury, the excessive generation of reactive oxygen radicals, polymorphonuclear sequestration in pulmonary microvasculature, increased endothelial permeability and interstitial edema have been suggested (15). Restoration of the blood supply to the ischemic tissue results in generation of reactive oxygen species (ROS). Excessive production of ROS causes lipid peroxidation in cell membranes and oxidative damage to DNA and proteins (16). A number of agents, such as N-acetylcystein, calcium dobesilate, aprotinin, erdosteine or magnesium sulfate have been proposed to be useful against lung injury induced by I/R (17–21).

AM is a potent vasodilating peptide that is expressed in tissues relevant to cardiovascular and renal function, such as the heart, kidney, aorta, lung and brain (22). Several mechanisms have been proposed for the protective effects of AM. It has been shown to possess a number of biological effects in addition to its well-known vasoactive activity (23). These include cell proliferation, differentiation, and oxidative stress-induced cell death inhibition (24–26).

Talero et al suggested that AM treatment prevented the formation of malondialdehyde, an indicator of lipid peroxidation, which reflects reduced oxidative stress. They have indicated that, AM has potential anti-inflammatory actions in the development of experimental pleurisy, attenuating nuclear factor-κB activation, and the expression of proinflammatory cytokines and inducible nitric oxide synthase enzyme (27). These effects may explain the decrease in neutrophil migration and oxidative stress during the course of the inflammatory response. Adrenomedullin has also been shown to play important roles in cardiovascular and renal diseases via suppression of oxidative stress production through activation of second messengers cAMP and nitric oxide cGMP-dependent signaling pathways (22).

VEGF is a potent regulator of vascular permeability, a key player in angiogenesis, and a survival factor for endothelial cells. Mura et al suggested that VEGF produced in type II alveolar epithelial cells plays an important role in maintaining alveolar structure in adults and in mediating acute lung injury by regulating pulmonary permeability and inflammation (28).

Considering the beneficial effects of AM and VEGF, the present study investigated the anti-oxidative effects of these agents in a model of skeletal muscle I/R-induced acute lung injury in rats. In this study, we have used a novel measurement method to evaluate the extent of oxidative stress in rat lungs after skeletal muscle I/R. This provides a useful method for the rapid evaluation of the TAS and TOS, which are valuable parameters in conditions involving oxidative stress. TOS indicates the total oxidative products in tissue. Oxidative products such as ROS, reactive nitrogen species, hydrochloric acid, malondialdehyde, and lipid peroxides constitute TOS (11). In our study, TOS levels significantly increased after aortic I/R. We also found that I/R plus AM and I/R plus VEGF significantly reduced the levels of TOS. TAS levels significantly increased in Group I/R+AM. Also I/R plus VEGF increased the levels of TAS, but this increase was not statistically significant. Our findings are consistent with previous papers reporting the antioxidant effects of AM on animal models of remote organ injury induced by aortic I/R (16, 29, 30). The mechanism of protective effect of AM against lung injury cannot be explained by only its antioxidative effect, since I/R injury is a complex process. Histopathologic and antiinflammatory effects of the peptid should also be investigated. The regulator and angiogenetic effects of VEGF have been suggested in previous studies (7, 10, 28). We have hypothesized that it may also have an antioxidative effect on lung injury to some extent. Our findings need to be supported by further studies evaluating different oxidative parameters.

Conclusion

The results of this study demonstrate that administration of AM and VEGF have antioxidative effects against lung injury induced by skeletal muscle I/R. However, further studies also evaluating histological and other biochemical parameters are required to confirm this finding and to elucidate the exact mechanism of action before clinical use.

References

1. Avci G, Kadioglu, Sehirli AO et al. Curcumin protects against ischemia/reperfusion injury in rat skeletal muscle. J Surg Res 2012; 172: e39.

2. Beyersdorf F, Unger A, Wildhirt A. Studies of reperfusion injury in skeletal muscle: preserved cellular viability after extended periods of warm ischemia. J Cardiovasc Surg 1991; 32: 664.

3. Eltzschig HK, Collard CD. Vascular ischemia and reperfusion injury. Br Med Bull 2004; 70 (1): 71.

4. Erre GL, Passiu G. Antioxidant effect of Iloprost: current knowledge and therapeutic implications for systemic sclerosis. Reumatism 2009; 61 (2): 90–97.

5. Lessiani G, Vazzana N, Cuccurullo C et al. Inflammation, oxidative stress and platelet activation in aspirin treated critical limb ischaemia: Beneficial effects of iloprost. Thromb Haemost 2011; 105: 321–328.

6. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005; 38: 1103.

7. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 2004; 37: 277.

8. Groeneveld AB, Raijmakers PG, Rauwerda JA, Hack CE. The inflammatory response to vascular surgery associated ischemia and reperfusion in man: effect on postoperative pulmonary function. Eur J Vasc Endovasc Surg 1997; 14: 351.

9. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg 2002; 10: 620.

10. Wijnen MH, Roumen RM, Vader HL, Goris RJ. A multi antioxidant supplementation reduces damage from ischaemia reperfusion in patients after lower torso ischaemia. A randomised trial. Eur J Vasc Endovasc Surg 2002; 23 (6): 486–490.

