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Research Article

Ghrelin Decreases Angiogenesis, HIF-1α and VEGF Protein Levels in Chronic Hypoxia in Lung Tissue of Male Rats

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Abstract

Purpose: Hypoxia is a condition of decreased availability of oxygen. When cells are exposed to a low oxygen environment, they impel the hypoxia responses to adapt to new situation. The hypoxia response leads to the activation of various cellular signaling pathways. The aim of this study was to evaluate the effect of ghrelin on angiogenesis, Hypoxia-Inducible-Factor-1α (HIF-1) and Vascular endothelial growth factor (VEGF) levels in normobaric hypoxia situation.

Methods: Twenty four animals were divided into 4 groups (n=6): control (C), ghrelin (Gh), hypoxia (H), and hypoxic animals that received ghrelin (H+Gh). Hypoxia (11%) was induced by an Environmental Chamber System GO2 Altitude. Animals in ghrelin groups received a subcutaneous injection of ghrelin (150 μg/kg/day) for 14 days.

Results: Our results showed that hypoxia significantly ($p<0.05$) increased angiogenesis without any significant changes on HIF-1 and VEGF levels, whereas ghrelin significantly ($p<0.05$) decreased angiogenesis, expression of HIF-1 and VEGF in this condition. Ghrelin administration did not show any significant changes in normal conditions.

Conclusion: Ghrelin had no effect on angiogenesis, expression of HIF-1 and VEGF in normal oxygen conditions but it reduced angiogenesis process in lung tissue with reducing the level of HIF and VEGF in hypoxic condition. Therefore, effect of ghrelin on angiogenesis could be related to blood oxygen level.

Introduction

Hypoxia is a common characteristic of many respiratory diseases resulting from inadequate alveolar ventilation, such as chronic obstructive lung disease or pulmonary edema due to heart failure or acute lung injury.[1] When cells are exposed to a low oxygen environment that is a mortal stress[1,2] they impel the hypoxia responses to adapt to new situation. The hypoxia response leads to the activation of various cellular signaling pathways involved in metabolism, cell survival and respiration.[2] Therefore, cells shift to the glycolysis pathway and induce the expression of glycolytic enzyme and inhibit enzymes leading to the tricarboxylic acid (TCA) cycle, such as pyruvate dehydrogenase for energy production and to reduce consumption of oxygen.[2] Hypoxia-Inducible-Factor-1α (HIF-1α) is one of the most important signaling pathways that are activated during hypoxia response.[2] HIF pathway plays an essential role in cellular and systemic oxygen homeostasis.[2] HIF is a heterodimeric transcription factor that is created of two subunits, α and β.[3] The protein firmness, subcellular localization and transcriptional power of the HIF-α subunits can be affected by oxygen levels.[3] HIF-1α protein is degraded at normal oxygen by process of the von Hippel-Lindau pVHL-mediated ubiquitin-proteasome pathway, but hypoxia blocks degradation.[3] HIF-1 regulates the expression of many hypoxia responsive genes.[2] HIF-1 target genes contribute in some processes, including encoding proliferation/survival factors, glucose transporters, glycolytic enzymes, and angiogenic factors,[3] such as VEGF.[3,4]

Vascular endothelial growth factor (VEGF) is mainly expressed in the lung tissue.[5] This protein increases oxygen availability via process of erythropoiesis and angiogenesis.[6] In many tissues, hypoxia is a strong inducer of expression of VEGF mRNA.[7] Hypoxia, via the activation of HIF-1, is a major regulator on VEGF.[6]

Blood vessel development is characterized by two distinct biologic processes, vasculogenesis, and angiogenesis.[8] Vasculogenesis is largely restricted to embryonic development. Angiogenesis, the process of new blood vessel formation from preexisting vessels, is responsible for the generation of neovasculature in adult life.[9] There are several immunohistochemical markers that can identify endothelial cells including antibodies that recognize epitopes on CD31.[10] CD31 or platelet endothelial cell adhesion molecule-1 (PECAM-1), a member of the immunoglobulin superfamily in

