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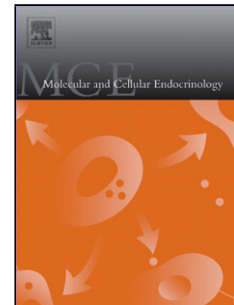
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Ghrelin in cardiovascular disease and atherogenesis

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Abstract

Although initially associated with regulation of appetite, the cardiovascular system has also been recognized as a potentially important target for ghrelin. Moreover, a limited number of clinical studies suggest a role for ghrelin in the treatment of congestive heart failure. So far reported cardiovascular effects of growth hormone secretagogues and/or ghrelin include lowering of peripheral resistance, either direct at the vascular level and/or by modulating sympathetic nervous activity. Other observed effects indicate possible improvement of contractility and cardioprotective effects both *in vivo* and *in vitro*. Taken together, these results offer an interesting perspective on the future where further studies aiming at evaluating a role of GHS and ghrelin in the treatment of cardiovascular disease are warranted.

Introduction

Growth hormone secretagogues (GHS) are small synthetic compounds, either peptides or non-peptides, with the ability to stimulate growth hormone (GH) secretion from the pituitary (Bowers *et al.*, 1977). It has been demonstrated that GHS act mainly through the GHS receptor (GHS-R) which is a G-protein-coupled receptor with seven transmembrane domains (Howard *et al.*, 1996). Studies using *in situ* hybridization have shown expression of the GHS-R in pituitary, hypothalamus and hippocampus and the identification of this orphan receptor prompted an active search for the natural ligand (Kojima & Kangawa, 2006). A couple of years later, the endogenous ligand for the GHS-R was purified from rat stomach and named ghrelin (Kojima *et al.*, 1999). Ghrelin was identified as a 28-amino acid peptide with an *n*-octanoylation of the serine-3 residue and this modulation has been found to be critical for the classical effects of ghrelin (Kojima *et al.*, 1999). The highest level of ghrelin expression is in the gastric mucosa, although expression at low levels have been found in multiple organs and tissues (Kojima *et al.*, 1999).

Apart from the ability to stimulate GH secretion and to exert regulatory effects on appetite and metabolism, it has become increasingly evident that growth hormone secretagogues (GHS) and ghrelin have a number of effects on the cardiovascular system. The ghrelin system is present in both vascular and cardiac tissues, where it is implicated in various functions. Ghrelin has been shown to have protective effects by inhibiting cardiomyocyte and endothelial cell apoptosis (Baldanzi *et al.*, 2002), and to improve left ventricular (LV) function during ischemia-reperfusion (I/R) injury (Frascarelli *et al.*, 2003). In rats with heart failure (HF), ghrelin improves LV dysfunction and attenuates the development of cardiac cachexia (Nagaya 2001b). Similarly, in short term studies,

ghrelin improves cardiac function and decreases systemic vascular resistance in patients with chronic HF (Nagaya *et al.*, 2001c). In the vasculature, ghrelin exerts vasodilatory effects (Nagaya *et al.*, 2001a) and possibly anti-inflammatory effects that may be of potential importance for the development of atherosclerosis (Dixit *et al.*, 2004).

The present article will give an overview of the main cardiovascular actions of GHS and also discuss potential central effects which may have importance for the regulation of vascular tonus. An interesting and intriguing feature of the cardiovascular effects of GHS is that they may be targeted directly to the heart and vasculature rather than being mediated by an increased growth hormone secretion. Evidence to suggest this is the finding of GHS binding sites on cardiomyocytes and the fact that some of the effects of GHS can be expressed also in the absence of GH (Pettersson *et al.*, 2002). Finally, the potential use of GHS and ghrelin as therapeutic agents in heart failure and related cardiac cachexia will be discussed.

Vasoactive effects

A well-documented cardiovascular action of GHS is a vasodilatory effect observed in both clinical and experimental studies. In a study by Kangawa and collaborators, a single injection of ghrelin caused a significant decrease in blood pressure (Nagaya *et al.*, 2001a). In unpublished observations from our own laboratory, we have also measured a modest but significant decrease in systolic blood pressure in hypophysectomized (hx) rats treated with ghrelin for two weeks.

Several possible mechanisms for the vasodilatory effect of GHS have been suggested.

