Serum amyloid A induces endothelial dysfunction in porcine coronary arteries and human coronary artery endothelial cells

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Submitted 6 March 2008; accepted in final form 15 October 2008

Wang X, Chai H, Wang Z, Lin PH, Yao Q, Chen C. Serum amyloid A induces endothelial dysfunction in porcine coronary arteries and human coronary artery endothelial cells. Am J Physiol Heart Circ Physiol 295: H2399–H2408, 2008. First published October 17, 2008; doi:10.1152/ajpheart.00238.2008.—The objective of this study was to determine the effects and mechanisms of serum amyloid A (SAA) on coronary endothelial function. Porcine coronary arteries and human coronary arterial endothelial cells (HCAECs) were treated with SAA (0, 1, 10, or 25 µg/ml). Vasomotor reactivity was studied using a myograph tension system. SAA significantly reduced endothelium-dependent vasorelaxation of porcine coronary arteries in response to bradykinin in a concentration-dependent manner. SAA significantly decreased endothelial nitric oxide (NO) synthase (eNOS) mRNA and protein levels as well as NO bioavailability, whereas it increased ROS in both artery rings and HCAECs. In addition, the activities of internal antioxidant enzymes catalase and SOD were decreased in SAA-treated HCAECs. Bi-plex immunoassay analysis showed the activation of JNK, ERK2, and IkB-α after SAA treatment. Consequently, the antioxidants seleno-l-methionine and Mn(II) tetraphos (4-benzoic acid)porphyrin and specific inhibitors for JNK and ERK1/2 effectively blocked the SAA-induced eNOS mRNA decrease and SAA-induced decrease in endothelium-dependent vasorelaxation in porcine coronary arteries. Thus, SAA at clinically relevant concentrations causes endothelial dysfunction in both porcine coronary arteries and HCAECs through molecular mechanisms involving eNOS downregulation, oxidative stress, and activation of JNK and ERK1/2 as well as NF-κB. These findings suggest that SAA may contribute to the progression of coronary artery disease.

IT IS WELL KNOWN that inflammation plays a crucial role in the pathogenesis of atherosclerosis (29). Serum amyloid A (SAA) belongs to a family of the major acute-phase proteins in vertebrates and was discovered in 1971 as a principal constituent within the amyloid deposits of patients with persistent inflammation (31, 44, 47). Recent studies have shown that increased levels of SAA are strongly associated with many inflammation conditions including cardiovascular diseases (11, 22, 32). For example, SAA levels have also been correlated with the severity of human coronary artery atherosclerosis (32) and many cardiovascular risk factors including obesity, insulin resistance, diabetes (26), and rheumatoid arthritis (50). SAA can bind to extracellular vascular glycans and impair the ability of HDL to promote cholesterol efflux from macrophages (3).

Wang et al. (27) reported that circulating SAA levels, but not lipid levels, were strongly associated with the extent of aortic atherosclerosis in a mouse model, and SAA colocalized with apolipoprotein A-I and proteoglycans in atherosclerotic lesions. SAA may bind and transport cholesterol into aortic smooth muscle cells (28). Additionally, SAA also promotes monocyte chemotaxis and adhesion (4). These data suggest that SAA is a biomarker and biomediator for cardiovascular disease.

Decreased bioavailability of nitric oxide (NO) produced from endothelial NO synthase (eNOS) plays a crucial role in the development and progression of atherosclerosis (51). eNOS converts l-arginine to l-citrulline and NO in the endothelium. Modulation of eNOS gene expression or activity can, in turn, control the NO signaling (7). Impaired eNOS activity leads to a decrease in the relative bioavailability of NO, which not only impairs endothelium-dependent vasorelaxation but also activates other mechanisms that have an important role in the pathogenesis of atherosclerosis (36). Furthermore, eNOS and NO levels can be modulated by reactive oxygen species (ROS), which can result in the activation of various signaling pathways, including MAPKs (14, 18). In the vascular system, ROS may react with and reduce NO bioavailability, leading to the endothelial dysfunction found in a number of cardiovascular disease states, including hypertension, chronic heart failure, and atherosclerosis (42). However, the precise mechanisms by which endothelial cells maintain the physiological levels of NO and ROS, such as superoxide anion, are not completely understood.

