Growth-related oncogene-α induces endothelial dysfunction through oxidative stress and downregulation of eNOS in porcine coronary arteries

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Bechara C, Wang X, Chai H, Lin PH, Yao Q, Chen C. Growth-related oncogene-α induces endothelial dysfunction through oxidative stress and downregulation of eNOS in porcine coronary arteries. Am J Physiol Heart Circ Physiol 293: H3088–H3095, 2007. First published September 14, 2007; doi:10.1152/ajpheart.00473.2007.—Growth-related oncogene-α (GRO-α) is a member of the CXC chemokine family, which is involved in the inflammatory process including atherosclerosis. We hypothesized that GRO-α may affect endothelial functions in both porcine coronary arteries and human coronary artery endothelial cells (HCAECs). Vasomotor function was analyzed in response to thromboxane A2 analog U-46619 for contraction, bradykinin for endothelin-dependent vasorelaxation, and sodium nitroprusside (SNP) for endothelium-dependent vasorelaxation. In response to 10−8 M bradykinin, GRO-α (50 and 100 ng/ml) significantly reduced endothelin-dependent vasorelaxation by 34.73 and 48.8%, respectively, compared with controls (P < 0.05). There were no changes in response to U-46619 or SNP between treated and control groups. With the lucigenin-enhanced chemiluminescence assay, superoxide anion production in GRO-α-treated vessels (50 and 100 ng/ml) was significantly increased by 50 and 86%, respectively, compared with controls (P < 0.05). With real-time PCR analysis, endothelial nitric oxide synthase (eNOS) mRNA levels in porcine coronary arteries and HCAECs after GRO-α treatment were significantly decreased compared with controls (P < 0.05). The eNOS protein levels by both immunohistochemistry and Western blot analyses were also decreased in GRO-α-treated vessels. Antioxidant seleno-L-methionine and anti-GRO-α antibody effectively blocked these effects of GRO-α on both porcine coronary arteries and HCAECs. In addition, GRO-α immunoreactivity was substantially increased in the atherosclerotic regions compared with nonatherosclerotic regions in human coronary arteries. Thus GRO-α impairs endothelium-dependent vasorelaxation in porcine coronary arteries through a mechanism of overproduction of superoxide anion and downregulation of eNOS. GRO-α may contribute to human coronary artery disease.

chemokine; endothelial dysfunction; superoxide anion; antioxidant; seleno-L-methionine; coronary artery; atherosclerosis

GROWTH-RELATED ONCOGENE-α (GRO-α) is a 73-amino acid peptide initially isolated and characterized from malignant melanoma cells for its growth stimulatory activity (2, 33). Another name for GRO-α is melanoma growth stimulatory activity. Further studies indicate that many cell types and tissues also express GRO-α. GRO-α is referred to CXCL1 (CXCL1), which binds to chemokine receptor CXCR2 (28). GRO-α was shown to induce chemotaxis of neutrophils (16), T lymphocytes (21), and monocytes (36). The ability of GRO-α to trigger leukocyte chemotaxis was also demonstrated in mice using a mouse homologue of GRO-α, known as keratinocyte-derived chemokine (5).

High GRO-α levels were detected in aneurismal aortic tissues (31), and serum levels of GRO-α were increased in several inflammation conditions (24, 40). Since atherosclerosis is a chronic inflammatory process, GRO-α may contribute to this process. It has been shown that macrophages in atherosclerotic lesions express CXCR2, the GRO-α receptor (4). In addition, chimeric mice deficient in leukocyte CXCR2 had less atherosclerosis (4). On the other hand, GRO-α can induce endothelial cell chemotactic and proliferative activity in vitro and angiogenesis in vivo (25, 38). GRO-α is also involved in the thrombin-induced angiogenesis (7). Taken together, GRO-α may contribute to vascular lesion formation including atherosclerosis.

