Allicin Reverses Diabetes-Induced Dysfunction of Human Coronary Artery Endothelial Cells

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ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death in the United States, and is the major source of morbidity and mortality associated with diabetes mellitus. Because the incidence of diabetes continues to increase, reducing the risk of CVD in diabetes will continue to be a major focus of cardiovascular research. An early manifestation of diabetes-induced CVD is dysfunction of the vascular endothelium, as indicated by depressed production of NO. Our findings now demonstrate depressed activity of endothelial nitric oxide synthase (eNOS) in diabetes, and suggest that treating human coronary artery endothelial cells with allicin, the major bioactive organosulfur component in garlic extract, can restore NO production in these cells. Coronary artery endothelial cells (Lonza) were obtained from control (HCAEC) or diabetic (DHCAEC) donors, and NO production was measured by fluorescence microscopy via 4,5-diaminofluorescein diacetate. On average, NO production was depressed by 12.9% in DHCAEC compared to controls. Treating these cells for 20 minutes with 4µM allicin restored NO production by 32.9%. Further, immunoblot studies revealed that diabetes decreased expression of eNOS protein by 20.3%; however, allicin was able to reverse this effect of diabetes. On average, eNOS expression was increased by 26% by overnight exposure to 5µM allicin. Taken together, these data indicate that allicin improves endothelium-dependent NO production in diabetes by enhancing the expression and/or activity of eNOS in human coronary artery endothelial cells. These studies further suggest that this improved endothelial function likely contributes to the established health benefits of garlic consumption (e.g., lowering blood pressure), and also suggests a natural means of reducing the devastating consequences of diabetes on CVD. Future experiments are needed to identify the mechanism of allicin action on eNOS and in vascular endothelial cells.

INTRODUCTION

Diabetes mellitus is a consistent rising public health burden in the 21st century, and remains a major risk factor for the development of CVD. Currently, diabetes affects over 125 million people in the western world, and estimates of its prevalence are expected to surpass 300 million by 2025 due to a combination of poor diet and lifestyle. Natural garlic (*Allium sativum*) supplementation has been utilized as a remedy for thousands of years in treating various chronic diseases. Allicin (2-propene-1-sulfonothioic acid S-2 propenyl ester, diallyl thiosulfinate) is the primary active component in garlic, and current literature supports its therapeutic effects on lowering blood pressure, decreasing blood glucose levels, reducing atherosclerotic plaque formation, and modulating inflammation. This organosulfur compound rapidly crosses the cellular membrane and reacts with sulfhydryl groups on cellular proteins or with glutathione. It has been demonstrated that allicin increases intracellular levels of glutathione and phase II detoxifying enzymes that include glutamate-cysteine ligase and heme oxygenase-I in vascular endothelial cells. We tested the hypothesis that allicin could reverse diabetes-induced inhibition of NO production in human coronary artery endothelial cells, possibly by decreasing oxidative stress to lower CVD risk.

METHODS

Cell Culture: Human coronary artery endothelial cells (HCAEC) from healthy, Type-I, and Type II diabetic cell lines(DHCAEC-I; DHCAEC-II) were grown in EBM-2 basal media supplemented with EGM-2 MV (Lonza). Passage 4 cells were thawed and used for treatment. Cells were changed to phenol-free media 24 hours prior to imaging.
Allicin Treatment: Cells were incubated in 10µM 4,5-Diaminofluorescein diacetate (DAF-2DA) for 15 minutes at 37˚ C. Cells were then washed with KREBS solution and incubated for 20 minutes at 37˚ C. Afterwards, cells were incubated with L-NAME and images were obtained with EVOS microscope at 10X, 20X, 40X and analyzed using ImageJ software.
Immunoblotting: Cell lysates (20µg of protein) were analyzed by 8% Tris Glycine SDS polyacrylamide gel electrophoresis and transferred to PVDF membrane. The membranes were blocked with 5% milk + TBST 0.1% for 1 hour and then probed overnight with a anti-eNOS antibody (1:10000). Membranes were then incubated with species-specific HRP-conjugated secondary antibody (1:5000) followed by detection with ECL substrate. Quantitation of proteins was determined by densitometry analysis of scanned films using ImageJ software.
Mass Spectrometry: Proteins were extracted from cell lines and equal amounts were immunoprecipitated with anti-eNOS antibody or control goat IgG. The immune complexes were immobilized on the protein A/G ultralink resin. Trypsin digestion and MS analysis were performed at the proteomics and metabolomics core facility at the Georgia Cancer Center. Scaffold (version Scaffold_4.8.9, Proteome Software, Inc.) was used to validate LC-MS and LC-MS/MS-based peptides and protein identification. Peptide identifications were accepted if they could be established at >95.0% probability as specified by the Peptide Prophet algorithm.