Despite the clinical implications, limited biological functions of SAA have been reported, and the role and mechanisms of SAA in endothelial functions have not been fully elucidated. In the present study, we tested the hypothesis that SAA may induce endothelial dysfunction by downregulating eNOS expression through oxidative stress and MAPK activation. Specifically, the effects of SAA on eNOS mRNA and protein levels were determined in human coronary artery endothelial cells (HCAECs) and porcine coronary artery rings. NO bioavailability, ROS production, and the internal antioxidant enzymes catalase (CAT) and SOD as well as MAPK phosphorylation were investigated. This study may provide new insights into the mechanisms of SAA-related endothelial dysfunction and its association with cardiovascular disease.

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SERUM AMYLOID A INDUCES ENDOTHELIAL DYSFUNCTION

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MATERIALS AND METHODS

Chemicals and reagents. Recombinant human apolipoprotein SAA was obtained from Leinco Technologies (St. Louis, MO). The endothelin level in the SAA preparation was <1.0 EU/μg. DMEM, the thromboxane A2 analog U-46619, bradykinin, sodium nitroprusside (SNP), seleno-l-methionine (SeMet), N7-nitro-l-arginine methyl ester (l-NAME), and the Tri-Reagent kit were obtained from Sigma Chemical (St. Louis, Mo). The ERK1/2 inhibitor PD-98059, p38 inhibitor SB-239063, and JNK inhibitor SP-600125 were obtained from Calbiochem (San Diego, CA). Dihydroethidium (DHE), 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA), and lucigenin were purchased from Molecular Probes (Eugene, OR).

DMEM was obtained from Life Technologies (Grand Island, NY). The antibody against human eNOS was obtained from BD Transduction Laboratories (Lexington, KY). Biotinylated horse anti-mouse IgG and the avidin-biotin complex kit were obtained from Vector Laboratories (Lexington, KY). Biotinylated horse anti-mouse IgG and the avidin-biotin complex kit were obtained from Vector Labs (Burlingame, CA). Mn(III) tetrakis-(4-benzoic acid)phosphoryl (MnTBP) was purchased from A. G. Scientific (San Diego, CA).

Myograph model. Fresh porcine hearts were harvested from young adult farm pigs (6–7 mo old) at a local slaughterhouse, placed in a container filled with cold PBS solution, and immediately transported to the laboratory. Fresh porcine right coronary arteries were carefully dissected and cut into multiple 5-mm rings. The rings were then incubated in DMEM with 1, 10, or 25 μg/ml of SAA at 37°C and 5% CO2 for 24 h. The myograph tension system used in our laboratory has been previously described (10). Briefly, rings were suspended between the wires of the organ bath myograph chamber in 6 ml of Krebs solution, maintained at 37°C, and oxygenated with pure oxygen gas. Rings were slowly subjected stepwise to a predetermined optimal tension of 30 mN, and each ring was precontracted with the thromboxane A2 analog U-46619 (final concentration: 10−6 M), which precontracted vascular rings ~60% of the maximal contraction (see Supplemental Fig. S1). After 60–90 min of contraction, a relaxation curve was generated by adding 60 μl of five cumulative additions of the endothelium-dependent vasodilator bradykinin (final concentrations: 10−9, 10−8 M) every 3 min. In addition, SNP (final concentration: 10−6 M) was added into the organ bath, and endothelium-independent vasorelaxation was recorded. A separate experiment, serial concentrations of U-46619 and SNP were used.

Lucigenin-enhanced chemiluminescence assay. Levels of ROS produced by endothelial cells of porcine arteries were detected using the lucigenin-enhanced chemiluminescence method as previously described in our study (10). Six sets of vessel rings in each group were used. Briefly, rings were cut open longitudinally and trimmed into 5 × 5-mm pieces. An assay tube was filled with 5 μM lucigenin, and the vessel segment was placed endothelium side down in the tube to record signals from the endothelial layer. Time-based reading of the luminometer was recorded. Data, in relative light units (RLU) 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 using a caliper and was used to normalize the data for each sample. Final data are represented as RLU and adjusted to 1 × 106 cells/FACS tube. For ROS and NO staining, DHE (3 μM) and DAF-FM DA (10 μM) were added, respectively, and incubated in 37°C for 30 min. ROS and NO levels in cells were analyzed by FACS Calibur flow cytometry (Becton Dickinson). In each experiment, at least 10,000 events were analyzed.

