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The mechanism of flow-induced dilation in human adipose arterioles involves hydrogen peroxide during CAD

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Phillips SA, Hatoum OA, Gutierrez DD. The mechanism of flow-induced dilation in human adipose arterioles involves hydrogen peroxide during CAD. Am J Physiol Heart Circ Physiol 292: H93–H100, 2007. First published October 13, 2006; doi:10.1152/ajpheart.00819.2006.—Flow-induced dilation (FID) is a physiologically important stimulus regulating vascular tone and homeostasis of the peripheral circulation. This important endothelial mechanism of vasodilation occurs in virtually every vascular bed and, in large arterioles, it may be critical for preventing atherosclerosis through release of the endothelium-derived antiproliferative compounds nitric oxide (NO) and prostacyclin (PGI2) (21, 25, 39, 51). The studies implicating the role of NO (24) and PGI2 (26) in FID have been performed in animal models in the absence of coronary artery disease (CAD). However, no studies have evaluated the mechanism of FID in isolated human microvessels from human visceral adipose with or without CAD. Human adipose has been proposed as a source for endocrine and paracrine modulation of vascular function during the establishment of atherosclerosis, and therefore vessels in this region could serve as sentinels for endothelial alterations produced by the proatherosclerotic milieu (29, 46).

Recent evidence indicates a complex role for reactive oxygen species (ROS) in regulating vascular tone in disease. For example, in animals, cardiovascular disease and its risk factors are associated with impaired flow-induced release of NO due to production of ROS (27, 30). In contrast, FID in coronary arterioles from patients with CAD is maintained primarily by the ROS, hydrogen peroxide (H2O2), and epoxeicosatrienoic acid (33, 38). Other studies implicate H2O2 as an endothelium-derived hyperpolarizing factor (EDHF) in the mesenteric circulation of mice (36) and humans (17, 35). It is not known whether ROS-dependent mechanisms of vasodilation also occur in adipose during CAD.

Studies indicate that visceral fat accumulation is more strongly associated with cardiovascular risk factors such as hyperlipidemia, glucose intolerance, and endothelial dysfunction than other fat storage depots (20, 55). Since visceral obesity is a significant risk factor for the development of atherosclerosis and CAD, endothelial dysfunction in adipose tissue may be an early marker for the development of atherosclerosis (20). Therefore, the primary goals of this study were 1) determine the basic mechanism of FID in human visceral fat arterioles and 2) determine whether the mechanism of FID in the adipose circulation is altered by the presence of CAD.1

MATERIALS AND METHODS

Tissue acquisition. Discarded human visceral fat was obtained at the time of abdominal or thoracic surgery. Specimens were placed in cold (4°C) HEPES buffer solution. Arterioles were cleaned of connective tissue and prepared for continuous measurement of diameter as previously described (52). Vessels were grouped according to whether or not the patient had a history of CAD. The patient’s medical history (including previous diagnoses) was obtained from the surgical report by operating room nursing staff. Patients with CAD were identified by review of the past medical history and chart-documented diagnoses. Since the tissues used

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were otherwise discarded and deidentified during the surgical procedure, we were unable to ascertain the specific criteria used to make the diagnosis or quantify the severity and duration of disease. All studies were approved by the Institutional Review Board (IRB) Committee.

**General protocol and microvascular preparation.** In an organ chamber, vessels were cannulated with glass micropipettes (external tip diameter ~40 μm) filled with cold bicarbonate buffer (physiological salt solution) consisting of (in mM) 123 NaCl, 4.4 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 20 NaHCO₃, 1.2 KH₂PO₄, and 11 glucose. Both ends of the vessel were secured with 10-0 nylon Ethilon monofilament suture, and the vessel was maintained at an intraluminal pressure of 20 mmHg for 30 min. Each preparation was transferred to the stage of an inverted microscope (magnification ×200) attached to a video camera, video monitor, VCR recorder, and a video-measuring device (model VIA-100; Boeckeler). The external bathing medium was continuously superfused with heated buffer solution (pH = 7.4 ± 0.05, P0₂ = 140 ± 10 mmHg) aerated with a gas mixture of 21% O₂-5% CO₂-74% N₂ made fresh daily and maintained at 37°C. After pressure was slowly increased to 60 mmHg and maintained for 30 min during 10 min of exposure to intraluminal flow elicited by 100 cmH₂O gradient. Acquired images were analyzed for fluorescence intensity in arbitrary units per minute (AU/min) using NIH Image software, and the rate of superoxide generation was calculated as the change in arbitrary units of fluorescent intensity per minute over the 30-min period normalized to the value in the absence of flow.

