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Effects of Acute and Chronic Endurance Exercise on Intracellular Nitric Oxide in Putative Endothelial Progenitor Cells: Role of NAPDH Oxidase

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Effects of acute and chronic endurance exercise on intracellular nitric oxide in putative endothelial progenitor cells: role of NADPH oxidase

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Jenkins NT, Witkowski S, Spangenburg EE, Hagberg JM. Effects of acute and chronic endurance exercise on intracellular nitric oxide in putative endothelial progenitor cells: role of NADPH oxidase. Am J Physiol Heart Circ Physiol 297: H1798–H1805, 2009. First published August 28, 2009; doi:10.1152/ajpheart.00347.2009.—We sought to delineate the effects of acute and chronic exercise on the regulation of intracellular nitric oxide (NOi) production in putative endothelial progenitor cells (EPCs). Putative EPC colony-forming units (CFU-EC) were cultured from blood drawn before and after 30 min of treadmill exercise at 75% of maximal oxygen uptake in active (n = 8) and inactive (n = 8) men. CFU-EC were similar between groups at baseline, but increased after exercise in active men only (P = 0.04). CFU-EC expressed lower NADPH oxidase subunit gp91phox mRNA and elevated endothelial nitric oxide synthase mRNA in active relative to inactive men at baseline (P < 0.05). Acute exercise reduced gp91phox mRNA in CFU-EC of both groups (P < 0.05), whereas p47phox mRNA levels were reduced in the inactive group only (P = 0.02). There were no differences between groups or with acute exercise in xanthine oxidase, superoxide dismutase isoforms, or glutathione peroxidase-1 mRNA levels. NOi was significantly greater in CFU-EC of active men at baseline (P = 0.004). NOi increased in CFU-EC of inactive men with acute exercise, and in vitro experiments with apocynin indicated the increased NOi production was caused by suppression of NADPH oxidase. However, the increases in NOi with the different treatments in the inactive group did not reach the baseline levels in the active group (P < 0.05). We conclude that acute exercise increases NOi in cells generated by the CFU-EC assay through an NADPH oxidase-inhibition mechanism in sedentary men. However, differences due to chronic exercise must involve additional factors. Our findings support exercise as a means to improve putative EPC function and suggest a novel mechanism that may explain this effect.

physical activity; angiogenesis; oxidative stress

Bone marrow-derived progenitor cells with vasculogenic capacity, often termed putative endothelial progenitor cells (EPCs), have emerged as a novel cardiovascular (CV) disease risk factor because of their role in the maintenance of vascular endothelial integrity. Circulating levels of putative EPCs independently predict CV disease progression, CV events, and endothelial dysfunction (29, 40), and EPC number and function decline with physical inactivity (34). Thus the available data strongly implicate putative EPCs as potential targets in the primary prevention of CV disease through regular exercise.

Acute and chronic endurance exercise are thought to increase EPC number and their ability to secrete proangiogenic growth factors and/or incorporate into existing vascular endothelium (30, 34). A single exercise bout increases putative EPC number in humans (3), and exercise training interventions have increased putative EPC number and colony-forming units (CFU-EC) in healthy subjects (11) and, most frequently, in patients with CV disease risk factors or overt CV pathologies (19, 20, 27, 32). However, although these previous studies provided strong evidence of putative EPC involvement in the exercise-induced enhancements of vascular health, the data are generally associative, and mechanistic cause-effect relationships have not been established.

The signaling molecule nitric oxide (NO) plays a central role in the function of EPCs (36), as well as in mature endothelial cells (ECs) (25). Exercise training improves endothelial function by increasing vasodilatory NO release and endothelial nitric oxide synthase (eNOS) activity in ECs (19). Conversely, NO activity is markedly reduced in the presence of CV disease risk factors, including sedentary behavior (22). Physical inactivity causes endothelial dysfunction in part through impairment of eNOS (41) by upregulation of the pro-oxidant enzyme NADPH oxidase (22, 26), which generates deleterious superoxide anions and, via uncoupling of the eNOS reaction, the powerful oxidant peroxynitrite (16, 26). NADPH oxidase is one of the most important sources of oxidative stress in mature ECs throughout the CV system (25), and, although the available evidence indicates that the eNOS and NADPH oxidase pathways are active in putative EPCs (7, 42), they have not been adequately characterized with respect to alteration of putative EPC function by physical activity. It is plausible that a reduction in NO generation by increased NADPH oxidase activity is a cellular mechanism for impaired putative EPC function with a sedentary lifestyle.

