Ghrelin protects musculocutaneous tissue from ischemic necrosis by improving microvascular perfusion

F. Rezaeian,1,2 R. Wettstein,1 C. Scheuer,3 K. Bäumker,1 A. Bächle,3 B. Vollmar,4 M. D. Menger,3 and Y. Harder1,2
1Faculty of Medicine, University of Geneva, Geneva, Switzerland; 2Department of Plastic Surgery and Hand Surgery, Klinikum Rechts der Isar, Technische Universität, Munich; 3Institute for Clinical and Experimental Surgery, University of Saarland, Homburg/Saar; and 4Institute for Experimental Surgery, University of Rostock, Rostock, Germany

Accepted in final form 21 April 2011

Submitted 19 April 2010; accepted in final form 21 April 2011

Ghrelin is a 28-amino acid peptide first discovered in the rat stomach in 1999. It is also produced in the intestine, pancreas, pituitary gland, kidney, and placenta (12). Ghrelin has been identified as the endogenous ligand for growth hormone secretagogue receptors and emerged as the first circulating “hunger hormone” (19). Accordingly, this peptide stimulates food intake and regulates energy balance, metabolic adipose tissue homeostasis, gastric motility, and growth hormone release (1, 22, 30, 33).

Irrespective of its properties in the regulation of nutrition and the release of growth hormones, several studies indicate that ghrelin further exhibits a variety of cardiovascular activities, including the regulation of vascular tone, the improvement of endothelial dysfunction (10, 26), and the protection from ischemia-reperfusion injury and chronic heart failure (17, 21). Whether ghrelin can also prevent tissue necrosis in critically ischemic conditions has not been elucidated yet.

Beside the control of vascular functions, ghrelin has also been demonstrated to exert potent anti-inflammatory (2) and antiapoptotic actions (5). Surgical trauma induces inflammation with endothelial cell swelling and endothelial surface molecule activation, resulting in leukocyte-endothelial cell interactions and compromised microvascular blood perfusion (4, 8). Depending on the extent of surgical trauma and patient comorbidities, this may lead to delayed wound healing, wound dehiscence, and necrosis (20). Because ghrelin and its receptor are also expressed on microvascular endothelial cells and are capable of inducing cell migration, cell proliferation, and neoangiogenesis in vitro (15), the peptide may also prevent tissue necrosis in trauma-induced inflammation.

The aim of the present study was therefore to evaluate the effects and the mechanisms of perioperative ghrelin actions after surgical induction of critically ischemic conditions in musculocutaneous tissue of mice. The role of microcirculatory, inflammatory, cellular, and angiogenic mechanisms were analyzed by intravital epi-illumination fluorescence microscopy and protein profiling using Western blot analysis, including nitric oxide synthase (NOS), vascular endothelial growth factor (VEGF), and nuclear factor-kB (NF-kB).

MATERIALS AND METHODS

Animals. The experiments were conducted in accordance with the Swiss legislation on protection of animals, and the experimental protocol was approved by the Animal Ethics Committee of the canton of Geneva, Switzerland. A total of 62 mice [C57BL/6; 12–24 wk-old; 24–28 g body wt (BW); Zootechnie, Centre Médical Universitaire, Geneva, Switzerland] were used in the study. The animals were housed one per cage at a room temperature of 22–24°C, a relative humidity of 60–65%, and a 12-h day-night cycle. The animals had free access to standard pellet chow (Altrinom, Lage, Germany) and tap water ad libitum.

Anesthesia. The surgical and the repetitive intravital microscopic procedures were performed while the animals were under general anesthesia, induced by intraperitoneal injection of 0.1 ml saline solution per 10 g BW containing 90 mg/kg BW ketamine hydrochloride (Ketavel; Parke Davis, Freiburg, Germany) and 25 mg/kg BW dihydroxydiphenoxazone hydrochloride (Rompun; Bayer, Zürich, Switzerland).

Address for reprint requests and other correspondence: Y. Harder, Department of Plastic Surgery and Hand Surgery, Klinikum Rechts der Isar, Technische Universität, Ismaninger Strasse 22, D-81675 Munich, Germany (e-mail: yves.harder@yahoo.com).

http://www.ajpheart.org

First published December 9, 2011; doi:10.1152/ajpheart.00390.2010.