RESULTS

Allicin reverses diabetes-induced depression of NO Production in HCAEC

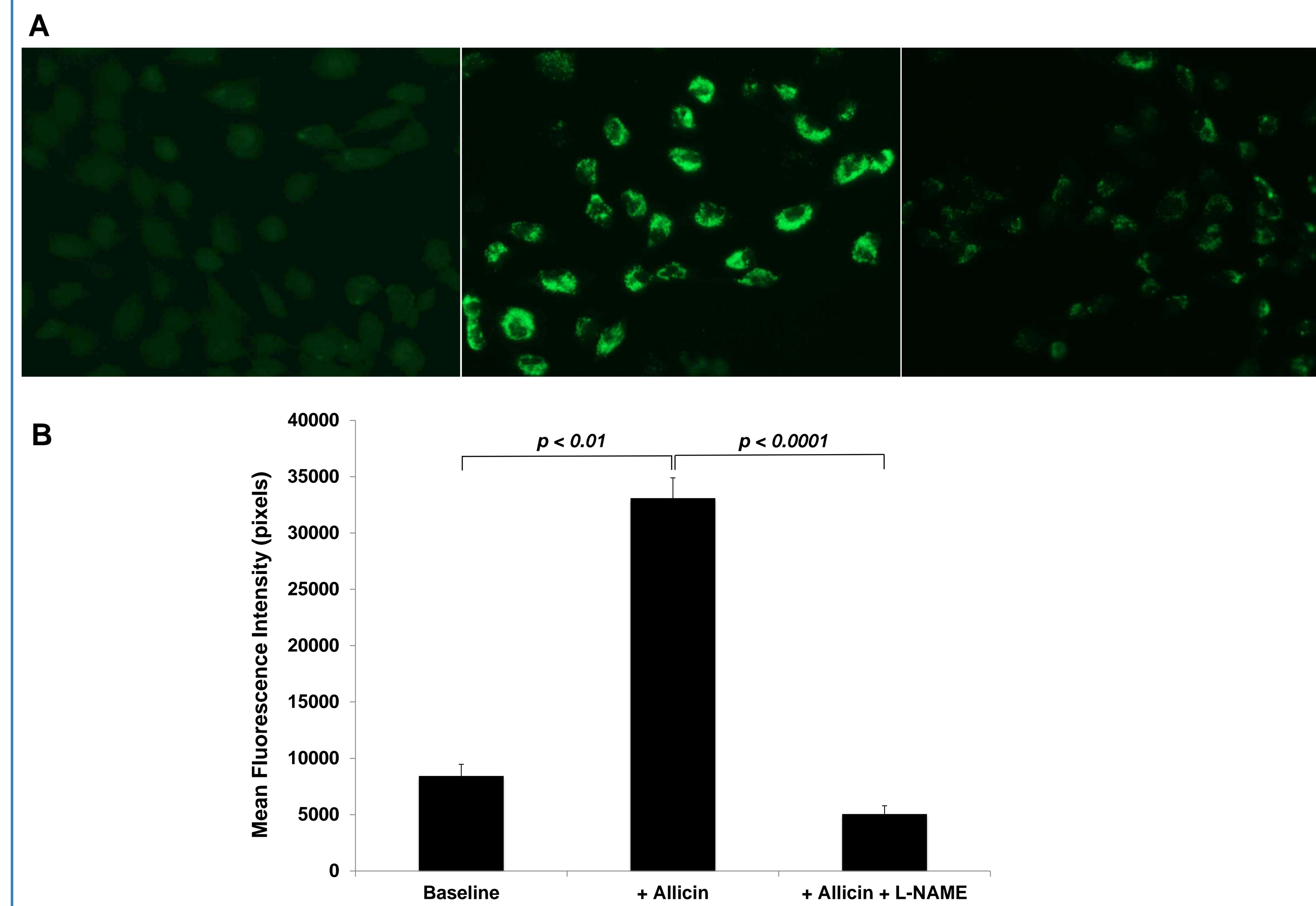


Figure 1. Effect of allicin on NO production and inhibition by L-NAME in DHCAEC-I cells. A. Representative images of NO production by DHCAEC-I cells untreated (left panel) allicin-treated (middle panel), and treated with allicin and L-NAME (right panel). B. Baseline DAF fluorescence was 8433 ± 6276, n=36 cells. Allicin increased mean fluorescence intensity by 292.39% from baseline (33090 ± 7705 n=18 cells). L-NAME, an inhibitor of eNOS activity, attenuated mean flourescence intensity to 5057± 5043, n=47 cells. This is a 554.34% decrease from allicin treatment. Data are expressed as mean ± SEM. Student's *t* test was performed.

