Microvascular Endothelial Dysfunction in Sedentary, Obese Humans Is Mediated by NADPH Oxidase

Influence of Exercise Training

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Objective—The objectives of this study were to determine the impact of in vivo reactive oxygen species (ROS) on microvascular endothelial function in obese human subjects and the efficacy of an aerobic exercise intervention on alleviating obesity-associated dysfunctionality.

Approach and Results—Young, sedentary men and women were divided into lean (body mass index 18–25; n=14), intermediate (body mass index 28–32.5; n=13), and obese (body mass index 33–40; n=15) groups. A novel microdialysis technique was utilized to detect elevated interstitial hydrogen peroxide (H_2O_2) and superoxide levels in the vastus lateralis of obese compared with both lean and intermediate subjects. Nutritive blood flow was monitored in the vastus lateralis via the microdialysis-ethanol technique. A decrement in acetylcholine-stimulated blood flow revealed impaired microvascular endothelial function in the obese subjects. Perfusion of apocynin, an NADPH oxidase inhibitor, lowered (normalized) H_2O_2 and superoxide levels, and reversed microvascular endothelial dysfunction in obese subjects. After 8 weeks of exercise, H_2O_2 levels were decreased in the obese subjects and microvascular endothelial function in these subjects was restored to levels similar to lean subjects. Skeletal muscle protein expression of the NADPH oxidase subunits $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ was increased in obese relative to lean subjects, where $p22^{phox}$ and $p67^{phox}$ expression was attenuated by exercise training in obese subjects.

Conclusions—This study implicates NADPH oxidase as a source of excessive ROS production in skeletal muscle of obese individuals and links excessive NADPH oxidase–derived ROS to microvascular endothelial dysfunction in obesity. Furthermore, aerobic exercise training proved to be an effective strategy for alleviating these maladies. (***Arterioscler Thromb Vasc Biol*. 2016;36:2412-2420. DOI: 10.1161/ATVBAHA.116.308339.)**

Key Words: acetylcholine ■ hydrogen peroxide ■ microdialysis ■ obesity ■ superoxide

Worldwide obesity rates have risen to pandemic levels, as the number of overweight and obese individuals has recently been estimated at 2.1 billion.[1] Obesity has become a major cause of mortality,[2] whereas obesity greatly increases the relative risk of death from cardiovascular disease (CVD).[3] Impaired endothelium-dependent vasodilation is a key early step in atherosclerotic progression and is predictive of future cardiovascular risk.[4] Such impairments to microvascular resistance vessels lead to decreased capillary recruitment and have been shown to be exacerbated with increasing adiposity.[5,6] Microvascular dysfunction is thought to contribute to the development of hypertension because of increased peripheral vascular resistance[7] and progression of insulin resistance by limiting nutrient and insulin delivery to skeletal muscle,[8] both pathologies that could further compound CVD risk. Thus, obesity may promote microvascular dysfunction and the ensuing elevated cardiovascular risk.

Oxidative stress is a systemic feature of obesity that is well documented in clinical and experimental studies. Excessive reactive oxygen species (ROS) production can result in apoptosis and increased cellular permeability, which may promote inflammation, endothelial dysfunction, and vascular remodeling.[9] NADPH oxidase (Nox), in particular, is considered a prominent source of vascular-derived ROS and is known to promote endothelial dysfunction and play a pathophysiological role in hypertension, atherosclerosis, and diabetic microvascular complications.[10] Recently, we developed and validated a novel microdialysis technique to measure in vivo production of the ROS hydrogen peroxide (H_2O_2) and superoxide.[11] We developed this technique because direct measurement of oxidative stress in humans is extremely difficult, with investigators typically relying on indirect by-products of lipid peroxidation in plasma or urine,[12] or from in vitro measurements of ROS production from excised tissue.[13]

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Nonstandard Abbreviations and Acronyms	
AIT	aerobic interval training
CVD	cardiovascular disease
H_2O_2	hydrogen peroxide
Nox	NADPH oxidase
ROS	Reactive Oxygen Species
XO	xanthine oxidase

Aerobic exercise training has been found to promote far-reaching health benefits in multiple organ systems that extend well beyond reducing the traditional CVD risk factors.[14] Aerobic interval training (AIT), in particular, has been shown to induce improvements in aerobic capacity, endothelial function, and insulin signaling in patients with the metabolic syndrome.[15] Although exercise has been shown to induce ROS generation during exercise in an intensity- and duration-dependent manner, the overall net effect of chronic exercise training tends to promote a reduced oxidative burden.[14] Previous studies suggest that aerobic exercise training reduces oxidative stress and reverses endothelial dysfunction through an attenuation of Nox activity.[16,17] Given the important pathological role of Nox-derived ROS, we sought to investigate the role of Nox in microvascular endothelial dysfunction in skeletal muscle of obese individuals and to determine whether aerobic exercise training mitigates this dysfunction by modulating Nox or ROS scavenging. To accomplish these goals, we coupled our newly developed ROS measurement technique with our previously established microdialysis methodology of monitoring microvascular blood flow[18,19] to simultaneously measure in vivo ROS levels and microvascular endothelial function in skeletal muscle of human subjects.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Subject Characteristics

Subject characteristics are listed in Table 1. Body fat percentage was increased in the intermediate relative to the lean group ($P<0.001$) and the obese relative to the intermediate group ($P=0.005$). There were no group differences for fasting serum glucose levels; however, higher fasting insulin levels in the obese group ($P=0.007$) resulted in a higher HOMA-IR (homeostatic model of insulin resistance; $P=0.014$) compared with the lean group. Triglyceride levels seem higher in the intermediate than the obese group, although this trend did not reach statistical significance ($P=0.265$).