Theory of endothelial injury and endothelial dysfunction leading to atherosclerosis was introduced by Dr. Ross in the 1990s (34). Multiple causes of endothelial dysfunction have been proposed (13). Nitric oxide (NO) is a critical molecule in vascular physiology. It causes smooth muscle relaxation, inhibits platelet aggregation, and plays an important role in angiogenesis (12). NO is produced by three isoforms of the enzyme nitric oxide synthase (NOS), and endothelial NOS (eNOS) is constitutively expressed in normal endothelial cells. In pathological conditions, NO bioavailability can be reduced by either eNOS inhibition or depletion by chemical reaction with reactive oxygen species (ROS) such as superoxide anion. These could happen either exclusively or simultaneously in humans, resulting in endothelial dysfunction (27).

In the present study, we hypothesized that GRO-α may affect endothelial functions. To test this hypothesis, we studied the effects of GRO-α in porcine coronary arteries as well as human coronary artery endothelial cells (HCAECs). This study may suggest new biological functions and mechanisms of GRO-α in the vascular system, which could contribute to human coronary artery disease.

MATERIALS AND METHODS

Chemicals and reagents. DMSO, thromboxane A2 analog U-46619, bradykinin, sodium nitroprusside (SNP), seleno-L-methionine (SeMet), and TRI-reagent kit were obtained from Sigma Chemical (St. Louis, MO). DMEM was obtained from Life Technologies (Grand Island, NY). Recombinant human GRO-α and anti-GRO-α antibody were obtained from Biosource International (Camarillo, CA). Lucigenin was obtained from Molecular Probes (Eugene, OR). Antibody against human eNOS was obtained from BD Transduction Laboratories.
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Tissue harvest and cell culture. Fresh porcine hearts were harvested from farm pigs (6- to 8-mo-old males) at a local slaughterhouse as previously described from our studies (8, 9, 26). Briefly, porcine right coronary arteries were carefully dissected and cut into 5-mm rings. Several rings from each heart were allocated into groups: controls (DMEM), those treated with GRO-α (5, 50, and 100 ng/ml), and those treated with 50 ng/ml of GRO-α plus antioxidant SeMet (20 μM). Recombinant human GRO-α is produced in Escherichia coli, and the amount of endotoxin in the GRO-α vial is <0.1 ng/μl. To rule out potential effect of endotoxin, heat-inactivated GRO-α at 95°C for 30 min was used.

HCAECs were purchased from Cambrex (San Diego, CA). The cells were used at passages 6 to 7. Once cells grew to 80–90% confluence in 6-well culture plates, they were treated with DMEM as control or with GRO-α (5, 50, 100 ng/ml) for 24 h at 37°C. Cells were then harvested, and total mRNA was extracted for real-time polymerase chain reaction (PCR) study.

Myograph analysis. The myograph tension system used in our laboratory has been previously described (8, 9, 26). Briefly, the rings were cut open longitudinally and trimmed to fit between the wires of the organ bath chamber (Multi myograph system). The vessel segments were placed endothelium side down in the tubes containing the Krebs solution. After equilibration, each ring was precontracted with 20 ng/l of SNP (10 μM). After 60–90 min of contraction, the relaxation concentration-response curve was generated by adding 60 μl of five cumulative additions of the endothelium-dependent vasodilator bradykinin (10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, and 10⁻¹⁰ M) every 3 min. In addition, 60 μl of SNP (10⁻⁶ M) was added to the organ bath, and endothelium-independent vasorelaxation was recorded.

Detection of superoxide anion. Levels of superoxide anion produced by endothelial cells were detected using the lucigenin-enhanced chemiluminescence method as previously described in our studies (8, 9, 26). Briefly, the rings were cut open longitudinally and trimmed into 5 × 5-mm pieces. An assay tube (12 × 75 mm) was filled with 500 μl of buffer and 25 μl of lucigenin (final concentration 5 μM). The vessel segments were placed endothelium side down in the tubes so signals from the endothelial layer could be recorded. Time-based reading of the luminometer was recorded. The data, in relative light units per second for each sample, were averaged between 5 and 10 min. Values of blank tubes containing the same reagents as the vessel ring samples were subtracted from their corresponding vessel samples. The area of each vessel segment was measured with a caliper and was used to normalize the data for each sample. Final data were presented as RLU per second per square millimeter.

