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Original Contribution

Redox regulation of the signaling pathways leading to eNOS phosphorylation

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Abstract

Oxidative stress mediates positive and negative effects on physiological processes. Recent reports show that H_2O_2 induces phosphorylation and activation of endothelial nitric oxide synthase (eNOS) through an Akt-phosphorylation-dependent pathway. In this study, we assessed activation of eNOS and Akt by determining their phosphorylation status. Whereas moderate levels of H_2O_2 (100 μM) activated the Akt/eNOS pathway, higher levels (500 μM) did not, suggesting differential effects by differing levels of oxidative stress. We then found that two pro-oxidants with activity on sulfhydryl groups, 1-chloro-2,4-dinitrobenzene (CDNB) and diethyl maleate (DEM), blocked the phosphorylation events induced by 100 μM H_2O_2. GSH was not a target thiol in this system because buthionine sulfoximine did not inhibit this phosphorylation. However, down-regulation of cell membrane surface and intracellular free thiols was associated with the inhibition of phosphorylation, suggesting that oxidation of non-GSH thiols inhibits the H_2O_2-induced phosphorylation of eNOS and Akt. DTT reversed the inhibitory effects of CDNB and DEM on Akt phosphorylation and concomitantly restored cell surface thiol levels more efficiently than it restored intracellular thiols, suggesting a more prominent role for the former. Similarly, DEM and CDNB inhibited TNF-α-induced Akt and eNOS phosphorylation, suggesting that thiol modification is involved in eNOS inductive pathways. Our findings suggest that eNOS activation is exquisitely sensitive to regulation by redox and that cell surface thiols, other than glutathione, regulate signal transduction leading to phosphorylation of Akt and eNOS. Published by Elsevier Inc.

Keywords: Nitric oxide synthase; Thiols; Akt; Endothelial cells; Signal transduction; Xenotransplantation; Free radicals

Introduction

Nitric oxide (NO) is a potent multifunctional biological messenger with many regulatory and immunomodulatory activities. For example, NO mediates interferon-γ-dependent immune suppression of T cell proliferation and of antibody synthesis by $CD5^+$ B cells [1], inhibits proliferation of T cells [2–4] and nuclear factor κB activation in glial cells [5], and plays an immunosuppressive role during development of experimental graft-vs-host disease [1,3]. In addition to an inhibitory or proapoptotic role, NO has also been shown to enhance cellular survival [6–9]. We have previously reported that NO protects human natural killer (NK) cells from activation-induced cell death [10] and that, under certain oxidation–reduction (redox) conditions, protects porcine endothelial cells (PAEC) from being lysed by interleukin (IL)-2 stimulated human NK cells [11]. In the cardiovascular system, production of NO is crucial to maintaining homeostasis [12] and vascular tone, through its role in vasodilation, antiatherogenesis, inhibition of leukocyte–endothelial cell

Abbreviations: ALM, Alexa-maleimide; BAEC, bovine aortic endothelial cells; BSO, buthionine sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; CMFDA, 5-chloromethylfluorescein diacetate; cms-SH, cell membrane surface thiols; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DEM, diethyl maleate; DTNB, 5,5′-dithiobis-2-nitrobenzonic acid; eNOS, endothelial NOS; NOS, nitric oxide synthase; GSH, reduced type glutathione; ic-SH, intracellular thiols; IL, interleukin; NK, natural killer; PAEC, porcine aortic endothelial cells; PI3-K, phosphatidylinositol 3-kinase; TRX, thioredoxin.

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interactions, smooth muscle proliferation, and platelet aggregation [13].

NO is synthesized by a family of enzymes known as NO synthases (NOS). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are produced constitutively, whereas inducible NOS (iNOS) can be induced in a number of cell types by treatment with cytokines or other agents [14]. eNOS is constitutively expressed in endothelial cells and is activated under conditions of Ca^{2+} influx [15,16]. eNOS, but not iNOS or nNOS, was associated with NO production in the two systems (NK cells [10] and PAEC [11]) in which we observed a cytoprotective effect of NO, suggesting a role for eNOS in these protective processes. eNOS activation is induced by a number of stimulants, such as shear stress, estrogen, or cytokines, resulting in phosphorylation of human eNOS on Ser 1177 [17,18] (corresponding to Ser in the 1179 position in the highly homologous porcine eNOS [19,20]) through a pathway involving phosphatidylinositol 3-kinase (PI3-K), and the Ser/Thr kinase, Akt. It has recently been demonstrated that oxidative stress also induces eNOS phosphorylation through the PI3-K/Akt pathway [18]. However, it is unclear by what mechanism oxidative stress activates this signaling pathway.

