Oxidative stress-induced endothelial dysfunction contributes to cardiovascular disease

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ABSTRACT

Objective - Oxidative stress is supposed to be involved in cardiovascular pathology through vascular cell damage. Identification of oxidative stress markers can represent a method for early diagnosis of vascular dysfunction. Biomolecular mechanisms of the vascular damage remain partially understood.

Material and Methods - In vitro and ex vivo studies were performed in order to investigate the role of oxidative stress and the potential preventive action of antioxidant agents against activation, proliferation and/or apoptosis of human endothelial and vascular smooth muscle cells.

The expression and activity of oxidative stress enzymes, reactive oxygen species and O_2^- radicals, including NADPH oxidase isoforms were evaluated, as well as leukocyte adhesion assay and the expression of cell adhesion molecules. In addition, histological and immunohistochemical analysis of human aorta tissue were performed.

Results - We detected higher levels of oxidative stress markers during endothelial activation and leukocyte recruitment. In addition, oxidative stress was involved in the modulation of cell proliferation, vascular smooth cell phenotype, and cell apoptosis. Vascular and endothelial dysfunction by oxidative stress was mediated by NADPH oxidase 4 activity. The treatment with antioxidant agents such as ascorbic acid, NAC and NADPH oxidase 4 specific inhibitors prevented oxidative stress-induced vascular and endothelial dysfunction.

Conclusion- We demonstrated that oxidative stress is pivotal in vascular and endothelial dysfunction. Moreover, our data provide additional information about the role of oxidative stress in the pathogenesis of cardiovascular disease. The identification of those oxidative stress markers in vascular cells will allow an early diagnosis and an appropriate antioxidant therapeutic approach.

Keywords: vascular dysfunction, oxidative stress, inflammation, NADPH oxidase isoform 4.

INTRODUCTION

Cardiovascular diseases (CVD) are a group of diseases involving heart and blood vessels and represent the main cause of morbidity and mortality in the world.[1] Endothelial dysfunction play a pivotal role in the pathogenesis of several vascular and metabolic human diseases such as diabetes, stroke, peripheral vascular disease, and heart disease.[2] Endothelial activation is a pro-inflammatory and pro-coagulant state characterized by the expression of endothelial cell-surface adhesion molecules required for inflammatory cell recruitment.[2] Endothelial dysfunction is defined as a systemic, silent and reversible pathological state of the endothelium derived from a reduced NO bioavailability.[3] Increased oxidative stress is linked to endothelial dysfunction and plays a central role in the pathogenesis of CVD.[4] ROS generation by oxidative stress leads to mitochondrial damage, endothelial dysfunction and promotes leukocyte adhesion, inflammation, and thrombosis.[5] Identifying new oxidative stress markers is a research goal fundamental for prevention, clinical diagnosis and for the development of new therapies for endothelial dysfunction and related CVD.

Materials and Methods

In vitro experiences

Human umbilical vein endothelial cells (HUVECs; Cambrex, Milan, Italy) were grown in endothelial basal medium (EBM-2) containing 2% FBS (fetal bovine serum) and endothelial growth factor supplements (EGM-2 bullet kit, Cambrex). First-third **passage h**uman umbilical vein endothelial cells (HUVECs, Lonza, Italy) were serum deprived (0.1% FBS over-night) and treated or not with TNF-a (5 ng/mL for 4 hours in 0.1% FBS; Sigma-Aldrich, Milan, Italy). For antioxidant studies, cells were pre-treated for 24 h with N-acetyl cysteine (NAC, 100 µM; Sigma-Aldrich) or ascorbic acid (AS, 100 µM; Sigma-Aldrich) before serum deprivation and TNF-a adding. All antioxidants were dissolved in sterile distilled water (< 0.1% v/v final concentration).

For some inhibition studies, plumbagin (10 µM; Sigma-Aldrich), a specific inhibitor of NOX4, was added in serum-deprived or TNFa-stimulated HUVEC cultures.