Real-time PCR. Endothelial cells of porcine coronary arteries were collected by scraping the luminal surface with surgical blades. HCAECs were collected from the cultures by trypsin digestion. Total RNA was isolated by use of Tri-Reagent following the manufacturer’s instructions. Both porcine and human eNOS mRNA levels were determined by real-time PCR analysis as previously described in our studies (8, 9, 26). Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units vs. PCR cycle number during exponential amplification so that sample measurement comparisons were possible. The eNOS mRNA levels in each sample were calculated as 2^(ΔΔCt) and further normalized to GAPDH expression as [2^(ΔΔCtGAPDH) – ΔΔCt(ΔCtGAPDH)]

Western blot. Endothelial cells of porcine coronary artery rings were collected. Proteins were extracted with cell lysis buffer (Cell Signaling Technology, Danvers, MA). Equal amounts of total proteins (50 μg) were loaded onto 10% SDS-PAGE, fractionated by electrophoresis, and transferred to polyvinylidene difluoride membranes. The membrane was incubated with the primary anti-human eNOS antibody (1:2,000) at 4°C overnight. Bands were visualized with ECL plus Chemiluminescent Substrate (Amersham Biosciences). Band density was measured to quantify the relative expression of target proteins vs. β-actin (ImageJ software).

Immunohistochemistry. For detecting eNOS expression, treated or control porcine coronary artery rings were fixed in 10% neutral buffered formalin and embedded in paraffin. Cross sections were stained with monoclonal antibody against human eNOS (1:1,000) by avidin-biotin complex immunoperoxidase procedure (8, 9, 26). For detecting GRO-α expression, full-thickness arterial wall specimens of coronary arteries were obtained from four patients with or without atherosclerosis undergoing autopsy (National Disease Research Interchange, Philadelphia, PA). All samples were fixed in formalin and embedded in paraffin. Immunohistochemistry was done with anti-human GRO-α antibody (1:200) (Sigma), biotinylated secondary antibody, and avidin-biotin reaction using peroxidase enzyme (ABC kit; Vector Laboratories). Images were captured under microscope with an attached SPOT-KT digital camera and software (Diagnostic Instruments, Sterling Heights, MI).

Statistical analysis. Statistical analysis was performed on the Data Analysis tool of the Microsoft Excel program (Microsoft Office 2003, Microsoft, Seattle, WA). Data were expressed as means ± SE. Significant differences between means from the control and treated groups were determined by the paired Student’s t-test (two-tail). The final data points of all contractions and relaxations among different groups were also analyzed by ANOVA test. P < 0.05 was considered statistically significant.

RESULTS

GRO-α decreases endothelium-dependent vasorelaxation in porcine coronary arteries. Porcine coronary artery rings were cultured overnight with DMEM as control or treated with different GRO-α concentrations (5, 50, and 100 ng/ml). Contraction was achieved by using the thromboxane analog U-46619. The maximum contraction in all groups did not show statistical differences compared with controls (Fig. 1A). The endothelium-dependent vasorelaxation was induced by cumulative concentrations of bradykinin (Fig. 1B). For example, GRO-α treatment (50 and 100 ng/ml) significantly reduced the vasorelaxation in response to bradykinin (10⁻⁶ M) by 34.73 and 48.8%, respectively, compared with untreated vessel rings (Fig. 1C). Finally, the endothelium-independent vasorelaxation was induced with SNP (10⁻⁶ M). There were no changes in vasorelaxation among all GRO-α-treated groups compared with controls (Fig. 1D).

GRO-α increases superoxide anion production in porcine coronary artery endothelial cells. To determine whether oxidative stress could be involved in GRO-α-induced endothelial dysfunction, superoxide anion levels were detected by using the lucigenin-enhanced chemiluminescence assay. Porcine coronary artery rings were incubated overnight with DMEM (control) and different GRO-α concentrations (5, 50, and 100 ng/ml). GRO-α at 50 and 100 ng/ml significantly increased superoxide anion production by 50% and 86%, respectively, compared with controls (P < 0.05). GRO-α at 5 ng/ml did not reach statistical significance compared with controls (Fig. 2A). Superoxide anion production was almost completely reversed using SeMet (20 μM), and neither SeMet alone nor the heat-inactivated GRO-α (50 ng/ml) showed any increase in superoxide anion production compared with controls (Fig. 2B).

