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GHRELIN EFFECTS ON LOCAL RENIN ANGIOTENSIN FROM PULMONARY VESSELS

L. Vata[1], I. Dumitriu[1], M. Gurzu[2], S. Slatineanu[1], A. Vata[3], B. Gurzu[1]

[1]Department of Functional Sciences, [2]Department of General Pathology, [3]Department of Infectious Diseases "Gr T Popa" University of Medicine and Pharmacy - School of Medicine, Iasi, Romania

Abstract

Background. Published data sustain the participation of vascular renin angiotensin system (RAS) on alteration of pulmonary vessels reactivity during the allergic airway inflammation. Ghrelin is a growth hormone-releasing peptide involved in modulation of immune function.

Objective. This study aims to investigate the interaction between ghrelin and local RAS from rat pulmonary vessels during ovalbumin — induced allergic airway disease.

Methods. The angiotensinogen (AGT) — induced contractions were assessed on isolated pulmonary artery and veins from ovalbumin sensitized rats receiving either saline (OSR) or ghrelin (OSG) by endotracheal instillation. Experiments were performed in the absence or the presence of losartan, D-ALA^7, chymostatin and N$^\omega$-nitro-L-arginine methyl ester (L-NAME).

Results. The angiotensinogen (AGT) contractile effects mediated by AT1 receptors were lower with at least 25% on vessels from OSG than from OSR. The D-ALA^7 and L-NAME significantly increases the AGT - induced contraction on OSG. The amount of nitric oxide released after stimulation with angiotensinogen (AGT) is higher on OSG and it is blocked by D-ALA^7.

Conclusion. Our results suggested that pulmonary delivery of ghrelin could modulate the local RAS from pulmonary vessels, probably by promoting the angiotensin 1-7 mediated effects. These data sustained the existence of another possible way for ghrelin's beneficial effects on the lung.

Key words: ghrelin, angiotensinogen /angiotensin 1-7, rat pulmonary artery, rat pulmonary vein, nitric oxide, losartan, chymostatin

INTRODUCTION

Ghrelin was identified in 1999 (1) as the endogenous ligand of growth hormone secretagogue receptor 1a (GHSR-1a) described by Howard *et al*. in 1996

*Correspondence to: L. Vata MD, "Gr. T. Popa" University of Medicine and Pharmacy - Functional Sciences, Str Universitatii Nb. 16, Iasi 700115, Romania, Tel: 0232301691
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(2). Ghrelin has been viewed by many scientists as a central modulator of energy homeostasis and a regulator of the growth hormone secretion. Some studies revealed cardiovascular effects of ghrelin, including lowering of peripheral resistance, possible improvement of contractility and cardioprotective effects both *in vivo* and *in vitro* (3). There are only few reports about ghrelin roles on the lung. Research studies presented ghrelin as having antiinflamatory roles or reducing the magnitude of pulmonary hypertension (4,5).

Published reports showed that, in asthmatic lungs, the pulmonary artery inflammation (6, 7) and remodeling (8) are connected with increased pulmonary pressure (9) and pulmonary artery hyper responsiveness (10). On the other hand, the role of the renin angiotensin system (RAS) on alteration of reactivity, inflammation and remodeling of blood vessels is well documented (11).

The RAS has been initially viewed as a circulatory system but knowledge regarding the existence of tissular alternative pathways for angiotensin synthesis (12), independent from renin and/or angiotensin II converting enzyme, led to the identification of local RASs, including the vascular RAS (13). Even more, it is well known that angiotensins could modulate the vascular tonus by acting on multiple types of specific angiotensin receptors and producing either vasoconstriction [by activation of angiotensin (Ang) II type 1 ($AT_{1)}$ receptors] or vasodilatation [e.g. *via* Ang $_{1\text{-}7}$ ($AT_{1\text{-}7}$) receptors] (14). On the other hand, our previous data, on ovalbumin (OVA) sensitized rats, showed the inflammation induced activation of vascular RAS on pulmonary vessel walls, leading to a misbalance between vasoconstrictor and vasodilator effects of locally synthetized angiotensins (15,16).

Taking into account both the anti-inflammatory effects of ghrelin and our previous results, we studied the effects of intratracheally administered ghrelin on pulmonary arteries and veins RAS activity on OVA sensitized and challenged rats.