Induction of persistent ischemia in musculocutaneous tissue. The dorsal skinfold chamber preparation was used for the experiments (18). It includes musculocutaneous tissue, consisting of one layer of skin, subcutaneous tissue, and striated muscle, i.e., panniculus carnosus. This tissue is subjected to acute persistent ischemia, resulting in ~50% necrosis of the final chamber flap surface (7). Briefly, after depilation, an incision measuring 15 mm in width and 11 mm in height was made, leaving the tissue on the lateral side attached to the animal. The resulting rectangular area of tissue-oriented perpendicularly to the spine was fixed to the backside of the chamber’s frame and sutured back to the adjacent skin. Thereafter, the counterpart of the frame was mounted and the observation window was sealed with a coverglass for subsequent microscopy. After the preparation, the animals were allowed to recover from anesthesia and surgery for 24 h. The animals tolerated the surgical preparation and the chamber implantation well, as indicated by normal feeding and sleeping habits.

Intravital epifluorescence microscopy. For in vivo microscopic analysis, anesthetized animals were placed on a custom-made plexiglass frame and received intravenous retrobulbar injection of 0.05 ml FITC-labeled dextran (molecular weight: 150,000; 50 mg/ml saline; Sigma-Aldrich Chemie, Buchs, Switzerland) for intravascular contrast enhancement of the plasma and 0.05 ml rhodamine 6G (0.1 mg/ml saline; Sigma-Aldrich) for in vivo labeling of circulating leukocytes. Subsequently, the animals were positioned under a Zeiss Axiotech microscope (Zeiss, Feldbach, Switzerland) equipped with a 100-W mercury lamp and filter sets for ultraviolet (330- to 390-nm excitation and >430-nm emission wavelength), blue (450– 490 nm and >520 nm), and green (530–560 nm and >580 nm) light. Microscopic images were captured by a charge-coupled device video camera (Kappa CF 112; Zeiss) and transferred to a DVD recorder (Panasonic LQ-MD 800; Lucerne, Switzerland). All parameters were analyzed offline using a computer-assisted image analysis system (Cap-Image; Zeintl Software, Heidelberg, Germany; Ref. 11). Microscopy for analysis of microcirculation and tissue morphology within the striated muscle were performed at constant room temperature of ~23°C. Different objectives (×5, numerical aperture (NA) = 0.16; ×20, NA = 0.50; and ×100, NA = 1.00) were used for recordings.

Microcirculatory and cellular analysis. The window of the chamber was subdivided in three horizontal segments of equal width originating from the tissue’s base that resulted in a proximal, central, and distal area of tissue to be investigated. All parameters were evaluated within each segment.

At each observation time point, the tissue within the chamber’s window was first scanned in ×5 magnification to determine the area of nonperfused and necrotic tissue, respectively. These tissue areas were indicated by the complete lack of blood perfusion, i.e., the absence of intravascular fluorescent dye. The areas were measured planimetrically and are given in percent of the total area of tissue.

Microcirculatory parameters were measured in perfused tissue using FITC-labeled dextran 150,000 (Sigma-Aldrich). Easily identifiable branching patterns of second- or third-order arterioles and accompanying collecting venules and capillary fields were selected. Video printouts of these branching patterns using the ×5 and ×20 objective were made to relocate the same vessels for repetitive measurements during the 10-day observation period. 1) Arteriolar red blood cell velocity (RBCV; mm/s) was analyzed by Cap-Image (Zeintl Software) using the line shift method that is based on the measurement of the shift (mm) of an individual intravascular gray level pattern over time (s). 2) Arteriolar diameter (μm) was measured perpendicularly to the vessel path. 3) Volumetric blood flow (pl/s) was calculated in arterioles from RBCV and vessel cross-sectional area (π * r²) according to the equation of Gross and Aroesty, i.e., Q = V * π * r² assuming a cylindrical vessel geometry (6). Functional capillary density (cm²/cm²) was defined as the length of all RBC-perfused preexisting capillaries per observation field except the newly formed vessels.

