Neutrophil antimicrobial peptide α-defensin causes endothelial dysfunction in porcine coronary arteries

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Background: Defensins are cysteine-rich cationic polypeptides released from neutrophils that exhibit powerful antimicrobial activities. Because inflammation, including neutrophil infiltration and release of defensins, may play an important role in atherosclerosis and other vascular diseases, we determined whether α-defensin could cause endothelial dysfunction, a major initial event of atherosclerosis, in porcine coronary arteries.

Methods: Porcine coronary arteries were sliced into 5-mm rings and treated with different concentrations of human recombinant α-defensin for 24 hours. Vasomotor reactivity was studied by using a myograph system. Levels of superoxide anion were detected by the lucigenin-enhanced chemiluminescence method. Endothelial nitric oxide synthase (eNOS) messenger RNA (mRNA) and protein levels were determined by real-time polymerase chain reaction and immunohistochemistry analysis, respectively.

Results: Endothelium-dependent relaxation in response to bradykinin was significantly reduced by 40% for the rings treated with 1500 nM of α-defensin compared with controls (P < .05). Vessel contractility in response to the thromboxane A2 analogue U46619 and endothelium-independent relaxation in response to sodium nitroprusside were not affected with defensin treatment. In addition, the superoxide anion level at the endothelial layer of porcine coronary artery rings was significantly increased by 80% in the defensin-treated (1500 nM) vessels compared with controls (P < .05). Furthermore, the eNOS mRNA levels in endothelial cells isolated from the cultured rings treated with defensin (1500 nM) were significantly decreased by 27% compared with controls (P < .05). Immunoreactivity of eNOS in the defensin-treated vessel rings was also substantially reduced.

Conclusions: Defensin reduces the endothelium-dependent vasorelaxation. This effect is associated with increased superoxide radical production and decreased eNOS expression in porcine coronary arteries.

Clinical Relevance: Inflammation is an important mechanism of atherosclerosis and other vascular diseases. The roles and interactions of biomediators released from inflammatory cells are not fully understood, however. This study provides new information about effects and potential molecular mechanisms of a major neutrophil releasing factor, α-defensin, on endothelial dysfunction of porcine coronary arteries. Thus, targeting α-defensin and its associated molecular mechanisms may become a new strategy to prevent vascular diseases. (J Vasc Surg 2006;43:357-63.)

Atherosclerosis is a disease process that starts in fetal life,1 progresses over decades under the influence of genetic and environmental factors, and is associated with a huge financial and social cost in modern societies.2 Retention of low-density lipoproteins within the walls of blood vessels3 and the development of an intense local inflammation4,5 are among the most popular theories of noxious stimuli4,5 are among the most popular theories that attempt to unify observational experimental data with the clinical characteristics of the disease. It is accepted that the presence of endothelial dysfunction, regardless of etiology, is the predominant event in the pathogenesis of the atherosclerotic plaque.6

Several lines of evidence show that inflammatory cells are responsible for the impairment of endothelial cell functions in atherosclerosis via the release of a number of mediators.6,7 It is also known that anti-inflammatory agents such as aspirin or steroids can attenuate the inflammation-induced endothelial dysfunction.8 Neutrophils are present in high numbers in the serum of atherosclerotic individuals and within atherosclerotic plaques.9,10 Naturally occurring antimicrobial peptides stored in the azurophilic granules of neutrophils are released during infection and constitute an essential component of the innate immunity, but they have also been found in large quantities within atheromatous plaques in the cerebral and coronary circulation.12,13 The role of these peptides has now broadened from simply endogenous effectors of the innate immunity to multifunctional mediators that can modulate the local inflammatory response.14 Among these peptides, defensins constitute a class of cysteine-rich cationic polypeptide antibiotics that have been isolated from mammals, insects, and plants15 and have been reported to possess several proatherogenic properties.16

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In the present study, we hypothesized that α-defensin, which is present in large quantities in atheromatous lesions, can induce endothelial dysfunction. To test this hypothesis, we investigated the effects of defensin in a porcine coronary artery model. Potential mechanisms that explain the defensin-induced endothelial dysfunction via altered endothelial nitric oxide synthase (eNOS) levels or nitric oxide depletion by superoxide radicals were also studied. Our results suggest that α-defensin interferes with vasomotor reactivity and further supports the role of neutrophils in atherosclerosis.

