Original Report:
Cardiovascular Disease
and Risk Factors

Effect of Interleukin-10 and Laminar Shear Stress on Endothelial Nitric Oxide Synthase and Nitric Oxide in African American Human Umbilical Vein Endothelial Cells

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Background: African Americans have a predisposition to heightened systemic inflammation and a high prevalence of hypertension.

Objective: The purpose of this study was to evaluate the influence of interleukin-10 (IL-10) and laminar shear stress (LSS) on African American endothelial cells by measuring total endothelial nitric oxide synthase (eNOS) protein expression and its phosphorylated form (p-eNOS) at Serine 1177, and nitric oxide (NO) levels, in response to IL-10 incubation and high physiological levels of LSS, used as an *in vitro* mimetic for aerobic exercise training (AEXT).

Design: Human umbilical vein endothelial cells (HUVEC) from an African American donor were cultured. The experimental conditions included *Static*, *Static with IL-10 Incubation, LSS at 20 dynes/cm^2, and LSS at 20 dynes/cm^2 with IL-10 Incubation*. Western blotting was used to measure eNOS and p-eNOS protein expression in the cells. A modified Griess assay was used to measure NO metabolites in the cell culture media.

Results: There were significant increases in p-eNOS, eNOS, and NO in the *LSS at 20 dynes/cm^2* and *LSS at 20 dynes/cm^2 with IL-10 Incubation* experimental conditions when compared to the *Static* experimental condition. There were no other statistically significant differences demonstrating that IL-10 did not have an additive effect on eNOS activity in our study.

Conclusion: The significant increases in p-eNOS, eNOS, and NO as a result of LSS in African American HUVECs suggest that AEXT may be a viable, nonpharmacologic method to improve vascular inflammation status and vasodilation, and thereby contribute to hypertension reduction in the African American population. *Ethn Dis*. 2015;25(4):413-418; doi:10.18865/ed.25.4.413

Introduction

The preponderance of research on hypertension in ethnic populations supports the conclusion that African Americans have the highest prevalence of hypertension in the United States and in the world.[1,2] Earlier onset and increased severity of this pathology in African Americans leads to higher rates of morbidity and mortality when compared with other ethnic groups.[3,4] Hypertension has been linked to independent and interactive effects of multiple genetic and environmental factors.[3] One of these factors is inflammation of the vessel wall, a systemic pathological mechanism that can cause endothelial dysfunction, which may be antecedent to hypertension.[5,6]

The high incidence of hypertension in African Americans may be attributed to their predisposition to heightened systemic inflammation.[7-10] Long-term exposure of the endothelium to proinflammatory cytokines leads to endothelial dysfunction, which supports an environment favoring hypertension.[11,12] It is hypothesized that the intravascular balance between pro- and anti-inflammation plays a crucial role as a determinant of endothelial health.[13] Research data have demonstrated a positive association between hypertension and proinflammatory markers including C-reactive protein (CRP) and interleukin-6 (IL-6).[5,6] In contrast, elevated circulating levels of the anti-inflammatory cytokine interleukin-10 (IL-10) have been associated with improved endothelial function.[14,15] IL-10 potently inhibits proinflammatory cytokines such as IL-6 and tumor necrosis factor alpha (TNF-α), and the primary function of IL-10 seems to be to limit and ultimately terminate inflammatory responses.[16,17] Studies have also demonstrated that IL-10 is contributory in the up-regulation of endothelial nitric oxide synthase (eNOS) and subsequent bioavailability of nitric oxide (NO), a well-documented facilitator of vascular dilation that is critical for normal endothelial function.[15,18]

Keywords: African Americans, Endothelial Cells, Endothelial Dysfunction, Endothelial Nitric Oxide Synthase, Inflammation, Interleukin-10, Laminar Shear Stress, Nitric Oxide