The inflammatory response was analyzed by determining the number of rhodamine labeled leukocytes that adhered to the endothelial lining of postcapillary venules for a time period of ≥30 s. Venular leukocyte adherence is expressed as cells per millimeter squared endothelial surface.

With the use of the DNA-binding fluorochrome bisbenzimide H33342 (Sigma-Aldrich), apoptotic cells were identified by the characteristics of nuclear condensation, fragmentation and margination and are given as number per millimeters squared (32).

The microcirculatory analysis further included the evaluation of the angiogenic response, which was analyzed by the density of newly formed blood perfused microvessels. The vessel density was calculated as the length of all newly formed vessels divided by the area of observation and is given in centimeters per centimeters squared.

Western blot analysis. Viable, nonnecrotic skin at day 0, 1, 3, and 5 (n = 4 per group and time point) was harvested. For day 10, samples from two to three animals were pooled (n = 1). Tissue was homogenized in lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X 100, and 0.02% NaN₃), incubated for 30 min on ice, and centrifuged for another 30 min at 16,000 g (4°C). The supernatant was served as whole-protein fraction. Before use, the lysis buffer received a supplemental protease inhibitor cocktail (1:75 vol/ vol; Sigma-Aldrich) and further 0.2 mM PMSF. Protein concentrations were determined using the Lowry assay with BSA as standard (18). Equal amounts of protein per lane (15 μg) were separated discontinuously on 10% sodium dodecyl sulfate polyacrylamide gels under denaturing conditions and transferred to a polyvinylidenefluoride membrane (Bio-Rad, Munich, Germany). After blockade of nonspecific binding sites, membranes were incubated for 4 h with a rabbit polyclonal anti-mouse inducible NOS (iNOS) antibody (1:400; Abcam, Cambridge, UK), a rabbit polyclonal anti-mouse endothelial NOS (eNOS) antibody (1:300; BD Transduction, Heidelberg, Gem- any), a mouse monoclonal anti-mouse MCNA antibody (1:1,000; DAKO Cytomation, Hamburg, Germany), and a rabbit polyclonal antimouse NF-κB antibody (1:400; C20; Santa Cruz Biotechnology, Heidelberg, Germany), and a rabbit polyclonal anti-mouse VEGF antibody (1:75; A20; Santa Cruz Biotechnology). Each was followed by a secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody or sheep anti-mouse IgG antibody (1:5,000; GE Healthcare, Freiburg, Germany) for 1.5 h. Protein expression was visualized using luminol-enhanced chemiluminescence and exposure of membranes to blue light-sensitive autoradiography film (Hyperfilm ECL; GE Healthcare). Signals were densitometrically assessed (Gel-doc; Quantity One software; Bio-Rad) and normalized to β-actin signals (mouse monoclonal anti-β-actin antibody; 1:10,000, Sigma-Aldrich) to correct for unequal loading.

Test substance. Ghrelin (Charles River, WIGA, Sulzfeld, Germany), which has been shown to induce the nitric oxide activity in a dose-dependent manner (31), was admixed to 0.3 ml NaCl 0.9% to achieve a final dose of 40 μg/kg BW. The solution was stored at a temperature of ~8°C and defrosted to room temperature immediately before usage.

N’-nitro-l-arginine methyl ester (l-NAME; Sigma-Aldrich), a l-arginine analog that inhibits the NOS in a nonspecific manner, was dissolved in sterile saline at room temperature to a final dosage of 50 mg/kg BW and administered intravenously.

