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GHRELIN SIGNALING IN HUMAN MESENTERIC ARTERIES

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The hypothesis is that the ghrelin signal pathway consists of new participants including a local second mediator in human mesenteric arteries. The contractile force of isometric artery preparations was measured using a wire-myograph. Whole-cell patch clamp experiments were performed on freshly isolated single smooth muscle cells from the same tissue. After the addition of ghrelin (100 nmol) the outward potassium currents conducted through iberiotoxin-sensitive calcium-activated potassium channels with a large conductance were almost entirely abolished. The effect of ghrelin on potassium currents was insensitive to selective inhibitors of cAMP-dependent protein kinase and soluble guanylate cyclase, but was eliminated in the presence of des-octanoyl ghrelin and O-(octahydro-4,7-methano-1H-inden-5-yl) carbonopotassium dithioate (D-609). Ghrelin dose-dependently increased the force of contraction of native, endothelium-denuded and mostly of endothelium-denuded and treated with tetrodotoxin human mesenteric arteries preconstricted with 1 nmol endothelin-1. This effect of ghrelin was blocked when the bath solution contained 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), 4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine (PP2), D-609, 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide (GF109203x), pertussis toxin, 2-aminoethyl diphenylborinate (2-APB), indomethacin, (5Z,13E)-(9S,11S,15R)-9,15,Dihydroxy-11-fluoro-15-(2-indanyl)-16,17,18,19,20,pentanor-5,13-prostadienoic acid (AL-8810) - a non-selective prostanoid receptor antagonist, 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazolo (SC-560) - a selective cyclooxygenase 1 inhibitor, ozagrel - a selective thromboxane A_2 synthase inhibitor or T prostanoid receptor antagonist GR32191B. It is concluded that ghrelin increases the force of contraction of human mesenteric arteries by a novel mechanism that involves Src kinase, mitogen-activated protein kinase kinase (MEK), cyclooxygenase 1 and T prostanoid receptor agonist, most probably thromboxane A_2.

Key words: *grehlin signaling, hormone, orexigenic, prostaglaudin, smooth muscle, tromboxane*

INTRODUCTION

The peptide hormone ghrelin, initially isolated from rat stomach mucosa, was identified as an endogenous ligand of the growth hormone secretagogue (GHS) receptor (1). Ghrelin binds mainly to GHS receptor type 1a (GHS-R1a), which preferentially activates Gq-protein (2). However GHS-R1a/G_q/phospholipase C (PLC) signaling cannot explain all physiological effects of ghrelin. Thus, ghrelin activates adenylate cyclase (AC) and cAMP-dependent protein kinase (PKA) *i.e.* AC/cAMP/PKA signaling in endothelial and smooth muscle cells of human aorta (3, 4), as well as in pancreatic β-cells (5), while in GH3 rat pituitary tumor cells it modulates cGMP production and cGMP-dependent protein kinase (PKG) (6). Recent studies reveal new important participants - extracellular signal-regulated kinases 1 and 2 (ERK1/2), whose activities are enhanced by ghrelin in human endothelial cells (3, 7). GSH-R1a activates $G_{i/o}$-proteins, $G_{q/11}$-proteins and a β-arrestin sensitive pathway in the heterologous expression system human embryonic kidney 293 cells (8). Additionally, ghrelin increases the production of cyclooxygenase (COX) 2-derived prostaglandin E_2 (PGE_2) in oxyntic gland area of the rat stomach (9) and in human and rat colonic mucosa (10) *via* stimulated COX-2 expression (9, 10).

Ghrelin regulates human circulation. Injection of ghrelin significantly decreased mean arterial pressure without a change of the heart rate in healthy volunteers (11). Ghrelin dilates the sustained endothelin-1 (ET-1)-induced contraction of human mammary artery preparations most probably *via* direct effect on the smooth muscle cells (12, 13). A preliminary study of Dimitrova (14) showed that ghrelin increased the force of contraction of endothelium-denuded preparations of human mesenteric arteries. However, the mechanisms of both ghrelin-induced dilation and constriction in human vascular beds have not been explored yet.

The combination of endothelium-denuded artery preparations with inhibited neurotransmission and whole-cell patch clamp method is useful in investigating complex receptor-coupled transmissions in arterial smooth muscles, as it prevents the many influences of vascular wall non-muscle cells to a great extent.

Furthermore, hormones and local mediators often change the conductivity of ion channels in the cell membrane, which can be used as sensors for proper signaling. Our pilot study showed that ghrelin reduces the iberiotoxin-sensitive Ca^{2+}-activated potassium current ($I_{K(Ca)}$) elicited in freshly isolated smooth muscle cells of human mesenteric arteries *via* PLD- and PKC-dependent mechanism (15). The sarcoplasmic reticulum is also necessary for this signaling as the blockade of sarco-endoplasmic reticulum Ca^{2+} ATPase or IP_3-activated Ca^{2+} channels of internal Ca^{2+} stores inhibit the effect of ghrelin on $I_{K(Ca)}$ (15).

In the present study, we used pharmacological tools to identify the participants of ghrelin signaling and found a second mediator involved.

MATERIALS AND METHODS

The investigation conformed to the 'Declaration of Helsinki' 1975 (revised 1983). Mesenteric arteries were isolated from extracted specimens of human mesentery taken during abdominal surgery on patients - 63 men aged 64.6±1.5 years and 43 women aged 59.3±1.9 - and transported to the laboratory in ice-cold saline. Half of the patients were operated for malignant growths (carcinoma sigma) and the rest - for nonmalignant conditions.