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Increased vascular shear stress during aerobic exercise has been associated with favorable endothelial adaptations.[19-22] In addition, a high physiological level of laminar shear stress (LSS), used as an *in vitro* aerobic exercise mimetic, has been demonstrated to be important in protecting endothelial cells against inflammatory activation.[7,21,23,24] We previously evaluated the influence of aerobic exercise training (AEXT) on inflammation and endothelial health and reported that AEXT elicited positive improvements in inflammation and endothelial function evident by decreasing plasma concentrations of IL-6, and increasing plasma concentration of IL-10 and NO in our African American cohort.[25,26]

Despite the high prevalence of hypertension and predisposition to heightened systemic inflammation in African Americans, a paucity of research exists on the influence of AEXT on inflammation and endothelial health as a preventive measure to reduce the risk for hypertension and cardiovascular disease (CVD) in this population. Furthermore, the influence of IL-10 in combination with LSS on endothelial cells of African Americans has not been examined. Therefore, the purpose of this study was to evaluate the influence of IL-10 and LSS on African American endothelial cells by measuring total eNOS protein expression and its activated, phosphorylated form (p-eNOS) at Serine 1177, as well as NO levels, in African American endothelial cells in response to 24 hours of IL-10 incubation, high physiological levels of LSS, and a combination of IL-10 incubation with LSS.

Methods

The experiment consisted of the following four conditions: *Static, Static with IL-10 Incubation, LSS at 20 dynes/cm^2,* and *LSS at 20 dynes/cm^2 with IL-10 Incubation*. Human umbilical vein endothelial cells (HUVEC) from an African American donor were obtained from Lonza (Walkersville, Md.) and preserved in liquid nitrogen until time of culture. Experiments were conducted with HUVECs between passages 4-7. HUVECs were cultured in phenol red-free M199 media supplemented with 20% fetal bovine serum and endothelial cell growth supplement and grown in 10% gelatin-coated dishes. It has been demonstrated that the addition of phenol red to media could potentially interfere with nitrate and nitrite measurements, and that measurement of NO metabolites (NOx) is most reliable when levels are quantified from cells that have been cultured in phenol red-free media.[27,28] Cells were maintained at 37°C (98.6°F) and 5% CO_2 in tissue culture dishes and were examined daily for confluency and morphology observation. LSS was applied when cells reached 95%-100% confluency. The culture dishes were exposed to the four experimental conditions for a period of 24 hours. Recombinant human IL-10 was reconstituted and diluted to a final concentration of 2.0 ng/mL in the complete cell media as recommended (Sigma-Aldrich; SRP3071; St. Louis, Missouri). This dose was used for the incubation of cells in the tissue culture dishes that were included in the IL-10 experimental conditions.

Laminar Shear Stress

Confluent HUVECs grown in 100-mm tissue culture dishes were exposed for a period of 24 hours to LSS at 20 dynes/cm^2 using a rotating Teflon cone (cone and plate viscometer, 0.5 degree cone angle). Shear stress experiments were conducted in a cell incubator. Immediately following LSS application, both the static and LSS culture dishes were harvested for protein analysis. Radio-Immunoprecipitation Assay (RIPA) Buffer, protease inhibitor, and phosphatase inhibitor were used to enable cell lysis and to stabilize the protein solution in order to measure protein concentration in the cell lysate. Phenylmethylsulfonyl fluoride protease inhibitor was freshly added. Briefly, cells were washed twice with cold Dulbecco's phosphate buffered saline. A 300 µL volume of the RIPA cocktail was added and a rubber scraper was used to harvest adherent cells. Cell lysates were collected and centrifuged at 16,000 g for 20 minutes at 4°C (39.2°F). The top one-third portion of supernatant was collected and stored at -80°C (-112°F) until time of assay. A Bradford protein assay using Bio-Rad protein reagent was conducted to measure the protein concentration. A 5X sodium dodecyl sulfate (SDS) solution was made, and the protein-SDS samples were boiled for 3 minutes at 95°C (203°F) and frozen at -80°C (-112°F) until use.