**METHODS**

**Chemicals and reagents.** Thromboxane A₂ analogue U46619 (9,11-Dideoxy-11α,9β-epoxy methanoprostaglandin F₂α), bradykinin, sodium nitroprusside (SNP), phosphate-buffered saline (PBS) solution, and Tri-reagent kit were obtained from California. Antibodies against human eNOS were obtained from Bio-Rad Laboratories (Hercules, Calif). Antibody against human eNOS was obtained from Transduction Laboratories (Lexington, Ky). Tinylated horse antimouse immunoglobulin G and avidin-biotin complex immunoperoxidase procedure were obtained from Sigma Chemical Co (St. Louis, Mo). Human α-defensin, which is composed of three closely related gene products and is also referred to as human neutrophil peptides 1-3, was obtained from Phoenix Pharmaceuticals (Belmont, Calif). Luciferin was obtained from Molecular Probes (Eugene, Ore). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Life Technologies, Inc (Grand Island, NY). Antibiotic-antimycotic solution was obtained from Mediatech Inc (Herndon, Va). 

**Real-time polymerase chain reaction.** Porcine coronary artery rings were incubated with the medium only (controls) and the rings treated with 1500 nM of defensin were studied. The rings were cut open longitudinally and trimmed into approximately 5 × 5-mm pieces. They were then rinsed briefly in a modified Krebs N-(2-hydroxyethyl) piperazin-N’-(2-ethanesulphonic acid) (HEPES) buffer solution.

After the solutions were gently mixed, the vessel segments were placed with the endothelium side facing down in the tubes to record signals from the endothelial layer. Time-based reading of the luminometer was recorded by using the FB12 software. The obtained in relative light units per second (RLU/sec) for each sample and were averaged between 5 and 10 minutes. Values of blank tubes containing the same reagents were subtracted from those of their corresponding vessel samples. The area of each vessel segment was measured with a caliper and used to normalize the data for each ring sample. Final data are presented as RLU/sec/mm².

**Detection of superoxide anion.** Levels of superoxide anion produced by endothelial cells were detected by using the lucigenin-enhanced chemiluminescence method with a Sairius Luminometer and FB12 software (Berthold Detection System GmbH, Pforzheim, Germany). Vessel rings incubated with the medium only (controls) and the rings treated with 1500 nM of defensin were studied. The obtained in relative light units per second (RLU/sec) for each sample and were averaged between 5 and 10 minutes. Values of blank tubes containing the same reagents were subtracted from those of their corresponding vessel samples. The area of each vessel segment was measured with a caliper and used to normalize the data for each ring sample. Final data are presented as RLU/sec/mm².

**Tissue harvest and culture.** Fresh porcine hearts were harvested from farm pigs (6 to 8 months old) at a local slaughterhouse. The hearts were immediately rinsed with sterile PBS, the aortic arch and coronary arteries were perfused with cold PBS, and each was stored in ice-cold PBS for transport back to the laboratory. The right coronary arteries from the hearts were dissected, the perivascular connective tissue was removed, and the arteries were divided into 5-mm rings to be used in the experiments. Several rings from each heart were assigned into groups that included controls treated with only dimethylsulfoxide (DMSO), and those treated with defensin at 15, 150, and 1500 nM. The rings were incubated in DMEM with defensin or vehicle (DMSO) at 37°C and 5% carbon dioxide for 24 hours. An antibiotic-antimycotic solution (0.5%) was added to the tissue culture medium for all experiments.

