Regulation of Endothelial Nitric Oxide Production and Vascular Tone by Redox Factor-1

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Redox factor-1(ref-1) is a ubiquitously expressed protein with two known functions. It is an important regulator of cellular processes including cell survival, differentiation, and growth. Ref-1 has two functional domains. The C-terminal domain operates as an apurinic/apyrimidinic endonuclease(APE) in the base excision repair pathway. Ref-1, through its N-terminal domain promotes the DNA-binding activities of many redox-sensitive transcription factors such as AP-1. Cysteines at position 65 and 93 confer this reducing property on ref-1 and are essential for the redox regulation of transcriptional activity. The role of ref-1 in the regulation of endothelial NO production and endothelium-dependent vascular tone is not known.

To investigate whether ref-1 could influence basal, agonist-independent, eNOS activity and endothelial production of NO, ref-1 was over-expressed with a recombinant adenovirus encoding full-length ref-1(AdRef-1) in bovine aortic endothelial cells (BAEC), and accumulation of nitrite(NO_2^-) was measured by NO-specific absorbance. Infection of BAECs with AdRef-1 stimulated basal, steady state, endothelial NO production, and basal eNOS activity compared to cells infected with the control-galactosidase virus(Adgal). In contrast to wild-type ref-1 (ref-1 WT), expression of the redox-defective mutant of ref-1(cysteine to alanine mutations at codons 65 and 93: ref-1 C65/93A) did not increase nitrite accumulation. Moreover, expression of ref-1 WT, but not ref-1 C65/93A, led to an increase in eNOS activity. To determine the role of the PI-3K-Akt pathway in ref-1-stimulated NO production, and eNOS activation through calcium sensitization, a dominant-inhibitory form of Akt(AktAA) was adenoviral co-expressed with ref-1 in HUVECs. Ref-1-stimulated NO production, and eNOS activity were significantly inhibited. Similar results were obtained when eNOS-transfected COS-7 overexpressing ref-1 were treated with the PI-3K inhibitor wortmanin. Ref-1 stimulated calcium sensitization of eNOS was also inhibited by wortmanin. We also determined the effect of ref-1 over-expression on Akt activity and eNOS phosphorylation at S1177. In human umbilical vein endothelial cells(HUVECs) infected with AdRef-1, Akt activity was increased, and eNOS was also phosphorylated. In addition, to test whether PI-3K is required for ref-1-stimulated eNOS phosphorylation, a dominant inhibitory form of the p85 regulatory subunit of PI-3K(p85DN) was adenovirally co-expressed in AdRef-1-infected HUVECs, and eNOS and Akt phosphorylation determined. Ref-1-stimulated eNOS phosphorylation and Akt phosphorylation was significantly inhibited by inhibition of PI-3K. The proto-oncogene H-ras lies upstream of PI-3K and Akt. Activation of H-ras leads to sequential activation of PI-3K and Akt. We therefore also investigated the involvement of H-ras in ref-1-stimulated NO production and eNOS activation. In COS-7 cells expressing eNOS, inhibiting endogenous H-ras activity by expression of a dominant negative allele H-rasN17, suppressed ref-1-stimulated NO production and eNOS activity.

To test the involvement of ref-1 in endothelium-dependent, NO-mediated, relaxation of intact blood vessels, we examined the effect of ref-1 overexpre-

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ssion in the endothelium of rat aorta on acetylcholine-induced relaxations. Adenoviral gene transfer with AdRef-1 ex vivo in rat aortic segments resulted in endothelial-specific overexpression of ref-1, and augmented acetylcholine-induced vasorelaxation of isolated aortic rings, without affecting nitroprusside -stimulated, endothelium-independent relaxation. To investigate whether endogenous ref-1 plays a role in regulating endothelial function and vascular tone, we characterized vascular responses in ref-1 heterozygous(ref-1 -/+) mice and their wild-type(WT) littermates. In comparison to their WT littermates, ref-1 +/- mice were found to have a significant impairment in acetylcholine-induced, endothelium-dependent relaxation, significantly reduced serum nitrite levels, and a marked increase in resting mean arterial blood pressure.

In summary, our data demonstrate a novel role for ref-1 in the regulation of endothelial NO production, endothelium-dependent vascular tone, and systemic blood pressure. The reducing property of ref-1 is critical for this function. In addition, our findings implicate H-ras/PI-3K/Akt-dependent calcium sensitization as the mechanism through which ref-1 exerts its effects on the endothelium. Since endothelial NO has been implicated in vascular gene expression, angiogenesis, and architectural changes associated with atherosclerotic vascular disease and hypertension, the ref-1-stimulated increase in eNOS activity might represent a major determinant of vessel wall structure and function.