Measurements of CAT and SOD activity. HCAECs were cultured with 10% FCS for 24 h. In all groups, cells cultured in EGM-2 alone were used as negative controls.

Western blot analysis. Total proteins were isolated from HCAECs using the Tri-Reagent kit. Equal amounts of total proteins were loaded using 10% SDS-PAGE, fractionated by electrophoresis, and transferred to nitrocellulose membranes. The membrane was incubated with the primary antibody against human eNOS at 4°C overnight. Dilutions of 1:1,000 for eNOS monoclonal antibody and 1:10,000 for β-actin monoclonal antibody were used. Bands were visualized with ECL Plus Chemiluminescent substrate (Amersham Biosciences). Densitometric measurements were performed to quantify the relative expression of eNOS proteins versus β-actin (AlphaEaseFC software).

Nitrite detection. NO levels released from vessel rings and HCAECs were determined by measuring the accumulation of its stable degradation products, nitrite and nitrate (Griess Reaction NO Assay kit, Calbiochem). Nitrate is reduced to nitrite by nitrate reductase. Thus, total nitrite levels represent total NO levels. Porcine coronary rings and HCAECs were cultured with or without SAA for 24 h. The supernatant was collected, and total nitrite levels were measured. Absorbance of the samples was determined at 540-nm wavelength and compared with standard solutions. The amount of nitrite detected was normalized to the area of the cultured rings (mM/mg/m2) or total proteins of HCAECs (in μM/mg).

Flow cytometry. Cells were harvested with 0.02% trypsin-EDTA and adjusted to 1 × 106 cells/FACS tube. For ROS and NO staining, DHE (3 μM) and DAF-FM DA (10 μM) were added, respectively, and incubated in 37°C for 30 min. ROS and NO levels in cells were analyzed by FACS Calibur flow cytometry (Becton Dickinson). In each experiment, at least 10,000 events were analyzed.

Measurements of CAT and SOD activity. HCAECs were homogenized and centrifuged in HEPES buffer (pH 7.4) containing 1 mM EDTA. CAT and SOD enzyme activities were measured with commercial enzyme assay kits (Cayman Chemical) following the manufacturer’s protocols. CAT and SOD enzyme activities were calculated from the average absorbance of each sample using the equations provided in the kit manuals. Final data for CAT activity are presented as means ± SE (nmol·min−1·mg−1); final data for SOD are presented as means ± SE (U/ml).
cluded four positive controls provided by Bio-Rad to monitor detector stability and specimen and sample integrity. Final data were analyzed and presented as the ratio of phosphoprotein to total protein for each MAPK and for iκB-α (average of triplicates).

Statistical analysis. Statistical analysis was completed by comparing the data from treatment and control groups using Student’s t-test (two-tailed). The vasomotor reactivity in response to serial concentrations of vasoactive drugs with multiple groups and data points was analyzed by ANOVA (two-tailed) followed by Bonferroni-Dunn’s post hoc test (Minitab software, Sigma Breakthrough Technologies, San Marcos, TX). P values of <0.05 were considered statistically significant. Experimental values are reported as means ± SE.

RESULTS

SAA decreases endothelium-dependent vasorelaxation in porcine coronary arteries. Endothelial dysfunction plays a crucial role in the development and progression of atherosclerosis. We first tested the effects of SAA on vasomotor functions in porcine coronary arteries by a myograph system including vessel contraction (U-46619), endothelium-dependent (bradykinin) relaxation assays, and endothelium-independent (SNP) relaxation assays. Maximal contraction in response to U-46619 was not different between SAA treatment groups (controls (SNP) relaxation assays). Maximal contraction in response to bradykinin at 10^{-5} M, endothelium-dependent vasorelaxation of SAA-treated groups in the presence of L-NAME, indicating that SAA treatment of SAA (10 μg/ml) for 24 h, porcine coronary arteries were preincubated with L-NAME (100 μM) for 30 min. Rings were then precontracted with U-46619 (3 × 10^{-8} M) and relaxed with bradykinin (10^{-9}–10^{-5} M). In response to bradykinin at 10^{-5} M, endothelium-dependent vasorelaxation of SAA-treated or untreated rings was significantly blocked by L-NAME compared with those without L-NAME treatment (P < 0.05; Fig. 1D), indicating a major role of NO in bradykinin-induced vasorelaxation of porcine coronary arteries. However, there were no significant differences of endothelium-dependent vasorelaxation between SAA-treated and untreated groups in the presence of L-NAME, indicating that SAA mainly affects the eNOS system but not prostaglandin and other endothelium-derived hyperpolarizing factors. In addition, endothelium-independent vasorelaxation in response to 10^{-6} M SNP showed no significant differences between L-NAME-treated and control groups (data not shown).