To evaluate the in vitro production of peroxide, vessels were loaded with 5 × 10⁻⁶ M 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Similar to HE, microvessels were incubated with and without a cell-permeable PEG-CAT (500 U/ml) to identify that portion of fluorescence specifically attributable to H₂O₂. DCFH oxidizes rapidly to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS (30). DCF fluorescence was excited by light at 488 nm and visualized utilizing fluorescence microscopy described above.

**Materials.** Endothelin-1 (Peninsula, San Carlos, CA) was prepared in saline with 1% bovine serum albumin. Other chemicals were obtained from Sigma. Indo was dissolved in 0.2 M Na₂CO₃. MnTBAP was dissolved in ethanol. Other agents were prepared in distilled water. Final molar concentrations of agents in the organ chambers are reported. None of the pharmacological antagonists or inhibitors produced significant changes in baseline microvessel diameter and resulted in less than a 1% change in total volume.

**Statistical analysis.** All data are expressed as means ± SE. Dilation to flow is expressed as a percentage, with 100% representing the change from constricted diameter to the maximal diameter at 60 cmH₂O intraluminal pressure (usually in the presence of papaverine). Differences in maximal diameter measurements between groups were determined with a Student’s t-test. Responses to flow were assessed with a two-factor, repeated-measures ANOVA to determine the effect of a treatment on this response. A multiple stepwise regression analysis was used to determine the influence of age, sex, and underlying disease on vasodilation and on the vessel dose-treatment responses. Covariate adjustment for the presence of disease (e.g., diabetes or hypertension) or other variables (e.g., age) was used to adjust for the influence of these variables on the degree and mechanism of vasodilation. Statistical significance was defined as a value of P < 0.05.

**RESULTS**

Visceral adipose resistance arteries from 63 patients were utilized for this study. The average internal resting diameter was 158 ± 5 μm at 60 mmHg intraluminal pressure. Table 1 summarizes demographics of the patients in this study.

**Responses of arterioles to flow.** Figure 1 shows the response of isolated visceral fat arterioles to intraluminal flow. Arterioles from patients with [maximal dilation (MD): 54 ± 8%; n = 13] and without (MD: 65 ± 9%; n = 12) CAD demonstrated significant and similar dilation to intraluminal flow

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Age values are means ± SE. CAD, coronary artery disease.
These studies are consistent with the observation that FID is maintained in coronary resistance arteries of patients with CAD (39). In separate experiments, removal of the endothelium nearly abolished FID both in patients with and without CAD (Fig. 1B; n/H11005 5). FID was eliminated in arterioles from patients with CAD in the presence of 30 mM KCl (MD: 76 ± 3% vs. 57 ± 11% control MD: 7.9 ± 14%; n/H11005 6). These results are consistent with previous studies of the coronary and skeletal muscle circulations indicating that FID in this preparation is endothelium dependent and that the maintained FID in microvessels from human subjects with CAD is possibly due to EDHF (25, 39).

Role of NOS and cyclooxygenase metabolites in FID. Figure 2 demonstrates that FID in vessels from patients without CAD was reduced in the presence of l-NAME (MD: 25.2 ± 6%; n/H11005 11 vs. control MD: 75 ± 5%) alone or in combination with Indo (MD: 70.1 ± 8%; n/H11005 6) or in combination with Indo (MD: 58.1 ± 12%; n/H11005 6) on FID in vessels from subjects with CAD.

Role of ROS in FID. To evaluate the role of H2O2 in mediating flow-induced relaxation of human visceral adipose arterioles, the H2O2 scavenger PEG-CAT was used. Vessels from CAD patients that were incubated with PEG-CAT, which selectively cleaves H2O2 forming water (35), constricted during intraluminal flow (MD: -14 ± 13; n/H11005 7; P < 0.05 vs. control without PEG-CAT; Fig. 3A). In contrast, PEG-CAT had no effect (MD: 57 ± 11% control vs. 58 ± 12% PEG-CAT; n/H11005 6) on FID in vessels from patients without CAD (Fig. 3B). These data suggest that H2O2 mediates the maintained FID in visceral adipose arterioles of patients with CAD, consistent with previous studies examining coronary resistance arteries (38). In contrast, incubation of vessels in PEG-SOD had no effect on FID (Fig. 4), supporting the conclusion that H2O2 plays a primary role in the dilation to flow during CAD. Incubation with MnTBAP did reduce FID in visceral arterioles from patients with CAD (MD: 76 ± 3% control vs. 7.4 ± 8% MnTBAP), consistent with its dual role as both an SOD mimic and scavenger of H2O2.

