Periadventitial adipose tissue impairs coronary endothelial function via PKC-β-dependent phosphorylation of nitric oxide synthase

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Periadventitial adipose tissue impairs coronary endothelial function via PKC-β-dependent phosphorylation of nitric oxide synthase. Am J Physiol Heart Circ Physiol 297: H460–H465, 2009. First published May 29, 2009; doi:10.1152/ajpheart.00116.2009. —Endogenous periadventitial adipose-derived factors have been shown to contribute to coronary vascular regulation by impairing endothelial function through a direct inhibition of endothelial nitric oxide synthase (eNOS). However, our understanding of the underlying mechanisms remains uncertain. Accordingly, this study was designed to test the hypothesis that periadventitial adipose tissue releases agents that attenuate coronary vascular regulation by impairing endothelial function through a protein kinase C (PKC)-dependent mechanism. Isometric tension studies were conducted on isolated canine circumflex coronary arteries with and without natural amounts of periadventitial adipose tissue. Adipose tissue significantly diminished coronary endothelial-dependent vasodilation and nitric oxide production in response to bradykinin and acetylcholine. The selective inhibition of endothelial PKC-β with ruboxistaurin (1 μM) abolished the adipose-induced impairment of bradykinin-mediated coronary vasodilatation and the endothelial production of nitric oxide. Western blot analysis revealed a significant increase in eNOS phosphorylation at the inhibitory residue Thr495 in arteries exposed to periadventitial adipose tissue. This site-specific phosphorylation of eNOS was prevented by the inhibition of PKC-β. These data demonstrate that periadventitial adipose-derived factors impair coronary endothelial nitric oxide production via a PKC-β-dependent, site-specific phosphorylation of eNOS at Thr495.

IN RECENT YEARS, investigators have increasingly recognized adipose tissue as both an active endocrine and paracrine organ. The production of adipose-derived cytokines (adipokines) has been well documented to influence many physiological and pathophysiological conditions (28). Specifically, adipokine production has been shown to influence a number of pathogenic pathways, including chemotaxis (21), inflammation (24), smooth muscle proliferation (3), and other key mediators of atherogenesis (28). Although adipokines have been proposed to be the molecular link between obesity and cardiovascular disease (28), the exact relationship between adipose tissue and vascular function remains uncertain. Studies have implicated the surrounding periadventitial adipose tissue as a local source of adipokines that contribute to both vascular function and disease (9, 20, 23, 34). Coronary atherosclerotic disease frequently occurs in large arteries encased by adipose tissue (23), whereas the opposite is true of arterial segments located underneath myocardial bridges lacking periadventitial adipose tissue (22). Together, these observations suggest that vascular function and disease are partly dependent on the presence of periadventitial adipose tissue.

Recent work from our laboratory documented that periadventitial adipose tissue significantly impaired coronary endothelial function in response to bradykinin both in vitro and in vivo, further implicating local adipose tissue in the initiation and pathogenesis of coronary vascular disease (39). In particular, we documented that adipose-derived factors diminished endothelial nitric oxide (NO) production through the direct inhibition of NO synthase. This effect was independent of changes in oxidative stress and peroxide-mediated vasodilation and was not related to alterations in smooth muscle responsiveness to NO. These findings are important because dysfunction and injury of the vascular endothelium are widely accepted to be a critical precursor to the development of atherosclerosis (42). However, no investigation has successfully identified the mechanisms by which periadventitial adipose-derived factor(s) impair coronary endothelial function.

Our current understanding of vascular disease would suggest that periadventitial adipose tissue may serve as a paracrine source of harmful inflammatory adipokines. However, few studies have characterized how periadventitial adipose tissue contributes to normal, healthy coronary endothelial function. Recent findings call attention to specific adipokines such as interleukins, TNF-α, and leptin as potential periadventitial adipose-derived factors altering endothelial function (9, 39, 46). Although promising, these investigations have yet to clearly identify the exact paracrine mediators of coronary endothelial impairment. Furthermore, the task of identifying key mediators from periadventitial adipose tissue without additional experimental evidence may prove to be arduous. Accordingly, the purpose of the present study was to delineate a common signaling pathway for periadventitial adipose tissue-induced endothelial dysfunction.

