Growth-Related Oncogene-Alpha Induces Endothelial Dysfunction through Oxidative Stress and Downregulation of eNOS in Porcine Coronary Arteries

Carlos Bechara, MD; Xinwen Wang, MD, PhD; Hong Chai, MD, PhD; Peter H. Lin, MD; Qizhi Yao, MD, PhD; Changyi Chen, MD, PhD.*

Molecular Surgeon Research Center
Division of Vascular Surgery and Endovascular Therapy
Michael E. DeBakey Department of Surgery
Baylor College of Medicine, Houston, Texas

Running head: GRO-Alpha Induces Endothelial Dysfunction

*Address correspondence to:
Changyi (Johnny) Chen, M.D., Ph.D.
Michael E. DeBakey Department of Surgery
Baylor College of Medicine
One Baylor Plaza, Mail stop: NAB-2010
Houston, TX 77030
Phone: (713) 798-4401
Fax: (713) 798-6633
Email: jchen@bcm.tmc.edu
Summary

Growth-related oncogene alpha (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 analogue U46619 for contraction, bradykinin for endothelium-dependent vasorelaxation, and sodium nitroprusside (SNP) for endothelium-independent vasorelaxation. In response to 10^{-6} M bradykinin, GRO-α (50 and 100 ng/ml) significantly reduced endothelium-dependent vasorelaxation by 34.73% and 48.8%, respectively, compared with controls (P < 0.05). There were no changes in response to U46619 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 to non-atherosclerotic regions in human coronary arteries. Thus, GRO-α impairs endothelium-dependent vasorelaxation in porcine coronary arteries through a mechanism of over-production of superoxide anion and downregulation of eNOS. GRO-α may contribute to human coronary artery disease.

Key Words:
GRO-alpha, chemokine, endothelial dysfunction, eNOS, Superoxide anion, antioxidant, SeMet, coronary artery, atherosclerosis
INTRODUCTION

Growth-related oncogene alpha (GRO-α) is a 73-amino acid peptide initially isolated and characterized from malignant melanoma cells for its growth stimulatory activity (1,2). Another name for GRO-α is melanoma growth stimulatory activity (MGSA). Further studies indicate that many cell types and tissues also express GRO-α. GRO-α is referred to CXC ligand-1 (CXCL1), which binds to chemokine receptor CXCR2 (3). GRO-α was shown to induce chemotaxis of neutrophils (4), T-lymphocytes (5), and monocytes (6). 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 (KC) (7).

High GRO-α levels were detected in aneurismal aortic tissues (8), and serum levels of GRO-α were increased in several inflammation conditions (9,10). 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 (11). In addition, chimeric mice deficient in leukocyte CXCR2 had less atherosclerosis (11). On the other hand, GRO-α can induce endothelial cell chemotactic and proliferative activity in vitro and angiogenesis in vivo (12,13). GRO-α is also involved in the thrombin-induced angiogenesis (14). 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 1990’s (15). Multiple causes of endothelial dysfunction have been proposed (16). 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 (17). 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 (18).

In the current 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. Dimethyl sulfoxide (DMSO), thromboxane A2 analogue U46619, bradykinin, sodium nitroprusside (SNP), seleno-L-methionine (SeMet), and Tri-reagent kit were obtained from Sigma Chemical (St. Louis, Mo). Dulbecco modified Eagle’s medium (DMEM) was obtained from Life Technologies, Inc (Grand Island, NY). Recombinant human GRO-α and anti-GRO-α antibody were obtained from Biosource International Inc (Camarillo, CA). Lucigenin was obtained from Molecular Probes (Eugene, Ore). Antibody against human eNOS was obtained from BD Transduction Laboratories (Lexington, KY). The biotinylated horse anti-mouse immunoglobulin G (IgG) and avidin-biotin complex kit were obtained from Vector Labs (Burlingame, Calif).

Tissue harvest and cell culture. Fresh porcine hearts were harvested from farm pigs (6 to 8 months old male) at a local slaughterhouse as previously described from our studies (19-21). 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 E.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 (HI) GRO-α at 95 °C for 30 min was used.

HCAECs were purchased from Cambrex (San Diego, CA). The cells were used at passage 6 to 7. Once cells grew to 80% to 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 (19-21). Briefly, the rings were cultured in the medium for 24 h and then were suspended between the wires of the organ bath chamber (Multi myograph system 700MO; Myo Technology, Aarhus N, Denmark) in 6 mL of Kreb’s solution. After equilibration, each ring was precontracted with 20 µl U46619 (10^-7 M). After 60 to 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^-9, 10^-8, 10^-7, 10^-6, and 10^-5 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 (O$_2^-$). Levels of superoxide anion produced by endothelial cells were detected using the lucigenin-enhanced chemiluminescence method as previously described in our studies (19-21). Briefly, the rings were cut open longitudinally and trimmed into 5x5-mm pieces. An assay tube (12x75 mm) was filled with 500 µl buffer and 25 µl 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 (RLU/sec) 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 using a caliper and was used to normalize the data for each sample. Final data were presented as RLU/sec/mm$^2$.

