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Effect of ghrelin on renal erythropoietin production in chronic hypoxic rats

[1]Feizi H, [2]Rajaee K, [3]Keyhanmanesh R, [4]Aliparasti MR, [5]Almasi S, [3]Alipour MR

[1]Department of Physiology, Hormozgan University of Medical Sciences; [2]Department of Physiology, Medical School, Tabriz University of Medical Sciences; [3]Tuberculosis and Lung Research Center, Tabriz University of Medical Sciences; [4]Drug Applied Research Center, Tabriz University of Medical Sciences; [5]Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
E-mail: alipourmr52@yahoo.com

Objective. Ghrelin, a 28 amino acid peptide, has diverse effects in body organs. Erythropoietin is a key mediator in increasing the red blood cells during hypoxia. Previously, we have shown that ghrelin has a polycythemic effect. In the present study, we evaluated the effect of ghrelin on erythropoietin gene expression with the aim to find out the mechanism of its effect.

Methods. Thirty two adult male Wistar rats were divided randomly into four groups. The hypoxic condition was induced by placing the rats into the hypoxic chamber with 11% oxygen for two weeks. Saline- and ghrelin-treated control rats remained in room with a regular air conditions. Erythropoietin gene expression was measured by real-time reverse transcription-polymerase chain reaction (RT-PCR). Plasma erythropoietin was measured by enzyme linked immunosorbent assay (ELISA).

Results. After 2-weeks of hypoxia, erythropoietin transcripts and erythropoietin plasma levels were significantly increased in hypoxic animals compared with control animals. Ghrelin treatment decreased both plasma erythropoietin and erythropoietin gene expression only in the hypoxic rats.

Conclusions. Our data indicate that ghrelin might induce polycythemia through an erythropoietin-independent manner. However, to confirm this hypothesis and to find out the precise mechanism of this phenomenon further investigations are needed.

Key Words: ghrelin, kidney, erythropoietin, chronic hypoxia

Ghrelin, first identified as a growth hormone releasing analogous, has diverse functions in different body parts (Van der Lely et al. 2004; Kojima and Kangava 2005). Among these actions, its cardiovascular effects, especially under conditions of hypoxia, are under investigation (Schwenke et al. 2008; Kishimoto et al. 2009; Alipour et al. 2011, 2012). Ghrelin receptors have been found in many organs including the kidneys (Mori et al. 2000; Davenport et al. 2005). Erythropoietin (Epo) is an essential glycoprotein hormone involved in the regulation of red blood cells production (Jelkmann 1986). It is produced by liver and kidney in response to hypoxia (Jacobson et al. 1957; Beru et al. 1986). The regulation of Epo gene expression occurs primarily at the mRNA level and it is under the control of transcriptional factors in which hypoxia-inducible factors (HIF) are the main factors mediating the renal response to hypoxia (Schuster et al. 1989; Goldberg et al. 1991; Nangaku and Eckardt 2007; Paliege et al. 2011). The plasma levels of Epo finally influence the rate of production of new erythrocytes by the bone marrow. Failure to increase the amount of circulating Epo in response to hypoxic stress can lead to anemia (Erslev 1991). Previously, we have shown that ghrelin has a potent polycythemic effect in hypoxic condition (Alipour et al.

Corresponding author: Mohammad Reza Alipour, Associate Professor of Physiology, Tuberculosis and Lung Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; phone/fax: +98-4113364664; e-mail: alipourmr52@yahoo.com.

2010). In the present study, we investigated the effect of ghrelin on erythropoietin production in the kidney to find out its assumed mechanism.

Materials and Methods

Animals and chronic hypoxia model design. All experiments were conducted in accordance with the ethical standards of the faculty of medicine, Tabriz University of Medical Sciences, Iran. Adult male Wistar rats (200-250 g) were housed in cages in a temperature and light-controlled environment and provided with food and water *ad libitum*. Animals were randomly divided into 4 groups namely, control (C), control with ghrelin (CG), hypoxic with saline (HS), and hypoxic with ghrelin (HG). Each group contains 8 rats. In (HS) and (HG) group of rats, hypoxia was induced by Environmental Chamber System GO2Altitude (Biomedtech Australia Pty. Ltd), which generates hypoxic air without any need to gas cylinder. HS and HG animals were placed into a ventilated chamber inflated with hypoxic air (11% O_2), simulated heights of 5150 m above the sea level. An O_2 sensor and controller were embedded into the chamber wall to monitor O_2 concentration. Animals were kept in the chamber continuously for two weeks, except the time of daily injections.

