Elevated peripheral blood mononuclear cell-derived superoxide production in healthy young black men

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Submitted 30 October 2014; accepted in final form 14 December 2014


Several studies have demonstrated that blacks exhibit elevations in systemic oxidative stress. However, the source(s) and mechanism(s) contributing to the elevation in oxidative stress remain unclear. Given that peripheral blood mononuclear cells (PBMCs) can be a major source of NADPH oxidase-derived superoxide production, we tested the hypothesis that young black men demonstrate greater superoxide production and NADPH oxidase expression in PBMCs compared with whites. PBMCs were freshly isolated from whole blood in young normotensive black (n = 18) and white (n = 16) men. Intracellular superoxide production in PBMCs was measured using dihydroethidium fluorescence, protein expression of NADPH oxidase subunits, gp91phox (membranous) and p47phox (cytosolic) in PBMCs were assessed using Western blot analysis, and plasma protein carbonyls were measured as a marker of systemic oxidative stress. Black men showed elevated intracellular superoxide production (4.3 ± 0.5 vs. 2.0 ± 0.6 relative fluorescence units; black men vs. white men, P < 0.05), increased protein expression for gp91phox and p47phox (e.g., p47phox, 1.1 ± 0.2; black men vs. white men, P < 0.05) in PBMCs and higher circulating protein carbonyl levels (22 ± 4 vs. 14 ± 2 nmol/ml; black men vs. white men, P < 0.05). Interestingly, a positive family history of hypertension in black men did not further enhance PBMC-derived intracellular superoxide production or NADPH oxidase subunit protein expression. These findings indicate that black men exhibit greater resting PBMC-derived superoxide production and an upregulation of the NADPH oxidase pathway with a possible contribution to increases in systemic oxidative stress.

Peripheral blood mononuclear cells (PBMCs) have recently been identified as one of the primary contributors to systemic reactive oxygen species (ROS) (20). Specifically, when circulating PBMCs, primarily monocytes, were selectively depleted in mice, systemic superoxide levels were significantly reduced following angiotensin II treatment. Restoration of these cells in the circulation reestablished the systemic oxidant status in these same animals indicating that PBMCs were the major source of systemic oxidative stress (20). Notably, this PBMC-derived increase in systemic ROS was mediated via gp91phox subunit, the catalytic membranous subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex (20). Indeed, angiotensin II is a primary mediator of NADPH oxidase-dependent ROS production (15, 20). Interestingly, several reports have shown that blacks exhibit enhanced activation of the renin-angiotensin system with increased plasma angiotensin II concentrations (9, 13, 17). These findings highlight a potential mechanism that may contribute to increases in PBMC-derived superoxide production and NADPH oxidase protein expression in blacks.

A recent cell culture study using commercially available primary endothelial cells (human umbilical vein endothelial cells) has reported that NADPH oxidase subunits, particularly gp91phox and p47phox are upregulated in human umbilical vein endothelial cells from blacks compared with whites (5). Importantly, endothelial cells typically produce low levels of ROS and, also, do not have the capacity to release superoxide and contribute to systemic oxidative stress (8). This is because they predominantly express the NOX4 isoform of NADPH oxidase, which is on intracellular organelles and not the cell membrane thereby, limiting its ability to extrude superoxide extracellularly (1). In contrast, PBMCs express the NOX2 isoform, which spans across the cell membrane and is capable of producing and releasing large quantities of superoxide (1, 8). However, whether superoxide production and NADPH oxidase subunit expression are elevated in circulating PBMCs from blacks is unknown.

With this background in mind, we tested the hypothesis that young black men would exhibit greater superoxide production and NADPH oxidase expression in freshly isolated PBMCs compared with young white men. Thus we compared basal levels of intracellular superoxide production and protein expression for gp91phox (membranous) and p47phox (cytoplasmic) subunits of NADPH oxidase enzyme complex in PBMCs between young black and white men. These subunits were chosen because gp91phox is the catalytic subunit and the primary membranous subunit of the NADPH oxidase enzyme complex, whereas p47phox plays an important role in the assembly of other subunits to the membrane because of its...
cytosolic location. In addition, in a subset of subjects, plasma protein carbonyl concentration was measured to compare systemic oxidative stress levels between groups. Lastly, because of the strong predictive influence of a family history of hypertension in blacks, we compared PBMC and systemic measures of oxidative stress between those black subjects with and without a family history of hypertension.