Experimental groups and protocol. A total of 62 animals were equipped with ischemic tissue within dorsal skinfold chambers. Thirty-two mice were assigned to four experimental groups of eight animals each: 1) ghrelin (40 μg/kg BW ip); 2) l-NAME (50mg/kg BW IV); 3) ghrelin and l-NAME (40 μg ghrelin/kg BW ip and 50 mg l-NAME/kg BW iv); and 4) control (0.3 ml NaCl 0.9% ip). All animals were treated with a total of eight doses of the substances 24, 12, and 0.5 h preoperatively as well as 0.5, 12, 24, 48, and 72 h postoperatively. In case of combined application of ghrelin and l-NAME, l-NAME was given 5 min before ghrelin at each time point. This sequence shall comprise drug administration before and after
induction of ischemia. Intravital fluorescence microscopic analyses of microhemodynamics, inflammation, apoptotic cell death, and angiogenesis were conducted at day 1, 3, 5, 7, and 10 after surgery. All animals were killed at the end of the experiment by administering 1 ml of the anesthetic. Finally, five additional animals each (ghrelin and CON) were killed at day 1, 3, and 5 after induction of ischemia to study the protein expression of iNOS, eNOS, VEGF, PCNA, and NFκB in viable tissue using Western blot analysis.

Surgery, microscopic examinations, as well as off-line analysis of the collected data were performed under blinded conditions.

Statistical analysis. All values are expressed as means ± SE. For comparisons between individual time points, ANOVA for repeated measures was performed, followed by the appropriate post hoc test (SigmaStat, Jandel; San Rafael, CA) that included measures to correct the alpha-error according to Bonferroni probabilities. Comparisons between the groups included ANOVA and the Student-Newmann-Keuls post hoc test. Differences were considered significant at $P < 0.05$.

RESULTS

Tissue necrosis. Macroscopic tissue morphology at day 10 after induction of ischemia of untreated animals displayed a

Fig. 1. Photomicrographs of dorsal skinfold chambers at day 10 after induction of ischemia (A–D). Note the red fringe (double arrowhead), representing a hyperemic response and microvascular remodeling and delineates the distally developed tissue necrosis (*; A). Administration of ghrelin (B) results in increased tissue survival, whereas additional administration of N-nitro-L-arginine methyl ester (L-NAME; C) shows an amount of tissue necrosis comparable to that of controls. Administration of L-NAME alone further increases the amount of tissue necrosis (D). Magnification = ×16. Time course of necrosis in controls (white squares) as well as in animals receiving ghrelin (grey circles), ghrelin and L-NAME (black circles), and L-NAME only (white circles). Note that ghrelin-treated animals show reduced perfusion failure already at day 1 after induction of ischemia compared with controls, resulting in increased tissue survival at day 10 (E); d, days. Data are means ± SE. *$P < 0.05$ vs. controls. †$P < 0.05$ vs. ghrelin.

Fig. 2. Intravital epi-illumination fluorescence microscopy displaying arterio (a)-venular (v) bundles in animals treated with ghrelin (A and B), L-NAME (C and D) or controls (E and F) at day 1 (A, C, and E) and day 10 (B, D, and F). Contrast enhancement with FITC-dextran 150,000. Magnification = ×80. Time course of arteriolar diameters within the critically perfused tissue area (G) of controls (white squares) as well as of animals receiving ghrelin (grey circles), ghrelin and L-NAME (black circles), and L-NAME only (white circles). Data are means ± SE. *$P < 0.05$ vs. controls. †$P < 0.05$ vs. ghrelin.
distinct demarcation between vital tissue proximally and necrosis distally, indicated by a red fringe, i.e., a zone of vasodilation and microvascular remodeling (Fig. 1A). Animals treated with ghrelin showed a considerable reduction of tissue necrosis (Fig. 1B), whereas coadministration of ghrelin and t-NAME did not improve tissue survival compared with saline-treated animals (Fig. 1C). Mice receiving t-NAME alone developed an even increased area of tissue necrosis compared with controls, which was found not significant (Fig. 1D).

At day 1, controls showed an initial microcirculatory perfusion failure of 41 ± 4%. At day 10, persisting microcirculatory failure resulted in a total necrotic tissue area of the final flap surface of 52 ± 2%. Treatment with ghrelin showed already at day 1 after induction of ischemia a significantly smaller area of nonperfused tissue of 8 ± 2% (P < 0.05 vs. control), resulting in a necrotic tissue area of 14 ± 2% at day 10 (P < 0.05; Fig. 1E). Tissue survival after coadministration of ghrelin and t-NAME was comparable to that of controls, whereas administration of t-NAME alone increased the amount of tissue necrosis compared with saline-treated animals.