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endothelial cells,[11] is found in large quantities on the surface of ECs.[10] Immunohistochemical detection of CD31 has been used extensively to quantify angiogenesis.[10,12]

In addition to hypoxia, some of hormones are effective on the expression of HIF or VEGF, such as thyroid hormones,[13] growth hormone,[14] GnRH,[15,16] estrogen,[17] LH, IGF-1, TNF-alpha.[18]

Ghrelin is a 28-amino acid peptide hormone that is found in the secretory granules of X/A-like cells in gastric mucosa.[19] It is also produced by other tissues such as kidney, pancreas, placenta, testis, pituitary, lung, and hypothalamus.[20,21] Ghrelin is mainly considered by researchers for its physiological effects such as; glucose homeostasis,[20] growth hormone secretion,[22] appetite stimulation and adipogenesis.[22] It also affects cell proliferation and survival,[20] blood cells production,[23] and reproduction.[24]

Since both hypoxia and hormones can affect induction of angiogenesis and expression of HIF, VEGF proteins we decided to examine the effect of ghrelin on these proteins and angiogenesis in normal and hypoxic conditions.

Materials and Methods

Animals

The Ethic Committee for Animal Experiments at Tabriz University of Medical Sciences approved the study plan, and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. In this study, 24 adult male Wistar rats (200-250 g) were used that obtained from animal house of faculty of Medicine. Animals were kept in a temperature (20-22°^{C}) and light-controlled (12 hours light- darkness) environment and provided with food and water ad libitum. Twenty four hours before the start of the study, animals were transported to the laboratory in order to comply with the environment.

Animals were randomly divided into 4 groups (n=6):

- Control (C): animals that were placed in room air (21% O_2)
- Ghrelin (Gh): animals that received ghrelin (150 µg/kg/day, i.p) for two weeks
- Hypoxia (H): animals exposed to O_2 11% for two weeks
- Hypoxia with ghrelin (H + Gh): animals exposed to O_2 11% and received ghrelin (150 µg/kg/day, i.p) for two weeks

Ghrelin was obtained from the Tocris Bioscience Co. (Bristol, UK) and dissolved in saline as vehicle.

Hypoxia induction (normobaric)

Animals in hypoxic groups (H and H+Gh) were placed into the hypoxia chamber (Environmental Chamber System GO_2 Altitude, Biomedtech Australia Pty. Ltd). This chamber induced O_2 11%. An oxygen sensor was embedded in the chamber to monitor O_2 concentration. Animals were kept in the chamber all the time for two weeks except for 20 min/day to clean cages, perform daily injections and placing water and food.[22]

Tissue sampling and protein measurement

At the end of hypoxic period, rats were anesthetized with an i.p injection of ketamine (80 mg/kg) and xylasin (5 mg/kg) and sacrificed. Then lung tissues were removed and after quick freezing with nitrogen, all tissues transferred to -70 °C temperature until HIF-1α and VEGF measurement.

Tissue samples were weighted, homogenized in PBS (pH 7.2-7.4) and centrifuged for 20 min at the speed of 3000 rpm and 4°C. Then supernatants were removed and VEGF and HIF-1α proteins were extracted. VEGF and HIF-1α levels were measured using sandwich rat ELISA kits according to the manufacturer's instructions (Glory Science co., Ltd, USA, VEGF catalog: 30634, HIF-1α catalog: 95692).

Immunostaining for PECAM-1/ CD31

For investigation of angiogenesis, lung tissues were fixed in 10% formalin immediately after excision. Then, serial 3µm thick sections were cut from them and floated onto charged glass slides.[10] Tissue sections were deparaffinized in xylene and dehydrated in a graded series of ethanol. Slides were incubated sequentially in proteinase K and treated by 0.3% hydrogen peroxide for blocking endogenous peroxidase activity. Sections were overlaid by primary antibody CD31 (Santa Cruz, USA) –a marker of angiogenesis - and incubated at +4°^{C} overnight. Sections were then washed and incubated with standard avidin–biotin complex (ABC; Santa Cruz) according to the manufacturer's instructions. Then slides were incubated in DAB (di-amino-benzidine, Santa Cruz) - as the chromagen- and counterstained with Mayer's hematoxylin. Finally, sections were cleared in xylene, mounted with Entellan and assessed by light microscope (Olympus BX 40, Japan). For assessment of immunostaining, the intensity of the staining was scored as 0 (<10%), 1 (10-25%), 2 (25-50%), 3 (50 -75%) and 4 (75-100%).[25]

Statistical analysis

The data were analyzed using SPSS version 16.0. Results were reported as mean ± S.E.M. For testing differences between the groups, data were analyzed by one-way ANOVA followed by LSD tests and $p< 0.05$ was considered as significant.