MATERIAL AND METHODS

The experiments were conducted in 150 age-matched Wistar male rats sensitized as described by Dumitriu and colleagues (17). All the experiments described here were performed in compliance with the European Communities Council Directive 86/609/EEC and Ordinance No. 37/2002 of the Romanian Government. After the second OVA administration the rats were randomly divided in two groups: the control group (OSR) and the ghrelin treated group (OSG). In the last week of sensitization, ghrelin (0.1 mM, on OSG) or saline (on OSR) were given intratracheally (50 μL, 3 times) as previously described (18). The diagram of the study design is presented in Fig.1. After termination of the sensitization protocol, the rats were decapitated and exsanguinated. The rat pulmonary arteries (PA) and veins (PV) were rapidly removed, cleaned and cut into 1-2 mm wide rings. Individual rings were then mounted between tungsten (50μm) wires organ bath. Individual rings were then mounted in a MYO-01 MYOGRAPH SYSTEM

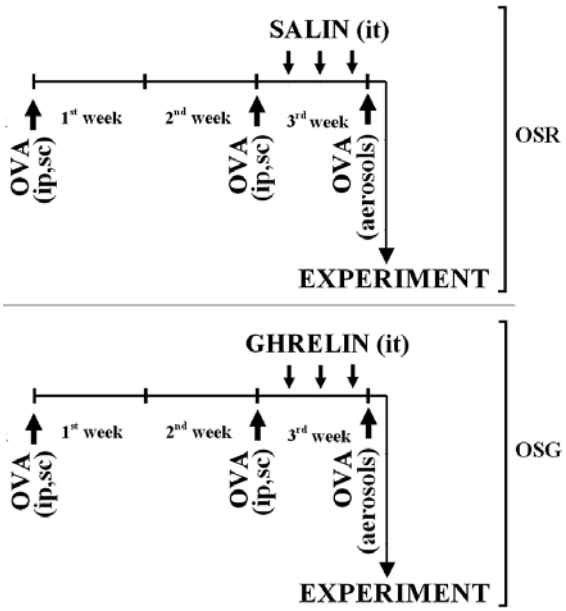

Figure 1. Timelines describing the study design. OVA: ovalbumin; 1st week, 2nd week, 3rd week: sensitization weeks. In the 3rd week, the rats were treated intratracheally (it) with ghrelin (OSG) or with saline (OSR). The experiment was performed *in vitro* on pulmonary vessels from these rats. it: intratracheal; ip: intraperitoneal; sc: subcutaneous.

(Experimetria LTD., Budapest, Hungary) and changes in vessel tension were recorded and analyzed by ISOSYS data acquisition system (Experimetria LTD., Budapest, Hungary). The tissue organ bath contained the Krebs—Henseleit solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.6, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.5. The Krebs-Henseleit buffer was maintained at 37°C, and bubbled continuously with a mixture of 95% O_2 and 5% CO_2 (pH=7.2—7.4). A resting tension of 0.5g for PA and 0.2g for PV was applied to each ring and then allowed to equilibrate for 45-60 minutes before starting the experiment. The bathing medium was renewed every 15 minutes. After the equilibration period, vessel rings were initially stimulated with 40 mM KCl as a standard stimulus. The functional integrity of the endothelium was assessed by testing the degree of relaxation produced by adding 10 μM acetylcholine (ACh) to 1 μM phenylephrine pre-contracted rings. The rings that produced less than 50% relaxation in response to ACh were discarded. Before starting to administer the studied substances, vascular rings were pre-treated with OVA (100 mg/mL). After re-equilibration in Krebs solution for 45 min, we quantified angiotensinogen (AGT) — induced contractions in the absence or in the presence (15 minute pre-incubation) of losartan (LOS), an AT_1 receptor antagonist), D-ALA^7 (an AT1-7 receptor antagonist), chymostatin (a chymase inhibitor) or N^{ω}nitro-L-arginine methyl ester (L-NAME, a nitric oxide synthases inhibitor). Results are expressed as percentage of control contraction induced by 40 mM KCl (mean ± S.E.M, n=6). The statistic significance was tested

using one-way analysis of variance (ANOVA), completed by the Bonferroni method (SigmaStat software, Jandel Corporation). $p<0.05$ was considered statistically significant.