**Arteriolar perfusion.** Within the critically perfused central tissue area, ghrelin induced a considerable arteriolar and venular dilation compared with controls already at day 1 after induction of ischemia (Figs. 2, A, B, E, F, and G). In contrast, in all animals receiving t-NAME (P < 0.05; Fig. 2G) microvascular diameters remained unaffected (Fig. 2, C and D). In addition, ghrelin also significantly increased RBCV (P < 0.05; Table 1). As a consequence, ghrelin was capable of significantly augmenting arteriolar blood flow after induction of ischemia (P < 0.05, Table 1). Although total arteriolar blood flow increased in all experimental groups during the 10-day observation period, values comparable to those seen in ghrelin-treated animals at day 1 were observed in saline-treated and ghrelin-treated and t-NAME-treated animals at earliest at day 5 to day 7 (Table 1).

**Capillary perfusion.** During the entire 10-day observation period, ghrelin-treated animals showed a ~50–60% higher functional capillary density within the critically perfused tissue compared with saline-treated controls (P < 0.05; Table 1). In contrast, all mice treated with t-NAME demonstrated a significantly lower nutritive perfusion compared with controls (P < 0.05; Table 1).

**iNOS and eNOS expression.** Ghrelin administration induced an upregulation of iNOS, which was found significant at day 1 and 3 after induction of ischemia compared with saline-treated controls (Fig. 3, A and C). In contrast, no significant difference could be observed in eNOS expression between ghrelin-treated animals and the control group over 10 days (Fig. 3, B and C).

**Vascular remodeling and angiogenesis.** Within the critically perfused tissue of saline-treated controls, the capillary architecture did not change considerably, except for the development of capillaries with irregular diameters indicating microvascular remodeling.

![Fig. 3. Western blot analysis of the 10-day time course of inducible nitric oxide synthase (iNOS; A and C) and endothelial nitric oxide synthase (eNOS; B and C) expression after induction of ischemia in saline-treated controls (Con, white bars) and ghrelin-treated animals (Ghr, grey bars). Note the significantly increased iNOS expression during the early time course (A) and the higher eNOS expression during the later time course (B and C) after ghrelin treatment compared with controls. Results from day 10 are from a separate experiment and bars represent a single measurement (A, B, and C). Data are means ± SE. *P < 0.05 vs. controls.

### Table 1. Microhemodynamic parameters within the critically perfused tissue area

<table>
<thead>
<tr>
<th>Group</th>
<th>1 Day</th>
<th>3 Days</th>
<th>5 Days</th>
<th>7 Days</th>
<th>10 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.54 ± 0.11</td>
<td>0.75 ± 0.16</td>
<td>0.95 ± 0.16</td>
<td>1.17 ± 0.2</td>
<td>1.07 ± 0.17</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>1.01 ± 0.14*</td>
<td>1.17 ± 0.11*</td>
<td>1.34 ± 0.07*</td>
<td>1.43 ± 0.07*</td>
<td>1.54 ± 0.08*</td>
</tr>
<tr>
<td>Ghrelin and t-NAME</td>
<td>0.47 ± 0.16†</td>
<td>1.00 ± 0.09†</td>
<td>0.99 ± 0.12†</td>
<td>1.18 ± 0.12†</td>
<td>1.31 ± 0.01†</td>
</tr>
<tr>
<td>t-NAME</td>
<td>0.49 ± 0.15†</td>
<td>0.71 ± 0.13†</td>
<td>0.78 ± 0.15†</td>
<td>0.82 ± 0.16†</td>
<td>0.70 ± 0.16†</td>
</tr>
</tbody>
</table>

Values are means ± SE. t-NAME, Nω-nitro-l-arginine methyl ester. *P < 0.05 vs. control. †P < 0.05 vs. ghrelin.
vascular remodeling. Ghrelin treatment was associated with induction of new capillaries, which were first observed at day 3 to 5 and which were found developing from parallel running striated muscle capillaries (Fig. 4B). Additional L-NAME application only partially counteracted ghrelin induced angiogenesis (Fig. 4C). In contrast, in saline-treated mice and L-NAME-treated animals the formation of new microvascular structures could not be observed (Fig. 4, A and C).

