Apurinic/Apyrmidinic Endonuclease 1 Regulates Endothelial NO Production and Vascular Tone

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Abstract—The dual-function protein apurinic/apyrmidinic endonuclease/redox factor-1 (APE1/ref-1) is essential for DNA repair and also governs the reductive activation of many redox-sensitive transcription factors. We examined the role of APE1/ref-1 in regulation of endothelium-dependent tone and systemic blood pressure. APE1/ref-$1^{+/-}$ mice have impaired endothelium-dependent vasorelaxation, reduced vascular NO levels, and are hypertensive. APE1/ref-1 upregulates H-ras expression and leads to H-ras–mediated, phosphoinositide-3 kinase/Akt kinase–dependent calcium sensitization of endothelial NO synthase (eNOS), stimulating NO production. The reducing property of APE1/ref-1 is essential for upregulation of H-ras and for the calcium sensitization of eNOS. These findings uncover a novel physiological role for APE1/ref-1 in regulating vascular tone by governance of eNOS activity and bioavailable NO. **(*Circ Res*. 2004;95:902-910.)**

Key Words: eNOS ■ redox ■ hypertension ■ H-ras

Impaired endothelium-dependent vascular relaxation is a prominent feature of highly prevalent vascular diseases such as atherosclerosis and hypertension. Such disorders share a common feature of increased oxidative stress, as evidenced by elevations of markers for oxidative DNA damage.[1,2] Among the endogenous mechanisms that repair oxidative DNA damage is the ubiquitously expressed apurinic/apyrimidinic endonuclease (APE1), also known as redox factor-1 (ref-1). APE1/ref-1 is a protein with dual function. It is an essential endonuclease in the base excision repair pathway of oxidatively damaged DNA, as well as having reducing properties that promote the binding of redox-sensitive transcription factors such as activator protein-1 to their cognate DNA sequences. The C-terminal domain of APE1/ref-1 displays endonuclease activity, whereas the N terminus has the reducing property.[3] Cysteines at position 65 and 93 confer this reducing property on APE1/ref-1.[4] Moreover, abrogation of the reducing property by deletion of the N terminus encompassing cysteines 65 and 93 does not interfere with its endonuclease function.[3] Therefore, the N termini and C termini of APE1/ref-1 are structurally and functionally independent.

Vascular APE1/ref-1 is upregulated in atherosclerosis and animal models of hypertension[2] (B.H.J., K.I., unpublished data, 2002). However, the functional relevance of this upregulation of APE1/ref-1 in pathological states is not known. Moreover, the role of endogenous APE1/ref-1 in vascular homeostasis under physiological conditions is also not known. The increase in vascular APE1/ref-1 in vascular pathologies begs the question whether this increase solely represents a response to vascular oxidative stress and DNA damage or whether it also serves in another capacity to try to compensate for impaired vascular function. Intrigued by the latter possibility, we hypothesized that in addition to its DNA repair function, APE1/ref-1 expression in the vascular wall serves to regulate vascular tone. In this report, we investigated the role and mechanism of APE1/ref-1 in governing endothelium-dependent NO production and vasomotor tone.

Materials and Methods

Cell Transfections and Immunoblotting

Cysteines 65 and 93 in human APE1/ref-1 were mutated using Quick Change site-directed mutagenesis kit (Stratagene). APE1/ref-1(ΔNLS) encodes APE1/ref-1 with a 35-aa deletion of the putative N-terminal nuclear localization signal. All mutations and deletions were verified by DNA sequencing. Wild-type (WT) APE1/ref-1, APE1/ref-1(ΔNLS), and APE1/ref-1(C65/93A) in DsRed expression vector were generated by standard cloning methods. H-rasN17 is the dominant-inhibitory mutant of H-ras. COS-7 cells were transfected with endothelial NO synthase (eNOS; 1 μg) and APE1/ref-1 plasmids (2 μg) using Lipofectamine 2000 (Invitrogen). All transfections were balanced for DNA amount using pcDNA3.1 or pDsRed.

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Twenty-four to 48 hours after transfection with DNA or infection with adenoviruses, expression and phosphorylation of proteins (50 μg) were determined by immunoblotting with antibodies to eNOS (SC-654; Santa Cruz Biotechnology), phosphoserine-1177 eNOS (phosphoserine-1179 for bovine eNOS), (9571; Cell Signaling), inducible NOS (iNOS; SC-650; Santa Cruz Biotechnology), APE1/ref-1 (SC-5572; Santa Cruz Biotechnology), Akt (9272; Cell Signaling), H-ras (SC-520; Santa Cruz Biotechnology), rac1 (05 to 389; Upstate Biotechnology), phosphoserine-473 Akt (9271; Cell Signaling), and hemagglutinin (HA) (1583816; Roche).

