

Original Article

The anti-apoptotic effect of ghrelin in the renal tissue of chronic hypoxic rats

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Abstract

Introduction: Ghrelin is an endogenous peptide that has diverse functions in the body. One of the newly recognized roles of this peptide is its antiapoptotic effect. However, this function has not been investigated in normobaric hypoxia- induced apoptosis in body organs. This study will examine the effect of ghrelin treatment on Bax-Bcl-2 gene expression in the kidney of chronic hypoxic rats.

Materials and Methods: Male Wistar rats in three experimental groups; normal, hypoxic+saline and hypoxic+ghrelin were used in this study. The expression level of Bax and Bcl-2 genes were measured by Real-Time PCR. The Bax/Bcl-2 ratio was also compared in three groups statistically.

Results: Chronic hypoxia decreased the Bcl-2 gene expression ($p < 0.01$), and increased the Bax/Bcl-2 ratio ($p < 0.05$). Ghrelin treatment decreased the Bax/Bcl-2 ratio through upregulation of Bcl-2 gene ($p < 0.05$).

Conclusion: It seems that ghrelin has an antiapoptotic effect on the kidney of animals that live in a chronic hypoxic state. Which could potentially introduce a therapeutic role for this peptide.

Keywords:

Ghrelin;
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Introduction

Apoptosis is a programmed form of cell death in which the cellular elements are degraded by a group of cysteine proteases (Orrenius, 1995, Elmore, 2007). Encountered with stress-causing situations such as glucocorticoids, heat, radiation, nutrient deprivation,

viral infection and increased intracellular calcium concentration, a damaged cell can release intracellular apoptotic signals (Elmore, 2007). It is notable that hypoxia is one of the well-known stressors, which triggers apoptosis in different cell types (Weinmann *et al.*, 2004). In the kidneys, most studies have been focused on the ischemic models to investigate the hypoxia- induced renal tissue damage and apoptosis

(Khan *et al.*, 1999, Zhao *et al.*, 2013). However, hypoxic environment could lead to the same injuries in the kidneys especially when living in chronic hypoxic state (Mazzali *et al.*, 2003). Ghrelin is an endogenous peptide that has diverse functions in the body (van der Lely *et al.*, 2004, Kojima and Kangawa, 2005). One of its newly demonstrated roles is its anti-apoptotic action (Baldanzi *et al.*, 2002, Chung *et al.*, 2007, Yang *et al.*, 2007, Zhang *et al.*, 2007, Granado *et al.*, 2009, Hwang *et al.*, 2009, Yang *et al.*, 2012). Recently, investigators have explored the beneficiary effects of ghrelin treatment regarding to chronic kidney disease (Cheung and Mak, 2009, Cheung and Mak, 2010, Suzuki *et al.*, 2013). Furthermore, it has been shown that ghrelin protects the renal tissue against the oxidative stress (Fujimura *et al.*, 2014, Khowailed *et al.*, 2015). Among the biochemical markers, the Bcl-2 family of proteins are one of the reliable indices that regulate the incidence of apoptosis (Jacobson and Raff, 1995). This family includes both pro-apoptotic and anti-apoptotic proteins with Bax and Bcl-2 as typical examples, respectively (Yang and Korsmeyer, 1996). In general, the Bax/Bcl-2 ratio could be used to measure the rate of apoptosis incidence. The greater the ratio the more probable is the occurrence of apoptosis (Salakou *et al.*, 2007).

Since the kidney could suffer due to hypoxia, based on the demonstrated and well-defined anti-apoptotic effect of ghrelin, the aim of this study was to evaluate the Bax/Bcl-2 gene expression ratio in the renal tissue of animals who live in normobaric hypoxia and determining the effect of ghrelin treatment on this ratio.

Materials and methods

Animals and chronic hypoxia induction

Male adult Wistar rats [200-250gr] were housed in cages in a temperature ($22 \pm 2^\circ\text{C}$) and light-controlled environment and provided with food and water *ad libitum*. Animals were randomly divided in three groups, including control [C], hypoxic with saline [H+S], and hypoxic with ghrelin [H+G]. Each group contains eight rats. Hypoxia was induced by placing animals in a ventilated chamber inflated by hypoxic air [O_2 11%]. An

O_2 sensor and controller were embedded in the chamber wall to monitor O_2 concentration. Animals were kept in the chamber all the time for two weeks except for daily injections. All experiments were organized in accordance with the ethical standards of the faculty of medicine, Tabriz University of Medical Sciences, Iran.