Results

Effects of hypoxia and ghrelin on HIF 1-α and VEGF protein levels in lung tissue

The effect of two weeks of ghrelin (150 µg/kg/day) treatment on HIF-1 α protein level in lung tissue in control and chronic hypoxia conditions showed that induction of hypoxia (O_2 11%) did not significantly change HIF1-α level in hypoxia group compared with control group (Figure 1).

HIF-1α protein levels in lung tissue in H+Gh group decreased significantly ($p<0.05$) compared to the control group. It also showed a significant ($p<0.05$) decrease when compared to hypoxia group.

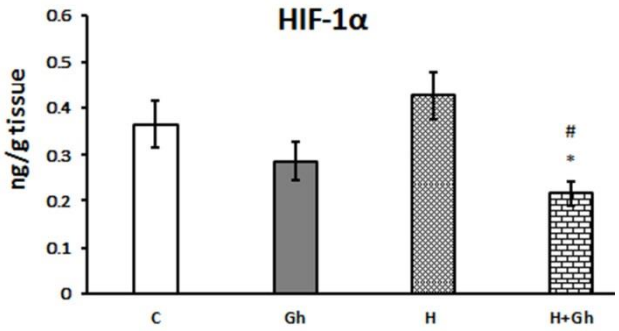

Figure 1. Effect of ghrelin on HIF-1 α level in lung tissue after 2 weeks in control, ghrelin, hypoxia and hypoxia plus ghrelin groups. Data are expressed as mean± SEM for 8 animals.*p<0.05 vs the control group, # p<0.05 vs the hypoxia group

Figure 2 depicts the effect of two weeks of ghrelin (150 µg/kg/day) treatment on VEGF protein level in lung tissue in control and chronic hypoxia conditions. Induction of hypoxia or treatment with ghrelin could not significantly change the levels of VEGF in lung tissue compared to control group. Interestingly, when ghrelin was administrated in the condition of hypoxia, it could significantly ($p<0.05$) reduce VEGF levels.

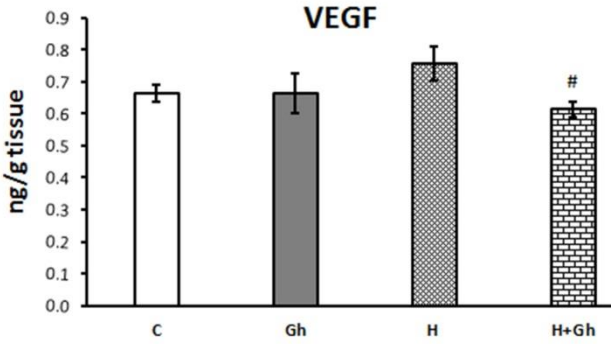

Figure 2. Effect of ghrelin on VEGF level in lung tissue after 2 weeks in control (c), ghrelin (Gh), hypoxia (H), and hypoxia plus ghrelin (H+Gh) groups. Data are expressed as mean± SEM for 8 animals. # p<0.05 vs the hypoxia group.

Effects of hypoxia and ghrelin on HIF angiogenesis in lung tissue

Immunostaining with CD31 marker was done for the assessment of angiogenesis in lung tissue (Figure 3). Brown stained tissues show CD-31 immunostained endothelial cells. Ghrelin treatment had no significant effect on angiogenesis, whereas exposure to hypoxia caused an extensive angiogenesis in lung tissue. Statistical analysis of immunohistochemical study revealed that angiogenesis was significantly ($p< 0.05$) increased in hypoxia or hypoxia +ghrelin groups compared to control group. Also, ghrelin treatment significantly ($p< 0.05$) reduced angiogenesis in hypoxia +ghrelin group compared with the hypoxia group.