**Myograph analysis.** The myograph tension system used in our laboratory has previously been described. After being cultured in the medium for 24 hours, rings were suspended between the wires of the organ bath chamber (Multi myograph system 700MO; Danish Myo Technology, Aarhus, Denmark) in 6 mL of Krebs solution, maintained at 37°C, and were continuously oxygenated. Rings were slowly subjected to a predetermined tension of 30 millinewton (mN) and allowed to equilibrate for 25 to 30 minutes. After equilibration, each ring was preconstricted with 20 μL of thromboxane A₂ analogue U46619 (10⁻⁷ M). After 60 to 90 minutes of contraction, a relaxation dose-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 minutes. At the end, 60 μL of SNP (10⁻⁶ M) was added into the organ bath, and endothelium-independent vasorelaxation was recorded. Contractility and percentage of relaxation were calculated based on the tension changes.

**Detection of superoxide anion.** Levels of superoxide anion produced by endothelial cells were detected by using the lucigenin-enhanced chemiluminescence method with a Sairius Luminometer and FB12 software (Berthold Detection System GmbH, Pforzheim, Germany). Vessel rings incubated with the medium only (controls) and the rings treated with 1500 nM of defensin were studied. The obtained in relative light units per second (RLU/sec) for each sample and were averaged between 5 and 10 minutes. Values of blank tubes containing the same reagents were subtracted from those of their corresponding vessel samples. The area of each vessel segment was measured with a caliper and used to normalize the data for each ring sample. Final data are presented as RLU/sec/mm².

**Porcine corona-ry artery rings were incubated with the medium only (controls) and the rings treated with 1500 nM of defensin were studied. The obtained in relative light units per second (RLU/sec) for each sample and were averaged between 5 and 10 minutes. Values of blank tubes containing the same reagents were subtracted from those of their corresponding vessel samples. The area of each vessel segment was measured with a caliper and used to normalize the data for each ring sample. Final data are presented as RLU/sec/mm².**
Sections were counterstained and viewed on an Olympus BX41 microscope (Olympus USA, Melville, NY). Images were captured with an attached SPOT-RT digital camera and software (Diagnostic Instruments, Sterling Heights, Mich).

**Statistical analysis.** A total of 18 hearts were harvested and used for this study. For each heart, the right coronary artery was isolated and cut into multiple 5-mm rings. The data of several coronary artery rings from each heart were averaged and represented as one data point for statistical analysis. Thus, the sample size is the number of hearts used. Among 18 hearts, 9 hearts were used for myographic analysis and the rings from each heart were randomly assigned to several groups of experiments. An additional nine hearts were used for real-time PCR (n = 6) and for superoxide anion detection and immunohistochemistry staining (n = 3). Statistical analysis was performed on the Data Analysis tool of the Microsoft Office 2000 Excel program (Microsoft Inc, Redmond, Wash.). Data were expressed as mean ± SE. Significant difference of data between control and treated groups was determined by paired Student’s t test (two-tailed). Comparison of vasorelaxation in response to a series of concentrations of bradykinin among the four groups was analyzed with the single-factor analysis of variance test. 

**RESULTS**

**Defensin impairs endothelium-dependent vasorelaxation in porcine coronary arteries.** Porcine coronary artery rings were cultured for 24 hours with a clinically relevant concentration of 15 (n = 6), 150 (n = 5), or 1500 nM (n = 9) of defensin and then subjected to physiologic contraction (U46619) as well as endothelium-dependent (bradykinin) and endothelium-independent (SNP) relaxation. In response to U46619, the contraction of the vessel rings was changed in a random manner unrelated to the concentration of defensin and was not statistically significant with that of controls (Fig 1). In response to bradykinin at 10−5 M, the endothelium-dependent relaxation compared with controls was reduced by 40% (P < .05) and 12% (P > .05) for the rings treated with 1500 and 150 nM of defensin, respectively. Rings treated with 15 nM of defensin demonstrated relaxation equalling that of controls (Fig 2). The endothelium-independent vasorelaxation in response to SNP was minimally changed in a random and statistically insignificant manner (P > .05) (Fig 3).