Subject characteristics before and after the exercise intervention are listed in Table II in the online-only Data Supplement. None of the groups lost weight, nor were there any changes in body fat percentage or fasting glucose or insulin levels. AIT did not alter blood lipid profiles in the intermediate or obese groups, although training significantly reduced total ($P=0.013$) and low-density lipoprotein ($P=0.007$) cholesterol levels in the lean group. AIT increased VO_{2peak} in the intermediate ($P=0.001$) and obese ($P<0.001$) groups, although the increase in the lean group did not reach statistical significance ($P=0.092$).

Table 1. Pretraining Subject Characteristics and Metabolic Parameters

	Lean	Intermediate	Obese
Age, y	23.8±1.0	29.0±2.0*	25.0±1.2
Sex, M/F	5/9	7/6	3/12
Race, AA/C	2/12	4/9	7/8
Height, m	1.70±0.03	1.73±0.03	1.68±0.02
Weight, kg	63.3±3.5	91.3±3.4*	103.7±3.4*,†
BMI, kg/m^2	21.6±0.6	30.1±0.4*	36.6±0.7*,†
Body fat, %	25.2±2.3	36.2±1.8*	46.0±1.2*,†
Glucose, mg/dL	86.1±2.4	91.7±1.9	89.7±1.8
Insulin, μIU/mL	7.1±0.8	14.1±3.5	18.7±2.9*
HOMA-IR	1.54±0.20	3.30±0.93	4.23±0.71*
Triglycerides, mg/dL	86.2±5.8	116±22.2	85.0±11.0
Cholesterol, mg/dL	160±8.1	161±6.1	154±8.6
HDL-C, mg/dL	57.3±3.7	42.8±3.8*	46.3±2.7
LDL-C, mg/dL	85.4±6.2	94.7±7.2	91.1±9.0
VO_{2peak}, mL/kg per min	35.3±1.9	30.3±1.6	22.9±1.0*,†

AA/C indicates African American/Caucasian; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model of insulin resistance; and LDL-C, low-density lipoprotein cholesterol.

*$P<0.05$ vs lean.

†$P<0.05$ vs intermediate.

Vascular Injury Markers

Pretraining serum markers of vascular injury are presented in Table 2. Serum concentrations of C-reactive protein ($P=0.003$), vascular cell adhesion molecule- ($P=0.021$), intracellular adhesion molecule-1 ($P=0.044$), E-selectin ($P<0.001$), and serum amyloid A ($P=0.004$) were higher in the obese relative to the lean group, with soluble intracellular adhesion molecule-3 higher in the obese relative to the intermediate group ($P=0.015$). There were no significant differences between the

Table 2. Pretraining Markers of Vascular Injury

	Lean	Intermediate	Obese
CRP, ng/mL	5.89±2.04	19.9±6.71	33.4±6.02*
VCAM-1, ng/mL	1.07±0.08	1.10±0.13	1.56±0.15*,†
ICAM-1, ng/mL	0.62±0.05	0.79±0.19	1.04±0.11*
sICAM-3, ng/mL	0.64±0.05	0.61±0.07	0.88±0.06†
E-selectin, ng/mL	5.69±2.10	13.0±2.93	20.0±2.06*
P-selectin, ng/mL	64.7±9.2	62.7±8.5	66.4±6.0
Thrombomodulin, ng/mL	3.77±0.46	3.36±0.41	3.47±0.15
SAA, ng/mL	12.4±3.8	16.0±4.6	36.0±5.8*,†

CRP indicates C-reactive protein; ICAM-1, intracellular adhesion molecule-1; SAA, serum amyloid A; sICAM-3, soluble intracellular adhesion molecule-3; and VCAM-1, vascular cell adhesion molecule-1.

*$P<0.05$ vs lean.

†$P<0.05$ vs intermediate.

intermediate and lean groups for any of the vascular injury markers. There were no AIT-induced changes for any vascular injury marker in any group (Table III in the online-only Data Supplement).

In Vivo ROS

Endogenous H_2O_2 was elevated in the obese group compared with both the lean and intermediate groups (P<0.001; Figure 1). There were no significant differences across groups for ROS measured in the apocynin perfused probe; however, apocynin significantly attenuated H_2O_2 only in the obese group (P<0.001). The increase in ROS signal on addition of superoxide dismutase to the perfusate, indicative of superoxide, was elevated in the obese group compared with both the lean (P=0.004) and intermediate (P=0.038) groups. The increased levels of H_2O_2 and superoxide in the obese group were independent of sex and race of the participants in each group.