One of the first studies on this subject demonstrated an antagonistic action of ghrelin on

the vasoconstrictor effect of endothelin-1 in isolated mammary arteries (Wiley & Davenport, 2002). Experiments using aortic ring preparations from GH deficient rats treated with ghrelin showed an increased maximal acetylcholine induced vasorelaxation compared with placebo and this vasodilatory effect was accompanied by an increase in endothelial nitric oxide synthase (eNOS) production (Shimizu *et al.*, 2003). Moreover, the vasoactive effect of ghrelin could be blocked by N(G)-nitro-L-arginine methyl ester, a nonselective NOS inhibitor, adding further support for a nitric oxide dependent pathway. However, the same research group has also reported possible vasodilatory effects independent of nitric oxide mechanisms (Okumura *et al.*, 2002).

The vasodilatory effects of ghrelin and GHS are not entirely consistent in the literature. This may possibly be due, at least in part, to differences in experimental models, administration modes, dosing and species. For instance, it has been recently shown that intracoronary infusion of acylated ghrelin (AG) decreases coronary blood flow without affecting other haemodynamic parameters or plasma levels of GH in a porcine experimental model (Grossini *et al.*, 2007). Moreover, when using pharmacological blocking agents, these authors showed that the coronary vasoconstriction induced by ghrelin was independent from muscarinic-cholinergic and alpha-adrenergic receptors stimulation, while probably involving the inhibition of a vasodilatory β 2-adrenergic receptor-mediated effect related to the release of nitric oxide. Thus, the vasoconstrictive effect of ghrelin was proposed to be expressed through inhibition of a tonic coronary β 2-adrenergic receptor-mediated, vasodilatory effect related to the release of nitric oxide (Grossini *et al.*, 2007).

Central effects associated with vascular tonus

Another interesting possible mechanism behind regulation of vascular tonus that has recently been reported is a central effect, involving decreased sympathetic nervous activity and resulting lower arterial pressure, observed after unilateral microinjection of ghrelin into the nucleus of the solitary tract of rats (Lin *et al.*, 2004). These findings are in line with a previous report describing decreased mean arterial blood pressure and sympathetic nerve activity (Matsumura *et al.*, 2002). In this study, peripheral and central administration of ghrelin to conscious rabbits were compared regarding arterial pressure and renal sympathetic activity. The authors suggest that intravenous injection of ghrelin acts, at least in part, on the central nervous system to decrease arterial pressure and renal sympathetic nerve activity, and that central ghrelin participates in the regulations of the sympathetic nerve activity to the kidney and the baroreceptor reflex in conscious rabbits. Central actions of endogenous AG have been recently demonstrated in conscious rats treated with the GHS-R antagonist [d-Lys-3]-GHRP-6 (Vlasova *et al.*, 2009). Mean arterial pressure and heart rate were dose-dependently elevated after subcutaneous (sc) administration of [d-Lys-3]-GHRP-6. Sympathetic blockade of both alpha-adrenoreceptors and beta1-adrenoreceptors abolished these haemodynamic alterations, suggesting that the increases of arterial pressure and heart rate induced by injection of ghrelin antagonist are, at least in part, due to the activation of the sympathetic nervous system. These results suggest that the use of the ghrelin antagonist system as a therapeutic target for reduction in food intake might lead to serious side effects, like elevated blood pressure in humans mostly already having an elevated blood pressure as part of their metabolic syndrome.

Vasodilatory and anti-inflammatory effects

Apart from vasodilatory effects, ghrelin may also have other vasoactive and anti-inflammatory properties. It has been shown that ghrelin has inhibitory effects on cytokine release and it activates nuclear factor- κ B and mononuclear cell binding in cultured human umbilical vein endothelial cells (HUVEC), which could potentially be important, should GHS be considered as therapeutic agents in conditions with atherosclerosis (Li *et al.*, 2004). Additional evidence for anti-inflammatory effects of ghrelin include the findings that T lymphocytes express both ghrelin and the GHS-R and that ghrelin secretion is increased when the T lymphocytes are activated (Dixit *et al.*, 2004). This may suggest a modulatory role on the immune system, since ghrelin was also reported to inhibit interleukin 1 β , interleukin-6 and tumor necrosis factor- α (TNF- α)(Dixit *et al.*, 2004). A summary of reported vasoactive effects are shown in Fig 1.