We propose that periadventitial adipose tissue attenuates coronary endothelial NO production via a protein kinase C (PKC)-β dependent, site-specific phosphorylation of endothelial NO synthase (eNOS) at Thr495. PKC is a known negative regulator of eNOS activity and NO production (38). Specifically, the phosphorylation of eNOS at the inhibitory Thr495 site significantly diminishes enzymatic activity by disrupting the binding of Ca2+/calmodulin to eNOS (16, 33). Further justification for our hypothesis is based on the well-documented role of PKC-β as a mediator of endothelial dysfunction (5, 7, 35, 46). Two recent clinical trials observed that the inhibition of this specific, predominately endothelial isoform of PKC markedly improved both macrovascular (35) and microvascular (7) endothelial function in patients with type II diabetes. Similarly, additional investigations suggest that endothelial impairment...
as a result of increased adiposity and/or adipokine release is potentially linked with PKC activity (5, 46). However, to date, no investigation has directly examined whether PKC activity contributes to periadventitial adipose-induced endothelial dysfunction.

METHODS

This investigation was approved by the Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Pub. No. 85-23, Revised 1996). Seven lean, mongrel dogs weighing between 20 and 30 kg were used for all experiments. Dogs were euthanized with a lethal intravenous dose of pentobarbital sodium (86 mg/kg body wt). After cardiac arrest was confirmed, a left lateral thoracotomy was performed to collect the heart.

Functional assessment of isolated epicardial coronary rings. Isolated coronary artery studies were performed as previously described (12, 25). Briefly, the left circumflex coronary arteries from lean dogs were dissected with or without the naturally surrounding periadventitial adipose tissue (~0.25 g adipose per ring). Arteries and periadventitial adipose tissue were always taken from the same animal and collected at the same time. Care was taken to isolate the same 2- to 3-cm proximal portion of the circumflex artery that is naturally surrounded by periadventitial adipose tissue. Arteries were cut into 3-mm rings and mounted in organ baths for isometric tension studies. To avoid any confounding differences between proximal and distal portions of the artery, the surrounding adipose was dissected off of every other arterial ring. The optimal length was found by assessing contraction to 60 mM KCl. The arteries were precontracted with the thromboxane A2 mimetic U-46619 (1 μM) to functionally assess endothelial function. Specifically, endothelial function was assessed by the addition of graded concentrations of bradykinin (0.1 nM–10 μM/L, n = 5) or acetylcholine (1 nM–10 μM/L, n = 4) to the tissue bath. Some arteries without adipose tissue were also incubated with the NO synthase inhibitor Nω-nitro-arginine methyl ester (L-NAME; 300 μM) before the dose-response experiments. In additional studies, bradykinin concentration responses were conducted in the presence of the general PKC inhibitor Ro-31-8220 (1 μM, n = 7) or the PKC-β-specific inhibitor ruboxistaurin (1 μM, n = 6). All results obtained during bradykinin dose-response experiments are reported as the percent relaxation for arterial rings from individual animals (Figs. 1 and 2). One hundred percent relaxation was defined as a return to the level of tension before thromboxane A2 mimic (U-46619) contraction.

Tissue collection and Western blot analysis. Coronary arteries from lean dogs (n = 3 dogs) were isolated and incubated in 5-mL organ baths for ~1 h at 4°C in Krebs-buffered solution. All arteries used for protein analysis were cleaned of periadventitial adipose tissue. Control arteries were allowed to incubate alone, whereas other arteries were incubated with either 3 g of periadventitial adipose (floating in the bath) or both adipose and ruboxistaurin. Following incubation, the arteries were immediately placed in liquid N2 and stored at −80°C for Western blot analysis as previously described (6, 13, 52). Following protein isolation, equivalent amounts of protein were loaded onto 10% acrylamide gels for electrophoresis and blotting. After being blocked for 1 h at ambient temperature, the membranes were incubated overnight at 4°C with primary antibodies directed against eNOS and phosphorylated eNOS Thr^695 (both 1:1,000; Affinity BioReagents and BD Transduction). The blots were washed and incubated with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase secondary antibodies (1:5,000; Santa Cruz Biotechnology) for 1.5 h at ambient temperature. The blots were then stripped and reprobed with β-actin antiserum (1:5,000; Santa Cruz Biotechnology) as the internal control. Immunoreactivity was visualized using an enhanced chemiluminescence Western blot analysis detection kit (GE Healthcare) and quantified by scanning densitometry (Quantity One 1-D Analysis Software; Bio-Rad).