SAA decreases eNOS expression and NO production in porcine coronary arteries and HCAECs. To investigate whether eNOS could be involved in SAA-induced vasomotor dysfunction in porcine coronary arteries, eNOS expression and NO production in both artery rings and HCAECs was analyzed by real-time PCR, immunohistochemistry, and Western blot analysis. Significant decreases of eNOS mRNA levels were observed in a concentration-dependent manner in response to SAA treatment. At 10 or 25 μg/ml SAA, eNOS mRNA levels of arterial rings showed significant decreases by 37% or 47%, respectively, compared with controls (Fig. 2A, P < 0.05). Immunohistochemistry staining also confirmed significant decreases in eNOS protein levels in endothelial layers of porcine arteries (Fig. 2B). Consistent with the effects of SAA on artery rings, both mRNA levels and protein levels of eNOS in HCAECs were significantly reduced with SAA treatment in a concentration-dependent manner (P < 0.05; Fig. 3). When cells were treated with
SAA (10 or 25 \( \mu \)g/ml) for 24 h, eNOS mRNA levels were decreased by 23% or 46%, respectively, compared with controls \((P < 0.05; \text{Fig. 3A})\). Moreover, HCAECs were treated with SAA in a time-dependent manner. As shown in Fig. 3B, when cells were cultured with SAA (10 \( \mu \)g/ml) for 6, 24, or 48 h, only 24- and 48-h treatment groups showed a significant reduction of eNOS mRNA levels compared with controls \((P < 0.01; \text{Fig. 3B})\). Furthermore, NO production was analyzed with the nitrite assay kit. NO production was substantially reduced in SAA-treated rings and HCAECs in a concentration-dependent manner. When treated with 10 \( \mu \)g/ml SAA, NO production was decreased by 21.3% and 26.8% in rings and HCAECs,
Cellular NO production was also demonstrated with the fluorescent dye DAF-FM DA and measured by flow cytometry. DAF-FM DA staining is a unique method to measure NO production in living cells or solutions (24). NO production was significantly reduced in SAA-treated cells in a concentration-dependent manner. SAA at 10 or 25 μg/ml concentration decreased NO-positive cell numbers by 25% or 34%, respectively, compared with controls (P < 0.05; Figs. 4A and B). In a separate experiment, L-NAME (100 μM) was incubated with HCAECs in the presence of 10 μg/ml SAA. As shown in Fig. 4C, NO production levels in HCAECs were substantially inhibited by l-NAME treatment.

SAA increases ROS production in both porcine coronary arteries and HCAECs. To investigate whether oxidative stress could play a role in SAA-induced endothelial dysfunction in the vascular system, ROS production from porcine coronary artery rings and HCAECs was analyzed with lucigenin-enhanced chemiluminescence and DHE staining, respectively. ROS levels from artery rings were significantly increased in SAA-treated vessels in a concentration-dependent manner with lucigenin-enhanced chemiluminescence assay. SAA treatment at 10 and 25 μg/ml increased ROS levels from vessel rings by 42% and 39%, respectively, compared with controls (P < 0.05; Fig. 5A). Moreover, differences in ROS levels were also compared with controls (P < 0.05; Fig. 5B). In a separate experiment, L-NAME (100 μM) was incubated with HCAECs in the presence of 10 μg/ml SAA. As shown in Fig. 5C, NO production levels in HCAECs were substantially inhibited by l-NAME treatment.

