EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE GENE (iNOS) STIMULATED BY HYDROGEN PEROXIDE IN HUMAN ENDOTHELIAL CELLS

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Abstract

Inducible nitric oxide synthase (iNOS) gene expresses a calcium calmudolin-independent enzyme which can catalyse NO production from L-arginine. The induction of iNOS activity has been demonstrated in a wide variety of cell types under stimulation with cytokines and lipopoly saccharide (LPS). Previous studies indicated that all nitric oxide synthases (NOS) activated in human umbilical vein endothelial cells (HUVEC) were calcium dependent. In this work we indicate that endothelial cells can express iNOS mRNA under stimulation by cytokines, LPS and H2O2. We also found that iNOS mRNA expression has increased by augmentation of H2O2 concentration and its expression depends on increasing in time. Therefore we conclude that two isoforms of NOS are expressed in the HUVEC and induction of iNOS enzyme by H2O2 and NO production in endothelial cells may represent a novel cellular defence mechanism against oxidative stress and infections.

Keywords: Nitric oxide; Inducible nitric oxide synthase; mRNA expression; Human endothelial cells

Introduction

Nitric oxide (NO), also described as endothelium-derived relaxing factor, has been demonstrated to play a key role in the control of vascular tone and peripheral blood flow. Under normal conditions, an endothelial isoform of nitric oxide synthase (ec-NOS) that produces NO and L-citrullin from L-arginine in the calcium-dependent way, generates NO which diffuses into the underlying vascular smooth muscle cells, activating intracellular guanylyl cyclase and causing relaxation of the vessel wall [1]. NO has other roles in endothelium including: inhibition of platelet aggregation [2], leukocyte adhesion [3] and smooth muscle proliferation [2].

In addition to ec-NOS that found in endothelium two isoforms have known which named n-cNOS, a neuronal isoform, and iNOS, an inducible calcium calmudolin-independent isoform, which expressed in macrophages and has a role in inflammatory conditions [4,6,20].

The induction of iNOS activity has been readily demostrated in a wide variety of cell types such as hepatocytes and smooth muscle cells under stimulation by cytokines and LPS [1]. During vascular response to injury, leukocytes activated by cytokines, produce massive amounts of reactive oxygen species after their adhesion to endothelial cells. In particular, hydrogen peroxide (H2O2) the dismutation product of the superoxide anion (O^{-2}) produced by superoxide dismutase (SOD) is profoundly implicated in the transcription of genes supporting the endothelial response to injury [7]. Modulatory effects of H2O2 have also been reported: low concentrations of H2O2 strongly enhance the inhibitory effect of NO on platelets [8] and enhance cytokin-induced NO synthesis [9]. On the other hand, excess H2O2 compromises the bioavailability of NO, reported for a clinical case of cerebral thrombotic disorder [10].

In this work we indicate that H2O2 increases iNOS mRNA expression in cultured human endothelial cells. We suggest that NO synthesized by iNOS in endothelial cells after stimulation by H2O2 may modulate the inflammatory process.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) (kindly provided from d`Alessio's Lab, Paris, France) were cultured in M19 and RPMI_1640 with 5% FCS (GIBCO-BRL), 100 U/ml penicillin, 100 µg/ml fungizon (GIBCO-BRL), endothelial cell growth factor containing heparin (SIGMA) 1 g/ml according to Jaffe [11].

Treatment of HUVECs with Cytokines

Endothelial cells, grown to confluence (passage 4-5), were stimulated 24 h with the following cytokines: human interferon-γ (IF-γ, 500 U/ml), interleukin-1β (IL-1β, 100 u/ml), tumor necrosis factor α (TNF-α, 5 ng/ml), all from R&D systems (Abingdon, UK), and lipopolysacchride (LPS, *Escherichia coli*, 5 µg/ml, SIGMA).