Contraction studies

Segments of mesenteric arteries were dissected, carefully cleaned of adipose and connective tissues and kept in ice-cold low Ca^{2+} solution containing (mmol): 118 NaCl, 5 KCl, 1.2 $MgCl_2$, 0.16 $CaCl_2$, 10 glucose, 1.2 Na_2HPO_4 and 24 HEPES. Arterial rings (2 mm long) were mounted on a wire-myograph for isometric tension recording DMT, model 410A (Danish Myo Technology, Aarhus, Denmark) whose chamber was filled with the same ice-cold low Ca^{2+} solution. After the mounting of the vessel rings, the organ bath solution was replaced with the same solution containing 2.5 mmol $CaCl_2$. The bath was heated up to 37°C and continually bubbled with carbogen (95% O_2 and 5% CO_2). The isometric force of contraction was recorded using the program Myodaq (DMT, Aarhus, Denmark). The arterial segments were equilibrated for 1 hour at 37°C in a buffer, which was changed at least 3 times during this equilibration period. In most experiments, the endothelium was removed by careful rubbing with a rat whisker. Then vessels were stretched to their optimal lumen diameter, corresponding to 90% of the passive diameter of the vessel at 100 mm Hg. The viability of the preparations was tested twice by application of 10 µmol noradrenaline. The integrity of the endothelium was tested with 10 µmol acetylcholine added to 10 µmol noradrenaline contracted rings. After the viability tests, the strips were contracted with 1 nmol ET-1, which produced relatively stable isometric contractions allowing the study of the effect of the increasing concentrations of ghrelin. The tension reached a steady state in about 40 minutes after the application of ET-1. Then ghrelin was applied to the bath in increasing concentrations of 10, 30, 100, 300 and 1000 nmol, *i.e*. starting from a value that is about 10 times higher than its plasma level. It led to a significant effect on native artery preparations (with endothelium and in the absence of TTX) at a 30-100 times higher concentration than in human circulation (11). A possible explanation of this result is that ghrelin has a low diffusion rate through the adventitia, which decreases its interaction with receptors of smooth muscle cells when applied to the bath solution. Other researchers have also used higher ghrelin concentrations while studying the effect of ghrelin on vascular preparations (12), probably due to the same reason. It is also possible that ghrelin reaches such values in the smooth muscle layer of the arterial wall due to its paracrine release from human vascular endothelium (16). Additionally, guinea pigs may have a higher plasma level of ghrelin if compared to humans or rats. The ghrelin-induced changes in tension were expressed as a percentage of the maximum tension elicited by 1 nmol ET-1. The influence of different pharmacological agents (inhibitors) on ghrelin effect was studied by means of their addition to the bath about 40 min after ET-1 application and incubated for about 30 min before the application of ghrelin. The effects of inhibitors were studied using several types *in vitro* preparations: i) native (untreated) preparations of small human mesenteric arteries; ii) endothelium-denuded preparations; iii) native preparations with tetrodotoxin (TTX, 300 nmol) and mainly iv) endothelium-denuded and TTX-treated preparations. The time control preparations were equally treated, but instead of ghrelin, an equal volume of solvent (deionised water) was added at the same time intervals. The inhibitors and antagonist were applied to block (to switch off) the studied enzyme or receptor activity and not to induce a partial inhibition only. This forced us to use a higher concentration of these substances. On the other hand, the possibility of a non-specific effect of the pharmacological tools restricted us to using them in lower concentrations. Thus, the aim to choose the optimal concentration of each substance for our experiments was not easy. For almost all of the blockers, however, there are at least several studies on arterial preparations *in vitro*, in some cases with dose-response curves and/or tests for cross-reaction. Besides, the choice of each concentration was based on our earlier experience with a significant part of the substances used either in electrophysiological or functional studies of different vascular beds.

Whole-cell patch-clamp experiments

This method has been described in detail elsewhere (17). In brief, whole-cell voltage-clamp experiments were performed on single smooth muscle cells, freshly isolated from human mesenteric arteries. The arteries were cut into 3 mm long pieces and placed in 0.1 mmol Ca^{2+}-containing physiological salt solution (PSS, for composition see below) warmed to 37°C and containing 1.5 mg ml^{-1} collagenase II, 1 mg ml^{-1} papain, 15 µl ml^{-1} elastase and 1 mg ml^{-1} albumin. After 30 to 35 min incubation at 37°C with continuous O_2 bubbling, the enzymes were washed away and the tissue pieces triturated 5 times in Ca^{2+}-free PSS using a pipette with a small tip opening. The remainder of the tissue was put back into the enzyme-containing solution for another 5 min and then carefully washed with Ca^{2+}-free PSS. Single smooth muscle cells were obtained by gentle trituration in 2 ml of the same Ca^{2+}-free solution. Cells could be stored for up to 8 hours in this solution at 4-6°C.

The external solution (PSS) for single-cell voltage experiments contained (in mmol): 126 NaCl, 5.6 KCl, 10 HEPES, 20 taurine, 20 glucose, 1.1 $MgCl_2$, 0.8 $CaCl_2$, 5 Na-pyruvate and pH was adjusted to 7.4 with NaOH. The same solution was used for the isolation of cells. The solutions in the recording pipette contained (in mmol): 125 KCl, 6 NaCl, 10 HEPES, 1 $MgCl_2$, 3 EGTA, 0.1 ATP, 5 Na-pyruvate, 5 succinate, 5 oxalacetate, 5 glucose and 2.15 $CaCl_2$ to give a calculated free Ca^{2+} of 200 nmol and pH was adjusted to 7.4 with KOH.