In order to examine the involvement of angiotensin $_{1-7}$ (Ang $_{1-7}$; 10 μM) on AGT — induced contractions, the nitric oxide (NO) release was measured using an APOLLO 4000 system (World Precision Instruments - WPI). The method was described by Simonsen (1999) (19). 30 μm diameter L-shaped electrodes (ISO-NOP30-L) were calibrated using S-Nitroso-N-acetyl-D, L-penicillamine according to the WPI technical indication. Results are expressed as percentage of the ACh — induced NO released on the same ring during examination of the endothelium integrity as described. The statistical significance was tested using one-way analysis of variance (ANOVA), completed by the Bonferroni method (SigmaStat software, Jandel Corporation). $p<0.05$ was considered statistically significant.

Rat ghrelin, angiotensinogen, acethylcholine, losartan, chymostatin, N^{ω}-nitro-L-arginine methyl ester, S-Nitroso-N-acetyl-D,L-penicillamine were all obtained from Sigma-Aldrich Inc. (St Luis, MO, US). Ang 1-7 and the antagonist D-Ala^7-Ang1-7 (D-ALA^7) were purchased from Phoenix Europe GmbH (Germany). All the other compounds used were of analytical grade.

RESULTS

The contractile effects of multiple doses of AGT (n = 6 for each dose) on pulmonary vessels are presented in Fig. 2.

First of all, the AGT — induced contractions were higher on pulmonary vessels from OSR then from OSG. On PA, the differences of contractile effects between OSR and OSG were statistically significant for 1 μM (35.21±3.38 *vs.* 18.43±0.93; $p<0.01$), 10 μM (46.41±2.72 *vs.* 29.91±4.13; $p<0.05$), 0.1 mM (55.77±3.58 *vs.* 35.36±2.67; $p<0.005$) and 1 mM (58.76±5.03 *vs.* 36.73±3.96; $p<0.05$). The contractile responses of PV were significantly different for 10 μM (28.57±1.74 *vs.* 20.68±2.39; $p<0.05$), 0.1 mM (34.84±1.65 *vs.* 26.14±1.72; $p<0.05$) and 1 mM (33.38±2.51 *vs.* 24.30±2.67; $p<0.05$). Contractile effects of AGT were more important on PA than on PV, but this is statistically significant on OSR. On OSG, only for the higher dose of AGT (1 mM) there was a significant difference between PA and PV ($p<0.05$). In our preliminary experiments, we used the same instillation protocol of ghrelin on normal rats. In absence of sensitization procedure, it was no significant difference of 10 μM AGT-induced contractions on PA (29.61±6.53 *vs*. 31.52±5.55, n=6; unpublished data) or PV (14.66±4.08 *vs*. 16.19±3.62, n=6; unpublished data) from ghrelin treated *vs*. untreated rats of isolated PA and PV.

The effects of the AT1 blocker losartan (LOS), the chimase inhibitor chymostatin (CST), the AT1-7 blocker D-ALA^7 and the NOS inhibitor L-NAME are presented in fig. 3A (pulmonary arteries) and 3B (pulmonary veins). LOS pre-treatment prevented AGT — induced contractions on all studied vessels