Treatment of HUVECs with Hydrogen Peroxide

Hydrogen peroxide (H2O2, SIGMA) was diluted with deoxygenated water from 9 M stock solution and kept sealed in airtight vials throughout the experiment. Endothelial cells grown to confluence were stimulated for 1 h with 50, 100 and 200 µM H2O2 added to complete culture medium containing 5% FCS. Culture medium was then replaced for 6-24 h with complete medium.

RNA Isolation and RT-PCR

Total RNA was isolated after induction, using RNAZOLB (BIOPROBE SYSTEMS). Briefly, confluent monolayers (10^6 cells) were overlaid with 1 ml RNAZOLB. The cells were homogenized and the RNA was extracted using 200 µl chloroform, precipitated in isopropanol, and resuspended in water. Each sample was quantified spectrophotometrically. Three µg of RNA was revers transcribed to complementary DNA (cDNA) with 5 µl of a bulk mix of a first strand cDNA synthesis kit (pharmacia-Biotech) using iNOS specific primers. The cDNA synthesis reaction was performed at 37°C for 1 h. The PCR was performed with Taq DNA polymerase (GIBCO-BRL) using the 10 µl cDNA reaction as template. The iNOS sense primer was 5´-GTG AGG ATC AA AAG TGG GG-3´, and the antisense primer, 5´-ACC TGC AGG TTG GAC CAC-3´. These primers amplify a 380 bp from the human iNOS cDNA [12]. To verify that equal amount of RNA were added to each RT-PCR reaction, primer for the abelson housekeeping gene were also used in each experiment in the same tube. These primers amplify a fragment of 319 bp [13]. Denaturation was performed at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min for 35 cycles. Amplified PCR products were detected by agarose gel (1.5%) stained by ethidium bromide.

Results and Discussion

First part of our study focused on expression of iNOS mRNA in human endothelial cells after stimulation with different molar concentrations of H2O2, LPS and combination of 3 cytokines. Stimulation with three different concentrations of H2O2 resulted in a dose dependent induction of iNOS mRNA at 24 h (Fig. 1). As showed in Figure 1, both LPS and combination of 3 cytokines can readily induce iNOS mRNA expression.

Kinetic studies showed that iNOS mRNA expression increased slightly between 6 and 24 h, as shown in Figure 2.

Our study shows that human endothelial cells express an inducible isoform of nitric oxide synthase as well as constitutive isoform which expression of it results in NO production in a basal level at normal conditions [1].

On the other hand, we suggest that this isoform play a key role in decreasing of injury caused by activated lymphocytes. After infection and trauma, circulating
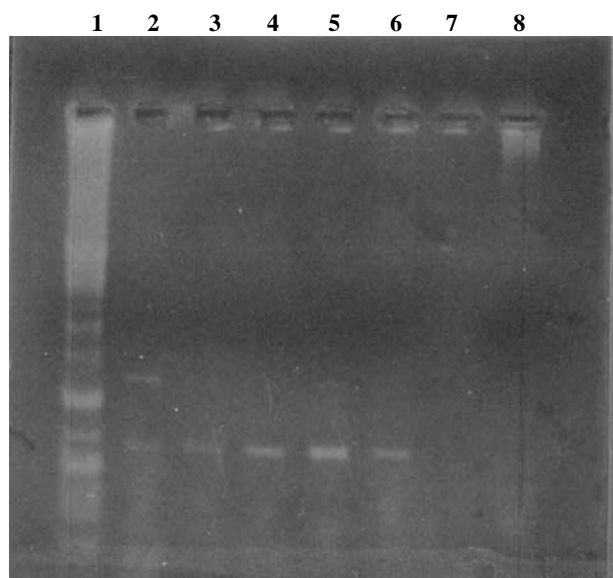