**Defensin increases superoxide anion production in porcine coronary arteries.** Oxidative stress is a central event in the development of endothelial dysfunction and vascular injury. To determine whether this mechanism is involved in defensin-induced vasomotor dysfunction, superoxide anion production was analyzed by a lucigenin-enhanced chemiluminescence assay (n = 3 for each group). The superoxide anion levels of the endothelial layer were significantly increased by 80% for the rings treated with defensin (1500 nM) compared with controls (P < .05) (Fig 4).

**Defensin reduces eNOS expression in porcine coronary arteries.** To determine whether eNOS expression was correlated with the reduction of endothelium-dependent vasorelaxation after treatment with defensin, porcine coronary artery rings were incubated with defensin (0, 150, and 1500 nM) (n = 6 for each group). The eNOS levels were then calculated using real-time PCR and were found to be significantly reduced by 27% in the rings treated with 1500 nM compared with controls (P < .05) (Fig 5). Immunohistochemistry staining showed a statistically significant reduction in expression by 40% compared with controls (n = 9) (*P < .05). Rings incubated with α-defensin (150 nM) demonstrated a 12% reduction in expression compared with controls. No change in the endothelium-dependent relaxation was seen in the rings treated with α-defensin (15 nM). Data are expressed as mean ± SE.

**Fig 1.** Effect of defensin on the contractility of porcine coronary arteries. Pig coronary artery rings were cultured with the medium dimethylsulfoxide (as control) or treated with α-defensin (15, 150, or 1500 nM) for 24 hours. Contraction of the vessel rings was achieved with addition of thromboxane A2 analogue U46619 (10−7 M). There was no specific pattern in the changes in contractility between rings treated with defensin and controls (n = 9) (P > .05). Data are shown as mean ± SE.

**Fig 2.** Effect of defensin on the endothelium-dependent relaxation of porcine coronary arteries. Precontracted vessels exposed to different defensin concentrations were tested for endothelium-dependent relaxation by the addition of bradykinin (10−9 to 10−5 M). The artery treated with α-defensin (1500 nM) showed a statistically significant reduction in relaxation by 40% compared with controls (n = 9) (*P < .05). Rings incubated with α-defensin (150 nM) demonstrated a 12% reduction in relaxation compared with controls. No change in the endothelium-dependent relaxation was seen in the rings treated with α-defensin (15 nM). Data are expressed as mean ± SE.

**Fig 3.** Effect of defensin on the endothelium-dependent relaxation of porcine coronary arteries. Pig coronary artery rings were cultured with the medium dimethylsulfoxide (as control) or treated with α-defensin (15, 150, or 1500 nM) for 24 hours. Contraction of the vessel rings was achieved with addition of thromboxane A2 analogue U46619 (10−7 M). There was no specific pattern in the changes in contractility between rings treated with defensin and controls (n = 9) (P > .05). Data are shown as mean ± SE.
hitochemistry analysis (n = 3) for eNOS was performed in porcine coronary artery rings using the avidin-biotin complex immunoperoxidase procedure. The noticed change in relaxation was random and not statistically significant (P > .05). Data are expressed as mean ± SE.

**DISCUSSION**

To our knowledge, this is the first study to demonstrate that α-defensin can cause endothelial dysfunction of porcine coronary arteries in association with increased superoxide anion production and decreased eNOS expression. Defensins are naturally occurring antibiotic polypeptides that are abundant in nature and exhibit remarkable antiviral, antibacterial, and antifungal activities. In humans, α-defensin has a length of 29 to 35 amino acids and is located in neutrophils and in the Paneth cells of the small intestine, where it is stored as pro-peptides. Defensins are incorporated into the cell membrane of prokaryotic organisms during the process of phagocytosis, disrupt ion fluxes, and promote cell lysis. In addition to their antimi-
crobial properties, they were recently proved to provide a link among lipid accumulation in the vessel wall, local inflammation, and endothelial cell perturbation. Defensins participate in the lipoprotein uptake and degradation in the vessel wall,12,21,22 interfere with vascular smooth muscle cells,23 possess antifibrinolytic activity,24,25 and regulate angiogenesis.26

The plasma concentration of defensin is <15 nM in healthy subjects, but levels as high as 50,000 nM have been recorded in patients with sepsis or bacterial meningitis.27 For our experiments, we selected a range of doses (15, 150, and 1500 nM) that mimic conditions of health and disease.