Direct application of H2O2 (10^-7 to 10^-3 M) produced a significant dose-dependent dilation in visceral fat arterioles (Fig. 2). Effect of nitric oxide (NO) synthase (NOS) inhibition and cyclooxygenase inhibition on FID of visceral fat arterioles. NOS inhibition with N^o-nitro-l-arginine methyl ester (l-NAME; 10^-4 M, P < 0.001) significantly reduced FID in patients without CAD (A; n/H11005 11). There was no additive effect of cyclooxygenase inhibition with indomethacin (Indo; 10^-5 M) and l-NAME on FID without CAD. There was no effect of l-NAME alone or combined with Indo on FID in visceral fat arterioles from patients with CAD (B; n/H11005 6). Values are means ± SE; n, number of vessels.
both in patients with (MD: 80.7 ± 4.4%; n = 8; P = not significant) and without (MD: 90.3 ± 1.5%; n = 7) CAD. To determine whether H$_2$O$_2$ generation is elevated during flow, we used DCF-DA fluorescence (47). In vessels from patients without CAD, H$_2$O$_2$ production during flow was minimal with (0.2 ± 0.01 AU/min; n = 4; see summarized data in Fig. 6) or without (0.3 ± 0.09 AU/min; n = 4; see summarized data in Fig. 6) PEG-CAT. However, flow induced a significant increase in DCF fluorescence in visceral fat arterioles from patients with CAD (1.2 ± 0.2 AU/min; n = 5; Fig. 6) vs. without CAD (0.3 ± 0.02 AU/min; n = 4; Fig. 6B), suggesting a role for H$_2$O$_2$ release during CAD.

We assessed superoxide production in visceral adipose arterioles using HE fluorescence microscopy (Fig. 7). Arteries from patients with CAD (2.0 ± 0.19 AU/min; n = 4; Fig. 7) exhibited a statistical increase in the rate of superoxide production during flow compared with vessels from patients without CAD (0.3 ± 0.09 AU/min; n = 4). There was no effect of PEG-CAT (0.3 ± 0.09 AU/min vs. control 0.2 ± 0.07 AU/min) on HE fluorescence demonstrating specificity.

Multiple stepwise regression analysis with covariate adjustment showed no influence on the vasodilatation to flow or H$_2$O$_2$ by underlying diseases (hypertension, hypercholesterolemia, diabetes mellitus, myocardial infarction, or congestive heart failure), sex, or age.

**DISCUSSION**

There are three major novel findings of this study. First, FID is mediated by NO in visceral adipose arterioles in patients without CAD. ROS contribute minimally to FID in these vessels. Second, in patients with CAD, the mechanism of FID in visceral adipose arterioles prominently involves ROS, specifically H$_2$O$_2$. Finally, flow-induced ROS generation is elevated in visceral adipose arterioles in patients with CAD versus non-CAD patients. Taken together, these results suggest that, in the presence of CAD, alternative dilator mechanisms involving vascular generation of H$_2$O$_2$ replace NO-mediated mechanisms of vascular relaxation in the adipose circulation and that the adverse effects of atherosclerotic cardiovascular disease extend to adipose.

FID is an important physiological regulator of tissue perfusion. The mediator of FID is vessel and species dependent but typically involves release of NO, PGI$_2$, and/or EDHF, which is often identified as a derivative of arachidonic acid (7) or ROS.
Previous studies suggest that endothelial NO and prostaglandins mediate FID in animal (23, 25) and human (21, 24, 39) resistance arteries. This has not been examined in adipose arterioles from patients with or without CAD. Altered endothelium-dependent FID is a hallmark of the development of cardiovascular disease and is an initiating event in the development of atherosclerotic heart disease (8). In this study, we demonstrate that visceral adipose responses to flow are NOS dependent. This finding is consistent with other studies indicating that FID of the human conduit artery (9) and forearm (45, 51) circulations are NOS dependent in vivo. Previous studies have determined that NO contributes to FID (39) in human coronary resistance arteries and to agonist-induced dilations of human subcutaneous adipose arteries (34) without coronary disease. However, this is the first study to our knowledge demonstrating NO-mediated FID in the isolated human adipose microcirculation. This observation is mechanistically consistent with studies linking the postprandial increase in adipose tissue blood flow in humans to NO (2).