NO production in HCAEC is mediated via eNOS activity

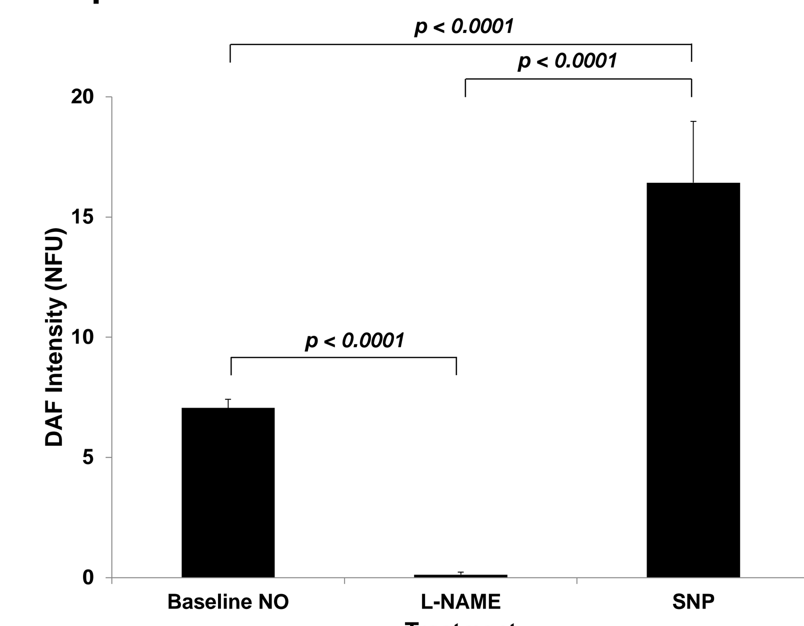


Figure 2. L-NAME, an inhibitor of eNOS, attenuated NO production and sodium nitroprusside (SNP) increased NO production in HCAEC. Baseline DAF fluorescence was 7.062 ± 0.3603 NFU, n=119 cells. L-NAME decreased intensity by 98.3% from baseline, (mean 0.1206 ± 0.111 NFU, n=41 cells.) Subsequent treatment with SNP, an exogenous NO donor, significantly increased DAF intensity to 16.43 ± 2.543 NFU, n=41 cells. This is a 13,524% increase from L-NAME and a 132.7% increase from baseline. Data analyzed using Student's *t* test.