Drug administration. Rats received a subcutaneous injection of either saline (0.1 ml) or ghrelin (150 μg/kg/day in 0.1 ml) (Schwenke et al. 2008) and then were placed into the hypoxic chamber. HS, CG and HG rats continued to receive daily injections of either saline or ghrelin during the 2-weeks. Ghrelin was obtained from the Tocris Bioscience Co. (Bristol, UK) and administered dissolved in saline serving as the vehicle.

RNA extraction and first-strand cDNA synthesis. For all animals, the right kidney was removed for RNA extraction under standard sterile surgical method. Total RNA was extracted from kidney using Trizol reagent (Invitrogen, USA) according to the manufacturer's description and treated with RNase-free DNase to remove any residual genomic DNA. Single stranded cDNAs were synthesized by incubating total RNA (1 μg) with RevertAid H Minus M-MuL V Reverse transcriptase (200 U), oligo-$[dT]_{18}$ primer (5 μM), Random Hexamer Primer (5 μM, dNTPs (1 mM), and RiboLock RNase-inhibitor (20 U) for 5 min at 25°C followed by 60 min at 42°C in a final volume of 20 μl. The reaction was terminated by heating at 70°C for 5 min.

Real-time relative quantitative RT-PCR. Quantitative real-time RT-PCR was done using the Corbett Life Science (Rotor-Gene 6000) System using 2 μl of a 3-fold diluted cDNA in each PCR reaction in a final volume of 20 μl. Each PCR reaction contained 5 pM of primers and 1×FastStart SYBR Green Master (Roche). Sequences of primers are listed in Table 1. PCR amplifications were performed by the following three cycle programs: 1) denaturation of cDNA (1 cycle: 95°C for 10 min); 2) amplification (40 cycles: 95°C for 15 s, 57°C for 30 s, 60°C for 34 s); 3) melting curve analysis (1 cycle: 60 to 95°C with temperature transition rate 1°C/s). Serial cDNA dilutions of a mixture of all samples were used to generate standard curves. β-actin (Actb) mRNA expression levels were used to calculate relative expression levels. All data are presented as ratio of the target gene/Actb. The relative quantification was performed by $2^{[-\Delta Ct]}$: Expression of target genes/Actb = $(1+E)^{-Ct}$ target gene/$(1+E)^{-Ct}$ Actb. The specificity of the PCR reactions was verified by generation of a melting curve analysis followed by gel electrophoresis, visualized by ethidium bromide staining.

Plasma erythropoietin measurement. All measurements were performed on blood samples from each animal separately and the acquired data are represented as mean ± SEM. Epo was measured by rat EPO ELISA kit (ref. DRE11406, Glory Science Co., Ltd, USA) using a reader (Statfax, Awareness, USA) at a wavelength of 450 nm. The intra-assay and inter-assay were 11.2% and 11.4%, respectively.

Hematocrit measurement. Hematocrit was measured using the standard microhematocrit method. Blood sampling was performed from tail of animals. Up to 2/3 the length of the microhematocrit tube (use 2 tubes for each sample, plain blue-ringed tube for anticoagulated blood, heparinized red-ringed tube for finger stick) was filled with blood sample and then sealed one

Table 1

Sequences of oligonucleotide primers

Gene	Forward Primer	Reverse Primer	Product size [bp]
Epo	GCT CCA ATC TTT GTG GCA TC	ATC CAT GTC TTG CCC CCT A	66
β-actin	TCCTCCTGAGCGCAAGTACTCT	GCTCAGTAACAGTCCGCCTAGAA	153

Epo – Erythropoietin

end with sealant of clay. Blood contained tubes were centrifuged at 12000xg for 5 min by a microhematocrit centrifuge. Finally, hematocrit was read in percent by using microhematocrit reader.

Statistical analysis. Expression of Epo was obtained through the Corbett Rotor-Gene 6000 and expressed as Ct (cycle threshold); ΔCt (Ct of target gene – Ct of housekeeping gene). The collected data was analyzed by statistical SPSS software, version 16. Variables were reported as means and standard errors. Data were analyzed by ANOVA to test differences between groups. For multiple comparisons where statistical significance was reached, according to equality of variances, post hoc analyses were performed using the Tukey tests. The value $p \leq 0.05$ was determined as the level of significance for all statistical analysis.