Inotropic effects

It has been shown that GHS can increase cardiac output during experimental conditions (Nagaya *et al.*, 2001b). However, it cannot be completely excluded that the observed increase in cardiac output is secondary to vasodilatory effects, rather than positive inotropic effects. Moreover, increased cardiac output and/or hypothetically increased contractility may be secondary to increased secretion and action of GH, which has been demonstrated to have positive inotropic effects (Strömer *et al.*, 1996, Tajima *et al.*, 1999). However, a few studies suggesting inotropic effects are of particular interest since improvement of systolic function was reported before changes in peripheral resistance

could be observed. An increased left ventricular ejection fraction (LVEF) in both healthy volunteers (Bisi *et al.*, 1999a) and in patients with growth hormone deficiency (GHD) (Bisi *et al.*, 1999b) was reported within fifteen minutes of iv hexarelin injection, a peptidic GHS, without a concomitant lowering of blood pressure. In a more recent study, ghrelin was administered sc to six healthy volunteers (Enomoto *et al.*, 2003). Ghrelin significantly increased LVEF and end-systolic volume thirty minutes after administration without significant changes in blood pressure or heart rate. At present there is no known mechanism for the inotropic actions of GHS, and experimental data addressing this topic are lacking. However, some of the positive effects on contractility, at least in non-GHD conditions, could be secondary to an increased GH secretion, which has been demonstrated to have inotropic effects in experimental models, possibly related to alterations in Ca^{2+} transients (Strömer *et al.*, 1996).

Cardioprotective effects against ischemia

Several reports from different research groups using a variety of experimental models indicate cardioprotective effects of GHS against ischemia. In one of the first studies addressing this topic, De Gennaro Colonna and collaborators used antiserum to GHRH in order to achieve GH deficiency in rats and then treated them with GH or hexarelin for two weeks (De Gennaro *et al.*, 1997). After killing the rats their hearts were subjected to retrograde aortic perfusion under ischemic conditions. As expected, control rats with GH deficiency showed a marked increase in left ventricular end diastolic pressure (LVEDP) and poor recovery of contractility after reperfusion. In contrast, rats treated with GH or hexarelin had normalized cardiac function. In a further study from the same research

group, hypophysectomized (hx) rats were pre-treated with GH or hexarelin which again protected the hearts from cardiac damage during ischemic reperfusion (I/R) *in vitro* (Locatelli *et al.*, 1999).

Interestingly, both of these studies suggest GHS effects independent of increased GH secretion since cardiovascular effects of hexarelin were observed in two different models of GHD.

Myocardial stunning is a clinical condition which can be defined as transient myocardial ischemia followed by reperfusion which causes a reversible cardiac dysfunction involving both systolic and diastolic function. A rabbit model was used to study possible protective effects of GH and GHS on myocardial stunning (Weekers *et al.*, 2000).

Animals were pretreated with GH or GH releasing peptide-2 (GHRP-2) for two weeks. Subsequently, hearts were perfused with blood and subjected to 15 minutes of ischemia followed by 80 minutes of reperfusion and compared with non-ischemic hearts. In this study there were significant effects of neither GH nor GHRP-2 on systolic function. However, a significant improvement of diastolic function was observed after pre-treatment with GHRP-2, suggesting a novel therapeutic possibility for myocardial stunning.

Several *in vitro* studies have been performed to elucidate responsible mechanisms for the protective effect of GHS against ischemia/reperfusion (I/R). The possible protective effect of ghrelin and the synthetic secretagogues hexarelin and MK-0677 were tested on isolated perfused rat hearts subjected to I/R (Frascarelli *et al.*, 2003). While hexarelin and AG significantly reduced infarct size, MK-0677 and UAG were ineffective. The protein kinase C inhibitor chelerythrine reduced the protection provided by hexarelin. Further