NO measurements. NO concentration was measured in isolated coronary arteries (n = 4 animals) before and during exposure to 400 nM bradykinin with or without the addition of periadventitial adipose tissue as previously described (39). NO was evaluated by a polargraphic technique, using a carbon fiber, recessed-tip glass microelectrode (51). This microelectrode has been previously demonstrated to not be sensitive to NO synthase inhibitors, including L-NAME and nitroglycerine formed by L-NAME when properly constructed with a Nafion barrier. eNOS blockade dramatically reduces the NO signal, indicating a measurement of endothelium-derived NO (10, 27). After the arterial rings were placed into a media-perfused organ bath, the tip of the NO microelectrode was positioned within the vessel lumen and pushed against the endothelial surface to ensure a stable, close contact. Baseline concentrations of NO were measured, followed by the response to bradykinin and a washout. The arterial rings were then treated with ruboxistaurin (1 μM) for ~30 min before repeating NO measurements. Finally, the periadventitial adipose tissue was added to the flowing media upstream of the artery for an additional 30 min, and a similar experimental protocol was conducted.

Statistical analyses. Data are presented as means ± SE. For isometric tension studies, a two-way ANOVA was used to test the effects of the periadventitial adipose and various doses of bradykinin, whereas t-test analysis was used to compare half-maximal effective concentration (EC50) values (Sigma Stat 3.0 Software). A two-way repeated-measures ANOVA was used to analyze Western blot densities and NO measurements. All experiments were analyzed per animal. When statistical differences were found, a Student-Newman-Keuls multiple comparison test was performed. The criterion for statistical significance was P < 0.05 in all tests.

RESULTS

Periadventitial adipose tissue and coronary endothelial function. To examine the effects of endogenous periadventitial adipose-derived factors on coronary endothelial function, iso-
metric tension studies were conducted in isolated coronary arteries with and without periadventitial adipose tissue. Consistent with our recent data (39), we found that the presence of periadventitial adipose tissue significantly decreased coronary endothelial-dependent vasodilation (Fig. 1). Specifically, periadventitial adipose attenuated arterial relaxation to both bradykinin in the concentration range from 1 to 320 nM ($P < 0.001$, Fig. 1B) and acetylcholine in the concentration range from 32 to 100 nM ($P < 0.001$, Fig. 1A). Adipose tissue also increased the EC$_{50}$ of bradykinin from 4.0 ± 1.2 to 14.7 ± 2.4 nM ($P < 0.01$) and of acetylcholine from 35.4 ± 10.5 to 89.7 ± 9.3 nM ($P < 0.05$). Periadventitial adipose tissue had no effect on the maximal vasodilatory response to bradykinin (98 ± 2%) or acetylcholine (88.9 ± 3%). Additional experiments with clean arterial rings in the presence of the NO synthase inhibitor l-NAME (300 µM) demonstrated that a pharmacological inhibition of NO production was similar to the effect of periadventitial adipose tissue alone. In particular, there was a modest difference between control arteries treated with l-NAME and arteries with periadventitial adipose tissue in response to acetylcholine ($P < 0.05$, Fig. 1A), whereas no difference was observed in response to bradykinin ($P = 0.81$, Fig. 1B).