Figure 1. RT-PCR product of iNOS gene in HUVECs stimulated by H2O2 at different concentrations. Lane 1 ladder 1 kb, lane 2 LPS, lane 3 H2O2 50 μm, lane 4 H2O2 100 μm, lane 5 H2O2 200 μm, lane 6 3cyto, lane 7 H2O, lane 8 control (unstimulated endothelial cells).
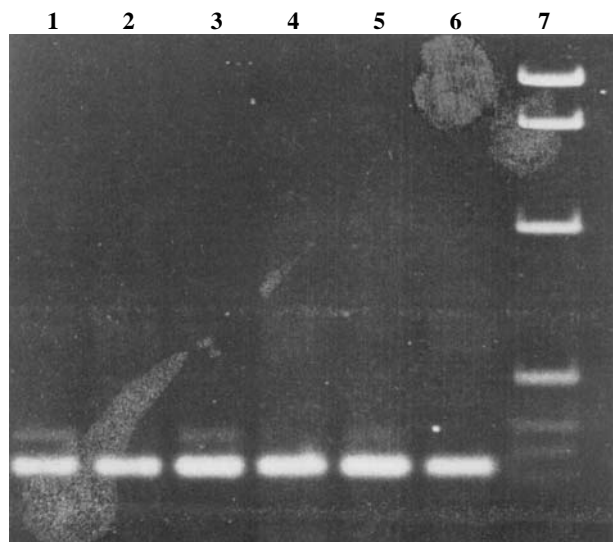

Figure 2. RT-PCR product of iNOS and abelson genes of HUVECs stimulated by 100 μm H2O2 at 6, 16, 24 h. Lane 1, 24 h, lane 2, 24 (untreated), lane 3, 16 h, lane 4, 16 h (untreated), lane 5, 6 h, lane 6, 6 h (untreated), lane 7, size marker.

leukocytes reach the site of injury by passing through the vascular endothelial monolayer. Inflammatory cytokines and reactive oxygen species such as H2O2 and bacterial LPS induce the endothelial expression of adhesion molecules for the recruitment of circulating leukocytes. Activated leukocytes may release peroxides. Cytokines, such as TNF-α increase the production of endogenous peroxides of mitochondrial origin, i.e., H2O2 [14]. Schuppe-koistinen and Jongking [15,16] have previously shown that glutathione peroxidase play a prominent role in protecting endothelial cells from inflammatory effectors and the latter are known to efficiently recycle the reduced form of glutathione (GSH).

The availability of GSH allows efficient detoxification of excess hydroperoxide which are secreted both intracellular and extracellular by activated leukocytes and restoration of endothelial compliance. This process is efficient over a limited time-course and plasma hydroperoxides have been shown to inactivate NO [10]. On the other hand NO enhances GSH in BAEC [17], there by reinforcing protection. Because persistent oxidative stress would progressively invalidate this protection, an additional mechanism may avoid marked endothelial dysfunction, in parallel to oxidant detoxification. The partial inhibition of ICAM-1/CD54 (Intra-cellular adhesion molecule-1) expression by endothelial cells would limit the recruitment of activated leukocytes, thus limiting oxidant toxicity observed during adhesion to endothelial cells.

Sadeghizadeh *et al.* [18] have shown that 1-h stimulation of HUVEC with increasing concentrations of H2O2 not only induced iNOS expression but also reduced ICAM-1/CD54 expression by 25%. Then it can be hypothesized that H2O2 initially is required for induction of transcription factors necessary for adhesive molecule expression and leukocyte recruitment throughout cell response to injury. As Roebuck *et al.* [19] have shown H2O2 activates ICAM-1/CD54 gene transcription through AP-1 elements within the ICAM-1/CD54 promoter, distinct form NFκ-B-mediated ICAM-1/CD54 expression by TNF-α [19]. Limiting extracellular peroxide import through low-regulation of adhesive molecule expression, as obtained by inducing iNOS gene expression, would avoid temporarily endothelial dysfunction, thus, permitting the endothelium to pursue its response to injury. It means that H2O2 can switch from the role of effector molecule to that of modulator of adhesion molecule.

In our opinion NO is an endogenous inhibitor of dysfunctional endothelium *in vitro* by reducing adhesion molecule expression and it depends on signaling by H2O2.

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