METHODS

General Procedures

Eighteen young normotensive black men and sixteen normotensive white men were studied. All subjects were nonsmokers and were recreationally active (≤3 days per wk) but not training competitively. Exclusion criteria included hypertension (resting blood pressure > 140/90 mmHg; minimum of 5 resting measurements), smoking, and any known cardiovascular, pulmonary, metabolic, or neurological disease. In addition, family history of hypertension was recorded for all subjects, defined as one or more biological parents having hypertension. After receiving a detailed verbal and written explanation of the intended experimental protocol, each subject provided written informed consent. All experimental procedures and protocols conformed to the Declaration of Helsinki and were approved by the University of Missouri Health Sciences Institutional Review Board.

On experimental days, subjects arrived at the laboratory in the morning following an overnight fast and without alcohol and physical activity for 24 h. Subjects were positioned supine in a quiet temperature-controlled room (22 to 23°C) and rested for 10 min. Resting arterial blood pressure and heart rate were measured by auscultation of the brachial artery of the right arm using an automated sphygmomanometer (Welch Allyn, Skaneatles Falls, NY). Following the measurement of cardiovascular parameters, 50 ml of blood was obtained from the antecubital vein and collected into sodium heparin tubes for the isolation of primary PBMCs via density gradient centrifugation, as previously described (4).

Detection of intracellular superoxide levels in PBMCs. Basal superoxide measurements were performed on freshly isolated primary PBMCs immediately upon isolation ex vivo to prevent any artifactual increase in superoxide production (4). The same number of PBMCs was used for all subjects. Briefly, ~1.5 × 10⁶ cells were incubated with 5 μM dihydroethidium (Life Technologies) for 60 min at 37°C, and superoxide fluorescence was determined using a fluorescent plate reader (Spectramax M2, Molecular Devices) at 544- and 612-nm excitation and emission wavelengths, respectively. The data are expressed as relative fluorescence units.

Assessment of protein expression in PBMCs by Western blot analysis. PBMCs were homogenized in lysis buffer, and the cell lysates (10 μg of protein) were separated by 4 to 15% SDS-PAGE and transferred to polyvinylidene fluoride membrane. Subsequently, the nonspecific sites on the membrane were blocked with 5% nonfat dry milk for 60 min and then probed with primary antibodies against gp91^phox^ (membranous) and p47^phox^ (cytoplasmic) subunits of NADPH oxidase enzyme, and angiotensin II type 1 receptor (AT,R) at 4°C overnight. AT,R was also probed because angiotensin II is a primary mediator of NADPH oxidase-dependent ROS production (15, 20), and several reports have shown that blacks exhibit increased plasma angiotensin II concentrations (9, 13, 17). All blots were incubated with horseradish peroxidase-conjugated anti-IgG and later detected by enhanced chemiluminescence. Tubulin was used as a loading control, and all protein data are expressed relative to that of tubulin. Eight samples were run on each gel per target. To minimize any operator bias, subjects were coded to de-identify their racial orientation. Although gels were run separately, the protein targets were all probed at the same time. Densitometric analysis of the bands was performed with image analysis software.

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<th>Table 1. Subject characteristics</th>
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Values are means ± SE. *P < 0.05 vs. white men.

Systemic measures of ROS and angiotensin II. In a subset of subjects (blacks, n = 8; whites, n = 5), additional blood was drawn and plasma was isolated to assess protein carbonyl concentration, a known marker of systemic oxidative stress, and angiotensin II levels using commercially available Protein Carbonyl Colorimetric Assay kit (Cayman Chemicals) and an enzyme immunoassay kit (Bertin Pharma), respectively. The plasma samples were flash frozen in liquid nitrogen and stored in −80°C until assayed.