Ghrelin Treatment

Subcutaneous injection of either saline [0.1 ml] or ghrelin [150 $\mu\text{g}/\text{kg}/\text{day}$ in 0.1 ml] was administrated for rats (Alipour *et al.*, 2011), which were then placed in the hypoxic chamber. H+S, and H+G 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 as the vehicle.

RNA Extraction and First-strand cDNA synthesis

For all animals, the kidney was removed for RNA extraction under standard sterile surgical method. Total RNA was extracted from kidney tissue 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 RiboLockRNase-inhibitor [20 U], for 5min 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 PCR was done using the Corbett Life Science [Rotor-Gene 6000] System is using 2 μL of a 3-fold diluted cDNA in each PCR reaction in a final volume of 20 μL . Each PCR reaction contained 150 nM of primers and 1 \times Fast Start SYBR Green Master [Roche]. Sequences of primers are listed in Table 1. PCR amplifications were performed by the

Table: Sequences of oligonucleotide primers

Gene	Forward Primer	Reverse Primer	Product Size [bp]
<i>Bax</i>	GACACCTGAGCTGACCTTGG	GAGGAAGTCCAGTGTCCAGC	310
<i>Bcl-2</i>	ATCGCTCTGTGGATGACTGAGTAC	AGAGACAGCCAGGAGAAATCAAAC	134
β -actin	TCCTCCTGAGCGCAAGTACTCT	GCTCAGTAACAGTCCGCCTAGAA	153

following three cycle programs: [1] denaturation of cDNA [1 cycle: 95°C for 10 min]; [2] amplification [40 cycles: 95°C for 15 Sec, 57°C for 30 Sec 60°C for 34 Sec for *Bcl2* gene and 60°C for 30 Sec 63°C to 34 Sec for *Bax* gene]; [3] melting curve analysis [1 cycle: 60 to 95°C with temperature transition rate 1°C/Sec]. β -actin [*Actb*] mRNA expression levels were used to calculate relative expression levels. The relative quantification was performed by $2^{-\Delta Ct}$. Expression of target genes/ β -actin = $[1+E]^{-Ct}$ target gene/ $[1+E]^{-Ct}$ β -actin. The specificity of the PCR reactions was verified by generation of a melting curve analysis followed by gel electrophoresis, visualized by ethidium bromide staining.

Standard Curve

Efficiency of RT-PCR reaction was determined by standard curve, which was derived from the 10-fold serial dilution of a positive PCR product by a customary RT-PCR. Logarithms of concentrations were plotted against target gene cycling threshold (Ct) of serial dilution. *Bcl2*, *Bax*, and *ACTB* efficiencies were 97%, 100 and 99% respectively.

Statistical analysis

Normal distribution of data was evaluated using Stata software with qnorm program version 11. Data was analyzed by statistical SPSS software, version 16. Variables that had normal distribution were reported as means and standard deviations. Medians were reported for the variables whose distribution deviated from the normal distribution. Differences between groups were evaluated using the Kruskal–Wallis test, and comparisons of gene expression levels between hypoxia or hypoxia with ghrelin and the control group was performed with the Mann–Whitney test. All tests

were two-tailed and a 5% significance level was applied.

Results

Comparison of *Bcl2* and *Bax* gene expression between hypoxic, hypoxic with ghrelin and normal kidney tissue

While *Bax* gene expression was observed in 100% of three groups, *Bcl2* gene expression was observed in 100%, 25% and 87.5% of the control, hypoxic with saline, and hypoxic with ghrelin groups, respectively. Median expression of *Bcl2* mRNA in the kidney tissue of the examined groups were different ($p=0.013$) (Figure2). However, there was no significant change in *Bax* mRNA expression among examined groups ($p=0.5$) (Figure 1).

Effect of chronic hypoxia on *Bcl-2* and *Bax* expression in the kidney

After 2-weeks of hypoxia, median expression of *Bcl2* mRNA in the kidney tissue was decreased compared to control animals ($P = 0.004$) (Fig.2). However, there were no significant differences in *Bax* mRNA expression between hypoxic and control groups ($p=0.328$) (Fig.1).