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 using Tri-Reagent following the manufacturer’s instructions. Both porcine and human eNOS mRNA levels were determined by real time PCR analysis as previous described in our studies (19-21). Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units (RFU) versus PCR cycle number during exponential amplification so that sample measurement comparisons were possible. The eNOS mRNA levels in each sample were calculated as $2^{(40 – Ct)}$ and further normalized to GAPDH expression as $[2^{(Ct_{[GAPDH]} – Ct_{[eNOS]})}]$.

Western blot. Endothelial cells of porcine coronary artery rings were collected. Proteins were extracted with cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA). Equal amount of total proteins (50 µg) were loaded onto 10% SDS-PAGE, fractionated by electrophoresis, and transferred to PVDF membranes. The membrane was incubated with the primary anti-human eNOS antibody (1:2000) 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 versus 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 (1:1000) using avidin-biotin complex immunoperoxidase procedure (19-21). For detecting GRO-α expression, full-thickness arterial wall specimens of coronary arteries were obtained from 4 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, St. Louis, MO), biotinylated secondary antibody, and avidin-biotin reaction using peroxidase enzyme (ABC kit; Vector Laboratories, Burlingham, CA). Images were captured under microscope with an attached SPOT-RT digital camera and software (Diagnostic Instruments, Sterling Heights, Mich).

Statistical analysis. Statistical analysis was performed on the Data Analysis tool of the Microsoft Excel program (Microsoft Office 2003, Microsoft Inc, Seattle, Wash). Data were expressed as mean ± standard error (SE). Significant difference of data between the control and treated groups was determined by the paired Student 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 using a thromboxane analogue U46619. 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 endothelial-independent vasorelaxation was induced using 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 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). 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). 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 adding anti-GRO-α antibody at 1:1 concentration to GRO-α, eNOS mRNA levels were reversed 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, while 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). In consistence 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. HI-GRO-α (50 ng/ml) did not affect endothelium-dependent vasorelaxation (Fig. 5). There were no significant differences of vessel contraction (U46619) 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, non-atherosclerotic 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 (23). 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 (24). Significant calcium mobilization was observed with \( \geq 1 \) 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 (25,26). However, these levels are significantly increased in many inflammation conditions (9, 10) such as acute pancreatitis (up to 3195 pg/ml) (27). 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 recently reported that high expression of GRO-α was detected in aneurismal aortic tissues (8). In the current 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 non-atherosclerotic 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 current 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 to vascular disease process. In our in vitro study, we only observed GRO-α-induced endothelial dysfunction for a short duration (24 h), while 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 pharmacologic agents have been studied extensively in our lab. In the current 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 nitric oxide (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 (28). 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 blocked the effect of GRO-α on endothelial vasomotor dysfunction. This clearly shows that GRO-α, by producing superoxide anion production, depletes NO contributing to endothelial dysfunction. In addition to depleting NO, 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 (29). 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 using 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, G protein coupled receptor, which stimulates eNOS to generate NO for vasodilation. Our current study and many other publications showed 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 (30) and prostanoids (30,31), which could also affect the vascular response to bradykinin. To differentiate eNOS from other vasoactive molecules, which contribute the endothelium-dependent vasorelaxation, using of eNOS inhibitor such as N^G^-monomethyl-L-arginine could be helpful. It could be a limitation that eNOS specific inhibitor was not included as controls in the current study. Another limitation could be that the potential role of CXCR2, GRO-α receptor, in endothelial dysfunction was not investigated in the current study.
Superoxide anion quenches NO to give peroxynitrite (32), and thereby reduces NO availability. Peroxynitrite also oxidizes tetrahydrobiopterin, a critical cofactor for eNOS, and causes eNOS uncoupling, thereby decreasing NO production (33). In addition, Superoxide anion can be converted to hydrogen peroxide, which could affect several endothelial functions such as cell proliferation and migration (34-36). Furthermore, ROS can regulate signal transduction pathways, which ultimately control gene expression and post-translational modification of proteins (37). We speculated that superoxide anion may act as the second-messenger molecule that reduces eNOS expression in GRO-α treated cells or vessel rings. Although elevation of superoxide anion induced by GRO-α in the current study could be present throughout the vessel wall, SNP-mediated vasodilation was not affected by GRO-α treatment. Exact reasons for this observation are unknown. However, we could speculate several possibilities. GRO-α might have a limited effect on vascular SMCs and thereby generate less superoxide anion compared with endothelial cells. Indeed, vascular endothelial cells are more sensitive to many cardiovascular risk factors than vascular SMCs. In addition, SNP might generate an excessive amount of NO which overcomes any negative effects of superoxide anion in the SMC layers.