Microvascular Endothelial Function

Resting microvascular nutritive blood flow was not different between any groups, nor was it significantly altered by apocynin perfusion (Figure 2A). Microvascular endothelial function, tested by acetylcholine-stimulated blood flow, was attenuated in the obese relative to the lean (P=0.016) and intermediate (P=0.044) groups (Figure 2B), which was not dependent on the race or sex distribution of subjects in each group. Apocynin coperfusion augmented acetylcholine-stimulated blood flow only in the obese group (P=0.041). Endothelium-independent vasodilatory function was tested by sodium nitroprusside–stimulated blood flow, which was not significantly different among any groups (Figure 2C).

Exercise Training Effects on ROS

Eight weeks of AIT decreased endogenous H_2O_2 in the obese group (P=0.033), whereas the lean and intermediate groups

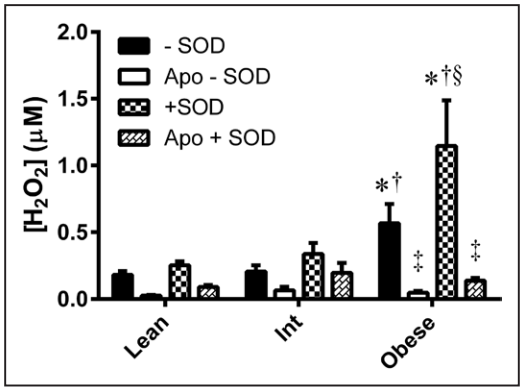

Figure 1. In vivo NADPH oxidase–mediated reactive oxygen species (ROS) in skeletal muscle of lean, overweight/mildly obese (Int), and obese subjects. ROS was measured without superoxide dismutase (–SOD) in the perfusate, indicative of H_2O_2 produced endogenously. ROS detected with SOD added (+SOD) to the perfusate is indicative of endogenous superoxide in addition to H_2O_2. ROS were also measured in the absence (–Apo) and presence of apocynin (+Apo), an NADPH oxidase inhibitor. Values are mean±SEM for n=13 to 15 subjects in each group. *P<0.05 vs lean group. †P<0.05 vs Int group. ‡Significant effect of apocynin (P<0.05). §Effect of SOD in obese significantly (P<0.05) greater than lean and Int groups.

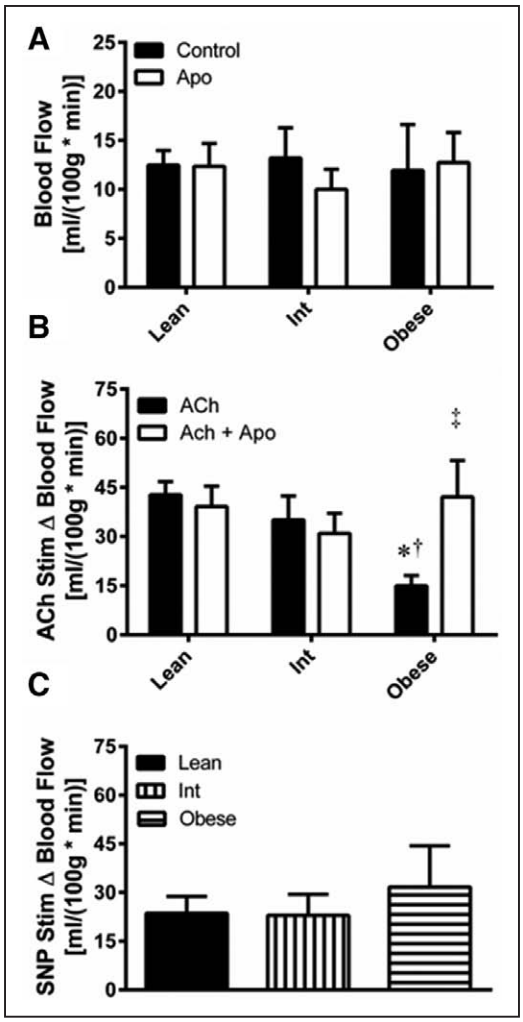

Figure 2. Association between obesity and NADPH oxidase–mediated skeletal muscle microvascular endothelial function. **A**, Basal microvascular blood flow was assessed in the absence (control) and presence (Apo) of apocynin in lean, overweight/mildly obese (Int), and obese subjects. **B**, Microvascular endothelial function was assessed as the change in blood flow from baseline (Δ blood flow) on the addition of acetylcholine (ACh) to the perfusate. **C**, Microvascular endothelium-independent blood flow was assessed by change in blood flow from baseline (Δ blood flow) on the addition of sodium nitroprusside (SNP) to the perfusate. Values are mean±SEM for n=13 to 15 subjects per group. *P<0.05 vs lean group. †P<0.05 vs Int group. ‡P<0.05 vs ACh only condition.

were unchanged (Figure 3A). However, the increase in total ROS signal on superoxide dismutase perfusion was not significantly changed by AIT in any group. Subtracting ROS concentrations measured in the apocynin perfused probe from that of the control probe revealed decreased Nox-mediated H_2O_2 after AIT in the obese group (P=0.019; Figure 3B). ROS detected in the apocynin perfused probe revealed no AIT-mediated changes in Nox-independent ROS (Figure 3C).