evidence for a protective action of ghrelin against cardiac I/R was provided by the study by Chang *et al.*, who showed that administration of ghrelin during reperfusion resulted in improvement in coronary flow, heart rate, left ventricular systolic pressure, and LVEDP, enhanced rates of left ventricular contraction and relaxation and reduced myocardial release of lactate dehydrogenase and myoglobin (Chang *et al.*, 2003). These results would suggest not only functional improvement but also protection from tissue injury, which may important clinical significance. Interestingly, the receptor-binding assay demonstrated that maximum binding capacity of ghrelin to sarcolemmal membranes was significantly increased after ischemia and was even further increased after I/R. Taken together, these data demonstrate that ghrelin and hexarelin exert specific cardioprotective effects against myocardial I/R injury, which are independent of GH secretion, and likely to involve binding to cardiovascular receptors, a process that is up regulated during I/R. An interesting aspect that has been raised is the possibility that cardioprotective effects may not be completely interchangeable between different GHS and ghrelin. Torsello and collaborators recently suggested that cardioprotective effects against ischemia after pretreatment with hexarelin could not be replicated by ghrelin (Torsello *et al.*, 2003). In this study, hx rats were pre-treated for seven days with either hexarelin or ghrelin and their hearts were subsequently subjected to ischemia/reperfusion *in vitro*. During ischemia there was a gradual increase of LVEDP pressure in hearts subjected to ischemia compared to controls. Pre-treatment with hexarelin but not ghrelin partially normalized LVEDP and protected the hearts from injury. It could be speculated that this differential effect of hexarelin and ghrelin might be in part due to different affinity to a variety of subtype GHS receptors. On the other hand, in a more recent study, ghrelin was reported

to be protective when added *in vitro* during ischemic reperfusion of isolated rat hearts (Chang *et al.*, 2004).

An intriguing possibility that some of the cardiovascular effects of GHS may be mediated through the CD36 receptor has been suggested (Bodart *et al.*, 2002). The CD36 receptor is multifunctional B-type scavenger receptor and binding of hexarelin was found to occur to a membrane protein in cardiomyocytes, identical to rat CD36 receptor. One observed GHS effect associated with activation of the CD36 receptor was coronary vasoconstriction (Bodart *et al.*, 2002). This is intriguing and in contrast to most other studies reporting vasodilatory effects of GHS. Whether the coronary vasoconstriction is specifically mediated by the CD36 receptor is not known and obviously more studies and better understanding of GHS binding sites are needed to clarify this.

Proliferative, antiapoptotic and metabolic effects on cardiomyocytes

Observations of GH-independent effects of GHS have prompted several studies on cultured cardiomyocytes to elucidate possible signalling mechanisms. In studies from our own laboratory, we have found that hexarelin and ghrelin increase thymidine incorporation, indicating increased proliferation of H9c2 cardiomyocytes in a dose-dependent and specific manner (Pettersson *et al.*, 2002). Moreover, binding studies on cardiomyocyte cell membranes revealed specific binding in the absence of detectable expression of the GHS-R1a, which would suggest an alternative binding site in the heart (Pettersson *et al.*, 2002).

Possible anti-apoptotic effects of GHS have also been studied on primary cardiomyocytes *in vitro* and cell survival signaling has been described (Baldanzi *et al.*, 2002). Both

acylated (AG) and unacylated acyl ghrelin UAG) had similar antiapoptotic effects on primary cardiomyocytes, H9c2 cardiomyocytes and endothelial cells with signaling via MAPK and PI3K/Akt dependent pathways (Baldanzi *et al.*, 2002).

In a recently published study, hexarelin was found to decrease apoptosis induced by angiotensin-II in cultured rat cardiomyocytes through inhibition of caspase-3 activity and expression of the proapoptotic protein Bax (Pang *et al.*, 2004). Moreover, Bcl-2 expression was increased, providing evidence for antiapoptotic effects of hexarelin through well-established anti-apoptotic signaling pathways. Thus, *in vitro* findings would support both proliferative and antiapoptotic effects of GHS involving well established cell survival signaling pathways.

Several later reports have further confirmed the antiapoptotic effect of ghrelin, independently of the method used to induce cell damage, also in different cell types through similar signalling pathways (Granata *et al.*, 2007). Iglesias *et al.* reported that ghrelin protects against the proapoptotic cytosine arabinoside in the mouse adult cardiomyocytes HL-1 cell line (Iglesias *et al.*, 2004). Moreover, it has been recently shown that, similarly to H9c2 cells, in HL-1 cardiomyocytes and primary cultured-mouse and rat cardiomyocytes, UAG recognizes specific binding sites and both AG and UAG exert antiapoptotic effects (Lear *et al.*, 2010). Interestingly, the same study showed that AG and UAG had opposing metabolic effects. Indeed, AG inhibited insulin-induced glucose uptake and had no effect on fatty acid uptake, whereas UAG reversed the AG inhibitory effect on glucose uptake and increased fatty acid uptake and glucose transporter-4 (GLUT4) translocation to the cell membrane (Lear *et al.*, 2010).