Chemicals

Most of the substances used for solution preparation were obtained from ICN (Irvine, CA, USA). NaOH, BSA, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC), pertussis toxin, indomethacin, O-(octahydro-4,7-methano-1H-inden-5-yl) carbonopotassium dithioate (D-609), collagenase type II, prostaglandine $F_{2\alpha}$($PGF_{2\alpha}$), (5Z,13E) - (9S,11S,15R)-

9,15,dihydroxy-11-fluoro-15-(2-indanyl)-16,17,18,19,20, pentanor - 5,13 - prostadienoic acid (AL-8810), (4Z)-7-[(1R,2R,3S,5S)-5-([1,1'-Biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid (GR32191B), 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole (SC-560) and ozagrel were obtained from Sigma-Aldrich (FOT, Sofia, Bulgaria). 1-H-[1,2,4] oxadiazole [4,3-]-quinoxalin-1-one (ODQ), cyclopiazonic acid, iberiotoxin, 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide (GF109203x), 1-(6-{[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5dione (U-73122), *n*-octanoyl ghrelin, desacyl ghrelin, tetrodotoxin, endothelin-1 and ryanodine were from Tocris Cookson (Bristol, UK). Elastase solution was from Serva (Heidelberg, Germany). Rp-cAMPS (Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine) was from Biolog (Bremen, Germany). 2-aminoethyl diphenylborinate (2-APB) was from Calbiochem (San Diego, CA, USA). 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene (U0126) and 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4,d] pyrimidine (PP2) were from Biaffin GmbH, Co KG (Kassel, Germany). O_2 (99.9% purity) and carbogen (95% O_2 and 5% CO_2) were from Technical Gases (Sofia, Bulgaria). All patch-clamp experiments were conducted at room temperature (21-24°C).

Data analysis

Current densities were expressed in pA/pF and plotted as functions of the potential applied to obtain data suitable for statistical analysis. The significance of differences between means was assessed using Tukey-Kramer multiple comparison test with $p<0.05$ regarded significant. The force of contraction was evaluated as a difference in tension (N/m) measured before ET-1 application and the plateau reached afterwards. The contractile effect of ghrelin was expressed as a percentage of the maximal ET-1 induced contraction, taken as 100%. Values were expressed as means±S.E.M. From five to ten human mesenteric arterial preparations (n) were included in the construction of each concentration-response curve of ghrelin. Data were subjected to a comparative statistical analysis one-way ANOVA with Bonferroni correction ($p<0.05$).

RESULTS

Octanoyl ghrelin, herein referred to as ghrelin, dose-dependently increased the force of contraction of isometric human mesenteric artery preparations constricted with ET-1 (*Fig. 1A*, n=9). The effect of ghrelin became more pronounced in endothelium-denuded human mesenteric arteries, *i.e.* with eliminated influence of endothelium-derived factors (*Fig. 1B*, n=8) and mostly in endothelium-denuded preparations treated with TTX (300 nmol) for inhibiting the neuronal action potential propagation (*Fig. 1C*, n=7). Next, we used endothelium-denuded and TTX-treated preparation for studying the ghrelin signaling in smooth muscle of human mesenteric arteries. Iberiotoxin (100 nmol), applied before ghrelin, inhibited its effect on endothelium-denuded and treated with TTX human mesenteric artery preparations (n=6, data not shown). Similarly, contractions induced by 40 mmol K^+-containing bath solution (*Fig. 1D*, n=4)

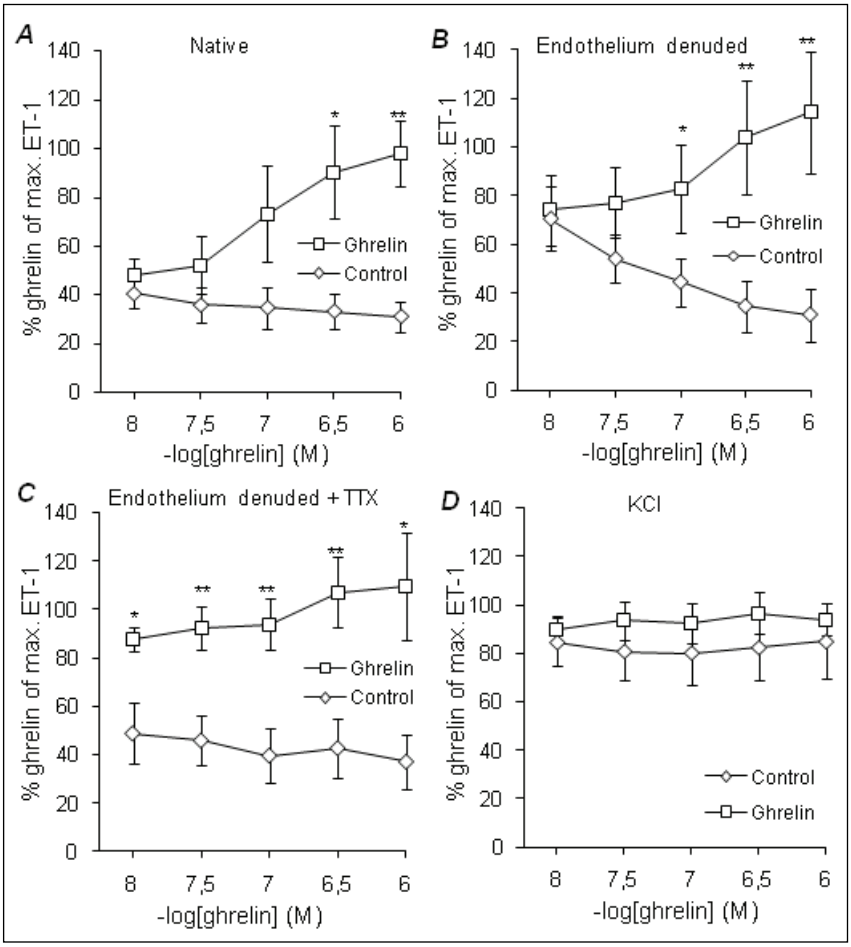

Fig. 1. Effect of ghrelin on human small mesenteric arteries contraction. (A) Original tension traces showing contractions induced by endothelin-1 (ET-1, 1 nmol) and the effect of subsequent application of increasing concentrations of ghrelin (10, 30, 100, 300 and 1000 nmol) or of solvent (time control). (B) Dose-response relationship of ghrelin-induced contractions of vessel preparations constricted with ET-1 (1 nmol). Artery tension after the application of ghrelin is expressed as a percentage of the maximal ET-1 evoked contraction. (C) Dose-response relationship of ghrelin-induced contractions of endothelium-denuded vessels preconstricted with ET-1 (1 nmol) or with 40 mM KCl-containing bath (D) and treated with TTX (300 nmol). * - $p<0.05$, ** - $p<0.01$.