Exercise Training Effects on Microvascular Endothelial Function

Eight weeks of AIT did not alter resting nutritive skeletal muscle blood flow in any group (Figure 4A), nor did it affect nutritive blood flow under apocynin perfusion (Figure 4B). AIT

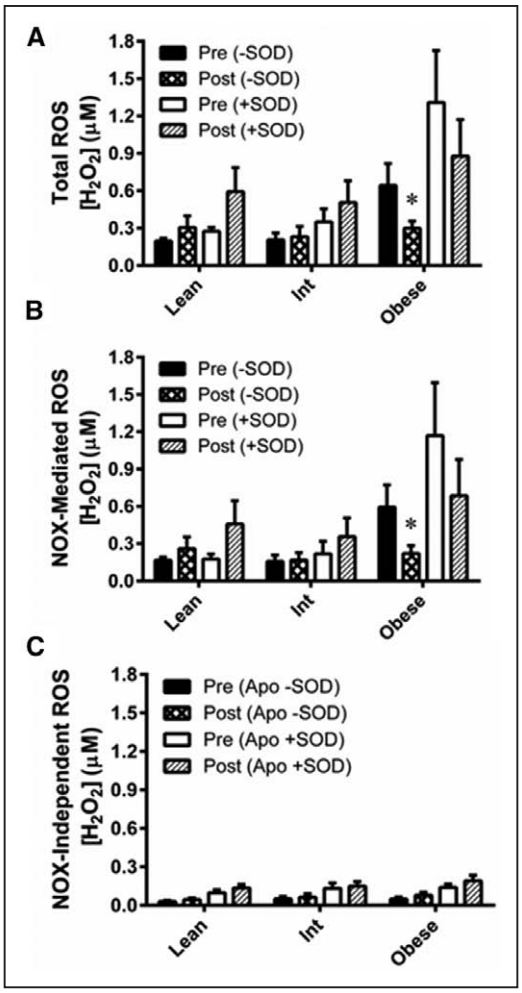

Figure 3. Effect of exercise training on NADPH oxidase–mediated ROS production. ROS were measured before (Pre) and after (Post) 8 weeks of aerobic exercise training in lean, overweight/mildly obese (Int), and obese individuals. **A**, ROS was measured without superoxide dismutase (–SOD) in the perfusate, indicative of H_2O_2 produced endogenously. ROS detected with SOD added (+SOD) to the perfusate is indicative of endogenous superoxide in addition to H_2O_2. **B**, NADPH oxidase (NOX)–mediated ROS were determined by calculation of the ROS that was inhibited by the NOX inhibitor apocynin (Apo). **C**, NOX-independent ROS were calculated as the fraction of ROS not inhibited by Apo. Values are mean±SEM for n=9 to 12 subjects in each group. **P*<0.05 vs pretraining.

augmented microvascular endothelial function in the obese (*P*=0.033) but had no effect on the lean or intermediate groups (Figure 4C). There were no AIT-induced changes in apocynin coperfused acetylcholine-stimulated blood flow in any group (Figure 4D). There were no AIT-mediated changes in endothelium-independent vasodilatory function in any group (Figure 4E).

Nox Subunit and Xanthine Oxidase Expression and Nox Activity

Representative images for Western blots ran against the Nox subunits gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, and xanthine oxidase (XO) in skeletal muscle samples from a subset of lean, and obese subjects are presented in Figure 5A. Densitometry analysis revealed a trend (*P*=0.06) for increased gp91^{phox} (Figure 5B) expression and significant (*P*<0.05) increases in expression for p22^{phox} (Figure 5C), p47^{phox} (Figure 5D), and p67^{phox} (Figure 5E) in obese relative to lean subjects. AIT significantly depressed p22^{phox} and p67^{phox} expression in obese subjects, although no AIT-induced changes were observed for gp91^{phox} or p47^{phox} expression. There were no obesity- or AIT-induced changes observed in XO expression (Figure 5F). Nox activity was significantly elevated in sedentary obese versus lean skeletal muscle samples, where AIT-induced increased Nox activity in the lean tissue but suppressed Nox activity in obese tissue (Figure 5G).

Discussion

In this study, we utilized a novel microdialysis technique to demonstrate that in vivo superoxide and H_2O_2 production are increased in skeletal muscle of obese relative to lean or overweight/mildly obese individuals. In addition, microvascular endothelial dysfunction was evident in these obese individuals relative to both of the other groups. A role for Nox in obesity-associated oxidative stress and microvascular endothelial dysfunction was confirmed by 3 independent experiments, in that ROS production and endothelial dysfunction were attenuated by apocynin perfusion, that immunoblot analysis demonstrated increased expression of Nox subunits gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} in obese skeletal muscle, and that Nox activity was increased in obese skeletal muscle samples. Finally, we demonstrated that 8 weeks of AIT attenuated H_2O_2 levels and reversed microvascular endothelial dysfunction in obese individuals, which coincided with decreased expression of 2 of the 4 Nox subunits investigated and decreased ex vivo Nox activity.