Impaired vasorelaxation in response to pharmacologic agents is a known indicator of vascular dysfunction.28 In current study, human α-defensin significantly impaired endothelium-dependent vasorelaxation in a dose-dependent manner. For the arteries incubated with 15, 150 and 1500 nM of defensin, the decrease in bradykinin-induced vasorelaxation compared with controls was 0%, 12%, and 40%, respectively, indicating defensin-induced endothelial dysfunction. It is noteworthy that in physiologic doses, defensin had no impact on endothelial cell function, a fact consistent with its role as a host-friendly agent of immune surveillance. Defensin had no effect on the SNP-induced vasorelaxation, indicating that its activity is largely endothelium-mediated.

Since defensin is dissolved in DMSO solution, we used an equal amount of DMSO as a vehicle control (negative control) in the experiments. For future studies, random-sequence cysteine-rich cationic polypeptide may be used for a much stronger negative control for defensins.

Another issue is the activity of human α-defensin on other species. Human α-defensin has biologic effects on rat vascular smooth muscle cells,29 but it has not been tested on porcine cells or tissues. Information about porcine α-defensin (genes and sequences) is also not available at this time. Our current study clearly demonstrates that human α-defensin does affect porcine coronary arteries. However, it is not clear whether the activity and potency of human α-defensin for human cells or tissues are different for porcine cells and tissues. Further investigations with human cells or tissues are warranted to address this important issue.

In recent years, we have established and characterized the in vitro culture model of porcine coronary artery rings for myograph analysis. Specially, endothelium-dependent vasorelaxation can be analyzed with a challenge of bradykinine, a potent vasodilator that acts through endothelial B2 kinin receptors to stimulate the release of nitric oxide through eNOS activation.30 We have used this model to study the effects of several clinical risk factors or molecules on endothelial functions. For examples, homocysteine (50 μM), resistin (40 ng/mL), progesterone (1 × 10⁻⁶ g/L), HIV Tat (10⁻⁷ M), ritonavir (15 μM), and lysocephatidlycholine (25 μM) significantly reduced the endothelium-dependent vasorelaxation of porcine coronary artery rings by 30%, 31, 30%, 18, 38%, 32 46%, 33 59%, 17 and 81%, 34 respectively. In current study, human α-defensin (1500 nM) significantly reduced endothelium-dependent vasorelaxation by 40% compared with controls. These molecules may independently contribute to vascular disease formation.

Imbalance in the production and catabolism of reactive oxygen species (ROS) generated during aerobic metabolism is known to lead to oxidative stress and contributes to vascular disease via a variety of mechanisms that include a state of continuous nitric oxide consumption and depletion,35,36 intracellular alkalinization,37 and regulation of gene transcription.38 In our study, an 80% increase of superoxide anion production was shown in the defensin-treated rings compared with controls, indicating that oxidative stress may be one of the predominant mechanisms in defensin-induced endothelial dysfunction.