**VEGF and PCNA expression.** VEGF expression was found marginally increased in ghrelin treated mice before induction of ischemia at day 0, compared with untreated controls. Yet, the ghrelin-induced VEGF upregulation at day 1, 3, and 5 was found to be statistically not significant. This increase of VEGF vanished at day 10 after induction of ischemia (Fig. 4, D and F).

Ghrelin-treated animals showed a significantly increased PCNA expression only at day 5, where angiogenesis was found in all ghrelin-treated mice. Saline-treated animals displayed a continuous decrease of PCNA expression over the 10-day observation period (Fig. 4, E and F).

**Leukocyte adherence.** In controls, persistent ischemia induced a considerable number of adhering leukocytes to the microvascular endothelium. All animals receiving ghrelin, either alone or together with L-NAME, demonstrated a significantly reduced leukocyte-endothelial interaction during the entire 10-day study period (P < 0.05; Fig. 5A). In contrast, L-NAME treatment alone could not attenuate leukocyte adhesion compared with saline-treated controls (Fig. 5A).

**Apoptotic cell death and NF-κB expression.** Ghrelin treatment significantly reduced apoptotic cell death within the critically ischemic tissue compared with saline-treated controls (P < 0.05; Fig. 5B). Additional administration of L-NAME did not affect this protective effect. In contrast, L-NAME alone showed comparably high numbers of apoptotic cells as observed in saline-treated control animals (Fig. 5B). The slightly increased expression of NF-κB in ghrelin treated mice was found statistically not significant, compared with untreated controls (Fig. 5, C and D).

**DISCUSSION**

The herein used model of persistent ischemia of musculocutaneous tissue is associated with a marked deterioration of capillary perfusion, resulting in ~50% tissue necrosis of the visible flap surface 10 days after surgery. The present study identifies ghrelin for the first time as a substance capable of protecting musculocutaneous tissue from ischemia-induced necrosis without reperfusion and reoxygenation. The tissue-protective effect is predominantly based on microcirculatory actions, mediated by the early upregulation of iNOS. This resulted in early arteriolar dilation, which maintained nutritive capillary perfusion, improving the survival of the critically perfused, ischemic tissue. Ghrelin also induced VEGF-associated neovascularization and attenuated the ischemia-associated leukocytic inflammatory response. However, these mechanisms may not have contributed to the ghrelin-mediated improvement of tissue survival, because the NOS blockade by L-NAME completely blunted the ghrelin-associated protection on tissue survival without significantly affecting the angiogenic and the anti-inflammatory actions.

Microhemodynamics including diameters, RBCV, and functional densities of the proximal area of the flap were similar to those observed within the proximal, central, and distal area of nonischemic chambers (7, 24). Treatment with ghrelin started 24 h before surgical induction of ischemia and continued until 3 days postoperatively, limiting tissue necrosis to only ~14%. Ghrelin provoked arteriolar dilation and increased arteriolar...
blood flow, guaranteeing adequate nutritive capillary perfusion. Ghrelin also upregulated iNOS and eNOS in an overlapping manner, whereas only the expression of iNOS was significant. The fact that blockade of NOS by L-NAME almost completely blunted the ghrelin-mediated improvement of both the microcirculation and tissue survival indicates that the protective action of ghrelin in critical ischemia is caused by the NO-mediated arteriolar dilation. These findings that have not been demonstrated so far in musculocutaneous tissue undergoing persistent ischemia are in line but extend the knowledge on the actions of ghrelin achieved from previous studies in bovine aortic endothelial cells (9) and the stomach (27). A NO-dependent dilatory effect of ghrelin has been reported both in vitro and in vivo in human umbilical veins and bovine aortas (34). Also, the ghrelin-induced upregulation of eNOS has been demonstrated to attenuate the development of pulmonary hypertension as well as the endothelial dysfunction in patients with metabolic syndrome (25, 29). Konturek et al. (13) have additionally shown that exogenous ghrelin administration protects gastric mucosa from posts ischemic reperfusion-induced lesions and also suggested that this protection is caused by NO-mediated hyperemia (13). Our in vivo experiments with repetitive intravital microscopy over almost 2 wk and NOS blockade now demonstrate that ghrelin is also protective in persistent ischemia of the skin without reoxygenation injury and that it acts by NO-mediated arteriolar dilation.