PKC and adipose-induced impairment of coronary endothelial function. To test the hypothesis that PKC-β mediates periadventitial adipose-induced endothelial impairment, we conducted additional isometric tension studies with different inhibitors of PKC. The administration of the general PKC inhibitor Ro-31-8220 (1 µM) or the PKC-β-specific inhibitor ruboxistaurin (1 µM) eliminated the effect of periadventitial adipose on bradykinin-induced coronary vasodilation (Fig. 2; $P = 0.49$ vs. 0.86, respectively). Both Ro-31-8220 and ruboxistaurin had no effect on the vasodilation of control arteries without adipose tissue as the EC$_{50}$ values were not statistically different (Ro-31-8220, EC$_{50}$ = 2.5 ± 1.3 nM; and ruboxistaurin, EC$_{50}$ = 2.7 ± 1.0 nM). The pretreatment with Ro-31-8220 and ruboxistaurin significantly enhanced endothelial-dependent vasodilation in arteries with periadventitial adipose tissue and maintained EC$_{50}$ values at control levels (Ro-31-8220 EC$_{50}$ = 2.4 ± 1.0 nM; and ruboxistaurin EC$_{50}$ = 2.2 ± 0.5 nM; $P < 0.001$ vs. untreated).

Periadventitial adipose and phosphorylation of eNOS. The incubation of coronary arteries with periadventitial adipose tissue significantly increased the degree of site-specific eNOS phosphorylation at the inhibitory Thr$^{495}$ residue (~140 kDa; Fig. 3; $P < 0.01$). This increase in eNOS phosphorylation was prevented by the pretreatment of arteries with ruboxistaurin ($P = 0.23$). No differences in expression of total eNOS or β-actin were noted between treatments (Fig. 3; $P = 0.30$ and 0.41, respectively). To assess the relative changes in the phosphorylation state of eNOS, the ratio of phosphorylated eNOS-Thr$^{495}$ to total eNOS was calculated. We found that the phosphorylated eNOS-Thr$^{495}$-to-total eNOS ratio was significantly elevated for arteries treated with periadventitial adipose tissue (Fig. 3; $P < 0.01$). This specific increase in the phosphorylation state of eNOS was prevented by the pretreatment of arteries with ruboxistaurin.

PKC, periadventitial adipose, and NO production. Additional experiments were also conducted to determine whether the inhibition of PKC-β improves coronary endothelial NO production in response to bradykinin in the presence of periadventitial adipose tissue. Previously, we documented that periadventitial adipose-derived factors significantly diminish both baseline and bradykinin-stimulated increases in coronary endothelial NO production ($P < 0.05$) (39). In the present study, we found that baseline NO concentration averaged 292 ± 65 nM in untreated control arteries. Under control conditions, the administration of bradykinin increased NO production by 193.3 ± 38 nM (Fig. 4). The pretreatment of arteries with ruboxistaurin did not significantly affect either baseline or bradykinin-stimulated NO production. In contrast, the addition of periadventitial adipose tissue dramatically de-
increased NO production, and bradykinin failed to significantly increase coronary NO production. Finally, the pretreatment of arteries with ruboxistaurin prevented this effect of periadventitial adipose tissue, as the increase in coronary NO production was similar to control conditions.

**DISCUSSION**

The present investigation was designed to elucidate the specific cellular/molecular mechanism by which periadventitial adipose tissue impairs coronary endothelial function. We hypothesized that PKC, a known negative regulator of eNOS, was a key mediator of adipose-induced endothelial dysfunction. The major new findings of this study include the following: 1) periadventitial adipose-derived factors impair both bradykinin and acetylcholine-mediated vasodilation and NO production through direct inhibition of eNOS activity (Fig. 1); 2) periadventitial adipose-induced impairment of endothelium-dependent vasodilation is similar to a pharmacological inhibition of NO synthase (Fig. 1); 3) selective inhibition of PKC-β prevents periadventitial adipose-induced endothelial dysfunction in isolated coronary arteries (Figs. 2 and 4); 4) factors released from periadventitial adipose tissue increase site-specific phosphorylation of eNOS at the inhibitory Thr495 residue (Fig. 3); 5) PKC-β inhibition significantly decreases the level of eNOS phosphorylation at Thr495, and 6) blockade of PKC-β prevents decreases in coronary endothelial NO production induced by periadventitial adipose tissue (Fig. 3). Taken together, these results indicate that periadventitial adipose tissue releases factor(s) that selectively impair endothelial-dependent dilation via a PKC-β-dependent phosphorylation of eNOS at Thr495.