GRO-α reduces eNOS expression in both porcine coronary arteries and HCAECs. Reducing eNOS and NO availability could be the important mechanism in endothelial dysfunction. Porcine coronary artery rings and HCAECs were cultured overnight with different GRO-α concentrations (5, 50, and 100 ng/ml)
ng/ml). The eNOS mRNA expression was quantified with real-time PCR and normalized to GAPDH mRNA levels, which had no significant changes in response to any experimental conditions such as treatment of GRO-α. The eNOS mRNA expression was decreased in a concentration-dependent fashion. In porcine coronary arteries, GRO-α at 50 and 100 ng/ml significantly decreased eNOS mRNA expression by 25 and 37%, respectively, compared with controls ($P < 0.05$).

**Fig. 1.** Effects of growth-related oncogene-α (GRO-α) on vasomotor functions in porcine coronary arteries. A: maximum contraction. Artery vessel rings were cultured overnight with or without different GRO-α concentrations. Contraction was achieved by use of a thromboxane analog, U-46619 ($10^{-7}$ M). The maximum contraction in all treated groups had no statistical difference compared with controls. B: endothelium (EC)-dependent vasorelaxation. The precontracted vessels were induced vasorelaxation by cumulative concentrations of bradykinin (from $10^{-9}$ to $10^{-6}$ M). GRO-α groups of 50 and 100 ng/ml showed a statistically significant reduction in vasorelaxation, in a concentration-dependent manner ($^*P < 0.05$, $^{**}P < 0.01$, $n = 6$, $t$-test). C: endothelium-dependent vasorelaxation in response to bradykinin ($10^{-6}$ M). GRO-α treatment (50 and 100 ng/ml) significantly reduced the vessel response to bradykinin by 34.73 and 48.8%, respectively, compared with untreated vessel rings ($^{**}P < 0.01$, $n = 12$, $t$-test). D: endothelium-independent vasorelaxation in response to sodium nitroprusside (SNP, $10^{-6}$ M). There was no change in vasorelaxation among all groups compared with controls. Data are expressed as means ± SE.

**Fig. 2.** Effects of GRO-α and seleno-L-methionine (SeMet) on superoxide anion production in porcine coronary arteries. A: superoxide anion levels were detected by the lucigenin-enhanced chemiluminescence assay. The data were adjusted per vessel ring area (mm$^2$) and expressed as relative light units (RLU) s$^{-1}$ mm$^{-2}$. Vessel rings were incubated overnight with or without GRO-α. GRO-α (50 and 100 ng/ml)-treated vessels showed an increase of superoxide anion production by 50 and 86% compared with controls ($^*P < 0.05$, $^{**}P < 0.01$, $n = 6$, $t$-test). B: heat inactivation and antioxidant SeMet. Porcine coronary artery rings were incubated overnight with or without GRO-α, heat-inactivated GRO-α, SeMet (20 μM), or 1:1 volume of SeMet: GRO-α. The GRO-α (50 ng/ml) group reached statistical significance compared with controls ($^*P < 0.05$, $n = 6$, $t$-test). The addition of SeMet almost completely reversed the GRO-α effect. Heat-inactivated GRO-α did not increase superoxide anion production compared with controls. GRO-α at 5 ng/ml did not show a significant effect. Heat-inactivated GRO-α (50 ng/ml) did not affect eNOS mRNA expression (Fig. 3A). By addition of anti-GRO-α antibody at 1:1 concentration to GRO-α, eNOS mRNA levels were re-

**Fig. 3A.** GRO-α induces endothelial dysfunction.
versed to the control level (Fig. 3B). In HCAECs, GRO-α at 50 and 100 ng/ml decreased eNOS mRNA expression by 36 and 48%, respectively, compared with controls (*P < 0.05). GRO-α at 5 ng/ml had no effect. Heat-inactivated GRO-α (50 ng/ml) did not show any decrease compared with controls (Fig. 3C). Anti-GRO-α antibody completely reversed the GRO-α-induced eNOS downregulation (Fig. 3D). Immunoreactivity of eNOS of the endothelial cells of GRO-α-treated vessel rings was substantially reduced compared with the control and to the group of heat-inactivated GRO-α, whereas SeMet could effectively block this effect of GRO-α (Fig. 4A). By Western blot analysis, GRO-α (50 ng/ml) treatment for 24 h significantly reduced eNOS protein levels by 40.68% in porcine artery rings compared with untreated vessels (*P < 0.05, Fig. 4B).