SAA decreases activities of CAT and SOD in HCAECs. Intrinsic antioxidants, including CAT and SOD, are present in the organism to protect it from oxidative stress (35). The activities of CAT and SOD were studied with assay kits (n = 3 for each). Both CAT and SOD activities in HCAECs were significantly decreased in a concentration-dependent manner in response to SAA treatment. SAA (10 and 25 μg/ml) treatment significantly reduced CAT activities in HCAECs by 19% and 37%, respectively, compared with controls (P < 0.05; Fig. 5D). Similarly, SOD activities were also reduced by 30% and 53%, respectively, compared with controls (P < 0.05; Fig. 5E). Coculture with the antioxidant SeMet (20 μM) effectively blocked the SAA-induced decrease of activities of both enzymes to control levels (P < 0.05; Fig. 5F). SeMet (20 μM) effectively blocked the SAA-induced decrease in eNOS expression, restoring the suppressive effect of SAA on eNOS mRNA levels by antioxidant SeMet was examined. As shown in Fig. 5F, SeMet (20 μM) effectively blocked the SAA-induced decrease in eNOS mRNA levels in HCAECs to control levels (P < 0.05).

SAA induces phosphorylation of JNK and ERK1/2 as well as IκB-α in HCAECs. To determine whether MAPKs could be involved in the signal transduction pathways of SAA-induced endothelial dysfunction, the activation status of three major MAPKs (JNK, ERK1/2, and p38) was determined by Bio-Plex immunoassay. Increased phosphorylation of JNK and ERK1/2, but not p38, was observed at 15–30 min after SAA (10 μg/ml) treatment by flow cytometric measurement of DHE staining. ROS levels were substantially increased by 42% compared with controls (Fig. 5C). Furthermore, ROS levels in HCAECs were markedly increased in the early stage of 10 μg/ml SAA treatment (30 min), and the antioxidant MnTBAP (SOD mimetic) effectively blocked the SAA-induced increase in ROS levels (Fig. 5G).

**Fig. 4. Effect of SAA on NO production in HCAECs.** SAA-treated HCAECs were stained with the fluorescent dye 4-amino-5-methylamino-2',7'-difluorohorescein diacetate (DAF-FM DA) and measured by flow cytometry. A and B: NO production was significantly reduced in SAA-treated HCAECs in a concentration-dependent manner. n = 3. *P < 0.05, controls (DMSO) compared with SAA. C: l-NAME (100 μM) was incubated with HCAECs in the presence of SAA. NO production levels in HCAECs were inhibited by l-NAME treatment.
treatment (Fig. 6A). To confirm the functional role of these MAPKs in SAA action, JNK inhibitor (SP-600125, 40 μM), ERK1/2 inhibitor (PD-98059, 40 μM), or p38 inhibitor (SB-239036, 1 μM) was used to pretreat HCAECs for 1 h before SAA (10 μg/ml) treatment for 24 h, and eNOS mRNA levels were determined with real-time PCR. Accordingly, JNK and ERK1/2 inhibitors, but not the p38 inhibitor, effectively blocked the SAA-induced eNOS decrease (*P < 0.05; Fig. 6B). However, these inhibitors alone did not show any effect on eNOS expression in HCAECs. Moreover, separate experiments showed that JNK and ERK1/2 inhibitors did not have a significant impact on SAA regulating CAT or SOD mRNA levels (Supplemental Fig. S3), indicating that the reduced CAT and SOD activities induced by SAA in HCAECs may be independent to ERK1/2 activation.

Additionally, ROS production in HCAECs pretreated with the ERK1/2 inhibitor was determined with DHE staining and flow cytometry analysis. SAA-increased ROS levels in HCAECs were not significantly affected by ERK1/2 inhibitor treatment (Fig. 5G), indicating that SAA-induced ROS production in HCAECs may be upstream of ERK1/2 activation or that SAA-induced ERK1/2 activation and ROS increase may be two parallel events contributing to endothelial dysfunction. Furthermore, to determine the role of ROS and MAPKs in SAA-induced endothelial dysfunction in porcine coronary arteries, the antioxidant MnTBAP (2 μM) or PD-98059 (40 μM) was used to pretreat artery rings followed by SAA (10 μg/ml) treatment for 24 h. As shown in Fig. 6D, in regard to the vasorelaxation in response to bradykinin (10−5 M), SAA significantly reduced endothelium-dependent vasorelaxation by 34% compared with untreated control rings (*P < 0.05, n = 4). Coculture of the antioxidant MnTBAP and SAA significantly increased the vasorelaxation by 31% compared with SAA-treated vessels (*P < 0.05). Coculture of the ERK
inhibitor and SAA also increased vasorelaxation by 24% compared with SAA-treated vessels; however, it did not reach a statistical difference ($P = 0.065$). Thus, both MnTBAP and the ERK1/2 inhibitor partially blocked the SAA-induced decrease in endothelium-dependent vasorelaxation in response to bradykinin in porcine coronary arteries, indicating that oxidative stress and ERK1/2 activation are involved in SAA-induced endothelial dysfunction. However, the maximal contraction and endothelium-independent vasorelaxation were not significantly different among these treatment groups (data not shown).