To address the growing prevalence of cardiovascular disease, recent investigations have attempted to better understand both the natural and pathophysiological relationship between periadventitial adipose tissue and vascular function. Initial investigations suggested that periadventitial adipose significantly attenuated the contractile responses of rat aorta (14, 18, 30, 45), rat mesenteric (17, 47), and human internal thoracic arteries (19) through the release of an adipose-derived relaxing factor. While these initial findings suggest a role for local adipose in regulating arterial tone, it is hard to ignore the mounting evidence that periadventitial adipose disrupts normal endothelial function and potentially contributes to atherogenesis (9, 20, 23, 34). Previous studies have documented that coronary periadventitial adipose tissue increases with obesity (37, 43, 44) and could serve as a link between inflammatory adipokines released during obesity and cardiovascular disease (2, 24, 32). In particular, recent work from our laboratory was the first to document that factor(s) released by coronary periadventitial adipose tissue rapidly inhibit NO production and concurrently attenuate coronary endothelial-dependent vasodilation (39). Importantly, we found that periadventitial adipose tissue had no effect on coronary smooth muscle response to NO, superoxide (O2·−) production, or hydrogen peroxide (H2O2)-mediated vasodilation.

The current findings confirm these previous observations (Figs. 1 and 4) and demonstrate that periadventitial adipose tissue results in a generalized impairment of eNOS activity that is not limited to specific endothelium-dependent vasodilators (i.e., impairment of both bradykinin of acetylcholine). We acknowledge that examining the regulation of NO production via agonists in which a majority of the dilation is not mediated by NO complicates the interpretation of these data. However, we chose to use these agonists because they are endogenous regulators of endothelial function that are classically used to examine the “physiological” regulation of coronary NO production. Importantly, our findings on the degree to which NO contributes to acetylcholine- and bradykinin-mediated dilation are consistent with those previously documented in isolated canine coronary arteries by the Vanhoutte laboratory (11). We submit that this limitation is tempered by the following: 1) adipose-induced impairment of endothelial-dependent dilatation is comparable with pharmacological inhibition of NO synthesis (Fig. 1); 2) our previous data demonstrate no additive effect of periadventitial adipose tissue + NO synthase blockade on bradykinin-mediated dilation (39); and 3) inhibition of PKC-β abolishes the effect of periadventitial adipose tissue on endothelial-dependent dilatation (Fig. 2), phosphorylation of eNOS at Thr495 (Fig. 3), and coronary endothelial NO production (Fig. 4). The credibility of the NO microelectrodes has been extensively addressed in previous studies by the Bohlen laboratory (5, 10, 27, 51). Additionally, the NO microelectrode data nicely correspond with the functional data from isolated coronary arteries with adipose and NO synthase inhibition with L-NAME and/or ruboxistaurin.

We hypothesize that the effects of periadventitial adipose tissue are unlikely to extend throughout the coronary circulation as only the conduit, nonresistance vessels have significant periadventitial adipose tissue. Furthermore, decreased NO production from these conduit arteries would not significantly affect vascular resistance since earlier studies have demonstrated that the blockade of NO synthesis alone produces little/no change in coronary blood flow (1, 4, 15). Our findings do importantly suggest that the periadventitial adipose tissue could be a potential contributor to endothelial dysfunction and atherosclerosis. However, future investigations are needed to more thoroughly examine this hypothesis in the setting of obesity.

