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Chronic nitric oxide deprivation induces an adaptive antioxidant status in human endothelial cells

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ABSTRACT

In a previous work, we showed an increased cell motility due to the accumulation and transcriptional activation of the Hypoxia Inducible Factor-1α (HIF-1α) and a reduced mitochondrial energy production in an *in vitro* model of endothelial dysfunction (ED) represented by human endothelial cells (ECs) chronically deprived of nitric oxide (NO) by L-NAME treatment. In the present study, in the attempt to unravel the pathway(s) linking NO deficiency to HIF-1α accumulation and activation, we focused our attention on Reactive Oxygen Species (ROS). We found that ROS were partially involved in HIF-1α stabilization, but not in the pro-migratory phenotype. Regarding mitochondrial dysfunction, it did not require neither ROS generation nor HIF-1α activity, and was not due to autophagy. Very interestingly, while acute treatment with L-NAME induced a transient increase in ROS formation, chronic NO deprivation by long term L-NAME exposure drastically reduced cellular ROS content giving rise to an antioxidant environment characterized by an increase in superoxide dismutase-2 (SOD-2) expression and activity, and by nuclear accumulation of the transcription factor NF-E2-related factor-2 (Nrf2). These results might have important implications for our understanding of the consequences of NO deprivation in endothelium behavior and in the onset of cardiovascular diseases.

1. Introduction

Nitric oxide (NO)[2] is an important signaling molecule involved in many physiological and pathological processes. As an example, the maintenance of vascular homeostasis crucially depends on the ability of endothelial cells (ECs) to produce NO, that by diffusing into neighboring smooth muscle cells induces vasorelaxation, thereby controlling blood pressure [1,2]. Endothelial NO also diffuses into platelets where it plays an antiaggregant activity that protects the cardiovascular system from thrombosis and acute events [2].

It is well known that endothelial dysfunction (ED), *i.e.* an impaired function of the endothelium coupled with a reduced release of NO, is a risk factor for atherosclerosis together with a list of conditions such as hypertension, hypercholesterolemia, smoking, diabetes, and the aging process itself [3]. These conditions are also associated with a significant increase in Reactive Oxygen Species (ROS) in the vascular wall, that may contribute to the establishment of ED and to the development of its late effect on cardiovascular system [4–10].

In addition to its effect on smooth muscle cells and platelets, endogenous NO also has important roles in EC physiology, stimulating their proliferation, migration and differentiation to form new blood vessels [11]. In particular, EC migration induced by angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) [12,13], endothelin [14] and oxytocin [15], depends on acute NO generation. It should be also mentioned that the concentration and timing of constitutive endothelial NO release appear to be of crucial importance in determining the final outcome on EC behavior. We have recently shown that the migratory behavior of Human Umbilical Vein ECs (HUVECs) is enhanced by a chronic NO deprivation induced by long term L-NAME treatment [16]. This behavior is mediated by a VEGF autocrine circuit set in motion by the nuclear accumulation and transcriptional activation of the Hypoxia Inducible Factor-1α (HIF-1α) [16]. Furthermore, chronic NO deprivation reduces the mitochondrial activity of HUVECs, showing an important role of the gas in the regulation of endothelial mitochondrial function. These results suggest that NO basal levels play an important homeostatic role in EC physiology, by exerting a tonic inhibitory effect on HIF-1α accumulation and by maintaining an adequate mitochondrial activity.

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[2] Abbreviations: AMPK, AMP-activated protein kinase; CM-DCFA, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate; ECs, endothelial cells; ED, endothelial dysfunction; eNOS, endothelial Nitric Oxide Synthase; HIF-1α, Hypoxia Inducible Factor-1α; HUVEC, Human Umbilical Vein Endothelial Cell; LC3, light chain 3 protein; L-NAME, N^G-nitro-L-arginine methyl ester; NAC, N-acetyl-cysteine; NO, nitric oxide; Nrf2, NF-E2-related factor-2; PHD, prolyl hydroxylase; ROS, Reactive Oxygen Species; SOD, superoxide dismutase; VEGF, Vascular Endothelial Growth Factor.

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In the present study, we have further investigated in our *in vitro* model of ED the effects of chronic NO deprivation on EC physiology, in the attempt to unravel the pathway(s) linking NO deprivation to HIF-1α accumulation and activity. We focused our attention on ROS, since their formation has been involved in HIF-1α stabilization in normoxia [17,18]. We found that ROS are only partially involved in the HIF-1α accumulation induced in HUVECs by long-term L-NAME treatment, and that their formation is not necessary for the pro-migratory phenotype observed in NO deprived ECs. Mitochondrial dysfunction as well does not depend on ROS generation or HIF-1α activity, and is not the result of mitophagic processes. Interestingly, chronic NO deficiency induces in HUVECs a cellular adaptive response represented by an antioxidant environment characterized by a reduction in cellular ROS content accompanied by an increase in the mitochondrial superoxide dismutase-2 (SOD-2) expression and activity, and by nuclear accumulation of the transcription factor NF-E2-related factor-2 (Nrf2). These results might contribute to a better knowledge of the endothelium behavior in the absence of NO, and finally to an improved comprehension of the molecular mechanisms involved in the onset of cardiovascular pathologies.