It has been reported that increased levels of endogenous ghrelin during the progression of doxorubicin-induced cardiomyopathy and heart failure might provide a compensatory self-protective mechanism (Xu *et al.*, 2005). The pathways responsible for this protective effect were investigated *in vitro* on primary cultured cardiomyocytes challenged with doxorubicin in the presence or absence of ghrelin or a TNF- α antagonist (Xu *et al.*, 2005). Ghrelin not only reduced apoptosis and markers of oxidative stress, but also increased antioxidative enzyme activity and retained mitochondrial membrane potential and energy metabolism. Moreover, ghrelin increased mitochondrial antiapoptosis-related gene protein expression, reduced cytoplasmic cytochrome C (Cyt C) release and strengthened the activation of NF-kappaB. Since all these effects were abrogated by the TNF- α antagonist, the authors suggested that the antioxidative and antiapoptotic effects of ghrelin are due to its ability to up regulate TNF- α and involve activation of the TNF- α /NF-kappaB pathways (Xu *et al.*, 2005).

Another potentially important effect of ghrelin on cardiomyocytes was recently reported and involves stimulation of 5'-AMP activated protein kinase (AMPK) (Kola *et al.*, 2005). This kinase has a central role in regulating energy supplies in cells and is activated in hypoxic conditions. Thus, a beneficial effect of ghrelin on cellular energy supply may contribute to the mechanism of cardioprotection.

Experimental and clinical use of ghrelin and GHS in heart failure

The various reported cardiovascular effects of GHS prompted studies to evaluate their potential role in the treatment of congestive heart failure (CHF). In a study from our laboratory, an established experimental approach using ligation of the left coronary artery

was used. Intact rats were subjected to experimental myocardial infarction and after four weeks of recovery, the rats were treated with two doses of hexarelin (10 or 100 $\mu\text{g}/\text{kg}\cdot\text{day}$, GH 2.5 $\text{mg}/\text{kg}\cdot\text{day}$) or saline for two weeks (Tivesten *et al.*, 2000). Cardiac structure and function were evaluated with echocardiography at baseline and at the end of the experiment. Stroke volume was significantly increased in both the high dose hexarelin group and GH group compared to control rats. Similar findings were also seen regarding cardiac output, which was also increased by hexarelin and GH. Both these parameters remained significantly elevated also when normalized to body weight. Interestingly, only GH but not hexarelin treatment significantly increased body weight and kidney IGF-I mRNA, suggesting that the effects of hexarelin were independent of GH.

As an alternative method to coronary artery ligation, electric pacing of the heart has been developed as a model for impaired cardiac function. King and collaborators induced rapid pacing at 240 bpm for three weeks in pigs (King *et al.*, 2001). An orally bioavailable GHS termed CP-424,391 was administered to one group of animals at initiation of pacing. At the end of the experiment after three weeks of pacing and treatment, GHS treated pigs had significantly higher fractional shortening and lower wall stress due to cardiac hypertrophy compared to control animals. Serum IGF-I was increased approximately two-fold which would suggest an effect at least partially mediated by an increased GH secretion.

Only a limited number of published studies have so far used ghrelin rather than synthetic GHS to study effects after myocardial injury. Kangawa and collaborators used rats with experimental myocardial infarction and treated them with ghrelin 100 μg daily for three

weeks (Nagaya *et al.*, 2001b). Echocardiography and catheterization were used to evaluate the effects of ghrelin, showing higher cardiac output and fractional shortening in ghrelin treated rats compared to controls.