Reverse transcriptase and Real-Time Polymerase Chain Reaction

Total RNA was extracted from treated cells by Trizol™ reagent (Invitrogen) and reverse transcriptase reaction performed.[6] Real-Time PCR was performed with gene-specific primers: human VCAM-1 sense 5'-TAAAATGCCTGGGAAGATGG-3' and antisense 5'-GGTGCTGCAAGTCAATGAGA-3', human ICAM-1 sense 5'-CAAGGCCTCAGTCAGTGTGA-3' and antisense 5'-CCTCTGGCTTCGTCAGAATC-3' and human Nox4 sense 5'-CTCAGCGGAATCAATCAGCTGTG-3' and antisense 5'-AGAGGAACACGACAATCAGCCTTAG-3'. Results were normalized against human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels: sense 5'-ACGGATTTGGTCGTATTGG-3' and antisense 5'-GATTTTGGAGGGATCTCGC-3'. For PCR amplification, iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Milan, Italy) was used and analyses carried out using iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad).[6] To verify the amplification specificity, for each gene, melting curve was analyzed and positive and negative controls checked. The results were reported as normalized fold expression of three independent experiments performed in triplicate.

Reactive oxygen species assay

Reactive oxygen species (ROS) levels in HUVECs were measured by 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H$_2$DCFDA) fluorescence method (Molecular Probes, Inc., Eugene, OR, USA), as described.[6] Fluorescence was monitored by analyzing at least 10,000 cells using a flow cytometer (Beckman Coulter, CA, USA). Results were expressed as the mean of three different experiments performed in triplicate.

Leukocyte adhesion assay

Human leukocytes were isolated from peripheral blood of volunteers (under written consent) by using Ficoll-Paque Plus (Amersham Pharmacia Biotech, NJ, USA) according to manufacture's guidelines and incubated with 2'7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (2 µM; Life Technologies Europe BV, Milan, Italy) for 45 min at 37 °C. Then leukocytes were counted using a hemocytometer, washed and layered (1x10^6/mL) on HUVEC monolayers (after treatments) for 1 h on a rocker plate. Non-adherent cells were removed during three gentle washing steps. Adhering cells were fixed in 2% glutaraldehyde and counted under a fluorescent microscope (E600 Eclipse; Nikon). Experiments were repeated in triplicate.

Small interfering RNA for Nox4

A 19-nucleotide small interfering RNA (siRNA) 3'-overhanged for human Nox4 (access NM_016931) was designed by using Block-IT^{TM} RNAi Designer (Invitrogen). The siNox4

sequence was: 5'-CCUCAGCAUCUGUUCUUAA-3', whereas a non-targeting siRNA sequence was used as control: 5'-CCTTACGTGTCTCTACTAA-3'. For transfection, HUVECs at 60-70% confluence were incubated with the siNox4-Oligofectamine complexes (Invitrogen) in antibiotic and serum-free medium, according to manufacture's guidelines. Depletion of Nox4 by siRNA was confirmed by Western blot.

Patients

For this study, twenty patients (mean=63.5 years, 73.75% male) undergoing surgical procedure in the Department of Cardiosurgery of Tor Vergata University for thoracic aortic aneurysm (TAA) were selected. As control, autoptic thoracic aorta tissue samples (n=10) from patients died for non-cardiovascular diseases were included (mean=49.1 years). The study was approved by the Local Ethics Committee and all patients gave written informed approval.

Immunohistochemical analysis

Serial four µm-thick paraffin section of 10% neutral-buffered formalin fixed aorta tissues were used for immunohistochemical studies. Sections were dewaxed, immersed in 3% H_2O_2 for endogen peroxidase blocking and, after antigen retrieval, sections were incubated with NOX4 antibody (Santa Cruz Biotecnology) with positive and negative controls.[7] For semiquantitative evaluation, 10 randomly selected fields along tunica media were analyzed, at 200X magnification, and staining intensity calculated using a grading system and expressed in arbitrary units, as reported.[7] Assessments were performed by two researchers in a blinded manner, with an interobserver reproducibility > 95%.

Statistical analysis

Data were expressed as the mean + standard error of mean (SEM). Student's t-test was used and values of p < 0.05 were considered statistically significant.

RESULTS

Antioxidant activity reduces leukocyte adhesion and oxidative stress in activated HUVECs

To better understand if the anti-inflammatory effect of antioxidants is mediated by their protective effect on endothelium, we studied their action on leukocyte adhesion in activated HUVECs. After TNF-a stimulation, leukocyte adhesion was increased (Fig. 1A, p < 0.001) as well as ICAM-1 and VCAM-1 mRNA levels (Fig. 1B-C, p < 0.001) compared with basal condition. The antioxidant pretreatment counteracted the TNF-a-induced leukocyte adhesion and molecule adhesion expression (p < 0.001 and p < 0.01, respectively).