Superoxide is a short-lived, highly reactive molecule with well-documented detrimental effects on vascular function driven by oxidative damage to lipids, proteins, and DNA, apoptosis, increased endothelial cell permeability, and quenching of NO bioavailability in the formation of peroxynitrite.[9,10] H_2O_2, in contrast, is less reactive and, thus, more stable and more readily permeates membranes. H_2O_2 is critical for redox-based signal transduction with several properties that may influence vascular function. H_2O_2 activates several signaling cascades that modulate vascular function, including angiogenesis, endothelial barrier dysfunction and apoptosis, and induction of inflammatory proteins.[20] Of particular interest, acute H_2O_2 exposure has been found to impair endothelium-dependent dilation of porcine coronary arterioles via induction of arginase activity.[21] Hellsten et al[22] have previously utilized a microdialysis approach based on cytochrome c reduction to demonstrate that superoxide levels are increased by exercise in human skeletal muscle. However, this study is the first to directly measure both H_2O_2 and superoxide levels in vivo in human skeletal muscle and to demonstrate that both ROS are elevated in obese subjects.

A recent study by Walther et al[23] demonstrated impaired brachial artery endothelial function and cutaneous microvascular endothelial function in obese subjects with the metabolic syndrome. This study extends these findings to the lower limb skeletal muscle microvasculature and adds an important role

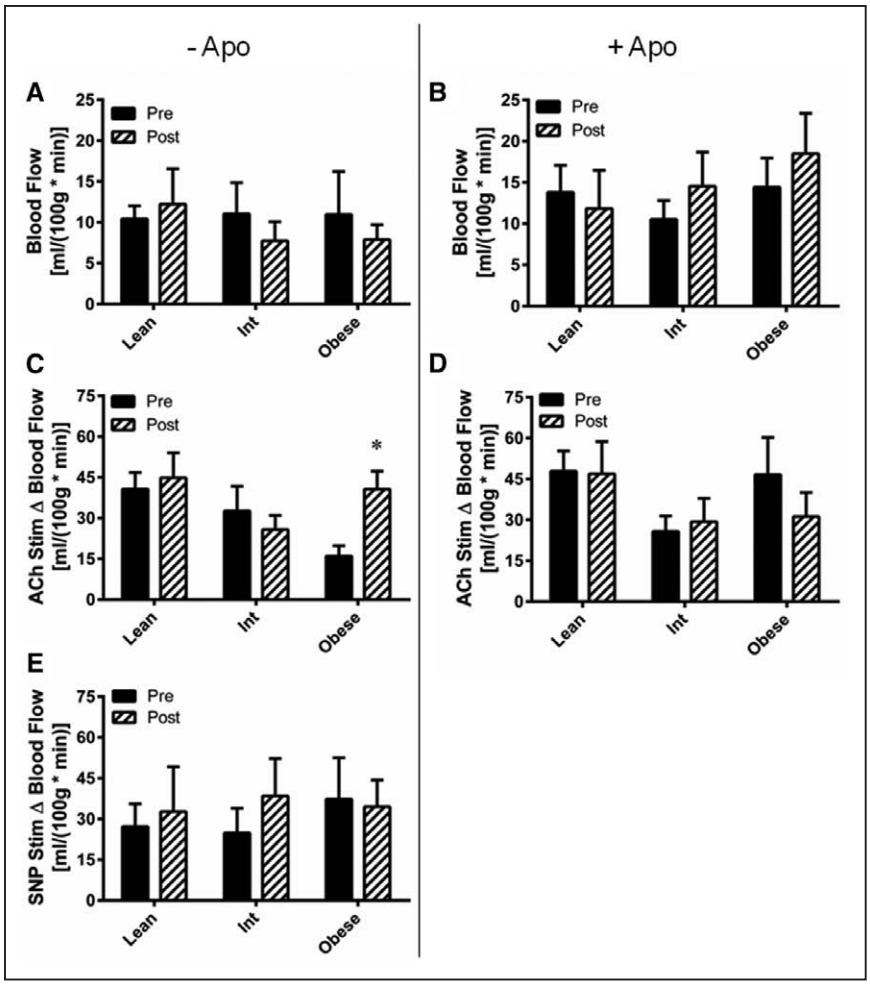

Figure 4. Exercise training effects on skeletal muscle microvascular endothelial function. Microvascular blood flow was assessed before (Pre) and after (Post) 8 weeks of aerobic exercise training in lean, overweight/mildly obese (Int), and obese subjects. **A**, Basal blood flow. **B**, Blood flow with apocynin perfusion. **C**, Microvascular endothelial function, assessed by change in blood flow from basal (Δ blood flow) in response to acetylcholine perfusion. **D**, Effect of NADPH oxidase on microvascular endothelial function, assessed by change in blood flow on acetylcholine addition to the apocynin perfused probe. **E**, Microvascular endothelium-independent blood flow was assessed by change in blood flow from baseline on addition of sodium nitroprusside (SNP) to the perfusate. Values are mean±SEM for n=9 to 12 subjects per group. **P*<0.05 vs pretraining.

