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Adenoviral-Mediated Ref-1 Overexpression Potentiates NO Production in Bradykinin-Stimulated Endothelial Cells

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The dual-function protein redox factor-1 (Ref-1) is essential for base excision repair of oxidatively damaged DNA and also governs the activation of many redox-sensitive transcription factors. We examined the role of Ref-1 in regulation of nitric oxide (NO) synthesis employing adenoviral-mediated overexpression of Ref-1 in bradykinin-stimulated endothelial cells. Intracellular NO was detected with the NO-sensitive fluorophore DAF-2. Overexpression of Ref-1 potentiates bradykinin-stimulated NO production in endothelial cells. And, cells infected with AdRef-1 showed higher fluorescence intensity compared with uninfected or AdDl312-infected cells. In parallel with this, overexpression of Ref-1 also stimulated endothelial NO synthase (eNOS) enzyme activity, compared with uninfected or AdDl312-infected cells, in bradykinin-stimulated cells as well as in unstimulated cells. These results suggest that Ref-1 implicates in endothelium-dependent vasorelaxation resulting from NO production in vascular system.

Key words – Ref-1, bradykinin, adenoviral gene transfer, NO, endothelial cells

Introduction

Nitric oxide (NO) is a multifunctional biomolecule involved in a variety of physiological and pathological process [16]. In contrast to the physiological activities, overproduction of NO has been associated with oxidative stress and with the pathophysiology of various diseases such as atherosclerosis, stroke, septic shock, autoimmune disease, and chronic inflammation [2,13]. In vascular system, NO acts as a major mediator of endothelium-dependent and arterial smooth muscle relaxation [7]. NO produced by endothelial NO synthase (eNOS) regulates vascular tone through a cGMP-dependent signaling pathway [23]. In addition, NO also has cGMP-independent effects within the vasculature, such as inhibition of leukocyte adhesion, that relies on its ability to inactivate or antagonize superoxide anions [8].

Redox factor-1 (Ref-1) (also known as APE [5], HAP1 [19], and APEX [20]) is a ubiquitous, multifactorial protein that is a redox-sensitive regulator of multiple transcription factors, including nuclear factor-κB (NF-κB), AP-1, c-myc, p53, Egr-1, and HIF-1α [9,10,15] as well as an apurinic/apyrimidinic endonuclease in the base excision repair pathway [5]. Ref-1 expression is selectively induced by non-toxic levels of a variety of reactive oxygen species (ROS), such as the superoxide anion, H_2O_2, and the hydroxyl radical, which are by-products of respiration [17]. We hypothesized that Ref-1 plays a critical role in the regulation of endothelial cell fate in response to pathophysiological stimuli. The present study demonstrates that Ref-1 overexpression potentiates NO production and eNOS enzyme activity in bradykinin-stimulated endothelial cells.

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Materials and Methods

Materials

Bradykinin (BK) and N^GMMA were purchased from Calbiochem Co. (La Jolla, CA, USA). Protease inhibitor cocktail, dimethyl sulfoxide (DMSO), trypan blue (0.4%), and AG 50W-X8 resin were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). DAF-2DA was purchased from Alexis Biochemicals (Carlsbad, CA, USA). Anti-Ref-1 and anti-eNOS antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary HRP-conjugated antibody, L-[^{14}C]arginine and ECL Western blotting kits were obtained from Amersham

Pharmacia Biotech (Piscataway, NJ, USA). Fetal bovine serum (FBS), HBSS and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD, USA).

Cell culture

Mouse aortic endothelial (MAE) cell line and HEK 293 cells were maintained in culture in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin (complete medium). Some experiments were performed using bovine aortic endothelial (BAE) cells. BAE cells were purchased from Clonetics (San Diego, CA, USA) and maintained in EGM-MV Bulletkit (Clonetics). Primary cells were plated in tissue culture flasks at a cell density of 5,000 cells/cm^2, and media were replaced every other day. Cells were subcultured on confluency, and cells in passages 3-10 were used in these experiments. Cells were cultured in either 60-mm dishes (for immunoblotting and NOS activity assays) or 35-mm dishes (for NO detection in intact cells).

