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Effect of Overexpressed Ref-1 on AKT Phosphorylation for NO Production in Mouse Aortic Endothelial Cell Line

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Redox factor-1 (Ref-1) is essential for repair of oxidatively damaged DNA and also govern the reductive activation of many transcription factors. In this study, we examined the effect of overexpressed Ref-1 on AKT activation for nitric oxide (NO) production in mouse aortic endothelial (MAE) cells. Adenoviral-mediated overexpression of Ref-1 enhanced NO production in unstimulated- as well as bradykinin-stimulated MAE cells. Importantly, forced overexpression of Ref-1 induced direct phosphorylation of AKT in cells. And, a PI3K inhibitor wortmannin completely abolished the increase in AKT phosphorylation by stimulation of bradykinin and/or overexpressed Ref-1. In addition, inhibition of AKT activity with HA-tagged activation-deficient AKT suppressed Ref-1-induced endothelial NO synthase (eNOS) phosphorylation and resulted in a corresponding inhibition of unstimulated- and bradykinin-stimulated NO production. These results suggest that Ref-1 stimulates direct phosphorylation of AKT for eNOS enzyme activity in murine endothelial cells.

Key words : Redox factor-1, mouse aortic endothelial, endothelial NO synthase, adenoviral vector, AKT

Introduction

Endothelium-derived nitric oxide (NO) exerts several vasoprotective activities including smooth muscle relaxation, inhibition of platelet activation, and regulation of endothelial cell permeability and adhesivity [1-3]. The endothelial NO synthase (eNOS) isoform is constitutively expressed and is activated upon an increase of intracellular calcium following cell stimulation with receptor-dependent stimuli such as thrombin and bradykinin or with receptor-independent stimuli like calcium ionophore [4]. Since NO interferes with key processes in atherogenesis [5], a lack of NO might promote the development of atherosclerosis [6]. The dysfunction of the endothelial NO pathway may involve impaired signal transduction mechanisms, decreased eNOS activity, reduced intracellular availability of L-arginine, or increased inactivation of NO by superoxide anions or oxidized low density lipoproteins [5,7]. Therefore, it is necessary to elucidate intracellular molecules involving in regulatory mechanisms for NO synthesis.

Redox factor-1 (Ref-1) is a ubiquitous 37 kD bifunctional protein that is transcriptionally upregulated in response to oxidative stresses [8]. It has two following important functions: 1) a nuclear reducing factor that promotes the DNA binding properties of many redox-sensitive transcription factors that regulate cell growth, differentiation, survival, and death including AP-1, NF-κB, p53, Egr-1, and c-Myb [9-11]; and 2) an endonuclease indispensable in the base excision repair pathway of damaged DNA, which are generated by oxidative damage [12]. However, it has not been known about the role of Ref-1 in the regulation of eNOS, and its importance in endothelial cells. Here, we report that adenoviral-mediated overexpression of Ref-1 stimulates AKT activation for NO production in unstimulated- and bradykinin-stimulated murine endothelial cells.

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Materials and Methods

Materials

Bradykinin, N^GMMA, wortmannin, protease inhibitor cocktail, dimethyl sulfoxide (DMSO), and trypan blue (0.4%) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). DAF-2DA was purchased from Alexis Biochemicals (Carlsbad, CA, USA). Anti-phospho-eNOS antibody (phosphoserine-1176 in the mouse eNOS sequence, corresponding to Ser-1177 in human eNOS), anti-phospho-AKT antibody (phosphothreonine-308 in the mouse AKT sequence, corresponding to Ser-473 in human AKT), anti-AKT antibody, and anti-hemagglutinin (HA) antibody

were purchased from Cell Signaling Technologies (Beverly, MA, USA). Anti-Ref-1 antibody and anti-eNOS antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary HRP-conjugated antibody 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

As described previously [13], experiments were performed using mouse aortic endothelial cell line (MAE). MAE cells and HEK 293 cells were maintained in culture in Dulbecco's modified Eagle's medium containing 10% FBS, penicillin, and streptomycin (complete medium). Cells were cultured in either 60-mm dishes for Western blot analysis or 35-mm dishes for NO detection in intact cells.