Allicin increases eNOS expression in DHCAEC-I

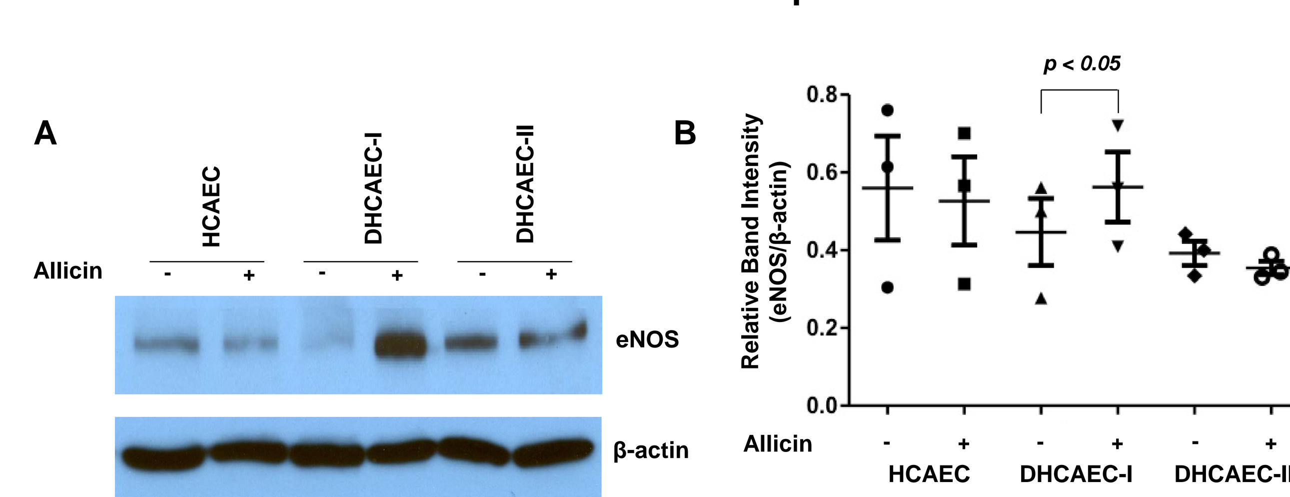


Figure 3. Allicin increased eNOS expression. A. Representative immunoblot analysis for eNOS in HCAEC, DHCAEC-I, and DHCAEC-II cell lines. β-actin was used as loading control. B. Quantification of eNOS expression in non-treated and allicin treated cell lines of HCAEC (n=3), DHCAEC-I (n=3), and DHCAEC (n=3). The data are expressed as mean ± SEM. Data analyzed using Student's *t* test.