Previous studies demonstrate that FID is reduced during cardiovascular disease (10, 23, 32). Cardiovascular disease states are known to reduce NO bioavailability by elevating the production of ROS, namely, superoxide (30). Many studies demonstrating reduced FID in the presence of CAD involve examination of large conduit arteries (8) that are much more prone to develop atherosclerosis than smaller arterioles. In contrast, in the current studies, there was a maintained arteriolar FID in arterioles in the presence of CAD (Fig. 1). In many cases, other endothelium-derived dilator substances compensate for the lack of NO release during flow (38) or agonist activation (17, 38, 54). This is consistent with the current observations since L-NAME plus Indo had no effect on FID in arterioles from patients with CAD. However, the ROS scavengers MnTBAP, a SOD mimetic, and PEG-CAT eliminated FID. This is consistent with previous studies where scavenging H_2O_2 with catalase blocked flow and agonist-induced dilation in both human (17, 35) and some animal (18) vascular beds.

SOD dismutates superoxide to H_2O_2; thus one might expect a SOD mimetic to increase H_2O_2 formation (31) as was observed in previous studies with Tiron (40). MnTBAP, however, has both superoxide- and H_2O_2-scavenging effects (11), which might explain its net inhibitory action on FID in this study (see RESULTS). However, a more specific scavenger of superoxide PEG SOD had no effect on FID in arterioles from CAD patients (Fig. 4), suggesting superoxide is not directly responsible for vasodilation to flow. Similarly, the more spe-
cific superoxide scavenger Tiron had no effect on FID in adipose arterioles of CAD patients (data not shown; n = 2).

Previous studies implicate NADPH oxidase as the major source of ROS during atherosclerosis (37, 50). Other likely contributors to ROS production during CAD are mitochondria, CYP-450, xanthine oxidase, and lipooxygenase and should be examined in future studies. Future studies should also test the role of the NADPH oxidase as well as other possible sources of ROS that may contribute to ROS formation in adipose arterioles during CAD.

H$_2$O$_2$ produced by the endothelium is known to stimulate smooth muscle relaxation via PG$_E_2$ release (53) and/or subsequent activation of Ca$^{2+}$-dependent K$^+$ channels (35). FID was reduced in the presence of KCl in CAD patients, implying potassium channel activation. Similar to previous studies showing increased ROS generation in patients with CAD (33, 50), flow-induced generation of H$_2$O$_2$ and superoxide fluorescence increased in adipose arterioles in patients with CAD more than in control patients without CAD. The effect of catalase on reducing H$_2$O$_2$ production confirms specificity of the DCFH fluorescence (17, 38). Taken together, these data emphasize the role of H$_2$O$_2$ in mediating FID in adipose arterioles and suggest that the increased H$_2$O$_2$ generation during CAD (33, 38) is supported by the adipose circulation in patients with coronary disease.

Although NO production was not assessed in this study, it is possible that the presence of NO in nondiseased states suppresses H$_2$O$_2$ formation by quenching generated superoxide (6). Interestingly, there was no apparent difference in H$_2$O$_2$ sensitivity of arterioles in patients with or without CAD, suggesting that alterations in mechanisms of FID are not related to enhanced sensitivity to H$_2$O$_2$ during CAD.

H$_2$O$_2$ acts as an EDHF in human mesenteric arteries (35, 40) and coronary microvessels (38). The possibility that EDHF's other than cyclooxygenase metabolites of arachidonic acid can contribute to FID in microvessels during CAD cannot be eliminated (39) since epoxyeicosatrienoic acid can be released during situations of elevated ROS when NO is reduced (43, 44). Alternatively, CYP-450 enzyme activity may generate ROS that contribute to FID during coronary disease (13) as observed in coronary arteries during heart disease (38). Future studies will need to determine the mechanism of H$_2$O$_2$ vasodilation in the human adipose circulation.