In order to confirm that endothelial dysfunction was characterized by the induction of oxidative stress, we investigated ROS accumulation in HUVECs. TNF-a stimulation induced higher ROS levels (Fig. 1D, p < 0.001) that antioxidant pretreatment counteracted (p<0.01). In accordance with previous reports,[6] we analyzed NOX4, the major NADPH oxidase subunit in HUVECs and responsible for ROS generation. As reported in Fig.4E, Nox4 mRNA levels increased after TNF-a stimulation (p < 0.001). This effect was counteracted by antioxidant pretreatment (p < 0.001).
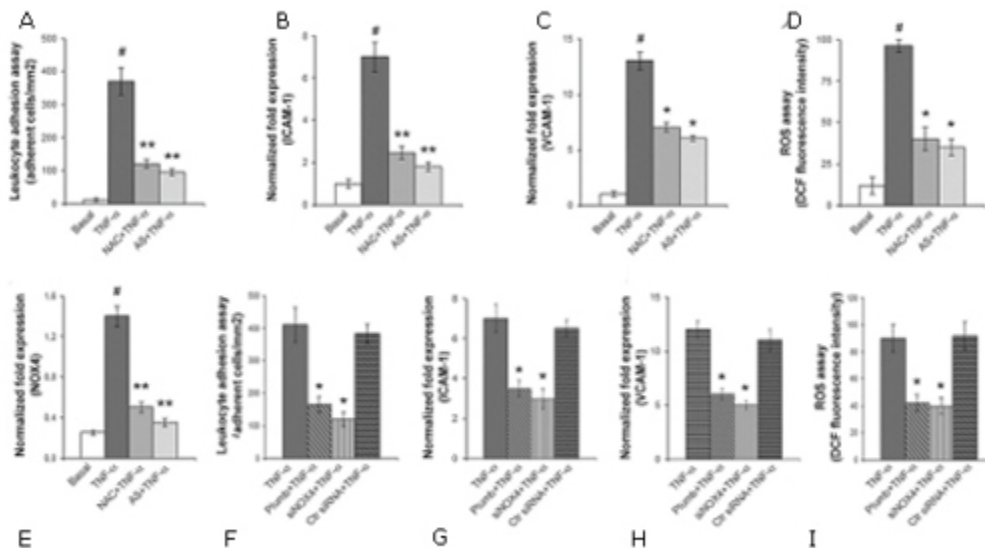
Fig. 1. Antioxidants reduce leukocyte recruitment, adhesion molecules and oxidative stress.

Figure 1. Antioxidants reduce leukocyte recruitment, adhesion molecules and oxidative stress. (A) Leukocyte adhesion assay in basal condition (2% FBS) or TNF-a-stimulated (5 ng/mL for 4 h in 0.1% FBS) HUVECs, with or without NAC (100 μM) or AS (100 μM) 24 h-pretreatment. (B,C) Bar graphs of Real-time PCR for ICAM-1 and VCAM-1 transcripts. (D) Ros assay by dichlorodihydrofluorescein intensity measurement. (E) Bar graphs of Real-time PCR for NOX4 transcripts. (F) Leukocyte adhesion assay in TNF-a-activated (5 ng/mL for 4 h in 0.1% FBS) HUVECs with or without plumbagin (Plu, 10μM), siNox4 or non-targeting siRNA (Ctr siRNA). (G,H) Bar graphs of Real-time PCR for ICAM-1 and VCAM-1 transcripts. *t*-Student: * and **, p < 0.01 and p < 0.001, respectively. Values are expressed as mean + SEM of three separate experiments.

Endothelial dysfunction is dependent on Nox4-mediated increase of oxidative stress

To verify if Nox4 activity was responsible for TNF-a-induced endothelial dysfunction, we used a specific Nox4 inhibitor plumbagin[8] and a siRNA for Nox4 (siNox4). The specific knockdown of Nox4 in HUVECs was assessed by blot (data not shown). Plumbagin and siNox4 counteracted

leukocyte adhesion in TNF-a-stimulated HUVEC cultures (Fig. 1F, p < 0.01), as well as the upregulation of ICAM-1 and VCAM-1 (Fig. 1G-H, p < 0.01). Plumbagin and siNox4 also prevented TNF-a-induced ROS generation (Fig. 1I, p < 0.01). Non-targeting siRNA (Ctr siRNA) induced no changes. These findings strongly suggest that oxidative stress-induced endothelial dysfunction is mediated, at least in part, by Nox4 activation.