Adenoviruses

The adenoviruses AdDl312 (null control virus), AdRef-1 (encoding full-length Ref-1), and AdAKT-AA (HA-tagged activation-deficient AKT) were used. All virus stocks were amplified in HEK 293 cells, purified on double cesium gradients, and titered using a standard plaque assay [14]. Infections were carried out at a multiplicity of infection (MOI) of 100 or 200 for 16 hr. Protein expression and biochemical or functional assays described below were carried out 24 hr after infection, as described previously [15]. All the viral vectors were a gift of Dr. Kaikobad Irani in The Johns Hopkins University School of Medicine.

NO measurement

Intracellular NO was measured in unstimulated or bradykinin-stimulated cells using the fluorophore 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 [16]. Briefly, the cells were incubated with serum-free medium containing DAF-2DA (10 μM) for 1 hr in the presence or absence of the NO synthase inhibitor N^GMMA, followed by bradykinin for 30 min. All samples were fixed 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. Fluorescence intensity was quantified in selected individual cells using MetaMorph software (Universal Imaging, Media, PA, USA). 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. To monitor the degree of phosphorylated eNOS and AKT, cells were washed twice with ice-cold PBS containing 1 mM Na_3VO_4 and incubated for 10 min on ice with 500 μl of lysis buffer containing 20 mM Tris, pH7.5, 2 mM EDTA, 2 mM EGTA, 1% v/v Triton X-100, 100 mM NaCl, 1 mM Na_3VO_4, 4 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and the complete protease inhibitor mixture. Cells were harvested by scraping and centrifuged at 4°C for 10 min in a microfuge. Samples were separated by SDS-PAGE. And, 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, with $p<0.05$ accepted as the level of significance.

Results and Discussion

Forced overexpression of Ref-1 promotes NO production in unstimulated or bradykinin-stimulated MAE cells

Vascular Ref-1 is upregulated in vascular disease such as atherosclerosis and hypertension [17,18]. However, the role of Ref-1 in vascular homeostasis under physiological and pathological conditions is not well known. The present study was undertaken to elucidate the molecular regulation of the signaling network by Ref-1 during endothelial cell-derived

NO synthesis. Here, we used a recombinant adenovirus encoding full-length Ref-1, AdRef-1, to overexpress Ref-1 in mouse aortic endothelial cell line, MAE.

As previously reported [13], infection of MAE cells with AdRef-1 resulted in significant overexpression of Ref-1 compared to cells infected with AdDl312 or uninfected, in MOI-dependent manner (data not shown). For the detection of intracellular NO, we used NO-sensitive fluorophore DAF-2DA. The *N*-nitrosation of DAF produces the highly green-fluorescent triazolofluoresceins. Bradykinin, an activator for NO synthesis in endothelial cells, produced high fluorescence intensity upon incubation with DAF-2. A NOS inhibitor N^GMMA totally inhibited the increase of fluoresence intensity, as expected (Fig. 1A). Overexpression of Ref-1 showed a significant increase of DAF-2 intensity on basal level in MAE cells. And, AdRef-1-infected cells showed higher fluoresence intensity by addition of bradykinin, compared with uninfected or AdDl312-infected cells, in MOI-depend-