Recombinant replication-deficient adenoviruses

The replication-deficient adenovirus, AdRef-1, encoding full-length Ref-1 was constructed through homologous recombination in HEK 293 cells, as described previously [1]. AdDl312 encodes an E1-deleted adenovirus without a transgene. AdDl-infected cells or uninfected cells were used as controls in all experiments. Adenoviral stocks were prepared in HEK 293 cells, purified on double cesium gradient, and titered using a standard plaque assay [25]. Infections were carried out at a multiplicity of infection (MOI) of 50, 100 or 200 for 16 hr. Protein expression and biochemical or functional assays described below were carried out 24 hr after infection.

Measurement of NOS activity

NOS activity was determined by measuring the conversion of radiolabeled L-arginine to L-citrulline [3]. Briefly, cell lysates were made in the presence of EDTA (0.1 mM) and EGTA (0.1 mM) to chelate free calcium released with cell disruption and incubated at 37°C for 10 min in 50 mM HEPES buffer (pH 7.4), 10 μM tetrahydrobiopterin, 1 mM dithiothreitol, 1 mM $CaCl_2$, 10 μg/ml calmodulin, 4 μM flavin mononucleotide, 4 μM flavin adenine dinucleotide, 0.5 μM L-argnine, 1 mM NADPH, and 0.2 μCi of L-[^{14}C]arginine in a final volume of 100 μl. The reaction was terminated by the addition of 200 μl of ice-cold HEPES buffer (100 mM, pH 5.5) containing 2 mM EDTA and 2 mM EGTA. Subsequently, L-[^{14}C]citrulline content was determined by column chromatographic separation and liquid scintillation counting, and activity was compared with background levels in the absence of calcium or in the presence of the NOS inhibitor N^GMMA (1 mM).

Detection of NO production in cells

NO production in intact cell was performed by fluorometric examination of cells loaded with DAF-2DA (4,5-diaminofluorescein diacetate), a membrane-permeant diacetate form of fluorescent indicator DAF-2 for NO. DAF-2 has been shown to form green-fluorescent trazolofluorescein by reacting with NO [12]. The cells were incubated with serum-free EGM-MV medium containing 10 μM DAF-2DA for 1 h in the presence or absence of the NO synthase inhibitor N^GMMA (1 mM), followed by bradykinin for 30 min. All samples were fixed in 2% paraformaldehyde for 3 min, and imaged immediately using a Zeiss confocal laser-scanning fluorescencemicroscope equipped with an excitation filter (488 nm), a dichroic mirror (505 nm), and an emission filter (515 nm). Typically, 10-15 cells from fluorescence images were identified in a field of view at 20X magnification, and changes in the integrated fluorescence intensity of each cell were monitored over time. Cell boundaries were drawn by using MetaMorph software (Universal Imaging, Media, PA), and fluorescence intensity was integrated over all pixels within the boundary of each individual cell. The fluorescence intensities within each experiment were normalized to a reference image of the basal state.

Western blot analysis

Equivalent amounts of total protein were loaded onto 10% SDS/PAGE. The gels were transferred to nitrocellulose membrane using an electroblotting apparatus (Bio-Rad, Richmond, CA, USA) and reacted with each antibody according to standard methods. Bound immunocomplexes were visualized on X-ray film by ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). β-actin was used as an internal control to monitor equal protein sample loading.

Statistical analysis

Three independent assays were performed. Statistical

values were expressed as the means ± standard deviation (S.D.) of the means obtained from each independent experiment. The results of the experimental and control groups were tested for statistical significance by a one-tailed Student's *t* test.

Results and Discussion

Ref-1 is upregulated in vascular disease such as atherosclerosis [4,14]. However, the functional relevance of this upregulation of Ref-1 in pathological states is not well known. Moreover, the role of endogeneous Ref-1 in vascular homeostasis under physiological conditions is also not known. These studies have provided an evidence establishing the existence of regulatory interactions between components of NO synthesis pathway and Ref-1. With the goal of examining the role of Ref-1 in NO synthesis, we used a recombinant adenovirus encoding full-length Ref-1, AdRef-1, to overexpress Ref-1 in endothelial cells. Adenoviral vectors are capable of gene transfer efficiencies approaching 100% in mouse aortic endothelial (MAE) cells. Infection of endothelial cells with AdRef-1 resulted in significant overexpression of Ref-1 compared to cells infected with AdDl312 or uninfected (Fig. 1), in MOI-dependent manner (data not shown).