Western Blotting

Levels of eNOS, p-eNOS, and alpha tubulin (α-tubulin) were analyzed by Western blotting with mouse monoclonal anti-eNOS (BD Biosciences; 610296; San

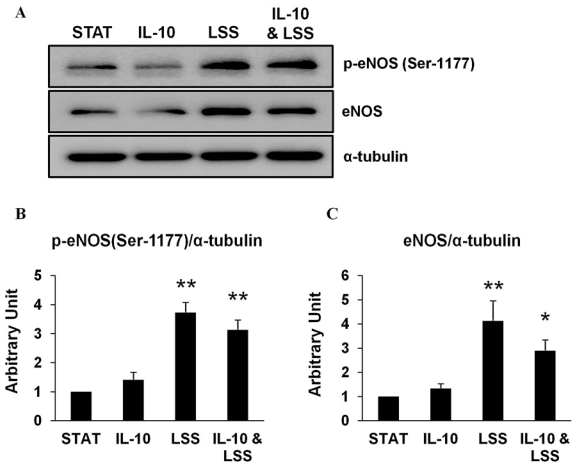

Figure 1. (A) Western blotting results from three independent experiments of phosphorylated endothelial nitric oxide synthase (p-eNOS) at Serine 1177 (Ser-1177) and endothelial nitric oxide synthase (eNOS) normalized to alpha tubulin (α-tubulin) for the four experimental conditions. STAT, *Static;* IL-10, *Static with Interleukin-10 (IL-10) Incubation;* LSS, *Laminar shear stress (LSS) at 20 dynes/cm²;* IL-10 & LSS, *LSS at 20 dynes/cm² with IL-10 Incubation*. (B) The bar graph depicts the results from densitometry analyses from western blotting experiments of p-eNOS at Ser-1177 normalized to α-tubulin for the four experimental conditions. (C) The bar graph depicts the results from densitometry analyses from western blotting experiments of eNOS normalized to α-tubulin for the four experimental conditions. Bars are expressed as mean ± standard error of the mean. *Denotes significant difference from STAT; $P<.05$. **Denotes significant differences from STAT; $P<.01$.

Jose, Calif.), mouse monoclonal anti-p-eNOS; s1177 (BD Biosciences; 612392; San Jose, Calif.), and mouse monoclonal anti-α-tubulin (Sigma-Aldrich; T9026; St. Louis, Missouri) antibodies, respectively. Alpha tubulin antibody was used as the loading control. Proteins were separated by SDS-Polyacrylamide Gel Electrophoresis on 10% gels and electrotransferred to polyvinylidene difluroide immobilion transfer membranes. Membranes were incubated overnight with a primary antibody at 4°C (39.2°F). After washing and incubating with a secondary antibody conjugated with horseradish peroxidase, total protein was detected by chemiluminescence. Band densitometry analyses were completed using ImageJ software (National Institutes of Health; Bethesda, Md.).

Nitric Oxide Assay

Cell culture supernatant was collected from the four experimental conditions and stored at -80°C (-112 °F) until the time of the assay. On the day of assay, all samples were centrifuged to remove particulates at 16,000g for 20 minutes at 4°C (39.2°F) and then ultrafiltered through a 10,000 molecular weight cut-off filter by microcentrifuge at 14,000 g for 20 minutes at 4°C (39.2°F). Concentrations of NOx (nitrite/nitrate) in the cell culture supernatant were determined using an assay based on the enzymatic conversion of nitrate to nitrite by nitrate reductase, followed by colorimetric detection of nitrite as an azo dye product of the Griess reaction (R&D Systems; Minneapolis, Minn.). The intra-assay CV value was 1.8%.

Statistical Analyses

Data are expressed as mean ± standard error of the mean. One-way analysis of variance followed by post-hoc testing with Fisher's least significant difference were used to assess differences across the four experimental conditions. Statistical significance was set at $P<.05$. All statistical analyses were performed using SPSS version 21.0 (SPSS Inc.; Chicago, Ill.).