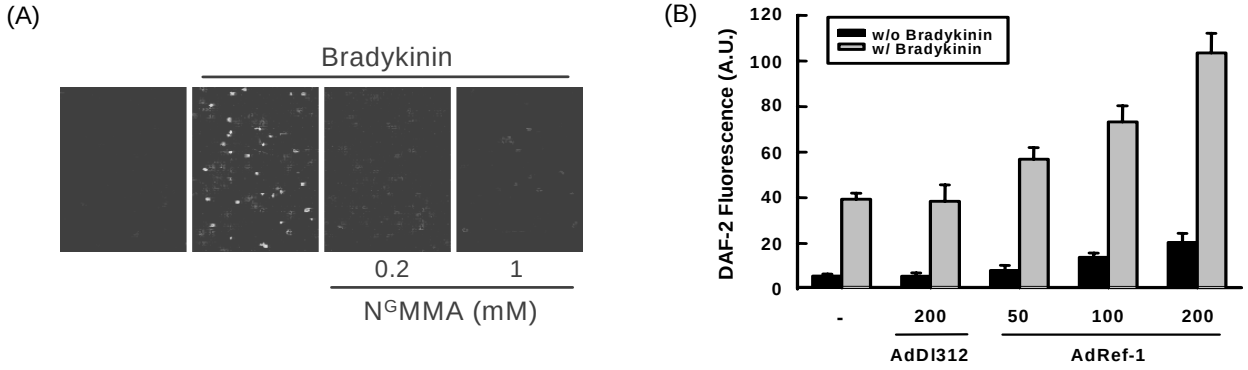

Fig. 1. Effect of overexpressed Ref-1 on NO production in unstimulated- and bradykinin-stimulated MAE cells. (A) Confocal microscope images of DAF-2, a NO specific fluorescent dye. MAE cells were loaded with DAF-2DA. Then cells were stimulated without or with bradykinin (1 μM) in the presence or absence of the NO synthase inhibitor N^GMMA (0.2 or 1 mM) for 30 min, washed, and fixed with 2% paraformaldehyde for 3 min. Images were obtained with a confocal laser-scanning fluorescence microscope. (B) Quantification of fluorescence images by DAF-2. Cells were infected with AdDl312 or AdRef-1, as indicated different MOIs, for 16 hr followed by loading with DAF-2DA. Then, cells were stimulated without or with bradykinin (1 μM) for 30 min, washed, and fixed with 2% paraformaldehyde for 3 min. Images were obtained with a confocal laser-scanning fluorescence microscope, and the density of green fluorescence was measured by analyzing the digital images using a computer as described in the "Materials and Methods". Values are mean±SD of 10-15 determinations.

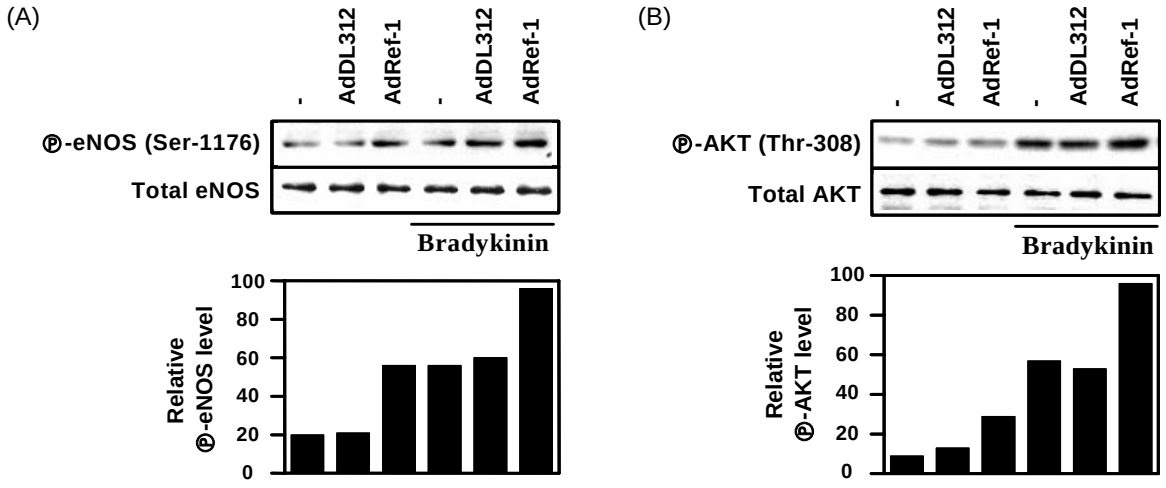

Fig. 2. Stimulation of the phosphorylation of eNOS and AKT by Ref-1 in MAE cells. Cells were infected with AdDl312 or AdRef-1, stimulated without or with bradykinin (1 μM) for 10 min. Cells were lysed, and the lysates were immunoblotted with appropriate antibodies. (A) anti-phospho-Ser-1176 eNOS and nonphosphospecific eNOS antibodies (B) anti-phospho-Thr-308 AKT and nonphosphospecific AKT antibodies. Results of scanning densitometric analysis of the Western blot are presented in the bar graph. The relative level of phosphorylation is normalized to the respective amount of total eNOS or AKT in each lane.