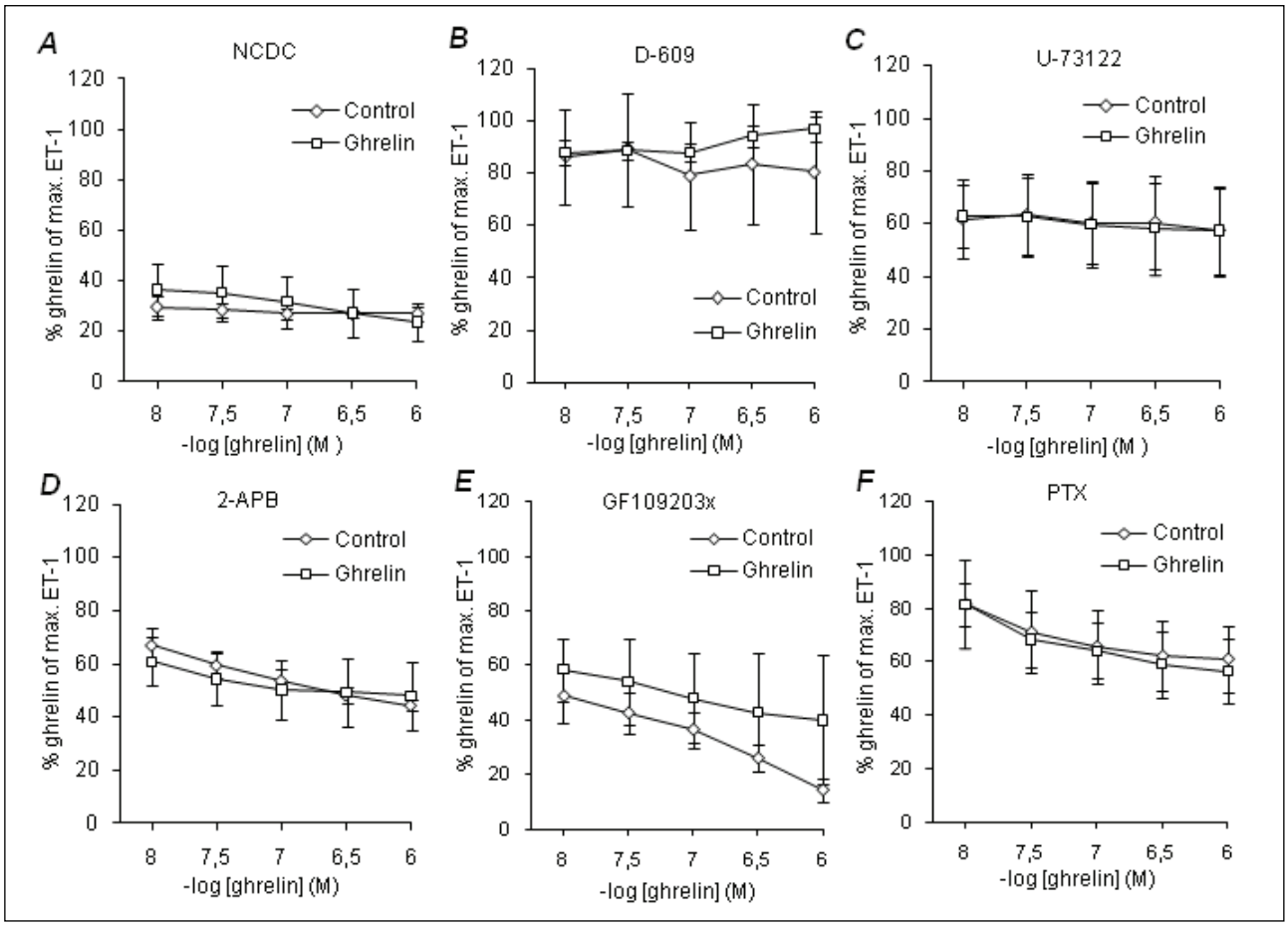

Fig. 2. PI-PLC, PC-PLC, IP3-induced Ca^{2+} release, PKC and G_i-protein mediate the ghrelin-induced increase of force of contraction. Time-controls of force of contractions of human mesenteric arteries were obtained in external solutions containing (A) NCDC (50 μmol), (B) D609 (10 μmol), (C) U-73122 (30 μmol), (D) 2-APB (100 μmol), (E) GF109203x (2 μmol) and (F) PTX (400 nmol). They all are presented with diamonds. Dose-response relationship of force of contraction after the addition of increasing doses of ghrelin to (A) NCDC-, (B) D609, (C) U-73122-, (D) 2-APB-, (E) GF109203x- or (F) PTX-containing bath solutions are illustrated with squares.

were insensitive to subsequent cumulative application of ghrelin. The control values of the force of contraction were increased after de-endothelization and at the time of the first and the second application were 40.82±6.06% *vs*. 70.59±9.77% and 36.01±7.23% *vs*. 54.05±8.12% of maximal ET-1 contractions respectively (compare *Fig. 1A* and *B*, diamonds). Additionally, the sensitivity of the force of contractions to ghrelin was enhanced: endothelium-denuded preparations respond significantly to 10^{-7} mol *versus* 3.10^{-7} mol ghrelin while endothelium-denuded and TTX-treated - from 10^{-8} mol.