Mass Spectrometry Analysis of eNOS Proteins Modified by Allicin

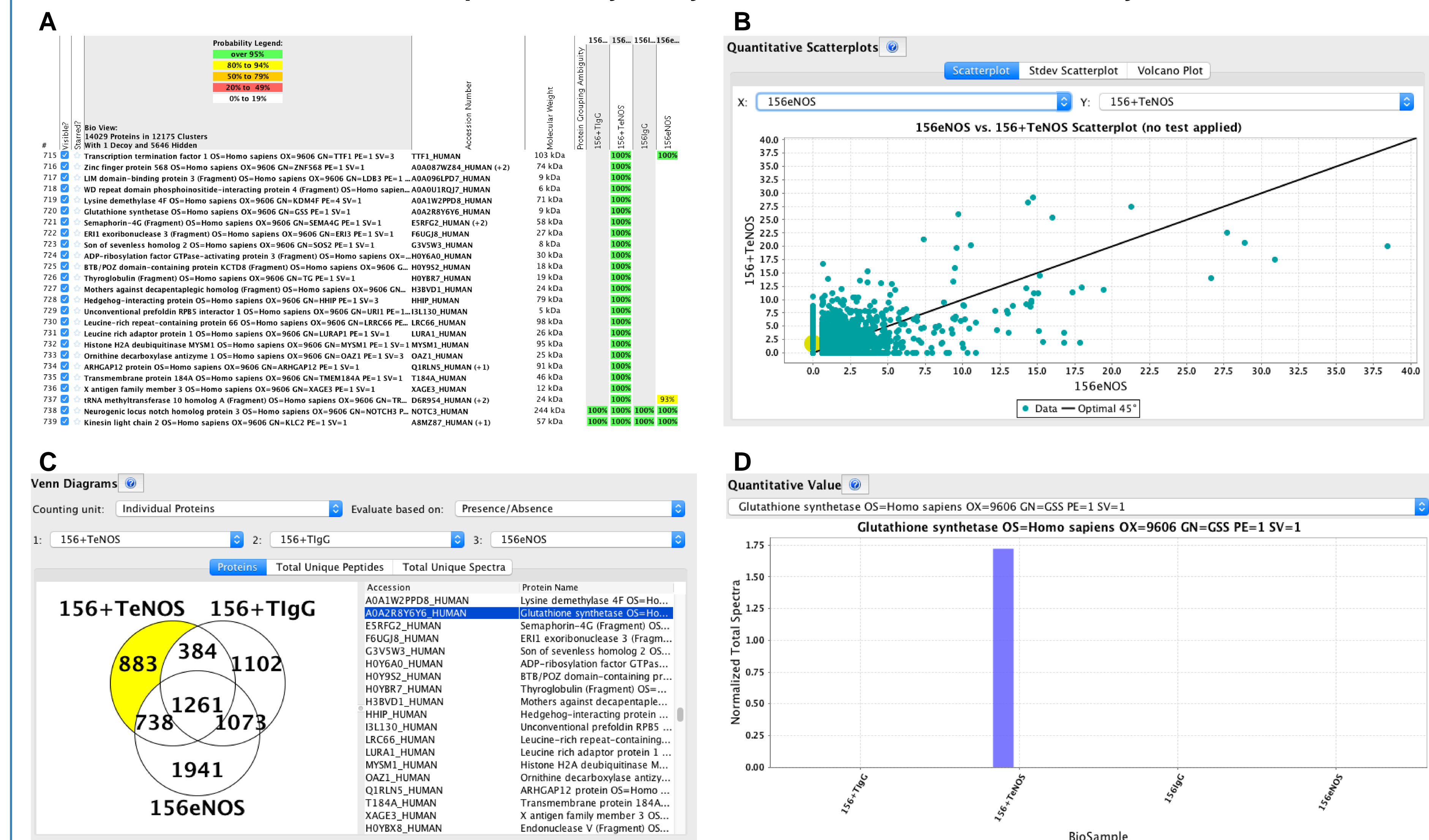


Figure 4. Analysis of eNOS immunocomplex induced by allicin treatment in DHCAEC-I cells by mass spectrometry (MS). To identify proteins, immunocomplexes were isolated from allicin-treated and non-treated DHCAEC-I cells using an anti-eNOS antibody and control IgG. LTQ Orbitrap MS was then used to analyze tryptic peptides generated following an immunoprecipitation. A. Highest ranking of proteins associated with eNOS complex induced by allicin. 156 indicated as DHCAEC-I cells. B. Quantitative scatterplot demonstrated proteins involved in the eNOS immunocomplex of non-treated versus allicin treated DHCAEC-I cells. C. Venn diagram representing the amount of proteins associated with the eNOS immunocomplex in each experimental group. 883 proteins are represented in the allicin specific immunocomplex of DHCAEC-I cells. D. Representative protein (glutathione synthetase, accession A0A2R8Y6Y6_HUMAN) specific for allicin induced eNOS immunocomplex.

SUMMARY

Our findings demonstrate that allicin:

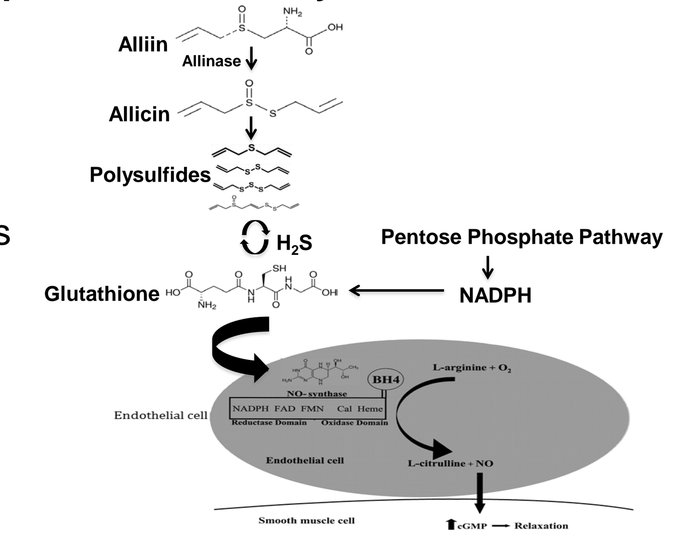
1.) increased NO production in DHCAEC-I cells

2.) increased eNOS expression in DHCAEC-I cells

3.) has no effect on eNOS expression on HCAEC and DHCAEC-II cells

4.) modified the eNOS immunocomplex in DHCAEC-I cells

Proposed Vasodilatory Mechanism of Allicin



CONCLUSION

Our data provide supporting evidence for the potential use of allicin as a preventative measure against CVD. We propose that allicin may aid in treatment of endothelial dysfunction associated with type-I diabetes or other conditions of high oxidative stress. Future research is needed to analyze the role of specific proteins involved in the eNOS immunocomplex induced by allicin.

ACKNOWLEDGEMENTS

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