Effect of ghrelin on *Bcl-2* and *Bax* gene expression during hypoxia

The median expression of *Bcl2* mRNA in the kidney tissue of the hypoxic + ghrelin group was increased compared to hypoxia + saline group ($p=0.024$) (Fig.2). Furthermore, there were no significant differences in

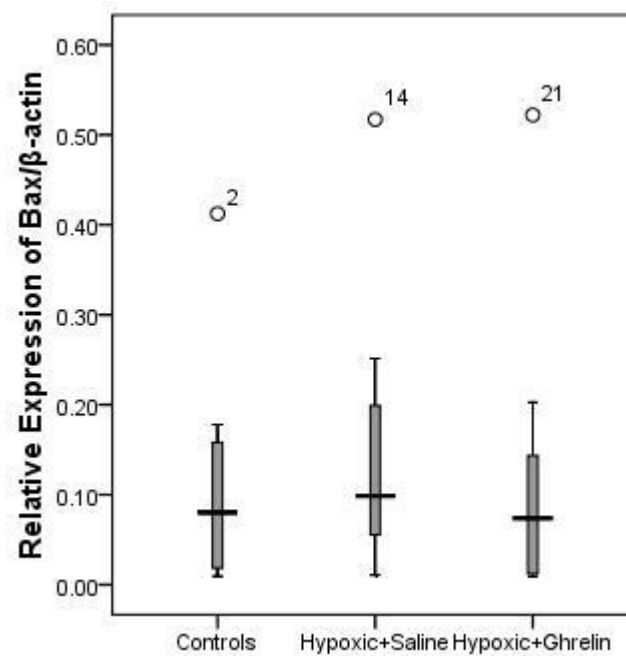


Fig. 1. Relative Quantitative RT-PCR of Bax to β -actin in the kidney tissue of experimental groups (n=8). H+S group versus Control, and H+G versus H+S group were compared statistically ($p < 0.05$).

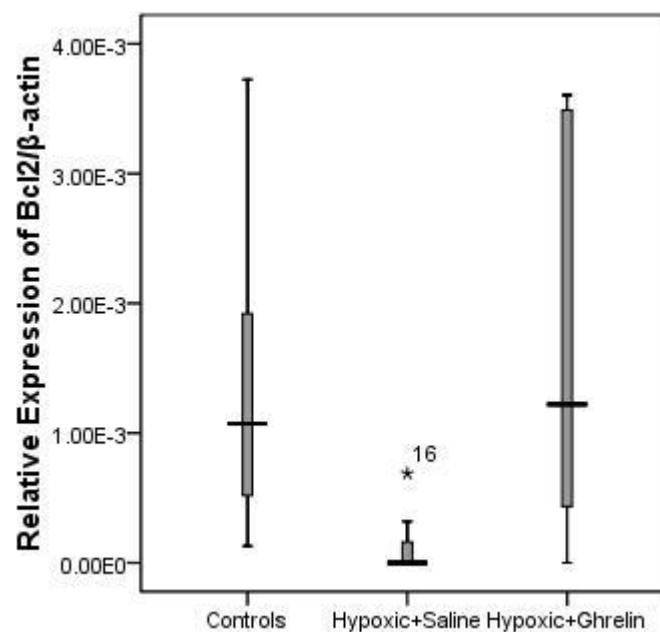


Fig. 2. Relative Quantitative RT-PCR of Bcl-2 to β -actin in the kidney tissue of experimental groups (n=8). H+S group versus Control, and H+G versus H+S group were compared statistically ($p < 0.05$).

Bax mRNA expression between these groups ($P=0.382$) (Fig.1).

Comparison of the Bax/Bcl-2 ratio between hypoxic, hypoxic with ghrelin and normal kidney tissue

The Bax/Bcl-2 expression ratio in the kidney tissue of the examined groups was significantly different ($p=0.035$) (Figure 3). The *Bax/Bcl-2 ratio* increased due to living in hypoxia in comparison with normal animals ($P=0.021$). Treatment by ghrelin decreased the Bax/Bcl-2 ratio in the hypoxia + ghrelin group compared