The application of increasing doses of ghrelin to external solution containing either NCDC (50 mol) (*Fig. 2A*, n=4) - a nonselective inhibitor of PLC, or D-609 (10 μmol) - a specific inhibitor of phosphatidylcholine-PLC (PC-PLC) (*Fig. 2B*, n=6), was without effect on the force of contraction. Similar was the result of the pretreatment with U-73122 (3 μmol) - a specific inhibitor of phosphatidylinositol-PLC (PI-PLC). The addition of ghrelin in the presence of U-73122 did not considerably change the force of contraction (*Fig. 2C*, n=5). When 2-APB - a selective and membrane permeable inhibitor of IP_3-induced Ca^{2+} release (100 μmol, n=5), and GF109203x (2 μmol, n=5) - a selective inhibitor of PKC, or pertussis toxin (PTX, 400 nmol) (*Fig. 3F*, n=4) were present in the bath solution, the addition of ghrelin did not significantly modify the force of contraction (*Fig. 2D-F*). The combination of cyclopiazonic acid (10 μmol) - a specific sarco-endoplasmic reticulum Ca^{2+}-ATPase inhibitor, and ryanodine (10 μmol) had a similar effect of inhibition of the ghrelin-dependent influence on the force of contraction (n=3, data not shown). The research into the effect of cyclopiazonic acid and ryanodine was not completed as they inhibited almost entirely the ET-1 induced contraction, which might notably alter the conditions in artery preparations.

The application of increasing concentrations of ghrelin to TTX- and ET-1-containing bath solution failed to influence significantly the force of contraction of endothelium-denuded human mesenteric arteries in the presence of PP2 (10 μmol) - a selective Src family kinase inhibitor (*Fig. 3A*, n=5), U0126 (10 μmol) - a highly selective inhibitor of mitogen-activated protein kinase kinase (MEK) *(Fig. 3B*, n=6), indomethacin (10 μmol) - a nonselective inhibitor of COX 1 and 2 (*Fig. 3C*, n=6), SC-560 - a selective COX-1 inhibitor (100 nmol) or AL-8810 (10 μmol) - a prostaglandin $F_{2\alpha}$ analog introduced as a specific antagonist of the prostaglandin (PG) $F_{2\alpha}$ receptor (FP receptor) (18) (*Fig. 3D*, n=5). In all these experiments the forces of contractions were almost indistinguishable if compared to time controls. Contractions induced by 1 mol $PGF_{2\alpha}$ (n=5, data not shown) also remained unchanged during the application of ghrelin. After that, we studied the participation of thromboxane A_2 pathway in ghrelin signaling using the same experimental design and applying an inhibitor of thromboxane synthase ozagrel (10 μmol, *Fig. 3E*, n=5) and a specific antagonist of T prostanoid (TP) receptor - GR32191B (3 μmol, *Fig. 3F*, n=6). GR32191B