Results

Hematocrit measurement. Average hematocrit of C, CG, HS and HG groups after two weeks are shown in Fig. 1. A significant polycythemia occurred in HS compared with C group ($p<0.0001$). Furthermore, ghrelin treatment in hypoxic animals lead to a significant elevation in hematocrit when compared with saline treated ones ($p<0.0001$). However, there was no a significant polycythemia in CG group compared with normal animals.

Effect of hypoxia on Epo gene expression. After 2-weeks hypoxia, Epo transcripts of hypoxic animals significantly increased compared with control animals ($p<0.001$) (Fig. 2.). Furthermore, data analysis showed that expression of Epo in kidney of HS animals increased about 2.42-fold compared with control group.

Effect of ghrelin on Epo gene expression during hypoxia. Ghrelin treatment did not change Epo gene expression in normal rats. However, during chronic hypoxia, ghrelin decreased Epo gene expression compared with saline-treated animals ($p<0.001$) (Fig. 2).

Plasma Epo measurements. In normal ghrelin-treated animals, plasma Epo did not change compared with control animals. After 2-weeks hypoxia, plasma levels of Epo increased significantly compared with control animals ($p<0.05$). In HG animals, plasma Epo significantly decreased when compared with HS rats ($p<0.001$) (Fig. 3).

Discussion

The results of this study indicate that the polycythemic effect of ghrelin could not be under potentiation of Epo gene expression. Since there is no correlation between ghrelin treatment and the amount of Epo gene expression, it seems that ghrelin induces the polycythemia independent of Epo. To prove this claim, the following points are remarkable: 1) although mild elevation in hematocrit was seen in normal ghrelin-treated animals, but there was not a significant change in renal Epo gene expression or plasma alteration; 2) in hypoxic animals

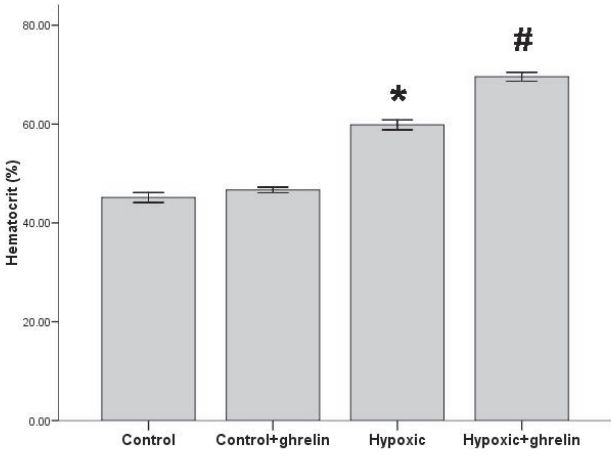

Fig. 1. Average hematocrit after two weeks in Control, Control with Ghrelin, Hypoxic with saline (HS), and Hypoxic with Ghrelin (HG) groups. Ghrelin was injected subcutaneously (150 μg/kg/day). Data are reported as mean ± SEM.
***p<0.0001 compared with normoxia**
#p<0.0001 compared with HS

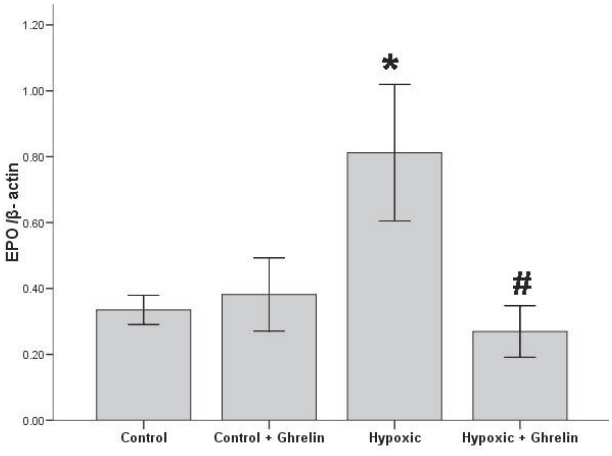

Fig. 2. Relative quantitative RT-PCR of erythropoietin to β-actin (n=8).
Data are presented as mean ± SEM.
***p<0.001 compared with normoxia**
#p<0.001 significant difference between chronic hypoxic rats treated with saline vs. ghrelin