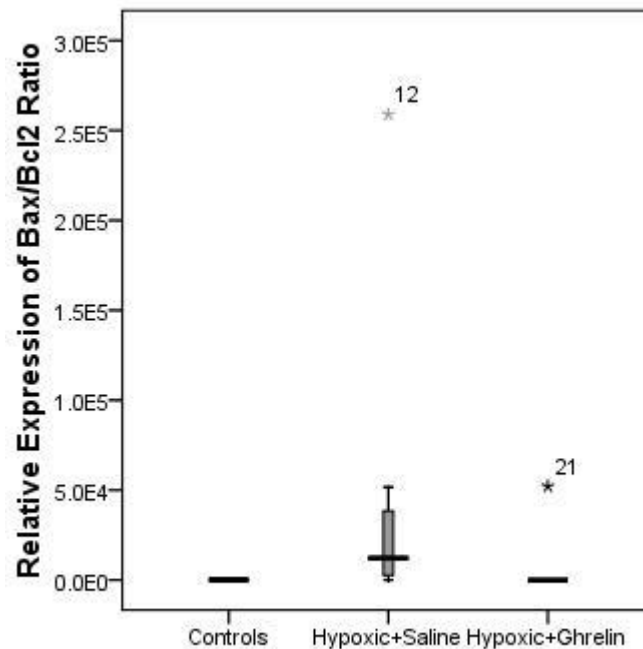


Fig. 3. Relative Quantitative RT-PCR representing the Bax / Bcl-2 ratio in the kidney tissue of experimental groups. H+S group versus Control, and H+G versus H+S group were compared statistically ($p < 0.05$).

to the hypoxia+saline group ($p=0.045$). However, there was no significant reduction in the Bax/Bcl-2 ratio in the hypoxia + ghrelin group compared to the normal animals ($P= 0.302$).

Discussion

Based on the results of the present study, although there was no significant effect on Bax gene expression, living under hypoxic conditions leads to a decrease in Bcl-2 gene expression and therefore, the elevation of the Bax/Bcl-2 ratio in the renal tissues. This could represent the cell drive to apoptosis due to remaining in general hypoxia. Previously, Khan and his colleagues showed hypoxia involvement in the pathogenesis of tubular atrophy, which induces renal tubular epithelial cell apoptosis in chronic renal disease (Khan *et al.*, 1999). Furthermore, our data are consistent with that one of [Nishikawa](#) and his colleagues in which they demonstrated that hypoxia decreases Bcl-2 mRNA and protein expression (Nishikawa *et al.*, 2006). The same kind of findings have been reported by [Yang](#) and his crew (Yang *et al.*, 2008). However, both the earlier researches were designed in an in vitro model of study whereas our study was performed in vivo.

In this study, ghrelin administration upregulated the Bcl-

2 gene and hence decreased the Bax/Bcl-2 ratio. As a result, it could be proposed that ghrelin has an anti-apoptotic effect in the renal tissue of animals exposed to chronic hypoxia. Although some research groups have declared that ghrelin induces the pro-apoptotic pathways (Kheradmand *et al.*, 2012), but many more studies have shown that ghrelin has protective roles against apoptosis. Yang *et al.* have shown that ghrelin inhibits apoptosis in PC12 cells through upregulation of heat-shock protein 70 (Yang *et al.*, 2007). Zhang and coworkers mentioned that ghrelin inhibited pancreatic beta cell line apoptosis, which mitogen-activated protein kinase/phosphoinositide 3-kinase pathways were involved (Zhang *et al.*, 2007). Another study by [Granado et.al](#), has demonstrated the protective role of this peptide against lactotrophs apoptosis in the pituitary of diabetic rats (Granado *et al.*, 2009). In addition, similar studies have presented the anti-apoptotic effect of ghrelin in the heart and brain tissue damages, regardless of the pathogenesis ([Baldanzi et al., 2002, \[Chung et al., 2007, \\[Hwang et al., 2009, \\\[Yang et al., 2012\\\\). Regarding the kidney, it has been revealed that ghrelin could protect this organ from different injuries. Cheung and his group have introduced ghrelin as a therapeutic agent in patients with end-stage renal disease \\\\(Cheung and Mak, 2009\\\\).\\\]\\\(#\\\)\\]\\(#\\)\]\(#\)](#)

Suzuki et al. have revealed the positive effects of the peptide due to chronic kidney disease cachexia (Suzuki *et al.*, 2013). The antioxidative effect, is another beneficial function of ghrelin seen in the kidney injuries (Fujimura *et al.*, 2014, Khowailed *et al.*, 2015). The presented data, to some extent, could justify the fact that ghrelin has a protective role against the probable hypoxic environment induced apoptosis. However, the mechanism by which this peptide has performed its novel role has not been investigated in this study.

Overall, these results, could lead to a therapeutic method based on ghrelins, especially in the kidney organ.

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Conflict of interest

The authors do not report any conflict of interest in this work.

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