Results from the present investigation indicate that periadventitial adipose-induced impairment of coronary endothelial NO production is mediated via a PKC-β-dependent pathway (Figs. 2–4). Earlier studies have documented a potential rela-
tion among obesity, PKC-β, and endothelial dysfunction (5, 7, 35, 46). In particular, Tinsley et al. (46) found that interleukin-1β (IL-1β), IL-6, and TNF-α can independently increase PKC activation, gap formation, and hyperpermeability across an endothelial monolayer. Likewise, the results from Bohlen’s laboratory (5) demonstrated that the inhibition of PKC-β substantially reversed endothelial dysfunction in Zucker obese rats, whereas recent clinical trials have documented an improved peripheral macrovascular endothelial function in patients with type II diabetes treated with ruboxistaurin (35). The present investigation extends these previous findings by implicating coronary periadventitial adipose tissue as an anatomic link between increased an PKC activity and endothelial impairment. The present findings, therefore, connect a proposed mediator of cardiovascular disease with a local adipose depot, further suggesting that periadventitial adipose-derived factors may contribute to the development of vascular dysfunction and disease.

Our data also indicate that periadventitial adipose tissue impairs coronary endothelial NO production via site-specific phosphorylation of eNOS by PKC-β at the key inhibitory residue Thr495. This finding agrees with previous investigations documenting that phosphorylation of eNOS at Thr495 by PKC decreases NO production and disrupts the protein-protein interaction between eNOS and calmodulin (16, 33, 36). Hence, periadventitial adipose-derived factors increase the basal phosphorylation state of eNOS-Thr495, thereby impairing coronary endothelial increases in NO production. However, the current findings extend these previous studies by implicating a direct role for the β-isof orm of PKC in eNOS phosphorylation. It is important to note that eNOS is regulated by a very complex network of kinases (protein kinases A, B, and C), phosphatases (protein phosphatases 1 and 2), cofactors (i.e., tetrahydrobiopterin, flavin mononucleotide, and nicotinamide adenine dinucleotide phosphate), protein-protein interactions, and subcellular localization (38). In general, many of these eNOS regulators have been shown to influence eNOS activity during disease states. In particular, the phosphorylation of the Ser1177 residue is thought to be among the most critical regulatory sites for eNOS activation (38). Furthermore, the disruption of Ser1177 residue (8) and the altered availability of tetrahydrobiopterin (41, 49, 50) have both been shown to increase eNOS production of O2−. These observations are in direct contrast to our recent data that found no involvement of O2− in periadventitial adipose-induced endothelial dysfunction (39). Although still uncertain, recent data also support a role for Ser116 as an inhibitory site of eNOS (when phosphorylated) (29). Hence, alternative mechanisms for periadventitial adipose-induced endothelial dysfunction exist. However, it is important to recognize that the PKC-β inhibitor ruboxistaurin prevented adipose-induced decreases in endothelial-dependent dilation and NO production (Figs. 2 and 4), as well as increases in eNOS-Thr495 phosphorylation (Fig. 3). Therefore, our present findings argue against a significant role for these alternative pathways.

To date, no investigation has successfully identified the periadventitial adipose-derived factor(s) that impair coronary endothelial function. However, the known association between PKC-β and endothelial dysfunction suggests that the active agent(s) released from periadventitial adipose tissue are likely harmful inflammatory adipokines that are known to be elevated during obesity. Importantly, results from Tinsley et al. (46) suggest a potential role for interleukins and TNF-α as periadventitial adipose-derived factors mediating endothelial dysfunction. These data are consistent with our recent findings which documented that periadventitial adipose tissue expresses appreciable concentrations of resistin, leptin, IL-1β, and TNF-α (39). Importantly, each of these factors has been shown to independently produce endothelial dysfunction (12, 25, 40, 46). In addition, other investigations have detected the presence of leptin, TNF-α, and other adipokines in coronary periadventitial adipose tissue (9, 39). The present results importantly implicate a common PKC-β-dependent pathway by which periadventitial adipose-derived mediators induce coronary endothelial dysfunction. In addition, our findings support the “outside to inside” (26, 31, 48) signaling paradigm for periadventitial adipose-derived factors in the pathogenesis of coronary vascular disease as well as PKC-β as a therapeutic target to improve endothelial function. Although our data establish a mechanistic link between periadventitial adipose tissue and diminished coronary endothelial NO production, additional studies are needed to examine the effects of periadventitial adipose-derived adipokines in the setting of obesity and the metabolic syndrome.

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