Statistical Analysis

Group comparisons between whites and blacks were conducted using unpaired Student’s t-tests. In addition, we also performed unpaired Student’s t-tests to examine the potential influence of family history of hypertension in blacks. The number of whites with a family history was only three, so we were not able to directly test this question within this group. In this regard, our subject population is representative of epidemiological data showing the greater prevalence of hypertension in blacks compared with whites (16, 18). Nevertheless, we performed analysis of covariance (ANCOVA) to determine if differences existed between the racial groups after controlling for known family history of hypertension. The corresponding covariate for each subject was determined as either negative (no family history of hypertension) or positive (one or more biological parents have...
hypertension). Results are presented as means ± SE. For all analyses, statistical significance was set at \( P < 0.05 \).

**RESULTS**

Table 1 presents general characteristics for all subjects. Although blacks were significantly younger compared with whites, the mean age difference was small (4 yr). There were no significant differences in body mass index, resting mean arterial pressure, or resting heart rate between groups. Plasma protein carbonyl and plasma angiotensin II levels were greater in blacks compared with whites. In addition, the number of blacks who had a positive family history of hypertension was greater than whites (50 vs. 19%).

Black men exhibited greater basal intracellular superoxide production in PBMCs compared with white men \( (P < 0.05) \); Fig. 1). Also, protein expression for gp91\( ^{phox} \) and p47\( ^{phox} \) was significantly upregulated in blacks \( (P < 0.05; \text{Fig. 2, A–C}) \). Likewise, protein expression for AT1R was also elevated in PBMCs of black compared with white men \( (P < 0.05; \text{Fig. 2, A and D}) \). Similar differences between groups were observed for both superoxide production and protein expression for NADPH oxidase subunits when controlling for family history of hypertension as a covariate (ANCOVA; \( P < 0.05 \)). In addition, there was no significant main effect of family history (ANCOVA; \( P > 0.05 \)). Indeed, basal superoxide production and NADPH oxidase subunit protein expression was not statistically different between blacks with and without a family history of hypertension (Fig. 3). Interestingly, blacks demonstrated a significantly positive relationship between protein expression for p47\( ^{phox} \) and intracellular PBMC-derived super-

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**Fig. 2.** A: individual contiguous Western blots side by side from 2 white and 2 black men showing protein expression of NADPH oxidase subunits (gp91\( ^{phox} \) and p47\( ^{phox} \)), angiotensin II type 1 receptor (AT1R), and tubulin in PBMCs. Mean data comparing protein expression of gp91\( ^{phox} \) \( (B) \) and p47\( ^{phox} \) \( (C) \), and AT1R \( (D) \) between white and black men as determined by densitometric analysis of Western blot are also shown. All protein data are expressed relative to that of tubulin. Values are means ± SE. *\( P < 0.05 \) vs. white men.

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**Fig. 3.** Mean data comparing PBMC-derived intracellular superoxide production \( (A) \), and protein expression for gp91\( ^{phox} \) \( (B) \) and p47\( ^{phox} \) \( (C) \) between black men with a positive or negative family history of hypertension \( (F/H \text{ HTN}) \). Superoxide data are expressed as relative fluorescence units, and all protein data are expressed relative to that of tubulin. Values are means ± SE.
oxide production ($R^2 = 0.44; P < 0.05$); however, no such relationship was observed in whites ($R^2 = 0.10; P > 0.05$).

**DISCUSSION**

Herein, we demonstrated for the first time that young black men exhibit greater basal intracellular superoxide production and protein expression for NADPH oxidase subunits in freshly isolated PBMCs compared with young white men. These findings are in parallel with greater levels of systemic oxidative stress as reflected by increased plasma protein carbonyl concentrations in blacks. Interestingly, such effects appear independent of family history of hypertension as no differences were observed between young black men with and without a positive family history of hypertension.