As mentioned earlier, recombinant GRO-α is produced using E. Coli and the endotoxin levels in original commercial GRO-α preparation is < 0.1 ng/µL. Even though the endotoxin level is minute after substantial dilutions in the experiments, we have included heat-inactivated GRO-α in this study to demonstrate the specificity of GRO-α because heat can denature and deactivate proteins, but not for endotoxin, which is heat-resistant. Indeed, heat-inactivated GRO-α did not show any effects on vasomotor dysfunction, superoxide production and eNOS mRNA expression. These data clearly demonstrate that the effects seen in this study are due to the GRO-α polypeptide, but not the endotoxin. We chose GRO-α 50 ng/ml for this specificity study because this concentration had a biological effect on coronary endothelial cells based on our data.

GRO-α is a 72-amino acid peptide which possesses 3 cysteines. However, the oxidative state of these cysteine residues in native and functional GRO-α is not known. It is possible that any proteins containing cysteine residues could be oxidized during isolation and purification. The oxidative state of these proteins could affect their biologic functions (38-40). In the current study, it is not clear whether commercially available recombinant human GRO-α is in the oxidation state or the free sulfhydryl state. It is also unknown whether oxidized cysteins in GRO-α could contribute to its impaired vasodilation and downregulation of eNOS. Further studies on this issue are warranted.

GRO-α is an interesting molecule with multifunction. It can induce growth, chemotaxis, and
metastasis of several cancer cell lines. Its relationship with vascular disease including atherosclerosis is largely unknown. This study clearly demonstrates that GRO-α causes endothelial dysfunction via oxidative stress and eNOS downregulation. How these data would be translated into the clinical practice is yet answered. Further investigations are warranted for both a physiologic or pathologic response of GRO-α.

In summary, to our knowledge, this is the first study that shows the association of GRO-α with vasomotor dysfunction by increasing superoxide anion production and decreasing eNOS expression in porcine coronary arteries and HCAECs. Antioxidant SeMet and anti-GRO-α antibody can effectively block these effects. GRO-α expression was increased in the atherosclerotic regions of human coronary arteries. This molecule could serve as a potential therapeutic target in patients at high risk for cardiovascular disease. GRO-α has been shown to be involved in the rolling to arrest phase of monocytes (41,42). Blocking GRO-α or its receptor may suppress monocyte arrest, thereby halting the transformation of monocytes into macrophage foam cells, the earliest step in atherosclerosis.

ACKNOWLEDGEMENTS

This work is partially supported by the National Institutes of Health (Lin: K08 HL076345; Yao: R21AI 49116 and R01DE15543; and Chen: R01HL61943, R01HL60135, R01HL65916, R01HL72716, and R01EB-002436) by the Michael E. DeBakey Department of Surgery, Baylor College of Medicine.

REFERENCES


FIGURE LEGENDS

Fig. 1. Effects of 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 using a thromboxane analogue U46619 (10^{-7}M). The maximum contraction in all treated groups had no statistical difference compared with controls. B. Endothelium-dependent vasorelaxation. The pre-contracted vessels were induced vasorelaxation by cumulative concentrations of bradykinin (from 10^{-9} to 10^{-5}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, n=12, ANOVA). 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 < 0.05, 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 mean ±SE.

Fig. 2. Effects of GRO-α and SeMet on superoxide anion production in porcine coronary arteries. A. Superoxide anion levels were detected using the lucigenin-enhanced chemiluminescence assay. The data were adjusted per vessel ring area (mm²) and expressed as relative light units (RLU/ sec/ mm²). 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, 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.