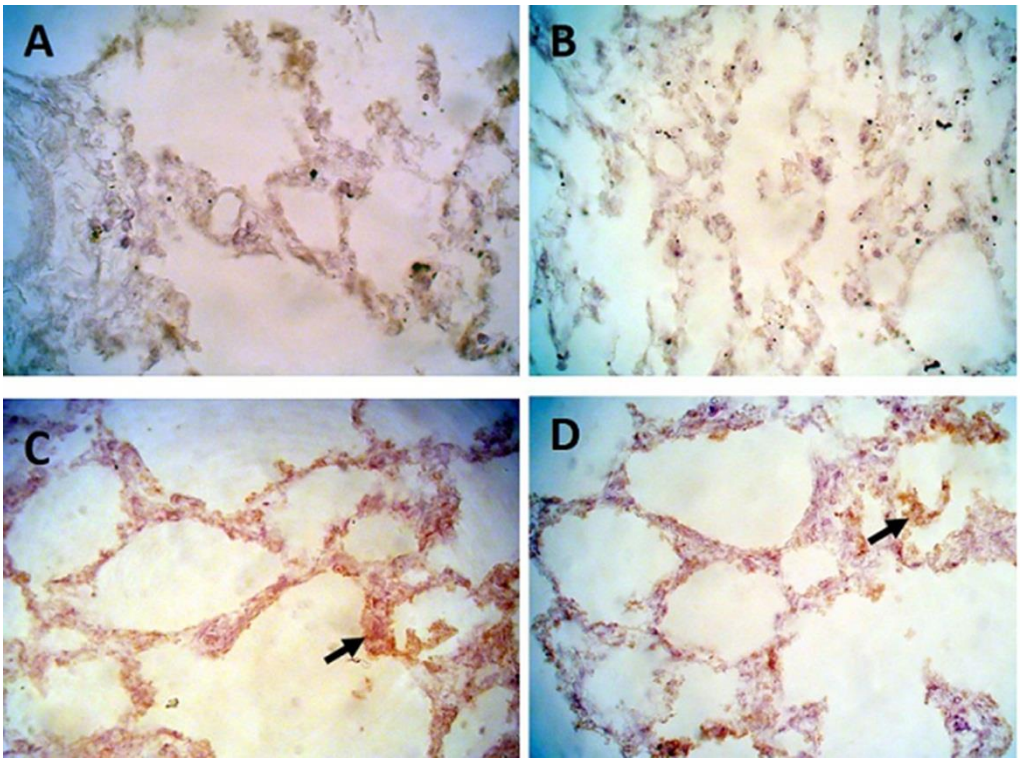

Figure 3. Immunohistochemical detection of CD31 in lung tissue. Brown stained tissues show CD-31 immunostained endothelial cells in (A): control, (B): ghrelin, (C): hypoxia and (D): Hypoxia+ghrelin. The intensity of immunostaining for CD31 (arrow head) increased both in H and H+Gh groups compared to control group. Treatment with ghrelin decreased angiogenesis compared to Hypoxia group.

Discussion

Our results for the first time showed that angiogenesis was increased in the lung tissue in hypoxia and ghrelin treatment had a depressing effect on angiogenesis process in this condition. Ghrelin treatment in hypoxic condition decreased HIF1- α level in lung tissue in comparison with both the control and the hypoxic groups. Ghrelin also decreased VEGF levels in hypoxic conditions in lung tissue in comparison with the hypoxic group.