The experience from clinical trials with GHS and ghrelin in conditions with impaired cardiac function is also very limited at present. Acute administration of hexarelin increased LVEF in patients with ischemic but not dilated cardiomyopathy (Broglia *et al.*, 2001). Moreover, in anaesthetized patients undergoing bypass surgery, short term hexarelin infusion was found to increase LVEF, cardiac output and mean arterial pressure without any changes in peripheral resistance (Broglia *et al.*, 2002). Another clinical trial where twelve patients with CHF were given a single iv infusion of human ghrelin or placebo demonstrated beneficial effects in the ghrelin group (Nagaya *et al.*, 2001c). Ghrelin significantly increased cardiac index, stroke volume index and decreased systemic vascular resistance within 60 minutes, all very favorable changes in the condition of CHF. Only one study with longer duration of treatment of patients with CHF has been published so far (Nagaya *et al.*, 2004). In this open study, ten patients with congestive heart failure of heterogeneous etiology were treated with ghrelin administered iv for three weeks. Ghrelin significantly increased LVEF, LV mass and decreased LV end-systolic volume. Moreover, ghrelin also increased peak workload and peak oxygen consumption during exercise which would indicate improved systolic function and exercise capacity. There was also a reported decrease in plasma norepinephrine whereas GH increased only transiently after a single ghrelin infusion and did not differ significantly between the groups after three weeks of ghrelin treatment. No serious adverse events were observed. Recently, ghrelin and GHS-R1a expression have been

investigated in different myocardial areas of patients with CHF as compared to heart-healthy subjects (Beiras-Fernandez *et al.*, 2010). The aim of this study was to better define the role of the ghrelin signaling system in the networks regulating cardiac function and its potential as a target for diagnosis and/or treatment of CHF. Ghrelin expression was found to be decreased in atria and ventricles of CHF hearts whereas GHS-R1a expression was increased. These findings suggested a possible association between myocardial expression of ghrelin and GHSR-1a and myocardial function. Ghrelin reduction in CHF hearts may reflect maladaptive processes, whereas GHS-R1a increase may represent a compensatory mechanism.

End-stage CHF is sometimes associated with cachexia which is a severe catabolic state characterized by weight loss and muscle wasting. Thus it is tempting to speculate that ghrelin and GHS may have multiple beneficial effects on this patient population by exerting both positive cardiovascular and orexigenic actions. To date there are no long-term clinical trials substantiating this hypothesis, although preliminary data suggest that administration of ghrelin increases muscle strength and lean body mass in patients with CHF (Nagaya *et al.*, 2004). It remains to be investigated further if GHS and ghrelin will have a role as therapeutic agents in the treatment of congestive heart failure and more long-term and placebo controlled trials are required. However, the clinical studies published to date certainly open interesting possibilities.

Summary

Although GHSs were initially recognized for their GH releasing properties, it has been recognized that the cardiovascular system is also a potentially important target for GHSs.

Moreover, a limited number of studies also indicate cardiovascular effects of ghrelin. So far reported cardiovascular effects of GHS and/or ghrelin include lowering of peripheral resistance, possible improvement of contractility and cardioprotective effects both *in vivo* and *in vitro*.

Taken together, these results offer an interesting perspective on the future where further studies aiming at evaluating a role of GHS and ghrelin in the treatment of cardiovascular disease are warranted.

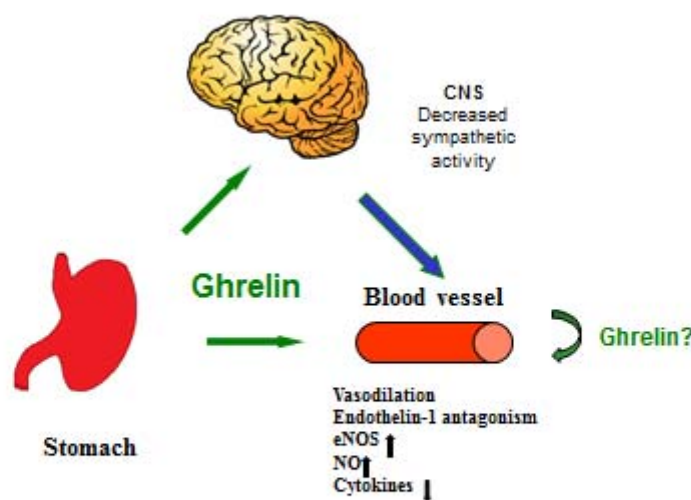


Fig. Schematic illustration of possible interactions between ghrelin, CNS and the vascular system. Exogenous GHS or ghrelin (systemic or locally produced in the cardiovascular system) can probably interact directly with blood vessels and antagonize the effect of the vasoconstrictor endothelin-1, increase eNOS expression and the production of the vasodilator NO. An alternative pathway may be through the CNS leading to decreased sympathetic nervous activity and an additive vasodilatory effect. Additional vasoactive effects that have been reported include cytokine inhibition.

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