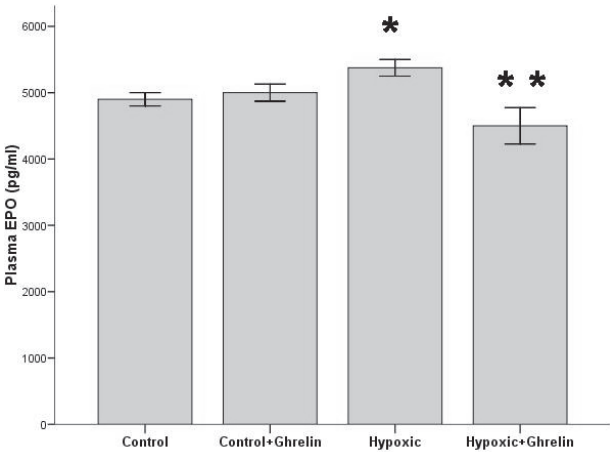

Fig. 3. Average erythropoietin after two weeks in Control, Control with Ghrelin, Hypoxic with saline (HS), and Hypoxic with Ghrelin (HG) groups. Ghrelin was injected subcutaneously (150 μg/kg/day). Data are reported as mean ± SEM.
***p<0.05 compared with control animals**
****p<0.001 significant difference between chronic hypoxic rats treated with saline vs. ghrelin.**

encountered a severe polycythemia, there was a significant increment in Epo gene expression but the elevation of plasma Epo was not as vivid as gene expression. It would be better to point out that the regulation of Epo levels during hypoxia occurs primarily at the transcriptional level. In response to hypoxia there would be an initial peak for plasma Epo and then it decreases but remains above baseline (Eckardt et al. 1990; Klausen et al. 1996). On the other hand, in hypoxic ghrelin-treated animals, although a severe polycythemia was seen but Epo gene expression and plasma level declined significantly compared with hypoxic animals. Since we measured all the variables at the end of the second week of hypoxia, it seems that ghrelin accompanies Epo synergistically leading to a more severe polycythemia compared with hypoxia alone. Thereafter, this potent polycythemia suppresses Epo gene expression vigorously. Overall, the problem is the real mechanism by which ghrelin induces polycythemia in an Epo-independent manner. One must remember that ghrelin is a growth hormone (GH) secretagogue and a hematopoietic effect has been found for GH secretagogue (Koo et al. 2001). So a mediator role for GH might be expected to elucidate this action of ghrelin. Whether ghrelin can establish red blood cell production directly or not is our future hypothesis. In our new projects, we will focus on spleen and hematopoietic stem cells, as probable targets for ghrelin, to find out the possible mechanisms of polycythemic effect of ghrelin especially during hypoxia.

It has been approved that tissue hypoxia results in the activation of a physiological stress response designed to increase erythropoiesis named as stress erythropoiesis (Socolovsky 2007). This phenomenon occurs in the adult spleen (Millot et al. 2010; Paulson et al. 2011). Furthermore, stress erythropoiesis, especially in the acute phase, could be promoted even in the Epo absence (Zeigler et al. 2010). In a study by Xia et al. (2004), it has been shown that ghrelin has a modulatory dose-dependent effect on proliferation of splenic T cells. Based on the present study in which we propose an Epo-independent erythropoiesis function for ghrelin, stress erythropoiesis in spleen is not unpredictable by ghrelin.

Suggesting splenic effect of ghrelin could be derived from literature data. Cytoplasmic protein Src homology-2 domain containing phosphatase-1 (SHP-1), a protein tyrosine phosphatase (PTP), has been previously introduced as an inhibitor of activation-promoting signaling cascades in hematopoietic cells (Tonks 2006; Lorenz 2009). Revealing the regulatory role of SHPS-1 in hematopoeisis, Ishikawa-Sekigami et al. (2006) indicated that it negatively regulates the phagocytosis of RBCs by splenic macrophages, thereby determining both the lifespan of individual RBCs and the number of circulating erythrocytes. It is notable that SHP-1 is activated by phosphorylation in the C-terminal Y536 residue of SHP-1 (Zhang et al. 2003). Regarding the fact that SHP-1 phosphorylation reaches its maximal levels under ghrelin stimulation (Lodeiro et al. 2011), it could be proposed that ghrelin might be involved in survival of RBCs when passing through the spleen.

Finally, some research groups have revealed that ghrelin interferes with stem cells differentiation and proliferation (De Vriese et al. 2005; Dixit et al. 2007; Togliatto et al. 2010; Yang et al. 2011). Based on the data of the present study, hematopoietic stem cells as a candidate target for ghrelin need to be further investigated.

In summary, it seems that ghrelin may induce polycythemia through an Epo-independent manner. However, understanding the precise mechanism of this phenomenon needs further investigations.

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