for the degree of obesity, as subjects with a body mass index of 28 to 32.5 were free of microvascular endothelial dysfunction as opposed to subjects with a body mass index of 33 to 40 in which microvascular endothelial dysfunction was evident. Recent large-scale population-based studies have concluded that noninvasive assessment of vascular function in conduit vessels adds little predictive value to CVD risk above the traditional CVD risk factors, whereas the infusion of acetylcholine into the resistance vasculature and assessment of vascular function in the resistance vessels improves CVD risk prediction beyond the Framingham risk score.[24–26] These studies highlight the applicability of stimulating endothelium-dependent dilation in the resistance vasculature for the assessment of cardiovascular health. In addition, 2 previous studies[6,27] demonstrate that brachial artery endothelial function is augmented by the infusion of the antioxidant ascorbic acid in obese individuals, further demonstrating the detrimental nature of acute ROS production in the obese human vasculature. A stated limitation of one of these studies was that local, physiologically relevant levels of ROS were not detectable with the methods used in the study.[27] The present study adds to these findings by detecting elevated in vivo H_2O_2 and superoxide concentrations in the obese cohort, as well as by demonstrating improved endothelial function in the resistance vasculature in this cohort by drastically attenuating ROS levels by apocynin perfusion, further implicating Nox as both a source of increased ROS and deterrent of vascular function in the resistance vasculature in the obese subjects. These findings are further corroborated by the increased skeletal muscle $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ content and ex vivo Nox activity in the obese subjects.

The finding that sodium nitroprusside–stimulated blood flow was not different between any of the groups indicates that the impairment of acetylcholine-stimulated blood flow in the obese was indeed because of impaired endothelium-dependent vasodilation, rather than the impairment of soluble guanylate cyclase-mediated smooth muscle relaxation. Furthermore, the finding that impaired acetylcholine-stimulated blood flow in the obese was reversed by apocynin perfusion implicates a role for Nox-mediated ROS in skeletal muscle microvascular endothelial dysfunction in human obesity. The observation that resting microvascular blood flow was not different between groups or altered by apocynin suggests that the regulation of resting microvascular blood flow is also controlled by factors in addition to ROS and NO. Nonetheless, Nox-mediated ROS seem to be significantly affecting NO production in the obese group, which can have important ramifications on CVD development. Nox is a likely important source of vascular ROS in human obesity, as expression of Nox subunits are elevated in venous endothelial cells obtained from obese human subjects.[28] Furthermore, Nox has previously been implicated in human vascular disease, as Nox

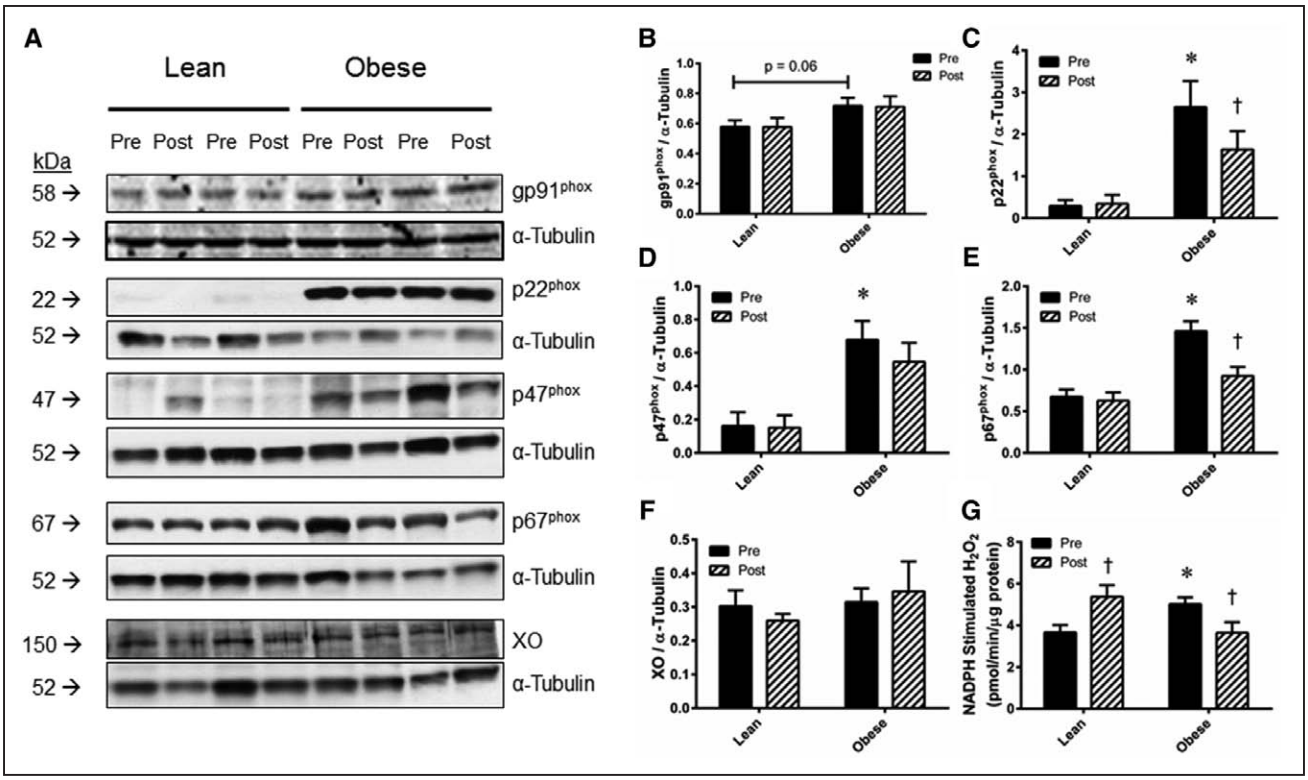

Figure 5. Protein expression of NADPH oxidase subunits, xanthine oxidase (XO), and NADPH oxidase activity in lean and obese skeletal muscle. **A**, Representative Western blot images for NADPH oxidase subunits gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, and XO from skeletal muscle obtained from lean and obese subjects before (Pre) and after (Post) 8 weeks of aerobic exercise training. Images of α-tubulin are provided for a loading control. Densitometry analyses for (**B**) gp91^{phox}, (**C**) p22^{phox}, (**D**) p47^{phox}, (**E**) p67^{phox}, and (**F**) XO expression normalized to α-tubulin. **G**, NADPH-stimulated H_2O_2 generation was measured as an index of NADPH oxidase activity. Values are mean±SEM for n=5 subjects per group. *P<0.05 vs lean group. †P<0.05 vs pretraining.