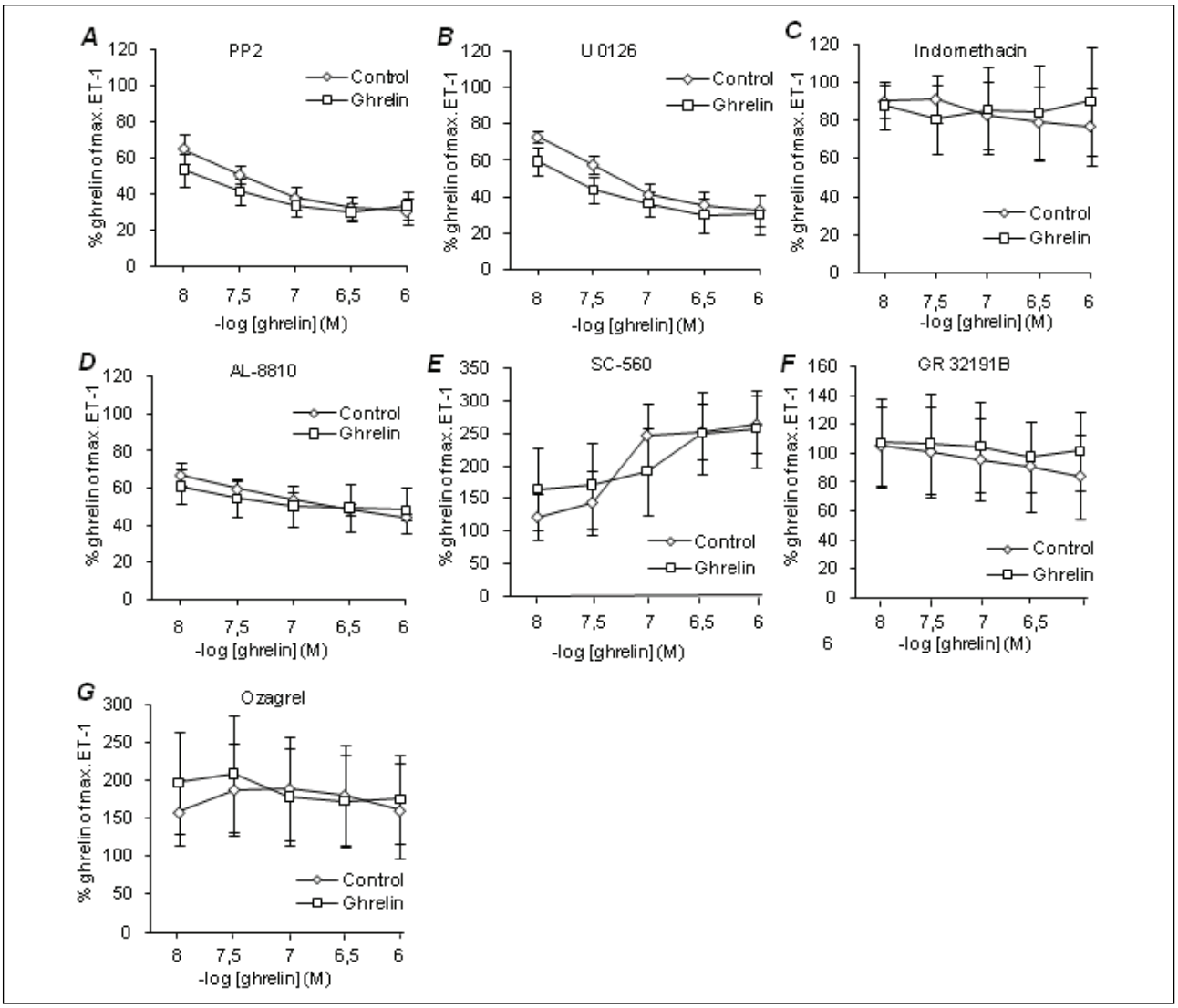

Fig. 3. Blockers of Src kinase, ERK1/2, COX1/2 and FP prostanoid receptor antagonist eliminated the effect of ghrelin. Time-controls of force of contractions of human mesenteric arteries were obtained in bath solutions containing (A) PP2 (10 μmol), (B) U0126 (10 μmol), (C) indomethacin (10 μmol), (D) AL-8810 (10 μmol), (E) SC-560 (100 nmol), (F) GR32191B (3 μmol) and ozagrel (10 μmol) are presented with diamonds. Dose-response relationships of force of contraction after the addition of increasing doses of ghrelin to (A) PP2-, (B) U0126-, (C) indomethacin-, (D) AL-8810-, (E) SC-560-, (F) GR32191B- and (G) ozagrel-containing bath solutions are illustrated with squares.

and ozagrel completely blocked the effect of ghrelin on endothelium-denuded human mesenteric artery preparations in the presence of TTX.

The addition of ghrelin (100 nmol) almost entirely inhibited the iberiotoxin-sensitive $I_{K(Ca)}$ recorded during a 500 ms depolarizing pulse to +40 mV from a holding potential of -50 mV (12). Rp-cAMPS (200 μmol), a specific membrane-permeable inhibitor of PKA, was without effect on the total outward potassium current (I_K) (n=5), while the subsequent addition of ghrelin (100 nmol) decreased I_K to the same degree as in the absence of this PKA inhibitor in single smooth muscle cells from human mesenteric arteries (n=5; $p<0.01$) (*Fig. 4A*). ODQ (5 μmol), a blocker of soluble guanylate cyclase failed to produce any changes in total I_K (n=6) (*Fig. 4B*). Subsequent application of ghrelin (100 nmol) inhibited I_K to the same degree as in ODQ-free bath (n=6; $p<0.01$). Indomethacin is a nonselective inhibitor of COX1/2. The addition of 100 nmol ghrelin to external solution containing 1 μmol indomethacin was without effect on the outward I_K. I_K remained at the same amplitudes as in the presence of indomethacin alone (*Fig. 4C*, n=5). Des-octanoyl ghrelin (100 nmol), an inhibitor of GHS-R1a type ghrelin receptors, was without effect on the total I_K (*Fig. 4D*, n=6). In the presence of des-octanoyl ghrelin, the addition of ghrelin (100 nmol) did not change the total I_K (n=6).

DISCUSSION

Ghrelin and des-octanoyl ghrelin are equipotent antagonists of ET-1 induced vasoconstriction of human mammary artery (13) while in single smooth muscle cells isolated from human mesenteric arteries des-octanoyl ghrelin blocks the ghrelin-induced inhibition of $I_{K(Ca)}$. This difference supposes the operation of more than one ghrelin receptors in human vascular beds - the des-octanoyl ghrelin-blockable GHS-R1a in human mesenteric arteries and another type in human mammary artery (for a review of ghrelin receptors see 19). Kleinz *et al*. (13) routinely applied indomethacin to exclude the possibility of