ent manner (Fig. 1B). This result suggest that overexpressed Ref-1 promotes NO production in basal- and bradykinin-stimulated murine endothelial cells.

Ref-1 enhances phosphorylation of eNOS and AKT in MAE cells

Endothelial NO production is regulated by an increase in eNOS activity primarily through intracellular Ca^{2+} influx [4]. And, fluid shear stress is also important pathway for eNOS activation, which is not inhibited by intracellular Ca^{2+} antagonists [19,20]. Therefore, the eNOS phosphorylation is thought to regulate enzyme activity in Ca^{2+}-dependent or -independent fashion [21-23]. Here, we analyzed the involvement of the AKT pathway in the regulation of murine eNOS activity. As shown in Fig. 2, overexpression of Ref-1 increased phosphorylation of eNOS and AKT in unstimulated- and bradykinin-stimulated MAE cells, without affecting eNOS and AKT protein levels. And, pretreatment of AdDl312- or AdRef-1-tansfected MAE cells with a phosphatidylinositol-3-kinase (PI3K) inhibitor wortmannin, which prevents the phosphorylation and activation of Akt, completely abolished the overexpressed Ref-1- and/or bradykinin-induced AKT phosphorylation (Fig. 3).

AKT directly regulates the Ref-1-mediated NO production in BK-stimulated MAE cells

Next, to confirm whether AKT could directly influence the production of NO in MAE cells, we used adenovirally the HA-tagged dominant-negative form of the AKT enzyme (AdAKT-AA). Cells were co-infected with AdAKT-AA and AdDl312 or AdRef-1. Infected cells showed significant expressions of AKT-AA and Ref-1 compared to cells infected with AdDl312 (Fig. 4A). As shown in Fig. 4B, inhibition of

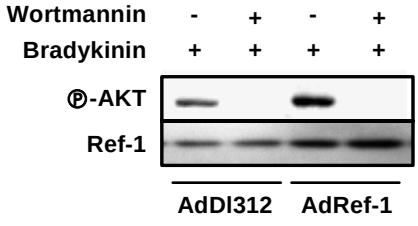

Fig. 3. Effect of wortmannin on bradykinin-stimulated AKT phosphorylation in MAE cells. Cells were pretreated with wortmannin (100 nM) for 30 min and then incubated with bradykinin (1 μM) for 10 min. Whole cell extracts were analyzed by Western blotting using phospho-AKT and Ref-1 antibodies. Equivalent protein loading was confirmed by Ponceau S staining.