Angiogenesis occurs in the pulmonary circulation during physiological adaptation and/or pathological mechanisms in lung disease.[26,27] Indeed, hypoxia is a condition of decreased O_2 levels that is seen in high altitudes and many respiratory diseases.[6] It can stimulate lung angiogenesis in adults,[26,28] through many factors or cytokines.[29] HIF-1α, which is known to regulate angiogenesis due to hypoxia in some tissues, is one of the most important mediators.[2,30] Vascular endothelial growth factor as a cytokine, [31] also increases in hypoxic condition,[32] and is a major factor in the control of angiogenesis.[18,28] There is a relation between HIF-1α and VEGF[3] and the involvement of HIF-1 in expression of VEGF gene due to hypoxia has been shown previously.[2,29]

The effect of certain hormones has been examined on the expression of HIF-1α, VEGF and angiogenesis process in normal oxygen conditions. Thyroid hormone in hepatocytes[13] and growth hormone in cerebral cortex increase HIF-1α protein levels.[33] Some other hormones like LH (luteinising hormone),[18] TNF α (tumor necrosis factor),[18] and IGF-1 (insulin like growth factor) [18] also have a positive effect on VEGF protein level in bovine granulosa cells.[17] Whereas, estrogen in MCF7 (Michigan Cancer Foundation-7) human breast cancer cells [17] and GnRH (gonadotrophin releasing hormone) in eutopic endometrial cell[15] have negative impact on VEGF level. Some other hormones such as endothelin, oxytocin, progesterone and growth hormone are ineffective on VEGF production.[18]

In the present study, treatment with ghrelin did not change density of blood vessels, HIF and VEGF levels in lung tissue in normal oxygen conditions. Studies that are conducted on the effect of ghrelin on angiogenesis are limited. Nevertheless, there are controversies in studies on the effect of ghrelin on angiogenesis that may be resulted from different experimental models.

On one hand, Conconia et al. indicated that ghrelin inhibits FGF-2-mediated angiogenesis in human umbilical vein endothelial cells (HUVECs)[34] and Tropea's study showed that ghrelin reduces VEGF expression in human ovarian luteal cells.[35] Moreover, Ahluwalia et al. showed that angiogenesis is decreased in the aging individuals (66 years and 90 years old) human microvascular endothelial cells (HMVECs) and treatment with exogenous ghrelin reversed impaired angiogenesis in aged HMVECs.[36] Aihua's study proposed that increasing effect of angiogenesis of ghrelin is probably via angiogenic growth factors such as VEGF and bFGF (basic fibroblast growth factor) in HMVEC.[37]

Our study also showed that angiogenesis, HIF and VEGF were reduced with ghrelin treatment in hypoxic conditions. It seems that ghrelin in hypoxic condition has reduced the angiogenesis through HIF and VEGF pathways.

In the present study, induction of hypoxia increased angiogenesis in lung tissue without any changes in the HIF and VEGF levels. This finding is in accordance with Srisuma et al. study that angiogenesis occurs in lung in ischemic condition without any changes in HIF and VEGF levels. Srisuma suggested that, these proteins are not essential for lung angiogenesis and hypoxia is not a necessary trigger for angiogenesis.[38]

There are some other mechanisms that are possibly involved in angiogenesis, such as inflammatory cells and Rho-kinase.[9,39] Alveolar hypoxia by itself can initiate inflammatory process in the respiratory compartment[6] and inflammation promotes neovascularization in lung tissue.[9] Also, studies have shown that hypoxia induces Rho kinase activation and Rho kinase induces angiogenesis in response to hypoxia.[39] More studies are needed to investigate the role of inflammatory products and Rho kinase in the induction of angiogenesis in hypoxia.

There are some possible mechanisms for angiogenesis reduction by ghrelin in hypoxic conditions: (1) FGF2 (fibroblast growth factor 2) increases tissue levels of VEGF and angiogenesis.[37,40] (2) Some inflammatory factors such as NFkb (nuclear factor kappa b) induce lung angiogenesis through activation of HIF and VEGF pathways[41,42] and, (3) Angiotensin II stimulates HIF production[43] and angiogenesis.[44] Ghrelin could inhibit production of FGF-2,[34] NFkb[45] and angiotensin II.[46]

In conclusion, ghrelin does not effectively modulate expression of HIF-1, VEGF, and angiogenesis in normoxic condition, whereas it reduces angiogenesis process in lung tissue possibly by reducing the level of HIF and VEGF in hypoxic condition. Future studies in this context may suggest ghrelin as a solution for treatment or control of certain diseases that angiogenesis is a negative trend for them.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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