A central event in the vascular cell homeostasis involves the fine regulation of nitric oxide bioavailability. Nitric oxide is generated by eNOS and is a potent vasodilator with multiple additional cardiovascular functions.39 eNOS is important in atherosclerotic disease.40,41 Patients with eNOS polymorphism that reduces enzymatic activity exhibit higher rates of myocardial events.42 We believe our data show that the changes in endothelium-dependent relaxation may also be due to the decrease in eNOS expression. Indeed, the real-time PCR showed a significant decrease of eNOS mRNA expression in defensin-treated vessels and agreed with data obtained from the immunohistochemistry studies. Real-time PCR is a very sensitive method to detect mRNA levels for many genes. Two most important quality control procedures are primer design of the gene of interest (specific) and a housekeeping gene (no change). We have developed these high standards for porcine eNOS and have published several studies related to eNOS downregulation.17,18,31-34

We believe that any statistically significant difference of mRNA downregulation normalized to GAPDH will have a biologic significance of eNOS-mediated functions. In current study, a 27% reduction of eNOS mRNA levels in defensin-treated (1500 nM) vessels compared with control vessels may account for the defensin-induced inhibition of endothelium-dependent vasorelaxation. eNOS immunoreactivity was reduced in defensin-treated (1500 nM) vessels by gross observation of color intensity. However, quantitation of eNOS immunoreactivity was not performed owing to technique difficulties and variability of staining. More reliable quantitative methods, such as Western blot for eNOS protein, may be warranted for studying the effect of human defensins on eNOS expression in porcine coronary arteries. These findings, together with the functional data obtained from myograph analysis, suggest a potential mechanism of defensin-induced damage to the endothelial cells via decreased eNOS expression and nitric oxide bioavailability.

Defensins are known to interfere with lipoprotein metabolism in endothelial cells and smooth muscle cells, favoring the intra- and extracellular lipoprotein (a) and low-density lipoprotein accumulation.12,21,22 Subsequent oxidation of these lipids has well known detrimental effects on the vasomotor properties of the vessel.43,44
Therefore, one could argue that the action of defensin on the vasculature is an indirect effect via altered lipid metabolism. In our model, α-defensin impaired endothelium-dependent relaxation in the absence of lipids, indicating a direct interaction at the endothelial cell level. Nassar et al.2,3 have shown that α-defensin binds to the low-density lipoprotein receptor-related protein/α2macroglobulin receptor, inhibits the mobilization of intracellular calcium (Ca++) promoted by phenylephrine, and ultimately interferes with the phenylephrine-induced smooth muscle cell contraction in denuded rat aortic rings. The authors, however, noted that defensin alone did not have any effect on Ca++ mobilization or contractility. The presence of intact endothelium seems to be a requisite for the manifestation of a direct defensin activity on vascular tone.

The precise role of defensins in the pathogenesis of endothelial dysfunction appears to be very intriguing, yet poorly defined. Defensins provide a unique link between two coexisting phenomena of arterial wall inflammation and lipid accumulation. Further study is necessary to decipher its role on atherosclerosis. For instance, defensin receptors have been identified on human umbilical vein endothelial cells and smooth muscle cells.21,23,25 However, the binding specificity, the downstream molecular events triggered by the defensin ligand, and the functional significance of the defensin-induced intracellular signal transduction pathways are not well understood. In addition, the significance of these observations in vivo has not been proved yet, and it is worth further investigation. Finally, little is known about factors that regulate defensin activity and influence its local concentration in the arterial wall.

CONCLUSION

We have demonstrated that human α-defensin can cause vasomotor dysfunction, increase superoxide anion production, and decrease eNOS expression in porcine coronary arteries. However, only human defensin at 1500 nM had a detrimental effect on endothelial functions of porcine coronary arteries. In the future, a large range of concentrations of human defensin should be used to study its effects on endothelium-dependent vasorelaxation, superoxide anion production, and eNOS expression, thereby better indicating clinical significance of defensins.

AUTHOR CONTRIBUTIONS

Conception and design: PK, HC, PHL, QY, ABL, CC
Data collection: PK, HC, QY, CC
Analysis and interpretation: PK, HC, PHL, QY, ABL, CC
Writing the article: PK, HC, QY, ABL, CC
Critical revision of the article: PK, HC, QY, CC
Final approval of the article: CC
Statistical analysis: PK, HC, CC
Obtained funding: CC
Overall responsibility: CC

REFERENCES