In most resting cells, the transcription factor NF-$\kappa$B is sequestered in the cytoplasm in an inactive form associated with inhibitory molecules such as I$k$B-$\alpha$. The phosphorylation status of I$k$B-$\alpha$ can release the inhibition of NF-$\kappa$B, thereby activating NF-$\kappa$B (34). After SAA (10 $\mu$g/ml) treatment, we observed a gradual increase of I$k$B-$\alpha$ phosphorylation levels, starting from 15 min and peaking at 60 min (Fig. 6C). These data suggest that SAA may activate NF-$\kappa$B.

**DISCUSSION**

It is not fully understood whether SAA can mediate cardiovascular pathogenesis, although its levels are associated with cardiovascular disease. The present study provides direct evidence for biological functions of SAA in the vascular system. We demonstrated a potential mechanism by which SAA causes endothelial dysfunction of porcine coronary arteries and HCAECs. SAA increases ROS production, decreases eNOS expression, and activates JNK and ERK1/2 MAPKs, which may function as signal transduction pathways for SAA’s action in endothelial cells. SAA could serve as a potential therapeutic target in patients with a high risk for cardiovascular disease.

Clinically, plasma levels of SAA can be determined by several techniques such as ELISA, radioimmunoassays, nephelometric assays, and dissociation-enhanced lanthanide fluorescence immunoassay. The sensitivity of these detection methods is different, and thereby plasma levels of SAA are variable among different reports due to the different methods used. In general, the plasma level of SAA in a healthy population without evidence of acute inflammation is ~2–4 µg/ml or less. However, plasma SAA levels can increase from 100 to 1,000 µg/ml in response to inflammation, viral or bacterial infections, neoplasia, and vasculitis (19, 32, 44, 47). Plasma SAA levels were significantly higher in patients with severe atherosclerosis than in normal individuals (11, 22, 32). For example, Johnson et al. (21) reported that a total of 705 women starting from 15 min and peaking at 60 min ($n = 3$). D: blocking effects of MnTBAP (SOD mimetic) or PD-98059 on the SAA (10 $\mu$g/ml)-induced decrease in endothelium-dependent relaxation in response to bradykinin (10$^{-8}$ M), $n = 4$. *P < 0.05, SAA compared with JNK inhi- bitor; + SAA, ERK1/2 inhibitor + SAA, or MnTBAP + SAA.