Results

Western blotting experiments were conducted to measure total eNOS

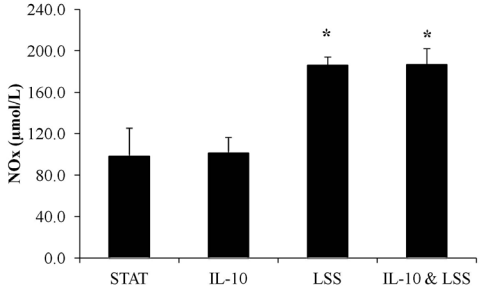

Figure 2. Total nitric oxide metabolite (NOx) concentrations from three independent samples of cell culture supernatant exposed to the four experimental conditions: STAT, *Static;* IL-10, *Static with Interleukin-10 (IL-10) Incubation;* LSS, *Laminar shear stress (LSS) at 20 dynes/cm²;* IL-10 & LSS, *LSS at 20 dynes/cm² with IL-10 Incubation.* Bars are expressed as mean ± standard error of the mean. *Denotes significant differences from STAT; $P<.05$.

protein expression and its phosphorylated form (p-eNOS) at Serine 1177 of the cells in the four experimental conditions with α-tubulin used as the loading control. Protein expression levels of both eNOS and p-eNOS were significantly increased in the *LSS at 20 dynes/cm^2* and *LSS at 20 dynes/cm^2 with IL-10 Incubation* experimental conditions when compared to the *Static* experimental condition. There were no significant differences between the *Static with IL-10 Incubation* and the *Static* condition or *the LSS at 20 dynes/cm^2 with IL-10 Incubation* and the *LSS at 20 dynes/cm^2* condition. These results are presented in Figure 1.

Total concentration of NOx, including nitrite and nitrate, was measured in cell culture supernatant, and concentration levels were determined using an assay based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. NOx concentration levels were significantly higher in the *LSS at 20 dynes/cm^2* and *LSS at 20 dynes/cm^2 with IL-10 Incubation* experimental conditions when compared with the *Static* experimental condition. There were no significant differences in the *Static with IL-10 Incubation* compared to the *Static* experimental condition or *the LSS at 20 dynes/cm^2 with IL-10 Incubation* compared to the *LSS at 20 dynes/cm^2* experimental condition. The results are presented in Figure 2.

Discussion

Research studies conducted on HUVECs have demonstrated that African American HUVECs have increased systemic inflammation, oxidative stress, and subsequent endothelial dysfunction when compared to Caucasian HUVECs.[7-10] It has been well-documented in the literature that oxidative stress and inflammation often occur simultaneously and have been linked to endothelial dysfunction and hypertension.[29-31] Elucidating the mechanisms relating to inflammation, endothelial dysfunction, and hypertension may be beneficial in developing preventive measures in reducing the CVD risk burden among the African American population. We have previously reported that African American endothelial cells had significantly greater levels of IL-6 protein expression and produced greater amounts of IL-6 in response to TNF-α, an inflammatory cytokine.[8] In addition, it was demonstrated that, compared with Caucasian endothelial cells, African American endothelial cells had significantly greater protein expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the principal source of reactive oxygen species in endothelial cells.[9] The findings from our studies suggest a heightened inflammatory and oxidative stress status in African American endothelial cells. Therefore, an intervention that can diminish this condition before endothelial dysfunction develops to the point where it manifests clinically may be very important.

A pivotal function of the endothelium altered by inflammation is NO-mediated regulation of vessel tone and blood flow, and this modification includes the reduction in the bioavailability of NO that impairs relaxation and contributes to endothelial dysfunction.[11,31,32] Research studies have demonstrated that NO exerts an anti-inflammatory influence by protecting endothelial cells against inflammatory activation.[33,34] In a study conducted on ethnic differences in endothelial function, the authors concluded that apparently healthy African Americans have impaired endothelial vasoreactivity when compared with apparently healthy Caucasians, and this disparity may be related to the increased inflammatory state demonstrated in African Americans.[35] This further emphasizes the importance of interventions for the African American population that target the bioavailability of NO, which has been demonstrated to be critically important for vasodilation and subsequent endothelial health.