Next, we assessed the role of Ref-1 in regulating NO synthesis of bradykinin-stimulated endothelial cells. Intracellular NO was detected with the NO-sensitive fluorophore DAF-2DA. The *N*-nitrosation of DAFs, yielding the highly green-fluorescent triazolofluoresceins, offers the advantages of specificity, sensitivity, and a simple protocol

Fig. 1. Demonstration of adenoviral-mediated gene transfer in mouse aortic endothelial (MAE) cells. Cells were infected with AdDl312 (200 MOI) or AdRef-1 (200 MOI) for 16 hr, and whole cell extracts were analyzed by western blotting using a Ref-1 antibody. β-actin was used as an internal control to monitor equal protein loading.

for the direct detection of NO (detection limit 5 nM). Cells were infected with AdDl312 or AdRef-1 as described in "Materials and Methods", loaded with DAF-2DA, and then stimulated with 1 μM bradykinin, the most notable activator for NO synthesis in endothelial cells, for 30 min. Overexpression of Ref-1 evoked increase of basal steady-state endothelial NO production compared with uninfected or AdDl312-infected cells. And, the agonist bradykinin increased NO synthesis in uninfected and AdDl312-infected endothelial cells. In comparison, cells infected with AdRef-1 showed higher intensity in bradykinin-induced fluorescence upon incubation with DAF-2 (Fig. 2A and B), indicating that overexpressed Ref-1 potentiates bradykinin-induced NO synthesis in endothelial cells. And N^GMMA, a known inhibitor of NOS, totally blocked the increase of fluorescence intensity, as expected. Overexpression of Ref-1 also enhanced NO production in bovine aortic endothelial (BAE) cells (Data not shown).

We also conducted the NOS enzyme assay to confirm whether Ref-1 regulates eNOS activity in endothelial cells. Bradykinin stimulated eNOS enzyme activity level in uninfected and AdDl312-infected cells. Moreover, NOS enzyme activity in response to bradykinin was higher in cells overexpressing Ref-1 (Fig. 3). Importantly, overexpression of Ref-1 stimulated eNOS enzyme activity in unstimulated cells, compared with uninfected or AdDl312-infected cells. This result was correlated with the slight increase of NO production in unstimulated AdRef-1-infected cells, as shown in Fig. 2A and B. This finding suggest that Ref-1 itself modulates eNOS catalytic activity in endothelial cells.

Ref-1 is described in the literature as a nuclear protein, and would therefore not be predicted to regulate cytoplasmic factors. However, Ref-1 is expressed in the cytoplasm of a variety of cell types, including endothelial cells, hepatocytes, and thyroid cells [6,22]. Moreover, numerous studies show that *in vivo* Ref-1 has a differential cellular and subcellular expression pattern suggesting a potential physiologic extra-nuclear role [6,11,18,24]. In endothelial cells, the oxidative stimulus led to a shift in the sub-cellular distribution of Ref-1. This re-distribution is in accordance with another report showing that exogenous H_2O_2 is a potent stimulus for nuclear translocation of cytoplasmic Ref-1 [21]. Although this study has focused on the role of Ref-1 in stimulating eNOS activity, the DNA repair function of Ref-1 may also play an important role in maintaining vascular homeostasis.