Angiogenesis was observed in all animals receiving ghrelin treatment, whereas in saline-treated controls and animals receiving L-NAME only newly formed microvessels could not be detected. This suggests that the persistent ischemic stimulus alone was not capable of inducing angiogenesis in this model. However, angiogenesis was associated with an increased VEGF expression that was observed even before the induction of ischemia. This association between ghrelin and VEGF upregulation has recently also been demonstrated in peritoneal fluid of patients with endometriosis (3). Further, Konturek at al. (13) reported an association between ghrelin-induced gas troprotection and VEGF-mediated angiogenesis based on the induction of the HIF-1α pathway. In fact, Li et al. (15) and Konturek et al. (13) were able to demonstrate angiogenesis after exogenous administration of ghrelin; however, these effects were demonstrated only in vitro. In the present study, we demonstrate for the first time that ghrelin induces new microvessels in vivo and that this angiogenic response is paralleled by an increased expression of PCNA at day 5 after surgery. So far, the involvement of ghrelin in cell proliferation associated with an increased PCNA expression has only been shown in nonmammalian species such as the chicken ovary (28). We could elucidate the role of ghrelin-mediated angiogenesis in the improvement of tissue survival. The additional administration of L-NAME in ghrelin-treated animals only minimally reduced the ghrelin-induced angiogenic response; however, it completely blunted the improvement of tissue survival. Thus the ghrelin-induced neovascularization, due to
its delayed appearance, is not the cause for the observed tissue protection.

Further, surgical trauma and induction of ischemia induced a considerable inflammatory response, reflected by an increased number of leukocytes adhering to the endothelium of postcapillary venules and an increased number of apoptotic cells. Li et al. (16) have previously demonstrated an anti-inflammatory property of ghrelin that results from inhibition of mononuclear cell binding and endotoxin-induced cytokine production in human endothelial cells in vitro and in rats in vivo. In the present study, we could confirm a NO-independent reduction of the inflammatory response after ghrelin treatment. We hypothesized a reduced cytokine response due to a down-regulation of NF-kB. Surprisingly, our protein profiling revealed an upregulation of NF-kB after induction of ischemia, which was not significant; thus a correlation between reduced inflammation and increased NF-kB expression remains unclear. However, the experiments with NOS blockade by L-NAME demonstrate that L-NAME although not abrogating the anti-inflammatory and antiapoptotic effect of ghrelin completely blunts the protection from tissue necrosis. Accordingly, the tissue protection of ghrelin in persistent ischemia is not mediated by its anti-inflammatory and antiapoptotic property. However, the coadministration of ghrelin and L-NAME similarly abrogated nutritive perfusion compared with L-NAME administration alone. Yet, coadministration showed a survival benefit of almost 10% compared with L-NAME administration alone. This nonsignificant survival benefit may indeed result from a combined ghrelin-induced NO-independent early anti-inflammatory and delayed angiogenic response, which could not be counteracted by inhibition of the NOS.

In conclusion, this functional study demonstrates that perioperative administration of ghrelin significantly improves survival of critically ischemic musculocutaneous tissue. This benefit is based on a NO-mediated arteriolar dilation that increases a sustained microcirculatory blood flow respectively capillary perfusion. We conclude that ghrelin may represent a simple noninvasive therapeutic approach to perioperatively protect musculocutaneous tissue from ischemic necrosis.

ACKNOWLEDGMENTS

We thank Freya Sandmann (Division of Plastic, Reconstructive and Esthetic Surgery, Geneva University Hospitals, Geneva, Switzerland) and Jean-François Egger (Department of Pathology, Geneva University Hospitals, Switzerland) for excellent technical assistance during the experiments.

GRANTS

This work was supported by the Swiss National Science Foundation (SNF-No. 3200BO-108408; to Y. Harder and R. Wettstein).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00390.2010 • www.ajpheart.org