activity and subunit expression have been found to be elevated in excised arterial segments from type II diabetic patients and coronary artery disease patients.[29,30] In addition, apocynin perfusion through microdialysis probes has previously been found to blunt local angiotensin II–mediated vasoconstriction in the human cutaneous microvasculature[31] and to restore local cutaneous vascular conductance in chronic kidney disease patients.[32] Thus, the findings in this study that apocynin delivery via microdialysis augments local microvascular function are not without precedent. Conversely, apocynin incubation has been shown to have no effect on flow-induced dilation in coronary resistance arteries from cardiopulmonary bypass patients.[33] The finding that Nox-mediated ROS and microvascular endothelial function were essentially unchanged in the overweight/mildly obese intermediate body mass index group relative to the lean group was unexpected and interesting. This intermediate group demonstrated normal levels of vascular cell adhesion molecule-1, intracellular adhesion molecule-1, and soluble intracellular adhesion molecule-3, which were all elevated in the obese group. The discrepancy in these inflammatory and atherogenic molecules between the obese and overweight/mildly obese subjects could have long-term consequences of the accelerated development of more severe vascular pathologies in the obese. However, that acute apocynin perfusion completely abrogated ROS levels and restored acetylcholine-stimulated blood flow implicates a more profound role for Nox in driving the microvascular endothelial dysfunction observed in this obese group. It is important to note that these obese individuals were young and free of overt disease. As such, it is possible that more factors than ROS may be involved in regulating microvascular function in more severe disease states. These data also implicate an important role for the degree of obesity, as no functional abnormalities were observed in the overweight/mildly obese intermediate body mass index group. Nonetheless, this study demonstrates a critical, acute role for Nox-mediated ROS in regulating skeletal muscle microvascular endothelial function in obese individuals.

A recent meta-analysis concluded that a large proportion of the protective effects of exercise on the vasculature occur independent of changes in the traditional CVD risk factors and are more likely explained by the direct effects of exercise on the arterial wall or cellular environment.[34] In this study, we observed significant decreases in both total and Nox-mediated H_2O_2 in the obese skeletal muscle extracellular environment post training. Despite no AIT-induced changes in the traditional cardiovascular risk factors, we observed augmented microvascular endothelial function in the obese group, whereas apocynin no longer affected endothelial function post training. These AIT-induced changes were unique to the obese group, as neither the lean nor intermediate groups demonstrated any changes in interstitial ROS levels or microvascular function post training. Previous studies suggest that exercise-induced improvements in endothelial function are inversely

proportional to pretraining functionality.[34] Given that the lean and intermediate groups demonstrated large pretraining vasodilatory responses to acetylcholine, failure to improve further was unsurprising. However, exercise-induced reversal of endothelial dysfunction through attenuation of Nox-derived ROS has been implicated in several contexts.[16,35–37] Exercise training has been shown to decrease Nox activity or subunit expression in coronary arteries of diet-induced obese rodents[36,37] and carotid arteries of aged rodents,[35] whereas apocynin improves endothelial function in sedentary but not exercise trained vessels in these studies. Importantly, patients with CAD who performed 4 weeks of aerobic exercise training before bypass surgery demonstrated reduced Nox activity and increased vasodilatory function in internal mammary artery segments relative to patients who remained sedentary before surgery.[16] These findings demonstrate an ability of Nox activity to be beneficially altered in severely diseased human arteries by relatively short-term exercise training.[16] This study adds to these findings by demonstrating an ability of exercise training to act in a restorative manner and reverse endothelial dysfunction in the lower limb microvasculature of the obese individuals, even those without overt vascular or metabolic disease.