Expression of NOX4 in human TAA

Semiquantitative evaluation of NOX4 expression revealed that the tunica media of TAA showed higher immunoreactivity compared with control aortas (Fig.2A-B, p<0.01). These findings strongly suggest that the degenerative process, occurring in TAA, is accompanied by Nox4 activation in smooth muscle cells of the tunica media.
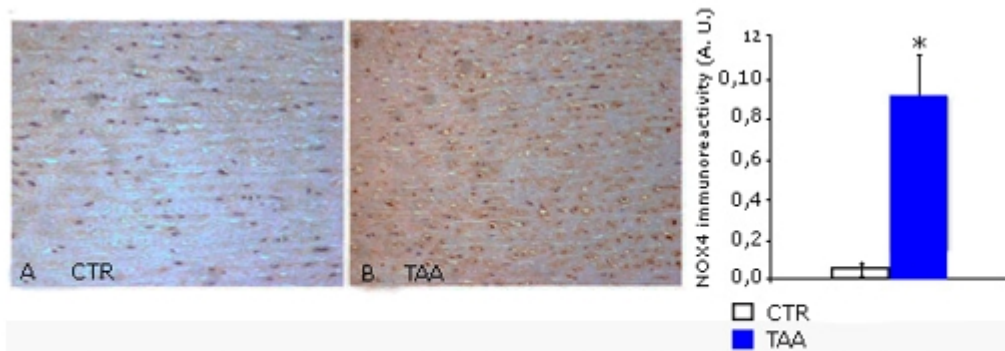
Fig. 2. Immunohistochemical evaluation of NOX4 expression in human TAA. (A,B) Representative images and semiquantitative evaluation of Nox4 immunostaining on human aortas from TAA patients and control group. t-Student: *, p < 0.01. Values are expressed as mean + SEM of three separate experiments.

DISCUSSION

In the present work, we investigated the mechanisms through which antioxidants can revert endothelial dysfunction. We documented that antioxidant pretreatment counteracted TNF-a-induced endothelial dysfunction in HUVECs by reducing adhesion molecules, leukocyte recruitment, and ROS accumulation through NOX4 inhibition. The modulation of vascular function is essential to maintain circulation integrity and therefore homeostasis of tissue environments under physiological conditions.[9] ROS are a family of molecules including molecular oxygen and its derivatives produced in all aerobic cells.[10] Excessive vascular production of ROS, outstripping endogenous antioxidant defense mechanisms, has been implicated in oxidation of biological macromolecules, such as DNA, protein, carbohydrates, and lipids.[10] This condition has commonly been referred to as oxidant stress. An increasing body of evidence suggests that oxidant stress is involved in the pathogenesis of many cardiovascular

diseases, including hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure.[10] Oxidative stress and ROS production play a pivotal role in endothelial cell dysfunction and apoptosis in atherosclerosis, hypertension and heart failure.[10] Inflammatory stimuli increase cellular oxidative stress that is driven by mitochondrial and Nox-dependent ROS generation.[11] According to this mechanism, we demonstrated that endothelial dysfunction is associates with oxidative stress and a strong increase of Nox4 activity, the main endothelial Nox isoform.[6] Moreover, the specific inhibition of Nox4 counteracted oxidative stress-induced leukocyte recruitment and adhesion molecule expression, suggesting that oxidative stress-mediated endothelial dysfunction depends, at least in part, on Nox4 activity. Antioxidants and free radical scavengers, such as NAC and ascorbic acid, showed anti-inflammatory effects when used as co-adjuvants in the clinical management of atherosclerosis.[12] Here we clearly demonstrated that antioxidant treatment counteracted endothelial dysfunction through the inhibition of oxidative stress generated by Nox4-medited ROS generation. Moreover, we demonstrated the involvement of NOX4 activity in the degenerative and inflammatory process that occurs in TAA, supporting a pivotal role of NOX4 also in vascular smooth muscle cells of the tunica media.

CONCLUSION

In conclusion, these data demonstrated that the efficacy of antioxidants in the recovery of endothelial function, after its activation, is due to the reduction of inflammation through the inhibition of Nox4-mediated ROS generation and oxidative stress. The present findings indicate new possible targets and therapies to counteract endothelial dysfunction and cardiovascular diseases.

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Conflict of interest: The Authors declare no conflict of interest regarding the publication of this paper.