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Research Article

Ghrelin Administration Increases the Bax/Bcl-2 Gene Expression Ratio in the Heart of Chronic Hypoxic Rats

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Abstract

Purpose: Programmed cell death or apoptosis, is a biochemical procedure that initiates due to some conditions, including hypoxia. Bax and Bcl-2 are among the agents that regulate apoptosis. The amplification of the first one triggers the initiation of apoptosis, and the second one prevents it. Ghrelin is an endogenous peptide that antiapoptosis is its new effect. The aim of this study is to examine the effect of ghrelin on the Bax/Bcl-2 ratio.

Methods: Twenty four wistar rats were divided randomly in three groups; control, hypoxic + saline and hypoxic + ghrelin. Hypoxic animals lived in O2 11% for 2 weeks and received either saline or ghrelin subcutaneously daily. The bax and Bcl-2 gene expression were measured by Real-Time RT-PCR.

Results: Chronic hypoxia increased the Bax gene expression significantly compared with normal animals (P = 0.008), but the Bcl-2 was not affected by hypoxia. The Bax/Bcl-2 ratio also amplified significantly (P=0.005). Ghrelin administration significantly increased the Bax/Bcl-2 ratio in the hypoxic animals compared to the hypoxic + saline and normal groups (p=0.042 and P= 0.001, respectively).

Conclusion: In the present study, animals' treatment with ghrelin leads to an increment of Bax/Bcl-2 ratio, which indicates a controversy related to cardioprotection of ghrelin.

Introduction

The process of programmed cell death, or apoptosis, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms.[1] In the apoptotic cells, biochemical changes such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition are some of the diagnostic presentations.[1] Among these parameters, Bcl-2, a gene located at chromosome 18q21, encodes a protein that blocks programmed cell death, while the bax protein is a member of the bcl-2 family that promotes apoptosis.[2,3] Generally, one of the standard ways to determine the vulnerability of a cell to apoptosis is the estimating of bax to bcl-2 ratio.[4,5] Hypoxia by inducing apoptosis as well as necrosis is a well-known cause of cell death.[6] In the cardiac myocytes, a possible role for Bcl-2 and Bax proteins has been proposed in hypoxia-induced apoptosis.[7,8] Ghrelin, a 28 amino-acid peptide, has diverse physiological functions in the body.[9-11] One of its recently found actions is the anti apoptotic effects.[12-14] This effect of ghrelin, at least in the cortical nerons, to some extent has been attributed to regulating of Bcl-2 family.[15] In the case of cardiomyocytes, it has been shown that ghrelin can protect these cells from different types of injuries, including apoptosis.[16-19] However, the effect of ghrelin on the apoptosis rate in the cardiac tissue of animals lived in chronic hypoxia has not been explored yet.

Base on this background, the aim of the present study is evaluating the effect of ghrelin on the Bax/Bcl-2 ratio in the heart of chronic hypoxic rats.

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. Male adult wistar rats [200-250gr] were housed in cages in a temperature 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 8 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 a chamber wall to monitor O_2 concentration. Animals were kept in the chamber all the time for two weeks except for daily injections.

Drug administration

Rats received a sc injection of either saline [0.1 ml] or ghrelin [150 µg/kg/day in 0.1 ml],[20] and were then placed

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into the hypoxic chamber. H+S, and H+G rats continued to receive daily injections of either saline or ghrelin during the 2-wks. 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 heart was removed for RNA extraction under standard sterile surgical method. Total RNA was extracted from heart 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 [1µg] 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 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 × 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 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 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.

Table 1. Sequences of oligonucleotide primers

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

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 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 heart tissue

In spite of the wide range of individual values of Bcl2 or Bax, median expression of Bax mRNA in heart tissue of the examined groups were different (p=0.001) (Figure 1). However, there were no significant differences in Bcl2 mRNA expression between examing groups (p=0.617) (Figure 2).