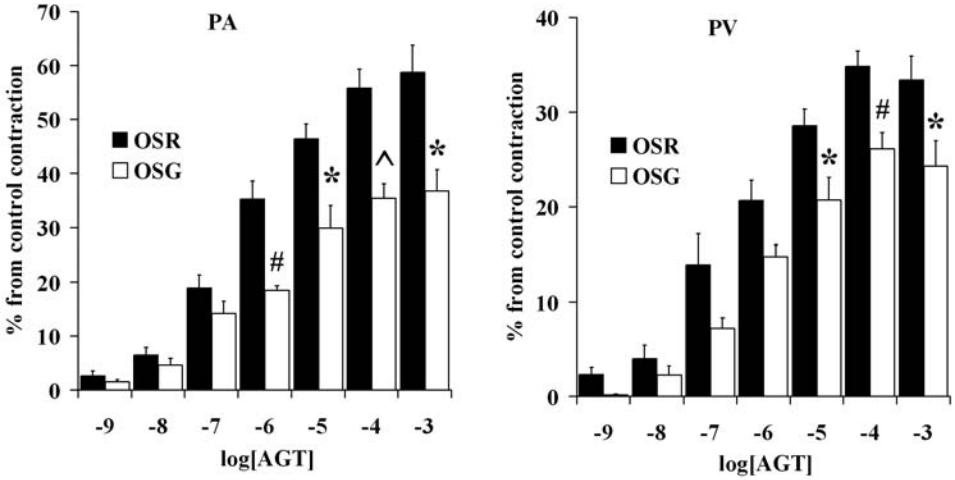

Figure 2. The contractile effects of multiple doses of angiotensinogen (AGT; 1nM - 1mM) on isolated pulmonary arteries (PA) and veins (PV) with intact endothelium form control ovalbumine sensitized rats (OSR; filled bars) and ovalbumine sensitized rats treated with ghrelin (OSG; empty bars). *: $p<0.05$ on OSG as compared with OSR; #: $p<0.01$ on OSG as compared with OSR; ^: $p<0.005$ on OSG as compared with OSR.

demonstrating the AT1R receptor dependency of obtained contractile responses. As we expected, the inhibition of chimase reduced the contractions induced by AGT, but these effects were significant only on OSR for PA (31.27±3.02 *vs*. 46.41±2.72 %, $p<0.01$; fig. 3A) and PV (9.35±1.82 *vs*. 28.57±1.74 %, $p<0.005$; fig. 3B). In contrast, the D-ALA7 and L-NAME had significant effects on OSG. Blocking of AT1-7 receptors increased the AGT — induced contractions on both PA (45.77±2.16% *vs* 29.91±4.13; $p<0.05$) and PV (29.29±1.30% *vs* 20.68±2.39; $p<0.05$) from OSG.

The inhibition of NOS with L-NAME increased the contractile effects of AGT by more than 60% for both PA and PV from OSG. On the other hand, the difference between OSR and OSG observed on AGT alone disappeared after the pretreatment with D-ALA^7. In the presence of L-NAME the AGT — induced contraction remained significantly higher on OSR as compared to OSG, but only on arteries (55.71±2.27% *vs.* 48.28±1.28%; $p<0.05$).

The NO release stimulated by AGT (10 μM) was compared to ACh - induced endothelial - dependent NO release because all used vessels belonged to diseased rats (ovalbumin sensitized) with different degrees of endothelial dysfunction (fig 4). The amount of NO released after stimulation with 1 μM AGT were higher on OSG than on OSR for both pulmonary arteries (62.57±6.38% *vs*. 14.90±2.76%, $p<0.001$) and veins (46.99±6.13% *vs.* 11.03±1.14%, $p<0.005$). The pretreatment with 0.1 mM D-ALA^7 prevented almost completely the stimulated release of NO. On the other hand, we have to notice that AGT — stimulated NO release was higher on pulmonary veins than on arteries, but the difference is not statistically significant.

DISCUSSION

Our results suggested that pulmonary delivery of ghrelin could prevent the activation of local RAS from pulmonary vessels of OVA sensitized rats probably by replacing, at least partially, the AT_1 — mediated vasoconstriction with AT_{1-7} — dependent vasodilatation.

The ghrelin and its receptor were found in many tissues, including lung parenchyma and pulmonary artery wall (20-24). There are few studies about the anti-inflammatory effects of ghrelin on lungs. Using an experimental model of sepsis Wu and colleagues (2007) showed that intravenous administration of ghrelin increased pulmonary levels of ghrelin, ameliorated lung histology, elevated lung blood flow, improved survival and decreased pulmonary levels of proinflammatory cytokines. Ghrelin attenuated pulmonary inflammation in LPS-induced acute lung injury and decreased production of proinflammatory cytokines (24). Even more, in cultured lung macrophages ghrelin suppressed LPS-induced expression of proinflammatory cytokines (25).

On our experimental model, the inflammation produced by ovalbumin sensitization could generate prominent changes in the vascular RAS, increasing the Ang II formation by a chymase — dependent mechanism on pulmonary vessels (15,26).