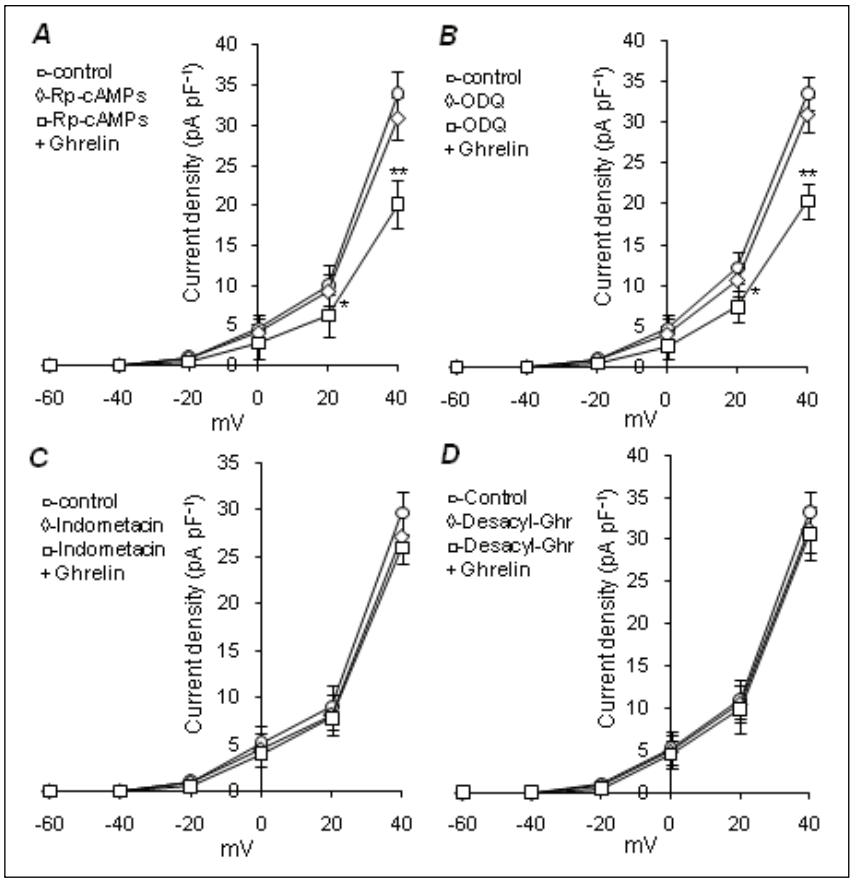

Fig. 4. Effect of inhibitors of PKA, soluble guanylate cyclase, COX1/2 and GHS-R1a on the ghrelin-induced reduction of I_K. *I-V* relationships of peak I_K under control conditions (circles), 14-16 min after the application of Rp-cAMPS (200 μmol) (A), ODQ (5 μmol) (B), indomethacin (1 μmol) (C) or des- octanoyl (des-acyl) ghrelin (100 nmol) (D) (diamonds) and after subsequent addition of 100 nmol ghrelin to (A) Rp-cAMPS-, (B) ODQ-, (C) indomethacin- or (D) des-octanoyl ghrelin-containing bath solutions respectively are presented with squares. Currents were evoked by pulses to test potentials in the range from -60 mV to +40 mV in 20 mV increments with 10 s pulse interval from a holding potential of -50 mV. * - $p<0.05$, ** - $p<0.01$.

endothelium dependent vasodilatation. This treatment however blocks not only endothelial COX1/2 but also those in the tunica media of the artery and thus eliminates the influence of COX1/2 downstream products generated in smooth muscle cells, as suggested by our study. Indeed, such a mechanism is unexpected in blood vessels but is reported in non-vascular smooth muscle (lower esophageal sphincter) that maintains mainly tonic type of contraction similarly to arteries (20). Therefore, it is still difficult to summarize the mechanisms of ghrelin effects on human arteries due to their opposite influences - relaxation and contraction, the different experimental conditions applied and the need for more detailed studies of the intracellular participants.

Most often GHS-R1a interacts with heterotrimeric $G_{q/11}$ proteins and stimulates the $G_{q/11}$/PI-PLC/IP_3+Ca^{2+}+DAG/PKC signaling (2). It was reported that the application of a specific PKC inhibitor entirely abolished the effect of ghrelin on $I_{K(Ca)}$, recorded in single smooth muscle cells of human mesenteric arteries (15). In our study the force of contraction of human mesenteric arteries did not respond to ghrelin application if iberiotoxin or GF109203x were present in the bath solution. Thus, ghrelin requires activation of PKC and suppresses K_{Ca} channels with a large conductance (BK_{Ca} channels) to increase the force of contraction.

Ghrelin regulates cAMP/PKA (3-5) and cGMP/PKG signaling (6), which can further influence ion channels. Han *et al*. (6) reported a ghrelin-induced reduction of voltage-gated I_K of rat anterior pituitary tumor (GH3) cells by a PKG-dependent mechanism and Kohno *et al*. (21) - a ghrelin-induced activation of N-type Ca^{2+} channels that required PKA. BK_{Ca} channels are involved in the formation of the spontaneous artery tone, counteract the elevation of the agonist-induced cytosolic free Ca^{2+} and participate in the relaxation induced by cAMP/PKA- and cGMP/PKG-coupled agonists (22). Therefore, we investigated the participation of both cyclic nucleotides using specific inhibitors Rp-cAMPS for PKA and ODQ for soluble guanylate cyclase. The presence of inhibitors of PKA or soluble guanylate cyclase in the bath solution did not prevent the effect of ghrelin on $I_{K(Ca)}$. We concluded that the second messengers cAMP and cGMP are not involved in the observed ghrelin-induced inhibition of $I_{K(Ca)}$. On the other hand, in human mesenteric arteries the effect of ghrelin on the force of contraction is blocked by pertussis toxin, which suggests the participation of G_i-proteins. In rat islet β-cells ghrelin decreases the insulin secretion by a $G_{\alpha i2}$-protein sensitive activation of voltage-gated K^+ channels and this effect is blocked by D-Lys^3-GHRP-6, a specific GHS-R1a inhibitor (23). Ghrelin inhibits BK_{Ca} channels in the guinea pig femoral artery *via* a pertussis toxin and GHS-R1a sensitive pathway (17). Ghrelin also activates G_i-protein in cell cultures (3, 8). All these data suggest that ghrelin/GHS-R1a/G_i-protein signaling is widespread and important for many tissues including human mesenteric arteries.

Ca^{2+} released from sarcoplasmic reticulum activates BK_{Ca} channels of smooth muscles cells (24). In human mesenteric arteries IP_3-sensitive Ca^{2+} release is essential for the ghrelin-induced decrease of $I_{K(Ca)}$ (15) and for the increase of the force of contraction. Additionally, IP_3-induced Ca^{2+} release from sarcoplasmic reticulum participates in the ET-1 evoked contraction of this vascular bed. Our pharmacological studies suggest that several DAG-producing phospholipases (PI-PLC and PC-PLC) are important in establishing the effect of ghrelin in human mesenteric arteries. We presume that PI-PLC is essential mainly for triggering the contraction (25) and for the IP_3-induced Ca^{2+} release-dependent translocation of PKC to the plasma membrane, while the sustained DAG producer PC-PLC (25) is responsible for the long lasting PKC activation. This suggestion is indirectly supported by the slowly developing inhibition (in 10-14 min) of $I_{K(Ca)}$ by ghrelin in this tissue (15).