**Fig. 6.** Effects of SAA on the phosphorylation of MAPKs and I$k$B-$\alpha$ in HCAECs. HCAECs were treated with SAA (10 $\mu$g/ml) for different times. Phosphorylated and total ERK2, JNK, and p38 as well as I$k$B-$\alpha$ were detected by Bio-Plex immunoassay. A: SAA (10 $\mu$g/ml) treatment increased the ratios of phosphorylated and total ERK1/2 and JNK, but not p38, at 15 and 30 min, respectively. B: to confirm the functional role of these MAPKs in SAA action, JNK inhibitor (SP-600125, 40 $\mu$M), ERK1/2 inhibitor (PD-98059, 40 $\mu$M), or p38 inhibitor (SB-239036, 1 $\mu$M) was used to pretreat HCAECs for 1 h before SAA (10 $\mu$g/ml) treatment. eNOS mRNA levels were analyzed with real-time PCR. JNK and ERK1/2 inhibitors, but not the p38 inhibitor, effectively blocked the SAA-induced eNOS decrease. C: SAA (10 $\mu$g/ml) treatment induced a gradual increase of I$k$B-$\alpha$ phosphorylation levels starting from 15 min and peaking at 60 min. $n = 3$. D: blocking effects of MnTBAP (SOD mimetic) or PD-98059 on the SAA (10 $\mu$g/ml)-induced decrease in endothelium-dependent relaxation in response to bradykinin (10$^{-8}$ M), $n = 4$. *P < 0.05, controls (DMSO) compared with SAA; #P < 0.05, SAA compared with JNK inhibitor + SAA, ERK1/2 inhibitor + SAA, or MnTBAP + SAA.
system is one of the most important components in the endog-
enuous defense against vascular injury, inflammation, and
thrombosis. Inhibition of eNOS accelerated atherosclerosis in
experimental animals (9). An impairment of endothelium-
dependent vasorelaxation is present in atherosclerotic vessels
even before vascular structural changes occur and represents
reduced eNOS-derived NO activity (23). Many cardiovascular
risk factors can directly reduce eNOS expression at transcription-
and/or posttranscriptional levels. For example, tetrahy-
drobiopterin (BH4) is a critical coenzyme of eNOS. When BH4
availability is reduced, eNOS decreases NO production,
whereas it increases superoxide anion production. This process
is termed “eNOS uncoupling” (45). In addition, TNF-α could
reduce eNOS mRNA stability through potential mechanisms of
unknown cytoplasmic proteins binding to eNOS mRNA 3′-
untranslated region (UTR) sequences (1, 25, 43). More re-
cently, we have shown that sCD40L also decreased eNOS
mRNA stability, and unknown cytoplasmic molecules were
able to bind to the eNOS mRNA 3′-UTR in sCD40L-treated
HCAECs (12). Furthermore, we performed a 95-micro-RNA
(miRNA) profiling experiment in HCAECs treated with
sCD40L or TNF-α for 24 h. sCD40L or TNF-α treatment
induced a specific expression pattern of 95 miRNAs, which
may be able to regulate eNOS mRNA translation or stability.
For examples, we found that sCD40L and TNF-α could in-
crease miR-221 levels by 38% and 47%, respectively (12).
miR-221 may have functions to regulate eNOS levels (46).
However, it is not clear whether SAA is able to regulate eNOS
expression at transcriptional and/or posttranslational levels in
endothelial cells.

The present study shows that SAA impairs endothelium-
dependent relaxation of porcine coronary arteries and down-
regulates eNOS expression in both porcine arteries and
HCAECs, indicating that SAA causes endothelial dysfunction
via damaging the NO/eNOS system. Moreover, we found that
endothelium-dependent vasorelaxation of either SAA-treated
or untreated rings was significantly blocked by 1-NAME.
Removal of NO by 1-NAME reduced vasorelaxation in re-
sponse to bradykinin in both SAA-treated and untreated rings.
These data suggest that SAA-induced endothelial dysfunction
is mainly through inhibition of NO production.

ROS are key mediators for vascular inflammation and
atherosgenesis via a variety of mechanisms, including a state of
continuous NO consumption and depletion, intracellular alka-
linization, and regulation of gene transcription (49). In the
present study, we found SAA induced a substantial increase of
ROS in both porcine coronary artery rings and HCAECs.
However, specific mechanisms of SAA-induced ROS produc-
tion are not well understood. Several enzymatic systems con-
tribute to ROS production in vascular endothelial cells, includ-
ing NA(D)PH oxidase, xanthine oxidase, uncoupled eNOS,
and the mitochondrial electron transport chain (16, 49).
NADPH oxidase is one of the best characterized ROS-gener-
ating enzymes in inflammation and in vascular cells. It has
been reported that SAA is able to induce the activation of
neutrophil NADPH oxidase with a resulting release of ROS
(6). Our previous study (48) has indicated that C-reactive
protein, an acute inflammatory protein, increased ROS produc-
tion in macrophage-derived foam cells, which may result
from mitochondrial dysfunction and upregulation of NADPH ox-
dase. In addition, decreased levels and/or activities of internal
antioxidant enzymes such as SOD and CAT may induce a
increase of ROS. SOD facilitates the formation of H2O2 from
superoxide anion, whereas CAT catalyzes the reaction of H2O2
to water (16).