Therefore, the purpose of this study was to determine the effects of acute and chronic endurance exercise on the intracellular environment of putative EPCs. We hypothesized that acute exercise and regular physical activity would increase CFU-EC intracellular NO (NOi) levels by upregulation of eNOS and suppression of NADPH oxidase. We also explored whether mRNA levels of other pro-oxidants (xanthine oxidase) and antioxidants [copper-zinc and manganese superoxide dismutases (CuZnSOD and MnSOD, respectively), and glutathione peroxidase-1 (GPX-1)] in CFU-EC were affected by acute exercise and regular physical activity.

METHODS

Screening. All participants were young, healthy, nonsmoking males with no history of CV or metabolic disease and were not taking prescription medications. The active group (n = 8) consisted of men age 18–30 yr with a history of >3 yr moderate- to high-intensity endurance exercise for 4 h/wk. These men were recruited from local running clubs in the University of Maryland area. The inactive group (n = 8) included young men of a similar age who reported ≤20 min endurance exercise ≤2 days/wk. Groups were matched for age, body mass index (BMI), body composition, and conventional CV risk factor profile. All participants provided written informed consent.
The endothelial lineage of these cells has been confirmed previously by immunocytochemical staining for von Willebrand factor, vascular endothelial growth factor receptor-2, and CD31 (10). Investigators trained in identification of colonies but blind to the status of the sample performed CFU-EC counts in four randomly chosen wells. The correlation between observers in our laboratory for CFU-EC counts was 0.98 (P < 0.001). To reduce assay variability, all experiments in this study were performed with the same stock and lot of Endocult basal medium and supplements.

**Gene expression by semiquantitative RT-PCR.** eNOS, NADPH oxidase (subunits gp91phox, p47phox, and p67phox), xanthine oxidase, CuZnSOD, MnSOD, and GPX-1 mRNA levels were measured using semiquantitative RT-PCR. Total RNA was extracted in quadruplicate from CFU-EC cultured for 5 days using the TRI reagent (Sigma-Aldrich, St. Louis, MO) according to previously described methods (31). RNA quantity was calculated from absorbance at 260 nm, and quality was verified by the 260- to 280-nm absorbance ratio. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s instructions. cDNA was amplified using specific primers under optimal thermal cycling conditions determined empirically for each target gene as previously described (31). The primer sequences and thermal cycling conditions are presented in Table 1. PCR products were separated by agarose gel electrophoresis and photographed under ultraviolet light. Signal intensities were semiquantified using the National Institutes of Health imaging software (ImageJ) and normalized to the signal for the reference gene 18S.

**Experimental blockade of NADPH oxidase activity.** NADPH oxidase activity in CFU-EC was inhibited using the drug apocynin (Sigma-Aldrich) in ex vivo experiments to determine if exercise increases CFU-EC NO, via an NADPH oxidase-dependent mechanism. Apocynin prevents assembly of the enzyme by reacting with thiol groups required for the translocation of the intracellular subunits p47phox and p67phox to the catalytic gp91phox subunit, in turn preventing the generation of deleterious superoxide radicals (33). Cells of active and inactive groups from before and after acute exercise were incubated from day 4 to day 5 of the CFU-EC assay with 100 μM apocynin in 1 ml Endocult medium. Cells treated with the same volume of the apocynin vehicle (10 μl PBS) in 1 ml Endocult medium were used as a control. The apocynin concentration and the 24-h treatment were empirically determined to produce detectable changes in NO. We aimed to not affect the early events of the assay such as adherence of the cells to the plate or initial differentiation into CFUs, but we did want to allow sufficient time for any measurable effects of

<table>
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<th>Amplicon Length, bp</th>
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<td></td>
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<td>53</td>
<td>30</td>
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</tbody>
</table>

eNOS, endothelial nitric oxide synthase; XO, xanthine oxidase; SOD, superoxide dismutase; GPX, glutathione peroxidase; F, forward; R, reverse.
aporessed on N02 to appear. An apocynin-induced increase in endothelium-dependent dilation and its NO component in mouse aorta was recently observed in as little as 60 min (6). Therefore, we believe the 24-h experiment was valid for our purposes. The experiment consisted of the following conditions for both active and inactive group CFU-EC: baseline-vehicle (BL-VEH), baseline-apocynin (BL-APO), exercise-vehicle (EX-VEH), and exercise-apocynin (EX-APO). These experiments were performed on CFU-EC from a subset of individuals (n = 3–5/group for each condition).