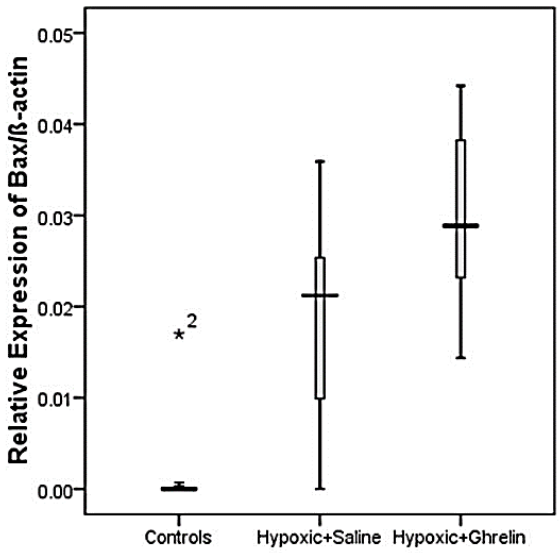

Figure 1. Relative Quantitative RT-PCR of Bax to β-actin in 3 experimental groups (n=8). Data are presented as median expression of Bax mRNA.

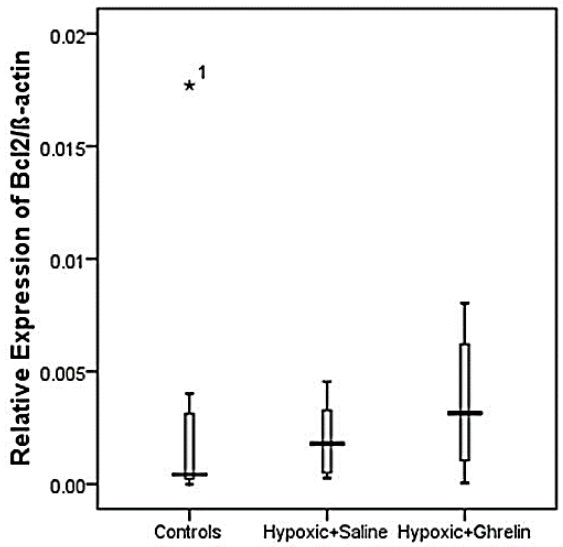

Figure 2. Relative Quantitative RT-PCR of Bcl-2 to β-actin in 3 experimental groups (n=8). Data are presented as median expression of Bax mRNA.

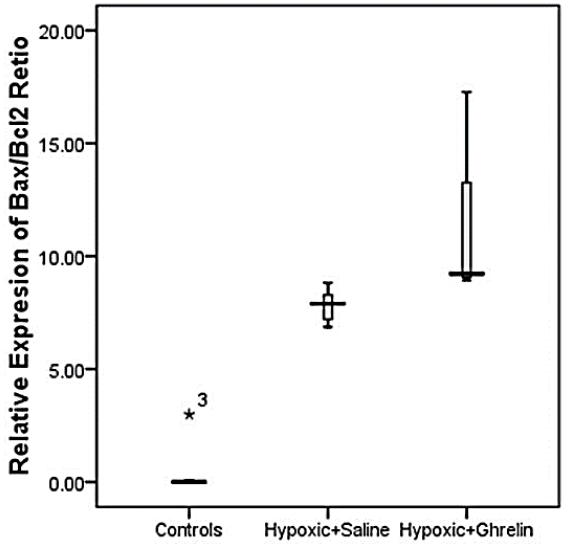

Figure 3. Relative Quantitative RT-PCR representing the Bax / Bcl-2 ratio in 3 experimental groups. Data are presented as median expression of each mRNA.

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

After 2-weeks of hypoxia, median expression of Bax mRNA in the heart tissue was increased compared to control animals (P = 0.008). However, there was no significant differences in Bcl2 mRNA expression between examine groups (p=0.674)

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

The median expression of Bax mRNA in the heart tissue of the hypoxic + ghrelin group was increased compared to hypoxia + saline group (p=0.093). However, this deference was not statistically significant. Furthermore, there were no significant differences in Bcl-2 mRNA expression between these groups (p=0.248).