Adenoviral Infections

A null adenovirus (AdDl312) and adenoviruses encoding β-galactosidase (Adβgal), full-length APE1/ref-1 (AdAPE1/ref-1), the constitutively active mutant of the small GTPase rac1 (Adrac1V12), the HA-tagged dominant-negative mutant of the p85 regulatory subunit of PI3-K (AdΔp85), and HA-tagged activation-deficient Akt (AdAktAA) were generated by homologous recombination in human embryonic kidney 293 cells, and have been described previously.[5,6] There was no difference in basal intracellular NO levels in endothelial cells infected with AdDl312 or Adβgal (supplemental Figure III, available in the online data supplement at http://circres.ahajournals.org), and these viruses were used interchangeably as controls. Human umbilical vein endothelial cells (HUVECs) or bovine aortic endothelial cells (BAECs) were infected with the indicated multiplicity of infection (moi; particle forming units per cell) of adenovirus for 18 hours. The virus was removed and cells incubated for another 24 hours. For measurement of basal NO production and eNOS activity, medium and cell lysates were collected 40 to 48 hours after viral infection.

eNOS Activity Assays

We used the conversion of ^{14}C-L-arginine to ^{14}C-L-citrulline to determine NOS activity in endothelial or COS-7 cell lysates (in lysis buffer containing phosphatase inhibitors) per manufacturer recommendations (NOS Detect Assay Kit; Stratagene). Appropriate amount of EDTA-buffered calcium was added to the incubation mixture to yield the desired free calcium concentration.

NO Measurement

Twenty-four to 48 hours after transfection, or 40 to 48 hours after adenoviral infection, media from cells were processed for measurement of the NO intermediary metabolite nitrite (NO_2^-) and nitrate (NO_3^-), the stable breakdown product of NO, by specific light absorbance per manufacturer recommendations (Calbiochem). Media were deproteinized using a 10-kDa cutoff filter. Absorbance from media of cells transfected with pcDNA3.1 was subtracted to control for background levels of $NO_2^- + NO_3^-$ found in the media. For serum NO measurements, 0.8 to 1.0 mL of whole blood was obtained by direct puncture of the right ventricle of anesthetized animals. The blood was kept at room temperature for 6 to 8 hours, after which it was centrifuged at 14 000 rpm for 15 minutes. The separated serum was processed for $NO_2^- + NO_3^-$ as above.

Intracellular NO was also measured in nonstimulated or bradykinin-stimulated BAECs using the NO-sensitive fluorophore diaminofluorescein-2 diacetate (DAF2-DA), according to manufacturer recommendations (Molecular Probes). Cells were imaged on a Zeiss laser scanning confocal microscope, and DAF2-DA fluorescence was quantified in randomly selected individual cells using MetaMorph software.

Vascular Reactivity and Blood Pressure Measurements

Rat thoracic aortic rings, 4- to 5-mm in length, were infected with 1×10^9 pfu of Adβgal or AdAPE1/ref-1 for 4 hours in PBS and then incubated in DMEM with 10% FBS at 37°C for an additional 24 to 48 hours. Krebs–Henseleit buffer–equilibrated rings were preconstricted with phenylephrine (PE; 0.3 μmol/L), and endothelium-dependent relaxations were observed with cumulative addition of acetylcholine (1 nM to 10 μmol/L). An identical protocol was used to assess endothelium-independent relaxant effects of the NO donor sodium nitroprusside (10 pM to 1 μmol/L). APE1/ref-1 overexpression in the endothelium was confirmed in frozen sections of adenovirus-infected aortic rings with a Vectastain ABC Kit (Vector Laboratories) and by Western blot in homogenates of whole aortas usingAPE1/ref-1 antibody.

WT and APE1/ref-$1^{+/-}$ mice from the same litter were genotyped by polymerase chain reaction as described previously.[7] Endothelium-dependent relaxation was assessed in 3- to 4-mm aortic rings obtained from male APE1/ref-$1^{+/-}$ and WT mice 12 to 16 weeks of age. Acetylcholine-induced relaxations were measured in rings that were preconstricted with PE (1 μmol/L). To determine basal NOS activity in WT and APE1/ref-$1^{+/-}$ mice, aortic rings were preconstricted with PE (1 μmol/L). N^G-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L) was then added to the bath. The increase in tension induced by NOS inhibition represents a measure of basal NOS activity. The percent change in tension with the addition of L-NAME was then calculated.

To measure systemic arterial blood pressure, carotid arteries of male WT and APE1/ref-$1^{+/-}$ mice anesthetized with ketamine (80 mg/kg) and xylazine (12 mg/kg) were cannulated with a PE-10 catheter serially connected to a pressure transducer, preamplifier, and analog-to-digital converter. After calibration of the system, blood pressure and heart rate were continuously recorded for 10 minutes. Data were analyzed using Labscribe software.