In the present study the ghrelin was delivered intratracheally 3 times, every 2 days during the last week of sensitization protocol to limit its actions on pulmonary level. Obtained results evidenced a decrease of AGT — induced contraction (fig. 2) in the pulmonary artery and veins from OSG as compared with OSR. In contrast, intratracheally administration of ghrelin did not modify significantly the angiotensinogen induced contractions on normal rats (*unpublished results*). According to published data the contractile responses to AGT are the results of AT1 receptors' activation by locally synthesized angiotensins (27). So, we utilized the contractile effect of AGT as an indirect marker of functionally active angiotensin' synthesis. In order to investigate how the pulmonary delivered ghrelin could modulate the RAS from pulmonary vessels from sensitized rats, we administered AGT in the presence of D-ALA^7, chymostatin and L-NAME.

Taking into consideration the chymase involvement on sensitization — induced activation of local RAS from pulmonary vessels (7,15,16,28) we verified if the chymostatin sensitive component of AGT — induced contraction was of the same extent in vessels from OSG as from OSR. As we can see in Fig. 3A and 3B, the chymostatin effects were significant only on vessel rings from OSR. On the other hand, chymostatin eliminated (fig. 3A and 3B) the difference between the AGT-induced contractions on OSR as compared with OSG. These data suggested a decrease of the chymase involvement on AGT metabolism in pulmonary vessels from sensitized rats as a result of pulmonary administration of ghrelin during the last week of sensitization protocol.

Another important issue about ghrelin is its positive effect on the endothelial function (29,30). The decreasing AGT — induced contractions could be mediated by restoring the endothelial function and the NO synthesis on pulmonary vessels. Indeed, the L-NAME pretreatment significantly increased the AGT contractile effects only on

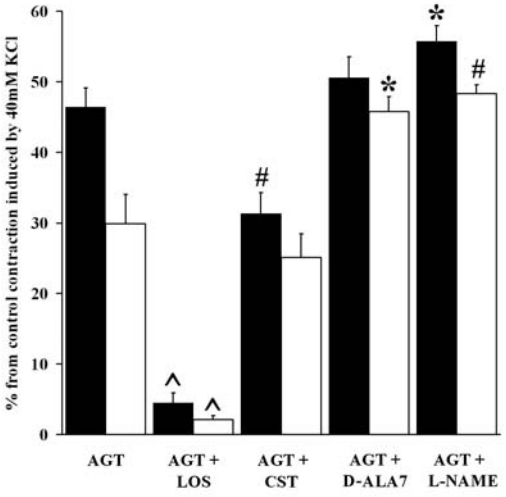

Figure 3A. The 10 μM angiotensinogen - induced contractions on isolated pulmonary arteries alone (AGT) or in the presence of 10 μM losartan (AGT + LOS); 10 μM chymostatin (AGT + CST), 10 μM D-ALA^7 (AGT + D-ALA7) and 100 μM L-NAME (AGT + L-NAME). The pulmonary arteries rings were obtained from control ovalbumine sensitized rats (OSR; filled bars) and ovalbumine sensitized rats treated with ghrelin (OSG; empty bars). *:p<0.05 as compared with AGT alone from the same group; #: p<0.01 as compared with AGT alone from the same group. ^: p<0.005 as compared with AGT alone from the same group.

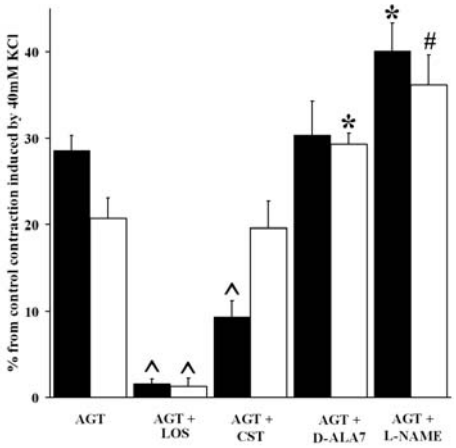

Figure 3B. The 10 μM angiotensinogen - induced contractions on isolated pulmonary veins alone (AGT) or in the presence of 10 μM losartan (AGT + LOS); 10 μM chymostatin (AGT + CST), 10 μM D-ALA^7 (AGT + D-ALA7) and 100 μM L-NAME (AGT + L-NAME). The pulmonary veins rings were obtained from control ovalbumine sensitized rats (OSR; filled bars) and ovalbumine sensitized rats treated with ghrelin (OSG; empty bars). *:p<0.05 as compared with AGT alone from the same group, ^: p<0.005 as compared with AGT alone from the same group.

pulmonary vessels from OSG by more than 60% (fig. 3A and 3B), revealing the involvement of NO synthesis in the modulation of AGT — vasoconstriction after the ghrelin treatment. Literature data showed that ghrelin attenuated pulmonary vascular remodeling and endothelial dysfunction during chronic hypoxia or monocrotalin — induced pulmonary hypertension (31,32). But, another possibility to implicate the stimulated NO synthesis in our obtained results is via Ang 1-7.