Comparison of Bcl2 and Bax gene expression between hypoxia with ghrelin and normal heart tissue

The expression of Bax transcripts in the heart tissue of the hypoxic with ghrelin group were increased compared to normal group (p<0.001). However, there were no significant differences in Bcl-2 mRNA expression between these groups (p=0.645).

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

The Bax/Bcl-2 expression ratio in the heart tissue of the examined groups was significantly different (p=0.001) (Figure 3).

Effect of chronic hypoxia on the Bax/Bcl-2 ratio in the heart

The *Bax/Bcl-2 ratio* increased due to living in hypoxia in comparison with normal animals (P=0.005).

Effect of ghrelin on the Bax/Bcl-2 ratio in the chronic hypoxia heart

Treatment by ghrelin significantly increased the Bax/Bcl-2 ratio in the hypoxia + ghrelin group compared to the hypoxia+saline group and normal group (p=0.042 and P= 0.001, respectively).

Discussion

According to the results of this study, living in chronic hypoxic condition leads to increase of the Bax/Bcl-2 ratio in the heart of animals. This ratio, as a reliable index for apoptosis,[4,5] can indicate the increase of cell death rate in cardiomyocytes, although it was not accompanied by histomorphological assessment. These findings are consistent with that found by Nishikawa and his coworkers.[21] A similar result has been presented by Yang and colleagues.[22] However, it is notable that the mentioned researches have been performed in an in-vitro model while our data validates the previous works through an in-vivo study. To date, many studies have demonstrated the antiapoptosis effect of ghrelin in different cell lines and by special mechanisms. For example, Yang et al. have shown that ghrelin diminishes apoptosis signal-regulating kinase 1 activity via upregulation of heat-shock protein 70.[12] Zhang and his colleagues have indicated that ghrelin reduces cell apoptosis in pancreatic beta cell line via interfering in mitogen-activated protein kinase/phosphoinositide 3-kinase pathways.[13] Granado and coworkers have mentioned that treatment by Ghrelin protect lactotrophs from apoptosis in a diabetic rat model.[14] Furthermore, the beneficiary effect of ghrelin treatment against cell apoptosis has been demonstrated in similar works about cardiac myocytes.[17-19] Conversely, ghrelin treatment in the present study enhanced the apoptosis index, the Bax/Bcl-2 ratio, in the heart of hypoxic animals. In contrast with our study, it would be better to emphasis

that much of the pointed out investigations were in vitro and the cause of apoptosis was not chronic hypoxia.

In response to hypoxia, cardiac myocytes switch from oxidative metabolism to anaerobic glycolysis.[23] In such condition, some research groups believe that glycolysis exaggerates the rate of cell death through acid production.[24,25] Kubasiak, and his colleagues demonstrated that hypoxia in the presence of acidosis- could be due to glycolysis- activates cardiac myocyte apoptosis, which is mediated by BNIP3, a Bcl-2 family protein.[26] Furthermore, Luo and coworkers mentioned that hypoxia inducible factor-1alpha induces activity of the glycolysis pathway in A549 cells, carcinomic human alveolar basal epithelial cells, and decreases the pH of the culture medium, resulting in increased cellular apoptosis.[27] Previously, we have shown that ghrelin administration in chronic hypoxic rats increases the gene expression of aldolase, a key enzyme of glycolisys, in the heart of these animals.[28] Although this finding could describe a positive role for ghrelin about the heart metabolism in hypoxia, but it seems that it may deteriorate the cardiomyocytes survival in the chronic state. We are not sure that ghrelin usage amplifies glycolysis pathway entirely leading to acid production in the heart, but it would be a possible reason for anti cardioprotection action of ghrelin showed in the present observation. However, finding the main mediator of this effect of ghrelin should be under scrutiny.

Conclusion

Taken together, besides the result of the present study, the positive roles of ghrelin in cardioprotection could not be denied. Since the condition of this study is special, it would be better to examine different doses of ghrelin in distinct O_2 pressures and also different durations of experience.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of interest in this work.

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