Detection of NOi in CFU-EC. NOi was measured using the DAF-FM diacetate fluorescent dye technique (Molecular Probes, Carlsbad, CA) as described by Drenning et al. (5), with minor modifications. DAF-FM diacetate is a pH-insensitive dye that emits fluorescence on reacting with an intermediate of NO during the spontaneous oxidation of NO to NO2. On day 5 of the CFU-EC assay, media were removed, and CFU-EC in triplicate wells were washed twice with 500 μl PBS, loaded with 500 μl PBS containing 10 μM DAF-FM diacetate, and incubated for 30 min at 37°C. Dyed cells were washed two times with PBS, and NOi fluorescence was measured using a multilabel plate reader (Wallac 1420 VICTOR2; PerkinElmer, Waltham, MA) with excitation and emission wavelengths of 488 and 535 nm, respectively. Every CFU-EC plate included the following controls: 1) duplicate wells of unloaded cells to serve as a control for cellular autofluorescence, 2) duplicate wells that contained no cells, but contained 10 μM DAF-FM in PBS to correct for any fluorescence resulting from the dye itself, and 3) duplicate wells of 500 μl PBS alone. Because the arbitrary-type fluorescence among these three control conditions were similar in pilot experiments (P = 0.7, data not shown), the average fluorescence value of the three controls was subtracted from each experimental value. The average within- and between-assay coefficients of variation for the arbitrary fluorescence were 5 and 9%, respectively, indicating good agreement in arbitrary fluorescence between wells on the same plate and among experiments conducted on different days. Data are expressed as fold difference from the mean for the active group’s CFU-EC in the BL-VEH condition.

In addition, we must point out that gene expression and NOi measurements were made on all cells present in the CFU-EC assay, and not the colonies alone. Thus the term “CFU-EC” must be interpreted to mean all cells cultured in the CFU-EC assay when we refer to our NOi and gene expression data.

Statistics. Between-group and acute exercise effects were analyzed by independent and paired-samples t-tests, respectively, according to a priori planned contrasts. Within- and between-group ANOVA with Dunnett’s post hoc tests were used to determine whether exercise and/or apocynin treatments increased NOi relative to BL-VEH. We used the α = 0.05 criterion for statistical significance. Data are presented as means ± SE unless indicated otherwise.

RESULTS

Active and inactive participants were successfully matched on the basis of age, BMI, and the standard CV risk factor profile, but differed significantly in terms of VO2max (Table 2).

CFU-EC counts. CFU-EC counts were not different between groups at baseline (P = 0.23; Fig. 1A), CFU-EC increased after exercise in the active group (P = 0.02), but did not change in the inactive group (P = 0.6; Fig. 1B).

Gene expression. eNOS mRNA levels were elevated in CFU-EC from active relative to inactive men by ~30% at baseline (P = 0.04) and by ~17% after exercise (P = 0.05), but did not change with exercise in either group (P = 0.4 for exercise-induced changes in both groups) (Fig. 2). gp91phox mRNA levels were ~44% lower in CFU-EC from active than inactive men at baseline (P = 0.02) and were reduced after acute exercise in both groups (active, P = 0.02; inactive, P = 0.04), with expression remaining significantly (P = 0.01) higher after exercise in CFU-EC of inactive compared with active men (Fig. 3A). p47phox mRNA levels did not differ between groups at baseline but decreased by ~20% after exercise in the inactive group (P = 0.02) (Fig. 3B). There were no differences between groups or with acute exercise in p67phox mRNA levels (Fig. 3C) or xanthine oxidase (Fig. 3D) mRNA levels (P > 0.05). There were also no differences between groups or with acute exercise in expression of the antioxidant genes CuZnSOD, MnSOD, or GPX-1 (Fig. 4, A–C; P > 0.05).