The connections between the Ang 1-7 and endothelial NO synthesis are well known (14). In order to verify the involvement of Ang (1-7) we blocked the Ang 1-7 specific receptors (AT1-7) by incubation of pulmonary vessel rings with D-ALA7. But, the D-ALA^7 amplificatory effects on AGT — induced contractions were comparable to those produced by L-NAME (fig 3A and 3B). These data suggested that the change of vascular responses to AGT by ghrelin treatment could be a result of the NO release induced by locally synthesized Ang 1-7. To check this hypothesis we used the real time analysis of associated variations in NO concentration and

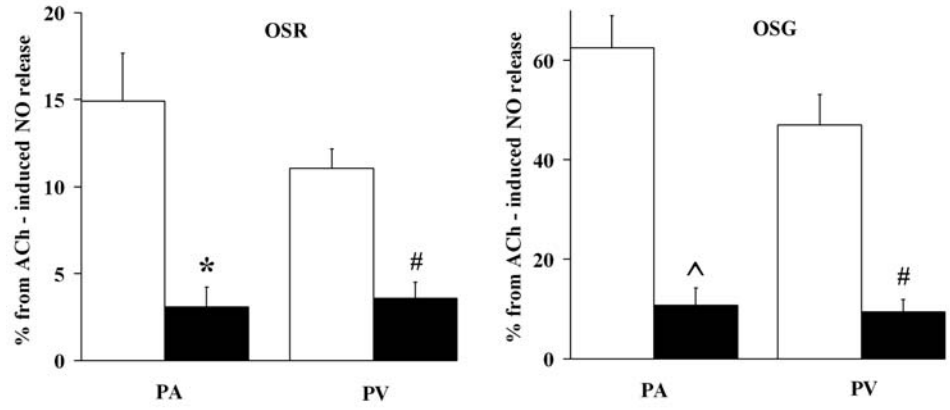

Figure 4. The angiotensinogen (AGT) - induced NO released on isolated pulmonary arteries (PA) and veins (PV) with intact endothelium in the absence (empty bars) and in presence (filled bars) of DALA7. OSR: ovalbumine sensitized rats. OSG : ovalbumine sensitized rats treated with ghrelin. *:$p<0.01$ as compared with results obtained in the absence of D-ALA7; #: $p<0.005$ as compared with results obtained in the absence of D-ALA7; ^: $p<0.001$ as compared with results obtained in the absence of D-ALA^7.

vasomotor responses by a NO-selective electrode introduced into the lumen of the mounted vascular ring as described previously (19). Experiments were conducted in absence and in presence of D-ALA^7. The AGT stimulated NO release was compared to ACh effect on the same ring. As shown in Fig. 4, the AGT — induced NO release was significantly higher in vessels from OSG and almost totally blocked by D-ALA^7.

These data sustain that, on isolated pulmonary vessels from ghrelin treated sensitized rats, the decrease of AGT — induced contraction could be a result of the decreasing chymase — dependent synthesis of vasoconstrictory angiotensin II and the increasing of AT1-7 — mediated production of NO.

In conclusion we demonstrated that intratracheally given ghrelin treatment decreases angiotensiongen — induced contractions of pulmonary arteries and veins in an ovalbumin — sensitized rat asthma model. The further use of AT1 and AT1-7 receptor antagonists, as well as a NOS inhibitor suggest that the ghrelin has an antagonistic activity upon the vasoconstrictive effects of locally synthesized angiotensin in lung vessels, by favoring AT1-7 vs AT1 — mediated activity and, implicitly, by increasing local NO production. This data sustain the possible existence of another way used by ghrelin for its protective actions at vascular level. At the same time, the possible role of ghrelin in developing new therapeutic tools for diseases that involved vascular inflammation is suggested but further studies aiming at elucidating the mechanisms implicated are needed.

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