Using different inhibitors we reveal several new enzymes participating in the ghrelin effect in human mesenteric arteries. Thus, the selective inhibition of MEK or Src kinase entirely blocks the ghrelin-induced contractions in endothelium-denuded human mesenteric arteries with suppressed neurotransmission. The nonselective COX1/2 inhibitor indomethacin eliminates either the ghrelin-induced constriction of human mesenteric arteries or the ghrelin-induced decrease of I_K in single smooth muscle cells isolated from the same tissue. All these data suggest the existence of a ghrelin-induced and pertussis toxin-sensitive mechanism, which increases the force of contraction by a consequent activation of Src kinase, MEK and ERK. Similar G_i-protein initiated signaling was reported for non-vascular tissues (for review see 26). Next, the stimulated ERK may activate the cytosolic PLA_2 (27), which increases vascular arachidonic acid production - the rate-limiting step for prostaglandin synthesis (28). COX1/2 transform this arachidonic acid into PGE_2, and then PGE_2 into PGH_2, which may further yield contracting prostaglandin or thromboxane, as reported for non-vascular smooth muscle (20). The addition of a selective COX-1 inhibitor blocks the effect of ghrelin on human mesenteric arteries, which may indicate the participation of COX-1 in ghrelin signaling. Next, we tried to identify COX-1 downstream messenger using AL-8810, a $PGF_{2\alpha}$ analog, introduced as a specific FP receptor antagonist (18). However, this pharmacological profile of AL-8810 was later doubted because its concentration of 10 μM, as used in our experiments, significantly decreased the force of contraction of isolated porcine ciliary artery induced by U46619 and only slightly suppressed the $PGF_{2\alpha}$-induced contractions of the same artery (29). For that reason we also applied ozagrel, a selective thromboxane A_2 synthase inhibitor (30) and the TP prostanoid receptor antagonist GR32191B (31). AL-8810, ozagrel and GR32191B inhibit the contractile effect of ghrelin on ET-1-contracted human mesenteric arteries. These data strongly support the suggestion that ghrelin stimulates the synthesis of the main physiological TP prostanoid receptor agonist, thromboxane A_2, into the smooth muscle layer of human mesenteric arteries.

It was reported that ghrelin receptor type GHR-R1a has the ability to oligomerize with prostanoid receptors, when they are transiently over-expressed in human embryonic kidney 293 cells (32). The same authors stated that this co-transfection significantly influenced GHR-R1a activity without changes in its affinity for ghrelin. Similarly, as an alternative explanation of our data, it is suggested that ghrelin first binds to GHS-R1a and then activates prostanoid receptor *via* a direct interaction in the existing GHS-R1a/prostanoid receptor heteromeric complex. If this is the case, the enzymes necessary to demonstrate the effect of ghrelin on the contractile activity (Src kinase, MEK, COX-1 and thromboxane synthase) only support the steady-state thromboxane A_2 production and are not additionally activated by ghrelin. The second explanation of our data, however, seems to be less probable as several new articles report a direct activation of ERK1/2 (3, 7, 33) and Src kinase (34) by ghrelin signaling.

Ghrelin decreases the mean arterial pressure of the rat by a COX-insensitive and NOS-sensitive mechanism (35). In rat mesenteric arteries both ghrelin and desacyl ghrelin evoke endothelium-dependent dilation by NOS- and COX-insensitive mechanism (36). Ghrelin inhibits the contraction of human aortic smooth muscle cells by cAMP/PKA pathway activation (4). Ghrelin and desacyl ghrelin antagonize the ET-1-induced contraction of human internal mammary artery (13). Ghrelin decreases the mean arterial pressure in humans as well (11). On the other hand, contractile effects of ghrelin were reported in guinea pig femoral (37) and renal (17) arteries and in rat coronary artery (38). Ghrelin increases the force of contraction of human mesenteric arteries partially constricted with ET-1. The effect is stronger in endothelium-denuded preparations and most pronounced in endothelium-denuded artery segments with blocked action potential propagation of perivascular neurons. These data point to a relaxing effect of ghrelin *via* endothelium and axonal projections in adventitia, which antagonize the direct and stronger contractile action of ghrelin on the smooth muscle layer of the vascular wall. Additionally, if compared to native preparations during the first half of the experiments, the higher force of contractions of endothelium-denuded human mesenteric arteries suggest that endothelium, as well as perivascular neurotransmission are functional, *i.e.* they are not badly damaged by the therapy before the surgical intervention or during the transportation. It can be concluded that ghrelin either increases or decreases the force of contraction of arteries depending on their type and the species. Thus, ghrelin and desacyl ghrelin may influence the artery resistance similarly to other regulators of the circulation with opposite effects on different vascular beds. For example, catecholamines redistribute the blood flow throughout the body, depending on the physiological needs, *via* different adrenergic receptors and intracellular mechanisms.

In summary, ghrelin has been shown to increase the force of contraction of human mesenteric arteries by a novel mechanism that requires active Src kinase, MEK, COX-1 and thromboxane synthase and that depends on the release of a local mediator - a T prostanoid receptor agonist. Additionally, our data suggest a novel physiological regulation, in which an empty stomach-initiated increase of ghrelin secretion reduces the abdominal circulation in adult humans until next meal.

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