11. Ozyurt H, Ozyurt B, Koca K et al. Caffeic acid phenethyl ester (CAPE) protects rat skeletal muscle againstischemia–reperfusion–induced oxidative stres. Vascul Pharmacol 2007; 47: 108–112.

12. Welbourn CR, Goldman G, Paterson IS et al. Pathophysiology of ischemia reperfusion injury: central role of the neutrophil. Br J Surg 1991; 78: 651.

13. Oyar EO, Kiris I, Gulmen S et al. The protective effect of adrenomedullin on renal injury, in a model of abdominal aorta cross–clamping. Thorac Cardiovasc Surg 2012; 60: 5.

14. Sotoudeh A, Takhtfooladi MA, Jahanshahi A et al. Effect of N–acetylcysteine on lung injury induced by skeletal muscle ischemia–reperfusion. Histopathological study in rat model. Acta Cir Bras 2012; 27 (2): 168.

15. Bozkurt AK, Konukoglu D, Ustundag N, Yuceyar L, Mayda AS. Calcium dobesilate ameliorates lung injury following lower limb ischemia/reperfusion. Drugs Exp Clin Res 2002; 28 (4): 127.

16. Koksal C, Bozkurt AK, Ustundag N et al. Attenuation of acute lung injury following lower limb ischemia/reperfusion: the pharmacological approach. J Cardiovasc Surg 2006; 47 (4): 445.

17. Sirmali M, Uz E, Sirmali R et al. The effects of erdosteine on lung injury induced by the ischemia–reperfusion of the hind–limbs in rats. J Surg Res 2008; 145 (2): 303.

18. Kao MC, Jan WC, Tsai PS, Wang TY, Huang CJ. Magnesium sulfate mitigates lung injury induced by bilateral lower limb ischemia–reperfusion in rats. J Surg Res. 2011; 171 (1): e97.

19. Fantone JC, Marasco WA, Elgas LJ, Ward PA. Stimulus specificity of prostaglandin inhibition of rabbit polymorphonuclear leukocyte lysosomal enzyme release and superoxide anion production. Am J Pathol 1984; 115 (1): 9–16.

20. Baltalarli A, Ozcan V, Bir F, Aybek H, Sacar M, Onem G, Goksin I, Demir S, Teke Z. Ascorbic acid (vitamin C) and iloprost attenuate the lung injury caused by ischemia/reperfusion of the lower extremities of rats. Ann Vasc Surg 2006; 20 (1): 49–55.

21. Bozok S, Ilhan G, Yilmaz Y et al. Protective effects of hyperbaric oxygen and iloprost on ischemia/reperfusion–induced lung injury in a rabbit model. Eur J Med Res 2012; 17: 14.

22. Fantone JC, Kinnes DA. Prostaglandin E1 and prostaglandin I2 modulation of superoxide production by human neutrophils. Biochem Biophys Res Commun 1983; 113: 506–312.

23. Simpson PJ, Mickelson J, Fantone JC, Gallagher KP, Lucchesi BR. Iloprost inhibits neutrophil function in vitro and in vivo and limits experimental infarct size in canine heart. Circulat Res 1987; 60: 666–673.

24. Kiris I, Tekin I, Yilmaz N, Sutcu R, Karahan N, Ocal A. Iloprost downregulates expression of adhesion molecules and reduces renal injury induced by abdominal aortic ischemia–reperfusion. Ann Vasc Surg 2009; 23: 212–223.

25. Bursch W, Taper HS, Somer MP, Meyer S, Putz B, Schulte–Hermann R. Histochemical and biochemical studies on the effect of the prostacyclin derivative Iloprost on CCl4–induced lipid peroxidation in rat liver and its significance for hepatoprotection. Hepatology 1989; 9: 830–838.

26. Yu AL, Fuchshofer R, Kampik A, Welge–Lüssen U. Effects of oxidative stress in trabecular meshwork cells are reduced by prostaglandin analogues. Invest Ophthalmol Vis Sci 2008; 49: 4872–4880.

27. Dedeoglu BD, Aytac E, Suzer O, Balci H, Uzun H, Seymen P et al. Donor heart preservation with Iloprost supplemented St. Thomas Hospital cardioplegic solution in isolated rat hearts. Prostaglandins Leukot Essent Fatty Acids 2008; 78: 415–421.

28. Ferrari R, Cargnoni A, Curello S, Boffa GM, Ceconi C. Effects of Iloprost (ZK 36374) on glutathione status during ischaemia and reperfusion of rabbit isolated hearts. Br J Pharmacol 1989; 98: 678–684.

29. Tassiopoulos A, Carlin RE, Gao Y. Role of nitric oxide and tumor necrosis factor on lung injury caused by ischemia/reperfusion of the lower extremities. J Vasc Surg 1997; 26: 647–656.

30. Vural KM, Öz MC. Endothelial adhesivity, pulmonary hemodynamics and nitric oxide synthesis in ischemiareperfusion. Eur J Cardiothorac Surg 2000; 18: 348–352.