NOi. Detectable NOi was ~56% greater in the cells cultured in the CFU-EC assay from active compared with inactive men (P = 0.004) (Fig. 5). In the active group, there were no significant differences among BL-VEH, BL-APO, EX-VEH, or EX-APO in CFU-EC NOi (ANOVA P > 0.05). In the inactive group, BL-APO (P = 0.04), EX-VEH (P = 0.04), and EX-APO (P = 0.02) all significantly increased NOi levels relative to the BL-VEH condition. However, there were no differences in NOi among these three experimental conditions for the inactive group (all P > 0.05). Additionally, inactive group NOi levels were significantly lower compared with the active group BL-VEH in all experimental conditions (BL-APO, EX-VEH, and EX-APO; all P < 0.05). Colony formation was not affected by VEH or APO treatments (data not shown); therefore, CFU-EC count data are from VEH or untreated samples.

DISCUSSION

The main findings of this study are 1) acute exercise increases CFU-EC NOi levels in sedentary individuals, 2) regular endurance exercise is associated with increased eNOS gene expression and NOi in CFU-EC, 3) the acute exercise effect on NOi is NADPH oxidase-dependent, and 4) the training effect on NOi appears to involve other mechanisms besides reduced NADPH oxidase activity. Importantly, these differences were observed between groups of healthy young men who were matched for age, BMI, and the standard CV risk factor profile,
and differed only in $V_{O2\max}$. Our findings support the notion that acute and chronic exercise improve putative EPC function and suggest a novel cellular mechanism that may explain this effect.

NADPH oxidase is regarded as one of the most important sources of oxidative stress in the CV system (9, 25, 26). ECs throughout the CV system express elevated NADPH oxidase in several pathological conditions associated with physical inactivity, including CV disease (1), hypertension (15), and diabetes (23). In putative EPCs, excessive NADPH oxidase-derived superoxide radicals promote premature cellular senescence and reduced proliferative capacity (13). Here, we show elevated expression of the NADPH oxidase catalytic subunit gp91$^{phox}$ gene in CFU-EC of healthy men who would be considered very healthy, and have excellent CV disease risk profiles, but who do not regularly perform endurance exercise. We show further that a single bout of exercise reduces mRNA expression of gp91$^{phox}$ and p47$^{phox}$ in CFU-EC NO$_i$ from these men. To our knowledge, no studies have assessed the effects of acute or chronic endurance exercise on putative EPC NADPH oxidase gene expression, but these data are consistent with a previous report of CV disease patients showing a reduction in gp91$^{phox}$ mRNA in coronary artery ECs following a 4-wk endurance training program (1).

Because eNOS uncoupling, and subsequent depletion of NO$_i$, is a consequence of elevated NADPH oxidase activity (16, 26), we tested whether the observed differences in eNOS and NADPH oxidase gene expression between groups and with acute exercise were corroborated by differences in CFU-EC NO$_i$. Consistent with the elevated baseline gp91$^{phox}$ and reduced eNOS mRNA levels in the inactive group, these individuals also displayed significantly lower NO$_i$ compared with the active group. The inactive group NO$_i$ levels increased with BL-APO, EX-VEH, and EX-APO treatments. Importantly, the effects of NADPH oxidase inhibition and acute exercise on NO$_i$ were nearly identical in magnitude and were not additive, as evidenced by no further increase in NO$_i$ in the EX-APO condition over either treatment alone. Furthermore, there was no acute exercise effect on eNOS mRNA in either group, despite a between-group difference at baseline. Together, these data indicate that, in inactive individuals, NO$_i$ mRNA levels in CFU-EC of healthy men who would be considered very healthy, and have excellent CV disease risk profiles, but who do not regularly perform endurance exercise. We show further that a single bout of exercise reduces mRNA expression of gp91$^{phox}$ and p47$^{phox}$ in CFU-EC NO$_i$ from these men. To our knowledge, no studies have assessed the effects of acute or chronic endurance exercise on putative EPC NADPH oxidase gene expression, but these data are consistent with a previous report of CV disease patients showing a reduction in gp91$^{phox}$ mRNA in coronary artery ECs following a 4-wk endurance training program (1).

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have observed a ceiling effect for the active individuals’ NOi levels.