Fig. 3. Effects of GRO-α and SeMet on eNOS mRNA levels in porcine coronary arteries and HCAECs. A. Porcine coronary artery rings (concentration-dependent). The rings were cultured overnight with or without GRO-α (5, 50 and 100 ng/ml), or heat-inactivated GRO-α (50 ng/ml). The eNOS mRNA levels were quantified with real-time PCR and normalized to GAPDH. GRO-α (50 and 100 ng/ml) significantly decreased eNOS mRNA levels by 25.3% and 37.40%, respectively, compared
with controls ($P < 0.05$, $n=6$, t-test). The heat-inactivated GRO-α did not affect eNOS mRNA expression. **B.** Porcine coronary artery rings (antibody blocking). Porcine coronary artery rings were cultured overnight with or without GRO-α or anti-GRO-α antibody. The eNOS mRNA expression was decreased as expected in the GRO-α 50 ng/ml group ($P <0.05$, $n=6$, t-test), and was completely reversed by adding the anti-GRO-α antibody. Anti-GRO-α antibody alone had no effect. **C.** HCAECs (concentration-dependent). HCAECs were cultured overnight with or without GRO-α or heat-inactivated GRO-α. GRO-α (50 and 100 ng/ml) decreased eNOS mRNA levels by 36% and 48%, respectively, compared with controls ($P < 0.05$, $n=6$, t-test). The heat-inactivated GRO-α did not affect eNOS mRNA expression. **D.** HCAECs (antibody blocking). The cells were cultured overnight with or without GRO-α or anti-GRO-α. The eNOS mRNA expression was decreased as expected in the GRO-α group ($P < 0.05$, $n=6$, t-test), and was completely reversed by adding the anti-GRO-α antibody. Anti-GRO-α antibody alone had no effect on eNOS mRNA levels.

**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).

**Fig. 5.** Effects of SeMet on the GRO-α-induced endothelial dysfunction in porcine coronary arteries. Vessel rings were cultured overnight with or without GRO-α (50 ng/ml), SeMet (20 µM) or HI-GRO-α (50 ng/ml). Contraction was achieved using a thromboxane analogue U46619 (10^{-7}M). Endothelium-independent vasorelaxation in response to bradykinin challenge was recorded. GRO-α reduced bradykinin-induced vasorelaxation of precontracted porcine coronary artery rings, while SeMet almost completely reversed this effect of GRO-α ($P < 0.05$, $n=6$, ANOVA). The data are shown as mean ±SE.

**Fig. 6.** Immunoreactivity of GRO-α in human coronary arteries. Atherosclerotic regions of human coronary arteries showed a strong immunoreactivity of GRO-α, while non-atherosclerotic 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. Dark brown color represents positive staining (arrows). Magnification (100X and 400X, respectively).
Fig. 1A. Maximal contraction
Fig. 1B. Porcine ring, relaxation

B.

- GRO-α (100 ng/ml)
- GRO-α (50 ng/ml)
- GRO-α (5 ng/ml)
- Control

EC-Dependent Relaxation (% of Pre-contraction)

Bradykinin (M)

Copyright Information
Fig. 1C. Porcine ring, relaxation
Fig. 1D. Porcine ring, SNP relaxation

D.

<table>
<thead>
<tr>
<th>GRO-α (ng/ml)</th>
<th>EC-Independent Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Control</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

The graph shows the EC-independent relaxation of SNP (SNP, 10^-6M) at different concentrations of GRO-α (ng/ml). The data indicates that there is no significant change in EC-independent relaxation with increasing GRO-α concentrations.
Fig. 2A. Pig vessels, superoxide anion

A. Superoxide Anion Levels (RUL/sec./mm²)

Control

GRO-α (ng/ml)

0 5 50 100

* **
Fig. 2B. Pig vessels, superoxide anion

B.

Superoxide Anion Levels (RUL/sec./mm²)

Control  |  GRO-α (50 ng/ml)  |  SeMet  |  GRO-α + SeMet  |  HI-GRO-α

* indicates significant difference.
Fig. 3A. eNOS mRNA in PCAECs

A.

Porcine eNOS mRNA Levels (Normalized to GADH)

0 0.001 0.002 0.003 0.004 0.005

Control 5 50 100

GRO-α (ng/ml)

HIGRO-α (50 ng/ml)
Fig. 3B. eNOS mRNA in ring PCAECs
Fig. 3C. eNOS mRNA in HCAECs

HCAEC eNOS mRNA Levels
(Normalized to GADH)

C.

GRO-α (ng/ml)
(50 ng/ml)

0 0.002 0.004 0.006 0.008 0.01 0.012 0.014

0 5 50 100

Control

* *
Fig. 3D. eNOS mRNA in HCAECs

- Control
- GRO-α (50 ng/ml)
- GRO-α + Anti-GRO Ab
- Anti-GRO Ab

HCAEC eNOS mRNA Levels (Normalized to GADH)

* indicates a significant difference compared to the control group.
Fig. 4A. Porcine artery eNOS immunostaining

A.

a). Control  

b). GRO-α

c). GRO-α + SeMet  

 d). HI-GRO-α
Fig. 4B. Porcine artery eNOS immunostaining

B.

Control  GRO-α

eNOS

β-Actin

eNOS Protein Level (eNOS/β-Actin Ratio)

Control  GRO-α

Copyright Information
Fig. 5. Porcine ring, relaxation

- - GRO-α (50 ng/ml)
- - GRO-α + SeMet
- - SeMet
- - Control
- - HI-GRO-α
Fig. 6. Human coronary artery, GRO-α staining