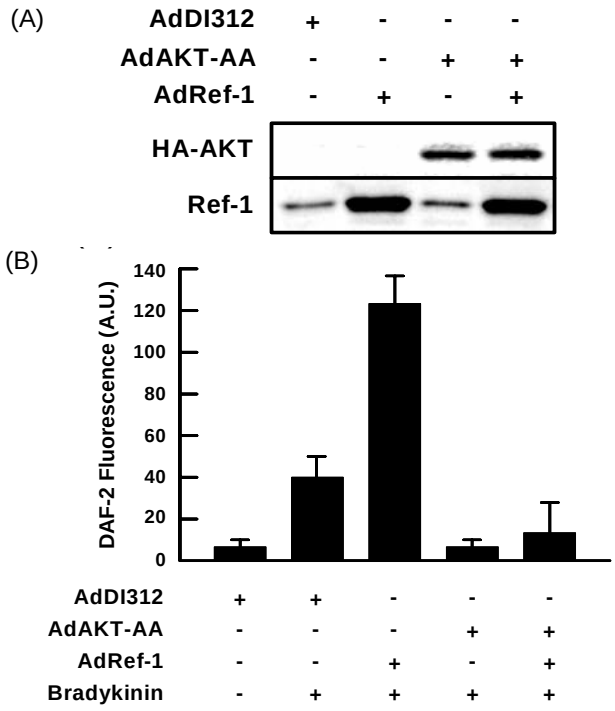

Fig. 4. Regulation of NO production by Ref-1-mediated AKT activation in bradykinin-stimulated MAE cells. (A) Western blot analysis of HA-AKT and Ref-1. Equivalent protein loading was confirmed by Ponceau S staining. (B) Quantification of fluorescence images by DAF-2. MAE cells were co-infected with adenoviruses AdDl312 (200 MOI) or AdRef-1 (100 MOI) and/or AdAKT-AA (100 MOI) followed by loading with DAF-2DA. Then, cells were stimulated without or with bradykinin (1 μM) for 30 min. Images were obtained with a confocal laser-scanning fluorescence microscope. The density of green fluorescence was measured by analyzing the digital images using a computer as described in the "Materials and Methods". Values are mean±SD of 10-15 determinations.

AKT activity suppresses bradykinin-stimulated NO production as well as Ref-1-mediated enhancement in bradykinin-stimulated NO production, in MAE cells. This finding suggests that overexpressed Ref-1 enhances eNOS enzyme activity via AKT in bradykinin-stimulated MAE cells.

Recently, it is reported that AKT can modulate NO production by direct phosphorylation of eNOS in human umbilical vein endothelial cells (HUVEC) [23]. Therefore, the eNOS activation by AKT might contribute to the important physiological role of AKT on vascular functions, including vasodilation, cell attachment, apoptosis, and inflammation. Ref-1 has a differential cellular and subcellular expression pattern suggesting a potential physiologic extra-nuclear role [24,25,26]. In endothelial cells, the oxidative stimulus led to

a shift in the sub-cellular distribution of Ref-1. 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.

In conclusion, the results presented in this report demonstrate that overexpressed Ref-1 stimulates direct phosphorylation of AKT, and enhances NO production via AKT activation in unstimulated- and bradykinin-stimulated MAE cells. And, this finding prompts future experiments of the underlying molecular mechanisms of cytoplasmic Ref-1 in the regulation of endothelial cell function in vascular system.

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초록 : 마우스 내피세포주 MAE의 NO 생성과정에서 과발현된 Ref-1의 AKT 활성 조절에 대한 연구

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Redox factor-1 (Ref-1)은 산화적으로 손상된 DNA의 복구와 세포내 산화환원에 민감한 전사인자들의 활성화에 필수적인 역할을 수행한다. 본 연구에서는 마우스 유래 혈관내피세포주 (MAE)에서 nitric oxide (NO) 생성과정에 관여하는 AKT 활성화의 측면에서 adenoviral vector를 사용하여 과발현된 Ref-1의 역할을 살펴보았다. NO 측정을 위하여 fluorophore DAF-2를 사용하였다. 과발현된 Ref-1은 bradykinin으로 자극한 세포뿐만 아니라 자극되지 않은 세포의 NO 생성도 증가시켰다. 놀랍게도 이 과발현된 Ref-1은 AKT의 직접적인 인산화를 유도하였으며, AKT 저해제로 널리 사용되는 wortmannin에 의해 반응이 억제되었다. 또한, Ref-1에 의한 직접적인 AKT 활성화를 증명하기 위하여 HA-tagged activation-deficient AKT를 과발현시키는 adenoviral vector를 사용하였다. 이 방법을 이용한 AKT 활성의 저해는 과발현된 Ref-1에 의한 NO 생성 및 bradykinin 자극에 의한 NO 생성을 억제하였다. 이들 결과는 Ref-1이 마우스 혈관내피세포에서 직접적인 AKT 인산화를 통하여 eNOS 활성화를 유도한다는 것을 의미한다.