Intracellular oxidative stress is predominantly controlled by the natural and ubiquitous thiol-reducing systems, the thioredoxin (TRX)/peroxiredoxin system or glutathione (reduced type glutathione: GSH)/glutathione-dependent peroxidase system. These systems control the intracellular redox state, regulating cell growth and cell death by regulating the activation of transcription factors, the sensitivity of cells to cytokines, and components of the apoptosis pathways. These systems function through oxidation or reduction of protein cysteine sulfhydryl (–SH) groups. Oxidation of SH on protein cysteine residues can cause conformational changes, particularly when the cysteines form either intermolecular or intramolecular disulfide bonds, and can be very important in modulation of signaling pathways [21].

Xenotransplantation of nonhuman organs into humans is being considered as a means to overcome the worldwide shortage of human organs, with pigs being the likely source animals [22]. Whereas the early stages of immunological rejection may be reduced by a variety of means, including genetic engineering of source animals, the later stages remain a serious problem [23]. Because vascular endothelial cells of vascularized xenografts are the primary targets of a recipient's immune response, and because we had observed that NO may provide a means to protect such target cells [11], we focused on the regulation of NO synthesis in PAEC. We now report that phosphorylation of Akt and eNOS is exquisitely redox-sensitive and that regulation through protein thiols is a key component of eNOS phosphorylation signaling in PAEC. We show that intracellular and, especially, cell membrane surface free thiols regulate Akt/eNOS phosphorylation signaling induced by oxidative stress via a pathway that does not involve intracellular glutathione.

Materials and methods

Antibodies and other reagents

Anti-phospho-eNOS (Ser 1177) polyclonal Ab, anti-phospho-Akt (Ser 473) polyclonal Ab, and anti-Akt polyclonal Ab were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). According to the Cell Signaling data sheet, the antibody to phospho-eNOS (Ser 1177) was raised against bovine aortic endothelial cells (BAEC) and detects two closely migrating bands by Western analysis, and the faster migrating band corresponds to phosphorylated eNOS and is more intense when lysates of BAEC are examined. In our hands, using PAEC, both bands are of similar intensity, but based on the data sheet, we have identified the faster band as phospho-eNOS. Polyclonal anti-eNOS was purchased from R&D Systems (Minneapolis, MN, USA). Anti-hTRX mAb (ADF-11 mAb; mouse IgG1) was provided by Redox Bio Science, Inc. (Kyoto, Japan). Alexa-maleimide (ALM)-488 and 5-chloromethylfluorescein diacetate (CMFDA) were purchased from Molecular Probes (Eugene, OR, USA), and buthionine sulfoximine (BSO), oxidized glutathione, diethyl maleate (DEM), and 1-chloro-2,4-dinitrobenzene (CDNB) were from Sigma. Human rTNF-α was from Peprotech (Rocky Hill, NJ, USA).

Cells and treatments

PAEC were obtained from BioWhittaker (now Cambrex Bioscience, Walkersville, MD, USA) and cultured in EBM2, a medium formulated for EC (Clonetics, Walkersville, MD, USA). PAEC were used between passages 5 and 13. We have observed that these PAEC continue to express CD31, even without stimulation, and CD106 on stimulation with TNF-α, on close to 100% of the cells in both cases (data not shown). Monolayers that were fully confluent were washed with HBSS without calcium (BioWhittaker) and cultured in DMEM including 10% FCS and BSO for 16 h, or with DEM or CDNB for 2 h, all at concentrations as indicated under Results. After treatment, cells were washed with PBS and incubated for 30 min in PBS with or without 0.1 mM hydrogen peroxide and with 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 22 mM Hepes. In some experiments cells were treated with recombinant human TRX at the concentrations indicated under Results. In these experiments, cells were treated with DEM or CDNB, washed, treated with TRX for 1 h, washed again, and treated with H_2O_2 as above. Cell viability was assessed using trypan blue dye exclusion.

Immunoblot analysis for phosphorylated and total Akt and eNOS

The phosphorylation status of Akt and eNOS was determined by immunoblot analysis using anti-phosphorylated Akt Ab and anti-phosphorylated human eNOS (Ser 1177) Ab (Cell Signaling). We found that the anti-human