2. Materials and methods

2.1. Cell cultures

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from freshly derived umbilical cords by digestion with collagenase as described by Jaffe et al. [19]. Umbilical cords were donated anonymously after informed consent according to national ethical legislation. Cells were routinely grown in 199 medium supplemented with 20% fetal bovine serum (FBS), 100 μg/ml endothelial cell growth supplement (ECGS) and 50 μg/ml heparin, and used at passages 2–7. Where indicated, HUVECs were treated with 5 mM N^G-nitro-L-arginine methyl ester (L-NAME) in 199 medium containing 10% FBS for 48 h preceding the experiments. The concentration of L-NAME was chosen according to Papapetropoulos et al. [13].

2.2. Determination of Reactive Oxygen Species (ROS)

HUVECs were plated at a density of 1.5×10^4 cells/well in a black 96-well microplate, and loaded for 30 min at 37 °C in the dark with the fluorescent dye 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CM-DCFA, 10 μM) in HBSS buffer (Hepes 25 mM pH 7.4, NaCl 120 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 25 mM, glucose 15 mM) containing 1% FBS. Afterwards, the cells were exposed to L-NAME (5 mM in HBSS), and fluorescence was assessed by means of a multiplate reader with excitation and emission wavelengths of 485 nm and 530 nm, respectively (Victor™, PerkinElmer, Waltham, MA, USA). In some experiments, cells were pretreated for 1 h with 5 mM N-acetyl-cysteine (NAC) in 199 medium containing 10% FBS. When ROS were determined in HUVECs chronically exposed to L-NAME, cells were loaded with CM-DCFA during the last 30 min of treatment.

2.3. Preparation and analysis of nuclear extracts

HUVECs, plated in 100-mm diameter Petri dishes, were washed with phosphate-buffered saline (PBS) and collected by scraping. The nuclear extracts, prepared as described in details in [16], were loaded on a 10% SDS-PAGE, and the immunoreactive bands were visualized by chemiluminescence (LiteAblot Plus, EuroClone, Italy) after overnight incubation with the anti-HIF-1α or the anti-Nrf2 antibody at a 1:500 dilution in 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween-20 (TBS-T). Densitometric analyses of the immunoblots were performed using the National Institute of Health (NIH) ImageJ program.

2.4. Cell migration assays

HUVEC migration was evaluated by means of chemotaxis experiments in a 48-well modified Boyden chamber as previously described [15,16].

2.5. Cell metabolism assays

HUVECs were plated at a density of 2×10^4 cells/well in 96-well microplates, and cell metabolism was assessed by means of a CellTiter 96® AQueous One Solution Reagent colorimetric assay (MTS, Promega, Madison, WI, USA). The total cellular ATP content was determined using a CellTiter-Glo® Luminescent Assay (Promega). Both assays were performed according to the manufacturer's instructions. Optical density at 490 nm (for MTS) and luminescence (for ATP) were measured using a multiplate spectrophotometer (Victor™).

2.6. Immunoblot analysis

HUVECs, plated in 35-mm diameter Petri dishes, were washed with PBS, and then directly lysed in SDS-PAGE sample buffer (62 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.04% bromophenol blue). Western blots were performed according to standard methods. Densitometric analyses of the immunoblots were performed using the NIH ImageJ program.

2.7. Cell transfection

HUVECs, plated at a density of 1.5×10^4 cell/well in a 96-well microplate, were transfected with the expression vector pcDNA3ARNTdelta_b (ΔARNT), coding for a dominant negative mutant form of the HIF-1β ARNT subunit, and the void vector pcDNA3 using for each well 0.05 μg DNA and 0.25 μl of the PepMute transfection reagent (SignaGen Laboratories, Rockville, MD, USA). Two hours after transfection, the culture medium was replaced with fresh medium, and the cells were exposed to L-NAME for the following 48 h. For small interfering RNA (siRNA) transfection, validated Stealth™ RNAi duplexes against human endothelial Nitric Oxide Synthase (eNOS) (GC content 48%) (Life Technologies) and Stealth™ RNAi negative control duplex (Medium GC Duplex, 48% GC content, Life Technologies) were individually transfected into HUVECs at a 10 nM concentration using PepMute siRNA transfection reagent following the manufacturer's instructions (SignaGen Laboratories). The ability of the RNAi molecules to knock-down eNOS expression was analyzed 48 h after transfection by western blot analysis.

2.8. Total RNA extraction for reverse transcription and quantitative real time PCR

Total RNA was extracted using the RNeasy® Mini Kit and accompanying QIAshredder™ (Qiagen, Hilden, Germany). To avoid DNA contamination of samples, a 15-min on column incubation was carried out with DNase I (Qiagen). Reverse transcription was performed using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Life Technologies). Quantitative real time PCR (RT-qPCR) reactions were run with the iQ SYBR Green SuperMix (Bio-Rad, Segrate, Italy) on an iCycler iQ Real-Time PCR Detection System (Bio-Rad) using 50 ng of cDNA. For calculation of results, the $2^{-\Delta\Delta Ct}$ method was used allowing normalization to 18S and to the calibrator which is set to a value of 1.

2.9. Superoxide dismutase and catalase assays

Superoxide dismutase (SOD) activity was measured on HUVEC lysates using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions. Catalase activity was determined according to Aebi [20].

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