Research data obtained from cell culture studies have demonstrated the beneficial effects of exercise on vascular health, which have been attributed to the increased exercise-induced shear stress.[7,21,23,24] The effect of LSS on cultured African American HUVECs has previously been reported, and significant improvements were demonstrated in eNOS protein expression and NO concentrations subsequent to high levels of LSS.[7] The results of our study demonstrate a similar outcome with significant increases in eNOS and p-eNOS protein expression, as well as concentrations of NO, subsequent to LSS; however, IL-10 did not provide an additive beneficial effect, as was originally hypothesized.

Other studies have examined the effect of IL-10 on eNOS protein expression and concentrations of NO. In a study conducted on mice, the authors reported that IL-10 exerted its anti-inflammatory influence by inhibiting the *in vivo* and *in vitro* adverse effects of TNF-α on the endothelium of the murine aorta by restoring the eNOS

protein expression that was reduced by TNF-α.[15] This study also showed that IL-10 without the presence of TNF-α had no effect on eNOS expression.[15] An additional study was conducted on HUVECs pre-incubated with TNF-α, and it was demonstrated that eNOS protein expression and NO production were increased in the cells subsequent to incubation with IL-10.[18] The authors concluded that in the presence of a pro-inflammatory stimulus, increased eNOS protein expression was mediated by the anti-inflammatory effect of IL-10.[18] An important commonality in both of these studies, that should be noted, is that IL-10 exerted its anti-inflammatory influence by increasing eNOS protein expression and NO in the presence of TNF-α, an inflammatory cytokine. These findings complement other *in vivo* studies which demonstrated that IL-10 exerted its anti-inflammatory effects in human subjects with diseases in which higher levels of inflammation are manifested.

In CVD patients with elevated plasma CRP levels, IL-10 has been associated with improved vasoreactivity and a more favorable prognosis, providing some evidence for the importance of IL-10 in endothelial health.[13,36] In addition, several studies have previously examined the effect of AEXT on circulating levels of IL-10 in subjects with type 2 diabetes and patients with CVD, and reported a significant increase in IL-10 subsequent to an AEXT intervention.[37-39] Furthermore, a review conducted by Batista et al on the role of TNF-α and IL-10 on the anti-inflammatory effect of AEXT included heart failure patients who exhibited elevated baseline levels of TNF-α.[37] The authors concluded that the anti-inflammatory effect induced by AEXT seemed to be primarily mediated by IL-10.[37]

In our study, the protein expression of p-eNOS and eNOS, as well as NOx concentration, were measured in HUVECs that were incubated with IL-10; however, the cultured cells were not previously exposed to any inflammatory medium, such as TNF-α. A future direction for an *in vitro* study may be to pre-incubate the cultured cells with TNF-α and subsequently measure eNOS protein expression and NOx concentration under the same four experimental conditions as our study.

Conclusion

The results of our study are novel because the effect of IL-10 incubation on protein expression of p-eNOS and eNOS in African American HUVECs has not been previously investigated. The primary findings of our study conducted on HUVECs suggest that IL-10 did not have an additive effect on total eNOS and p-eNOS. Although IL-10 had little effect in our study, p-eNOS and eNOS protein expression, as well as NO concentrations, were demonstrated to be significantly increased as a result of LSS in African American HUVECs. Therefore, AEXT may be a viable, non-pharmacologic method to improve vascular inflammation status and vasodilation, and thereby contribute to reducing hypertension and CVD risk in African Americans.

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Author Contributions

Research concept and design: Babbitt, Kim, Forrester, Brown, Park. Acquisition of data: Babbitt, Kim. Data analysis and interpretation: Babbitt, Kim, Forrester, Park. Manuscript draft: Babbitt. Statistical expertise: Babbitt. Acquisition of funding: Brown. Administrative: Babbitt, Kim, Forrester. Supervision: Babbitt, Park.

Statement of Human Rights

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

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