Superoxide Measurement in Aortic Rings

Dark-adapted lucigenin solution (5 μmol/L) was prepared in aerated Krebs–Hepes buffer. Aortic rings were immersed in lucigenin solution and chemiluminescence detected with a Monolight luminometer. The chemiluminescence signal was integrated over 5 minutes. Freshly prepared NADPH was added for 5 minutes where indicated.

Ras Activity

Twenty-four to 48 hours after transfection, total GTP-bound (active) H-ras was determined in lysates of COS-7 cells and HUVECs with an active ras pull-down assay using the ras-binding domain of raf-1 (Upstate Biotechnology) according to manufacturer recommendations. Total H-ras was detected in whole-cell lysates. Densitometric values of the active and total H-ras bands were quantified using Quantity One software (Bio-Rad).

Statistics

Each experiment was reproduced at least once. A representative experiment is shown. Bars represent mean±SEM. ANOVA and Student *t* test (two-sided) for unpaired values were used for statistical analysis.

Results

We first examined whether endogenous APE1/ref-1 participates in physiologic regulation of vascular tone. To do this, we used mice heterozygous for the APE1/ref-1 allele (APE1/ref-$1^{+/-}$) and their WT littermates. APE1/ref-$1^{+/-}$ mice have been reported previously to be highly susceptible to tumorigenesis when challenged with DNA-damaging agents[8] but have not been studied for any cardiovascular phenotype. The endothelium-dependent vasodilatory response to acetylcholine was measured in aortic rings of APE1/ref-$1^{+/-}$ and WT mice. Endothelium-dependent relaxation is markedly impaired in APE1/ref-$1^{+/-}$ mice relative to those from their WT littermates (Figure 1a). This difference in endothelium-dependent relaxation led us to explore possible differences in eNOS activity and basal endothelial NO production between the WT and APE1/ref-$1^{+/-}$ mice. To determine basal eNOS activity in vessels from APE1/ref-$1^{+/-}$ and WT mice, and therefore determine the role of APE1/ref-1 in