Elevations in NADPH oxidase activity and oxidative stress have been linked with the development of cardiovascular disease such as hypertension (1, 2, 6, 10–12, 14, 19). Such increases in NADPH oxidase activation have been shown to be mediated by several factors including angiotensin II, norepinephrine, and endothelin-1 (2, 4, 10). In this regard, we found a higher AT1R protein expression in PBMCs from young black men compared with white men. We also found that plasma concentrations of angiotensin II were elevated in blacks (Table 1). Although we cannot establish cause and effect, circulating angiotensin II may serve as a potential systemic trigger at the AT1R on PBMCs to activate the NADPH oxidase enzyme complex and increase superoxide production. This warrants further study. Indeed, the greater expression of NADPH oxidase subunits and elevated intracellular superoxide levels in PBMCs of young black men suggests this pathway may represent a viable target to reduce oxidative stress and its associated negative cardiovascular consequences. Along these lines, studies have shown that elevations in oxidative stress can contribute to a number of deleterious consequences that negatively impact cardiovascular health, including endothelial dysfunction, sympathoexcitation, apoptosis, increases in intracellular calcium, and fibrosis (6, 10, 19). Thus elevated oxidative stress in young black men may contribute to the greater predisposition for the development of cardiovascular disease in this group, and the present findings identify PBMCs as a potential target to reduce this burden. Importantly, although unlikely to circulate, the importance of greater superoxide release from PBMCs is that it is the progenitor of many other free radicals and ROS (7, 8). For example, following its release from PBMCs superoxide can be quickly dismutated to hydrogen peroxide, either spontaneously or via superoxide dismutase. In addition, it can also react with nitric oxide to form highly reactive peroxynitrite (7).

Interestingly, we did not detect a greater intracellular PBMC-derived superoxide production or NADPH oxidase subunit protein expression in the young black men who had a positive family history of hypertension compared with those with a negative family history. These findings suggest that, at least at a young age, family history does not appear to further enhance the elevated PBMC-derived superoxide production in young black men compared with white men. However, some consideration of sample size is warranted for the family history comparison. There are potential limitations to the conclusions regarding a lack of statistical difference between black men with and without a family history of hypertension in superoxide and NADPH oxidase subunits. Based on the data collected, greater subject numbers would be needed to achieve 80% power. For example, to fully examine the mean differences observed for superoxide, a total sample size of 78 would have been required. Given that determining the impact of family history of hypertension was not the primary goal of this study, we felt that these large numbers were unrealistic to obtain. Thus, in consideration of the potential for statistical type II error, caution should be used when interpreting these trends regarding family history of hypertension in blacks. Indeed, additional studies are warranted.

Consistent with our findings, Feariheller et al. (5) have shown an upregulation of NADPH oxidase subunits in blacks compared with whites using commercially available primary human umbilical vein endothelial cells. Importantly, superoxide released by the phagocytic NADPH oxidase present in PBMCs are much greater compared with that produced by non-phagocytic cells like endothelial cells (8). As such, the likelihood of endothelial cells contributing to an elevation in systemic oxidative stress is minimal. This is because PBMCs, specifically monocytes, predominantly express the NOX2 isoform of NADPH oxidase enzyme complex (1, 8), which is located on the cell membrane, making PBMCs a potential source of systemic superoxide (20). On the other hand, the NOX4 isoform is predominantly expressed in endothelial cells, and since the location of this NOX isoform is on intracellular organelles, it lacks the ability to extrude superoxide from the cell (1).

In summary, we report for first time a greater basal intracellular superoxide production and an upregulation of the NADPH oxidase pathway in PBMCs from young black men compared with white men.

**ACKNOWLEDGMENTS**

We thank Charla Jay for assistance with subject recruitment and blood draws.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grants R01-HL-093167 (to P. J. Fadel) and R15-HL-096103 (to D. M. Keller) and American Physiological Society Arthur C. Guyton Award for Excellence in Integrative Physiology (to P. J. Fadel).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**