The decrement in interstitial apocynin-inhibitable H_2O_2 induced by AIT in the obese is supported by AIT-induced decreases in $p22^{phox}$ and $p67^{phox}$ protein content and ex vivo Nox activity in skeletal muscle tissue in a subset of these subjects. Aside from Nox, there are several factors that could potentially influence interstitial ROS levels. It is possible that exercise training induces an upregulation in the H_2O_2 detoxification pathways in the obese, such as the glutathione and thioredoxin systems, catalase, and the Nrf2/Keap1 phase II antioxidant system.[38] As H_2O_2 has a longer half-life and is much more membrane permeable than superoxide, it is plausible that some of the H_2O_2 detected via microdialysis is mitochondria derived. In this context, it is also likely that exercise training in the obese subjects promotes a reduction in mitochondrial-derived ROS emission, which could manifest as reduced interstitial H_2O_2. We have previously demonstrated that AIT attenuates mitochondrial H_2O_2 emission in red gastrocnemius of high-fat, high sucrose-fed rats through an upregulation of the thioredoxin system.[39] In addition, Gram et al[13] recently demonstrated that exercise training attenuates mitochondrial H_2O_2 emission in skeletal muscle biopsy samples from subjects who had previously undergone leg immobilization. Importantly, excessive mitochondrial-derived ROS has been found to promote endothelial dysfunction.[40,41] There also remains a possibility that Nox-derived ROS stimulates mitochondrial ROS production.[42] Mitochondrial ROS production from Nox-overexpressing transgenic mice fed a high-fat diet is increased 3-fold over wild-type mice fed the same high-fat diet.[43] Thus, it remains possible that apocynin perfusion attenuates mitochondrial ROS production through the inhibition of ROS-induced ROS release. In addition, synergy between Nox- and XO-derived ROS has been demonstrated, whereby Nox inhibition prevents XO activation.[44] Both mitochondrial ROS and XO activity have been shown to be increased in nondiabetic obese subjects relative to lean counterparts.[45,46] Regardless of the enzymatic source of ROS, we observed a decrease in skeletal muscle H_2O_2 and apocynin inhibitable H_2O_2 after exercise training in the obese group, which coincided with improved microvascular endothelial function in these individuals.

Apocynin was the only Nox inhibitor used in this study; thus, all inferences made on the microvascular effects of Nox are derived from the influence of apocynin. Apocynin is reported to inhibit the assembly of $p47^{phox}$ and $p67^{phox}$ within the membrane complex and the activation of NADPH oxidase isoforms that require subunit translocation.[47] However, apocynin has been reported to be both an antioxidant[48] and a pro-oxidant.[49] Thus, we cannot be certain that the effects of apocynin were mediated specifically through Nox inhibition. All of the ROS measurements from the apocynin perfused probe were converted from fluorescence units to [H_2O_2] based off of an H_2O_2 standard curve constructed in the apocynin-containing perfusate. Thus, any possible H_2O_2 scavenging effect of apocynin or any possible interference of apocynin on the amplex ultrared assay would be accounted for by the standard curve. The marked inhibition of ROS by apocynin strongly suggests that apocynin does not have a pro-oxidant effect in this system. Despite the potential limitations of apocynin, it is by far the most commonly used Nox inhibitor available,[31,32,35,37,40] and at the concentrations used by use in these studies, it is unrealistic to expect that any potential antioxidant effect of this compound could explain the effects observed here. A few subjects from each group dropped out of the exercise program before completion; thus, data were not attained from these subjects for post-training measures. In addition, the muscle biopsy was an optional procedure to which only 5 subjects per group consented. Despite the small sample size, the marked elevation in Nox activity and expression of Nox subunits in the obese subjects conferred clear differences that were statistically significant. Furthermore, the obese subjects failed to lose weight in response to exercise training. There were no improvements in traditional cardiometabolic risk factors or inflammatory markers after exercise training, although there is evidence to suggest that weight loss is necessary for a reduction in fasting glucose, triglyceride, or cholesterol levels.[50,51] High-intensity exercise has been shown to induce an acute proinflammatory state, which may last for ≤72 hours after intense exercise.[52] Thus, any long-term anti-inflammatory effects of the exercise program could be masked by blood sampling 48 hours after the final exercise bout. It is noteworthy that exercise training altered the ROS levels, Nox activity and expression levels, and microvascular endothelial function without improvements in any of these inflammatory or traditional risk factors, which highlights the beneficial effects of exercise in the obese population, which extend well beyond these markers.

In conclusion, we demonstrate for the first time that in vivo ROS are elevated in skeletal muscle of obese human subjects. This study is the first utilization of the newly developed microdialysis approach to measure in vivo H_2O_2 and superoxide[11] and is the first demonstration of increased in vivo ROS in human skeletal muscle under a pathological condition. Perfusion of apocynin normalized ROS levels and reversed microvascular endothelial dysfunction in obese subjects, providing a mechanistic link between ROS and microvascular

function in the in vivo setting. In addition, AIT was proven effective at attenuating in vivo H_2O_2 and reversing microvascular endothelial dysfunction in the obese cohort, providing further evidence that AIT is a practical, clinically relevant means to alleviate these obesity-associated maladies. Furthermore, these obese subjects were young with normal blood chemistries of the traditional clinically evaluated cardiometabolic risk factors, although they were likely in an early state of CVD pathogenesis as evidenced by microvascular endothelial dysfunction. This study implicates Nox-mediated ROS as a potential target to prevent further CVD progression in obesity.

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Disclosures

None.

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Highlights

- A novel technique to directly measure in vivo H_2O_2 and superoxide revealed increased levels of both ROS in obese human subjects relative to lean and overweight/mildly obese subjects.
- Microvascular endothelial function was impaired in obese relative to lean and overweight/mildly obese subjects.
- NADPH oxidase inhibition normalized H_2O_2 and superoxide levels and reversed endothelial dysfunction in the obese subjects.
- Eight weeks of aerobic exercise training decreased H_2O_2 levels and improved microvascular endothelial function in the obese subjects.
- Skeletal muscle NADPH oxidase subunit expression and activity were increased in obese subjects, both of which were decreased with exercise training.