Most reports of acute exercise and exercise training effects on putative EPCs have enumerated cells by colony-forming potential in culture or flow cytometry. In general, exercise training increases putative EPC number and colony-forming potential (11, 19, 27, 32), but this has not always been the case (35). The available data are also equivocal as to whether an acute exercise bout increases putative EPCs, with some studies showing increased EPC number (18, 38) and colony formation (8, 18, 28) after acute exercise, but others showing no effect on putative EPC number (35) or colony formation (38). The most consistent effects of acute and chronic exercise have been observed in patient populations with overt endothelial dysfunction and CV pathologies (19, 27, 32). Of all these studies, the acute exercise CFU-EC data in the present study are most consistent with 1) a report in which CFU-EC increased in endurance-trained individuals after exercise of equivalent intensity and duration to that in the present study (30 min at ~80% VO2max) (18) and 2) another report showing no change in CFU-EC after exercise in a group of untrained, but healthy, men and women (38). Therefore, taken with previous data, the present study suggests a relationship between participant fitness and the response of putative EPC colony formation to acute exercise, but this hypothesis needs further attention.

However, the effects of exercise on the EPC intracellular environment may well be far more important than acute or chronic exercise effects on putative EPC number or colony formation. From our data, a reasonable working hypothesis is that the intracellular environment of a putative EPC may influence its functional capacity and its ability to affect endothelial function. NADPH oxidase and eNOS were chosen as target genes in this study because of their well-characterized role in the (dys)function of the endothelium throughout the CV system. Clearly, our evidence suggests that NOi production is altered in putative EPCs because of changes in activity, indicating an important effect of exercise on the EPC intracellular environment. Our evidence further suggests that an impaired intracellular redox state resulting from high NADPH oxidase activity may be detrimental to putative EPC function in young, healthy men with a sedentary lifestyle.

Our findings indicate a role for NADPH oxidase in the regulation of NOi in cells generated by the CFU-EC assay. However, additional factors were implicated in training-related differences and the response to acute exercise in inactive subjects. Thus we also investigated the expression of other genes involved in intracellular redox status. The findings that mRNA levels of antioxidant (CuZnSOD, MnSOD, and GPX-1) and pro-oxidant (xanthine oxidase) genes did not differ between groups or with acute exercise support the role for

Fig. 3. mRNA levels of the NADPH oxidase subunits gp91phox (A), p47phox (B), and p67phox (C) and xanthine oxidase (XO, D). *Significant difference between groups (P < 0.05). †Significant within-group difference (after vs. before acute exercise; P < 0.05).
NADPH oxidase as a key mediator of NOi dynamics. However, these data do not clarify the complete training-related or acute exercise-induced differences in NOi. Further work will be necessary to fully identify all of the factors explaining the differences in NOi regulation in putative EPCs between the trained vs. untrained states. We speculate that there may be differences in the activities or responsiveness of these or other oxidases or antioxidant systems that we did not detect at the mRNA level.

We must acknowledge three limitations of our study. First, NOi and gene expression were measured from all cells present in the dish, and not the colonies themselves. Fluorescence microscopy experiments have documented eNOS activity in cells generated by the CFU-EC assay (24), but the amount of NO release by the colonies relative to the other cells present in the dish has not been determined. Therefore, it is unclear if differences detected in NOi measures were the result of changes in NO production in the colonies themselves or changes in a subpopulation of cells outside the colonies that express eNOS and/or NADPH oxidase. However, the data of Hill and colleagues (10) suggest that it is the CFU-EC themselves that express endothelial markers, and the colonies have been repeatedly shown to take up acetylated low-density lipoprotein and bind to lectin, suggesting an endothelial phenotype. Second, there are conflicting reports about the identity of cells generated by the CFU-EC assay. An emerging hypothesis suggests that CFU-EC may be T cells (4) with a distinct angiogenic phenotype (12). There are also very recent data on the existence of a novel population of circulating CD31+ T cells that secrete angiogenic growth factors (17), which is one mechanism by which CFU-EC have been proposed to promote new vessel growth (12, 37). We believe our data provide new information on how acute and chronic endurance exercise may alter the function of these cells, but we must emphasize that the precise identities of these cells and other putative vasculogenic progenitors are still being elucidated. Finally, although it appears likely that the effect of acute exercise on NOi was...
NADPH oxidase-dependent based on our study design, we must be somewhat circumspect with this conclusion. We acknowledge that a number of other factors affect NO throughout the CV system (25).

In conclusion, we found a cross-sectional difference between active and inactive young men in NO in cells cultured in the CFU-EC assay that may be partly explained by elevated NADPH oxidase with physical inactivity. Acute exercise appears to attenuate some, but not all, of this difference through suppression of NADPH oxidase activity, indicating the existence of a training effect that must be the result of elevated eNOS and other factors. Our findings may have important implications for the role of putative EPCs in the maintenance of a healthy endothelium through physical activity.

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