Antioxidant SeMet effectively blocks GRO-α-induced endothelial dysfunction in porcine coronary arteries. Antioxidant SeMet is known to increase the activity of glutathione peroxidase in endothelial cells (22). To further confirm that oxidative stress is involved in GRO-α-induced endothelial dysfunction in porcine coronary arteries, SeMet was included in the vasomotor analysis (Fig. 5). Consistent with the data in Fig. 1, GRO-α at 50 ng/ml significantly decreased endothelium-dependent vasorelaxation in response to bradykinin compared with controls (*P < 0.05). SeMet (20 μM) almost completely blocked this effect of GRO-α. Heat-inactivated GRO-α (50 ng/ml) did not affect endothelium-dependent vasorelaxation (Fig. 5). There were no significant differences of vessel contraction (U-46619) and endothelium-independent vasorelaxation (SNP) (data not shown).

Increased expression of GRO-α in atherosclerotic regions of human coronary arteries. GRO-α was strongly expressed in atherosclerotic regions of human coronary arteries. However, nonatherosclerotic regions of the arteries showed no immunoreactivity of GRO-α. The increased signal of GRO-α was mainly located in intima and media areas of atherosclerotic plaques (Fig. 6).

DISCUSSION

Chemokines are gaining a worldwide attention for their roles in atherosclerosis, angiogenesis, tumor metastasis, and other inflammatory conditions (25). GRO-α may have potential effects on vascular disease formation because of its leukocyte recruitment to lesion sites. This is the first study, to our knowledge, demonstrating the clear association between GRO-α and endothelial dysfunction of porcine coronary artery endothelial cells via increased superoxide anion production and...
decreased eNOS expression. These effects were effectively reversed when SeMet was added to GRO-α and also when GRO-α was blocked with anti-GRO-α antibody.

The biological activity of GRO-α was previously determined by measuring the concentration-dependent mobilization of intracellular calcium (calcium flux) with human neutrophils (10). Significant calcium mobilization was observed with ≥1 ng/ml of recombinant human GRO-α. Information about porcine GRO-α is not available currently, and there are no studies testing the effects of human recombinant GRO-α on porcine arteries. Plasma levels of GRO-α have been reported to be 71–87 pg/ml in human healthy individuals (6, 23). However, these levels are significantly increased in many inflammation conditions (24, 40) such as acute pancreatitis (up to 3,195 pg/ml) (37). Inflammatory tissues may have much higher local levels of GRO-α than its plasma levels. Since atherosclerosis is a chronic inflammatory process, high GRO-α expression at vascular lesion sites may contribute to the atherogenic process. Indeed, Middleton et al. (31) recently reported that high expression of GRO-α was detected in aneurismal aortic tissues. In the present study, we studied GRO-α expression in human coronary arteries by immunohistology analysis. We found that GRO-α immunoreactivity was substantially increased in the atherosclerotic regions compared with nonatherosclerotic regions of human coronary arteries. However, exact tissue concentrations of GRO-α in any inflammatory condition are unknown. Three concentrations of GRO-α (5, 50, and 100 ng/ml) were used in the present study. Although these concentrations are much higher than its plasma levels in humans, they may be close to its tissue levels at the inflammatory sites and contribute

Fig. 4. Effect of GRO-α on eNOS protein levels in porcine coronary arteries. A: immunoreactivity. a: DMEM control showing a positive staining pattern of eNOS on the arterial wall endothelial layer. b: GRO-α (50 ng/ml) showing a significant reduction of the staining pattern of eNOS compared with the control. c: GRO-α (50 ng/ml) mixed with SeMet (20 μM) showing almost same immunostaining pattern seen in the control. d: heat-inactivated GRO-α (50 ng/ml) showing the same immunostaining seen in the control. B: Western blot. The vessel rings treated with GRO-α (50 ng/ml) for 24 h significantly decreased eNOS protein levels compared with untreated vessels (*P < 0.05, n = 3, t-test).
to vascular disease process. In our in vitro study, we only observed GRO-α-induced endothelial dysfunction for a short duration (24 h), whereas in chronic inflammatory conditions in humans, high levels of GRO-α could be increased and sustained for a long time, and GRO-α could have an accumulated effect on vascular damage.