Study limitations. Our results may be confounded by the multiple sites from which visceral adipose tissue (abdominal and thoracic) was obtained. However, we observed no site-specific differences in FID mechanisms, suggesting that the results may be generalizable to multiple fat stores. Tissue was obtained from discarded specimens so it was not possible, based on IRB requirements, to collect comprehensive data about the subjects. Patients with differing underlying medical conditions underwent surgery for multiple reasons and were likely taking a variety of medications. Therefore, it is difficult to control for all of these variables as well as disease severity including CAD.

It is important to note that, in the present study, a majority of the CAD subjects had coronary disease severe enough to necessitate surgical intervention. However, there was no difference in the physiological mechanisms of FID between those patients diagnosed with CAD when no surgical intervention was required. These results are consistent with previous studies indicating FID of the brachial artery was a sensitive indicator of CAD in patients with chest pain but did not predict the severity of angiographically assessed CAD (19). Our approach was to use a statistical design to ensure that factors we were able to assess (Table 1) did not contribute as confounders to the results presented. Although we cannot exclude an effect of other factors, such as residual medications in vivo on isolated microvessels, we believe that such influences on vascular tone are minimal since vessels are superfused with >200 ml of drug-free buffer solution before diameter measurements are made. In addition, the effects of applied pharmacological dilator agents are reversed by washing with fresh buffer, suggesting that residual medications are easily removed. Similarly, because our study design involved adipose tissue discarded during surgery, we did not quantify visceral fat volume or measure body mass index to identify obesity, which could influence the mechanisms or magnitude of vasodilation independent of the presence of CAD. Future studies will need to address the specific vascular abnormalities that may occur in obese subjects.

FID is an important physiological regulator of blood flow in all tissues. This study was limited to responses of arterioles from visceral fat, which is more closely associated with cardiovascular disease. Since we have previously determined that the mechanisms of dilation are different in visceral and subcutaneous fat (49), future studies should examine how this difference is affected by CAD and its risk factors. It will also be important to determine whether other pharmacological agonists utilize endothelial production of H$_2$O$_2$ as a mechanism of vasodilation during CAD.

Other oxidative metabolites may also be detected by DCFH methods of H$_2$O$_2$ measurement. However, PEG-CAT is highly specific for H$_2$O$_2$ ensuring specificity for DCFH. Recent studies have shown that HE is oxidized to 2-hydroxyethidium, another fluorescent product that is distinct from the red fluorescent product ethidium (57). It is possible that our results using fluorescent measurements are confounded by the oxidation of HE to ethidium and 2-hydroxyethidium in microvessels. However, HPLC has recently been used to more specifically quantify HE-stained cells and tissues (56). In future studies, these methods could be helpful in providing more quantitative assessment of ROS generation in the intact microcirculation. Although ROS generation cannot be specifically quantified, the modulation of signal by specific inhibitors (i.e., PEG-CAT) argue for involvement of H$_2$O$_2$ in FID.

Clinical implications. Cardiovascular morbidity and mortality are related to the distribution of adipose. The accumulation of visceral fat is closely associated with conduit artery endothelial dysfunction (4) and heart disease (41). Previous studies indicate that vascular dysfunction during obesity is related to the increased vascular ROS and inflammation (14, 20, 28) that play a vital role in the pathogenesis of atherosclerosis (16). Postprandial lipid accumulation and peroxidation occur in individuals with high visceral fat content (20), an effect that might be related to differences in inflammatory cytokine release from other energy depots (42).

Visceral fat utilizes NO-mediated mechanisms of vasodilation to pharmacological agonists, such as bradykinin, in healthy individuals (17, 49). Endogenously released NO reduces the rate of lipolysis in adipocytes (1), possibly through its scavenging effect on ROS (15). It is conceivable that

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endothelium-derived, flow-induced NO release also inhibits lipolysis in visceral fat and may represent an important auto-regulatory mechanism in the postprandial state when adipose tissue blood flow is augmented in part by NO. Alternatively, endothelial release of ROS may predispose or contribute to the development of atherosclerosis by altering postprandial adipose tissue blood flow. When ROS are elevated, NO scavenging may result from NO scavenging by ROS. Thus altered flow-induced endothelial generation of ROS may represent a pathophysiologic link between visceral adipose accumulation, metabolism, and risk of heart disease.

In summary, this study demonstrates that H2O2 mechanisms of FID predominate in human visceral adipose arterioles during CAD. NO mediates FID in the absence of heart disease. The mechanism of FID generation in visceral adipose arterioles during heart disease warrants further investigation.

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