Although endothelial dysfunction is a multifactorial process,
there is evidence to support that increased vascular ROS
production is likely to be an important step since superoxide
reacts rapidly with NO, resulting in the formation of perox-
ynitrite anion and loss of bioactivity of NO (30). ROS may
promote the oxidative degradation of BH4, leading to the
decreased production of NO (8). The present study dem-
strates that SAA significantly increased ROS production in
porcine coronary arteries and HCAECs, whereas it decreased
both CAT and SOD activities simultaneously in HCAECs.
SAA-induced decreases in CAT and SOD activities may be
responsible for the oxidative stress and reduction of NO bio-
availability, thereby attributing to endothelial dysfunction.
SAA stimulates TNF-α secretion from human T lymphocytes
by forming a SAA-extracellular matrix complex (37), and
TNF-α can induce oxidative stress (17). SAA at high concen-
trations (50 and 100 μg/ml) can stimulate neutrophil superox-
dide anion production (15). NO bioavailability is mainly depen-
dent on NO production by eNOS and the presence of ROS. A
decreased level of eNOS could directly reduce NO production.
Decreased levels of CAT and SOD could increase the accu-
mulation of ROS, which readily interact with NO to form
peroxynitrite (13), thereby reducing NO bioavailability. In-
creased ROS could also inhibit eNOS expression and activity
(40). Thus, it is possible that there may be a synergistic effect
between the loss of eNOS and loss of CAT and SOD reducing
NO bioavailability in response to SAA.

In addition to the detrimental effects of ROS, multiple
studies have demonstrated that elevated ROS could play a role
in various signaling pathways, among which are MAPKs,
including ERK, JNK, and p38. MAPKs can be activated by a
wide variety of stimuli, such as inflammation, growth factors,
and ROS. For example, ROS modulates the cardiomyocyte
response to ischemia-reperfusion through MAPK activation
(33). ROS scavengers inhibited the activities of ERK1/2 and
p38 in alveolar epithelial cells (53, 54). It is possible that
MAPK is involved in the onset of ROS production because
MAPK activation precedes ROS production. However, recent
evidence has indicated that ROS production is induced by a
mechanism independent of the activation of MAPKs (41). In
the present study, we demonstrated that the SAA-induced ROS
increase in HCAECs is not significantly affected by treatment
of the ERK1/2 inhibitor, although SAA can activate ERK1/2.
The reason for this observation is clear, and we speculate that
SAA-induced ROS production in HCAECs may be the up-
stream of ERK1/2 activation or that the SAA-induced ERK1/2
activation and ROS increase may be two parallel events con-
tributing to endothelial dysfunction. This interesting relation-
ship between ROS and MAPK activation in SAA-treated
vessels or cells warrants further investigation.

ROS may mediate the activation of MAPKs in a variety of
cells, leading to the activation of transcription factors such as
NF-κB, attributing to changes in gene expressions (39, 52).
ROS have been implicated in initiating inflammatory responses
in the lungs through the activation of transcription factors such
as NF-κB, causing chromatin remodeling and gene expression
of proinflammatory mediators (38). MAPK pathways have

AJP-Heart Circ Physiol • VOL 295 • DECEMBER 2008 • www.ajpheart.org
been involved in regulating these transcription factors. SAA has been found to activate MAPKs such as ERK1/2 and p38 in both HeLa and THP-1 cells in a CD36 and LIMPII analogous-1-dependent manner (5). SAA activates NF-κB and proinflammatory gene expression in human and murine intestinal epithelial cells (20). A recent study (55) has indicated that SAA rapidly induces the expression and activity of tissue factor and that the effects were mediated through the activation of MAPK and NF-κB. In the present study, increased phosphorylation of JNK, ERK1/2, and IkB-α was observed in SAA-treated HCAECs, and JNK and ERK1/2 inhibitors effectively blocked the SAA-induced eNOS decrease, indicating that the signal pathways of JNK and ERK1/2 as well as NF-κB may be involved in SAA’s action in endothelial cells.

In summary, the present study shows that SAA induces endothelial dysfunction of porcine coronary artery endothelial cells and HCAECs via increasing ROS production, decreasing eNOS expression, inhibiting CAT and SOD activities, and activating JNK and ERK1/2 MAPKs. Antioxidants or the EKR1/2 inhibitor effectively blocked the SAA-induced eNOS decrease, indicating that inhibition of oxidative stress and MAPK activation may have the potential to block SAA-induced endothelial dysfunction.

ACKNOWLEDGMENTS

Authors thank Dr. Esteban A. Henao for technical assistance.

GRANTS

This work was partially supported by National Institutes of Health Research Grants HL-076345 (to P. H. Lin), DE-15543 (to Q. Yao), AT-003094 (to Q. Yao), EB-002436 (to C. Chen), and HL-083471 (to C. Chen) as well as by the Grants HL-076345 (to P. H. Lin), DE-15543 (to Q. Yao), AT-003094 (to Q. Yao), and HL-083471 (to C. Chen).

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