Our study clearly demonstrates the effects of GRO-α on porcine coronary arteries as well as human coronary artery endothelial cells. Vasomotor dysfunction and response to pharmacological agents have been studied extensively in our laboratory. In the present study, GRO-α impaired endothelium-dependent vasorelaxation compared with controls in a concentration-dependent manner. The control used was DMEM, which is the same medium used to incubate GRO-α with the arteries. GRO-α had no effect on SNP-induced vasorelaxation or maximum contraction, indicating that its activity is mainly endothelium-dependent.

It is well documented that NO causes vasorelaxation as well as many other biological functions. Oxidative stress via oxygen free radical production such as superoxide anion reacts with NO resulting in its depletion and eventually leading to atherosclerosis (3). Oxidative stress was one of the mechanisms we explored to explain the endothelium-dependent vasomotor dysfunction caused by GRO-α. There was an increase by 86% with maximum GRO-α concentration (100 ng/ml) compared with the control. Furthermore, adding antioxidant SeMet (20 μM) effectively reversed the production of superoxide anion as well as blocking the effect of GRO-α on endothelial vasomotor dysfunction. This clearly shows that GRO-α, by producing superoxide anion production, depletes NO, thereby contributing to endothelial dysfunction. In addition to NO depletion, we tested the effects of GRO-α on the expression of eNOS, which is an enzyme that generates NO. It is well known that impaired eNOS activity could influence endothelial dysfunction and myocardial disease (30). In both porcine coronary arteries and HCAECs, eNOS expression was decreased in a concentration-dependent fashion compared with controls. These effects were reversed by blocking GRO-α 50 ng/ml with anti-GRO-α antibody at 1:1 concentration. These data clearly show that GRO-α not only decreases NO availability via superoxide anion production, but also by inhibiting eNOS expression. We noticed that GRO-α had a more potent effect on eNOS downregulation in HCAECs than in porcine coronary arteries. This could be due to GRO-α being a human recombinant peptide. In addition to decreasing eNOS mRNA levels in GRO-α-treated cells and vessels, decreased eNOS protein levels in the porcine coronary endothelium was depicted by immunohistochemical staining.
and Western blot analysis, which was also reversed by antioxidant SeMet.

As experimental design, it was hypothesized that GRO-α could reduce eNOS expression and functions, which were demonstrated by mRNA/protein levels and endothelium-dependent response to bradykinin, respectively. It is unknown whether GRO-α could affect the expression and functions of other vasoactive molecules such as endothelium-derived hyperpolarizing factor (EDHF) and prostanoids. Bradykinin is a potent endothelium-dependent vasodilator through interaction with endothelial kinin B2 receptor and G protein-coupled receptor, which stimulates eNOS to generate NO for vasodilation. Our present study and many other publications showed that eNOS levels are negatively correlated to the vessel response to bradykinin. However, it was reported that bradykinin could potentially stimulate endothelial cells to release other vasoactive molecules such as EDHF (10) and prostanoids (10, 19), which could also affect the vascular response to bradykinin. To differentiate eNOS from other vasoactive molecules, which contribute the endothelium-dependent vasorelaxation, use of eNOS inhibitor such as N\textsuperscript{\text{-}}\text{G}-monomethyl-L-arginine could be helpful. It could be a limitation that eNOS-specific inhibitor was not included as controls in the present study. Another limitation could be that the potential role of CXCR2, receptor, which stimulates eNOS to generate NO for vasodilation. This work is partially supported by National Institutes of Health Grants K08 HL076345 (P. H. Lin); R21AI 49116 and R01DE15543 (Q. Yao); and R01HL61943, R01HL60135, R01HL65916, R01HL72716, and R01EB-002436 (C. Chen) and by the Michael E. DeBakey Department of Surgery, Baylor College of Medicine.

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