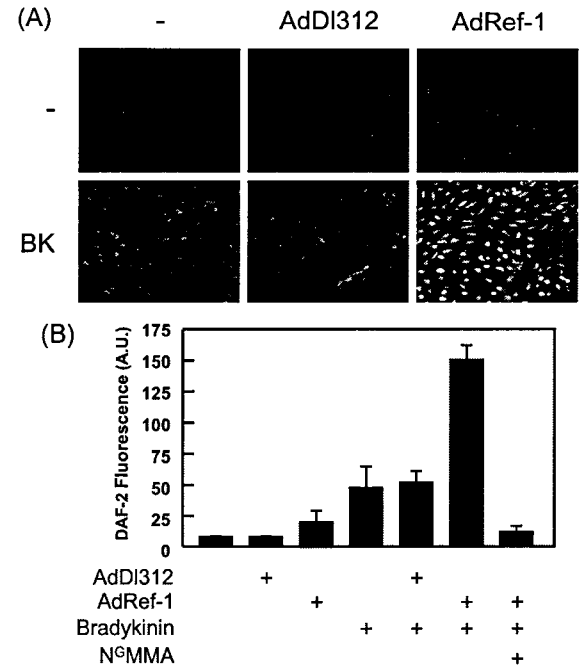

Fig. 2. Effect of Ref-1 on NO production in endothelial cells. MAE cells were infected with adenoviruses AdDl312 or AdRef-1 followed by loading with DAF-2DA. Then cells were stimulated without or with 1 μM bradykinin (BK) in the presence or absence of the NO synthase inhibitor N^GMMA (1 mM) for 30 min, washed, and fixed with 2% paraformaldehyde for 3 min. (A) Confocal microscope images of NO indicator DAF-2. Images were obtained with a confocal laser-scanning fluorescence microscope. (B) Fluorescence intensity. The density of greenfluorescence was measured by analyzing the digital images using a computer as described in the "Materials and Methods". Values are means ± S.D. of 10-15 determinations.

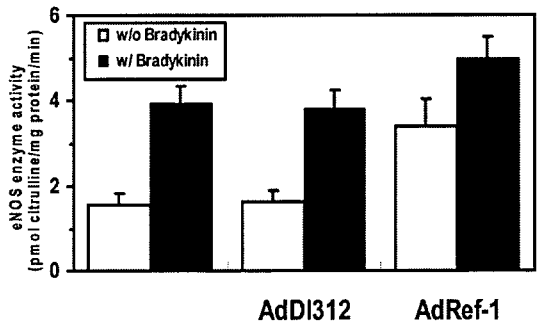

Fig. 3. Effect of Ref-1 on eNOS enzyme activity in unstimulated and bradykinin-stimulated cells. BAE cells were infected with AdDl312 or AdRef-1, stimulated without or with 1 μM bradykinin in the presence or absence of the NO synthase inhibitor N^GMMA (1 mM) for 30 min. [^{14}C]Citrulline formation was measured as described under "Materials and Methods". Values are means ± S.D. of triplicate experiments.

In conclusion, the results presented in this report demonstrate that Ref-1 potentiates NO synthesis in bradykinin-stimulated endothelial cells. The results also suggest that the level of intracellular Ref-1 may regulate directly eNOS activity for NO synthesis in bradykinin-stimulated endothelial cells as well as in unstimulated cells. And this finding prompts future experiments of the underlying molecular mechanisms of cytoplasmic Ref-1 in the regulation of NO synthesis and vasomotor tone in endothelial cells.

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초록 : Bradykinin으로 자극한 혈관내피세포에서 Ref-1의 세포내 과발현에 의한 NO 생성 증진 효과에 대한 연구

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Redox Factor-1 (Ref-1)은 손상된 DNA의 복구 및 많은 세포내 산화환원에 민감한 transcription factors의 활성화에 기여하는 양면의 역할을 수행하는 단백질이다. 본 연구에서는 혈관내피세포에서의 nitric oxide (NO) 생성과정에서 Ref-1의 역할을 살펴보았다. Ref-1의 세포내 과발현을 위하여 adenoviral vector를 사용하였고 bradykinin으로 자극한 혈관내피세포에서 생성되는 NO 측정을 위하여 fluorophore DAF-2를 사용하였다. Ref-1 과발현은 bradykinin으로 자극한 혈관내피세포의 NO 생성을 증가시켰다. 또한 자극되지 않은 Ref-1 과발현 세포는 viral vector로 감염되지 않은 그리고 control로 사용한 AdDl312로 감염된 세포보다 높은 fluorescence intensity를 나타내었다. 이와 비슷하게, Ref-1 과발현은 bradykinin으로 자극한 세포뿐만 아니라 자극하지 않은 세포에서도 감염되지 않은 그리고 AdDl312로 감염된 세포와 비교할 때 endothelial NO synthase (eNOS)의 활성을 크게 증가시켰다. 이는 Ref-1 자신이 eNOS의 효소활성을 직접 조절할 수 있다는 것을 의미한다. 결론적으로 Ref-1이 혈관계에서 NO 생성에 의해 기인되는 endothelium-dependent vasorelaxation에서 중요한 역할을 한다는 것을 시사한다.