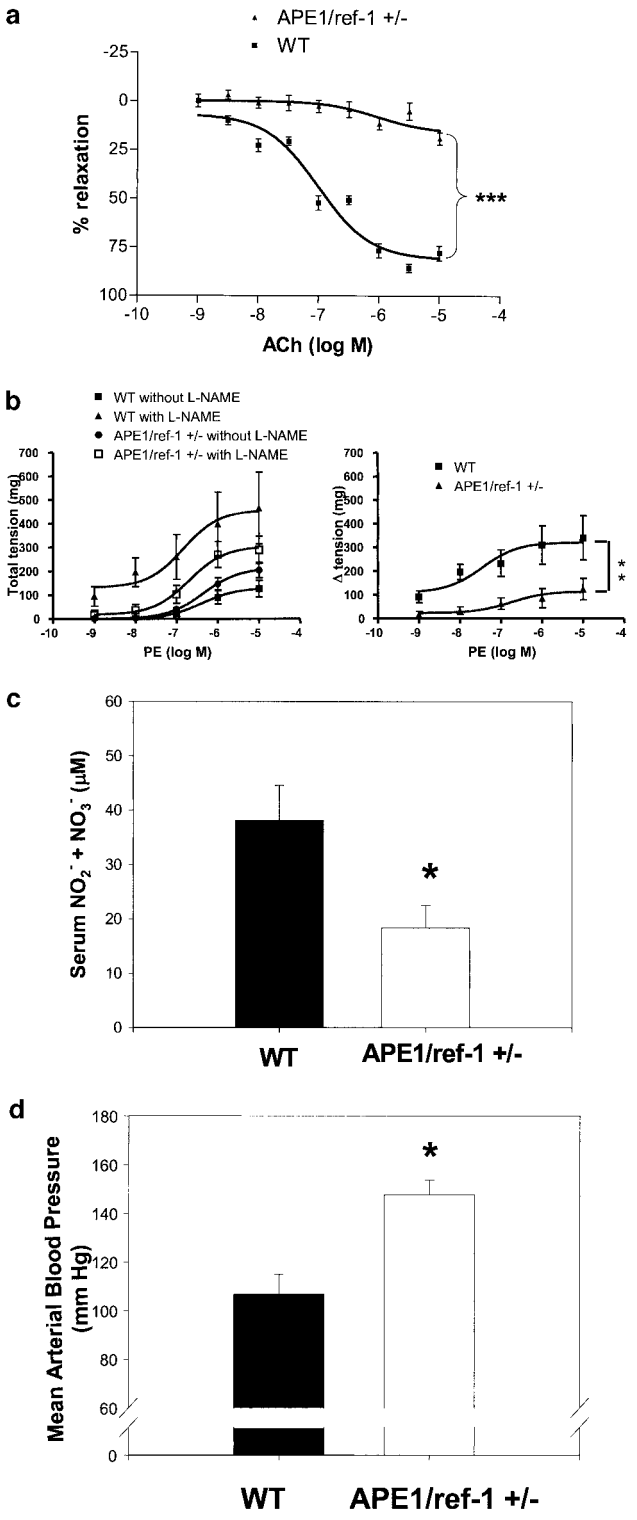

Figure 1. APE1/ref-1$^{+/-}$ mice have lower circulating NO, impaired endothelium-dependent relaxation, and are hypertensive. a, Acetylcholine-stimulated endothelium-dependent relaxation in aortic rings from WT and APE1/ref-1$^{+/-}$ mice. ***$P<0.001$ compared with WT (n=17). b, PE-induced tension in aortic rings from WT and APE1/ref-1$^{+/-}$ mice with and without addition of L-NAME (n=4). **$P<0.01$ WT vs APE1/ref-1$^{+/-}$. c, NO metabolites $NO_{2-}+NO_3^-$ in serum samples from APE1/ref-1$^{+/-}$ and WT mice (n=6). *$P<0.05$ WT vs APE1/ref-1$^{+/-}$. d, Mean carotid artery blood pressure in anesthetized APE1/ref-1$^{+/-}$ and WT mice (n=7). *$P<0.05$ WT vs APE1/ref-1$^{+/-}$.

regulating basal NO production, aortic rings were preconstricted with PE, and the increase in tension evoked by the addition of L-NAME, a nonspecific NOS inhibitor, was measured. This L-NAME–induced increase in vascular tone represents a measure of basal NOS activity. PE responses at each individual dose were significantly lower in WT than in APE1/ref-1$^{+/-}$ mice (Figure 1b). This suggests that basal depressor tone (a function of increased NOS activity and NO production) is higher in WT than in APE1/ref-1$^{+/-}$ mice, implying that differences in NOS activity are an important factor, although likely not the only factor, contributing to differences in contractile responses observed with PE between WT and APE1/ref-1$^{+/-}$ mice.

Next, we measured serum NO_2^- and NO_3^- levels (a sensitive reflection of basal endothelial NO formation in physiologic systems[9]) in APE1/ref-1$^{+/-}$ and WT mice. Basal serum NO metabolites are significantly lower in APE1/ref-1$^{+/-}$ mice relative to their WT littermates (Figure 1c). Because basal NO formation in the endothelium has been shown to participate in setting the resting tone of resistance vessels and thus regulating arterial blood pressure, we were also interested in determining the role of endogenous APE1/ref-1 in regulating systemic blood pressure. Resting mean arterial blood pressure under anesthetized conditions is markedly elevated in APE1/ref-1$^{+/-}$ mice compared with their WT littermates (Figure 1d). There is no difference between the resting heart rates of the two groups (WT 412±26 bpm; APE1/ref-1$^{+/-}$ 406±21 bpm). Together, these findings establish an important role for APE1/ref-1 in governing endothelium-dependent vascular tone and systemic arterial blood pressure.

Having established a role for endogenous APE1/ref-1 in controlling vascular tone, we then examined the effect of APE1/ref-1 overexpression on endothelium-dependent relaxation. Adenoviral gene transfer with AdAPE1/ref-1 ex vivo in rat aortic segments results in significant overexpression of APE1/ref-1 in the endothelium (Figure 2a). Control vessels were infected with an adenovirus encoding the inert *Escherichia coli* LacZ gene (Adβgal). Overexpression of APE1/ref-1 in the endothelium augments acetylcholine-induced vasorelaxation of isolated aortic rings (Figure 2b). Moreover, in the presence of L-NAME, the vasoconstrictor response to PE is significantly greater in vessels overexpressing APE1/ref-1 (Figure 2c), suggesting increased NOS activity and a larger pool of bioavailable NO in these vessels compared with control vessels. In contrast to acetylcholine-induced vasorelaxation, overexpression of APE1/ref-1 does not influence the direct endothelium-independent vasorelaxant effects of the NO donor sodium nitroprusside (Figure 2d).

Because bioavailable NO and endothelium-dependent relaxation are altered by changes in vascular oxidant levels, we wondered whether the vasodilatory effect of APE1/ref-1 is a reflection of decreased reactive oxygen species (ROS) production in the vessel wall. Therefore, we examined the consequence of endothelial APE1/ref-1 overexpression on vascular superoxide levels. A significant portion of vascular superoxide production is governed by the rac1-regulated NAD(P)H-dependent oxidase.[10] Basal- and NADPH-stimulated vascular superoxide, quantified by lucigenin-enhanced chemiluminescence, is not significantly different in aortic rings infected with AdAPE1/ref-1 when compared with those infected with the control Adβgal

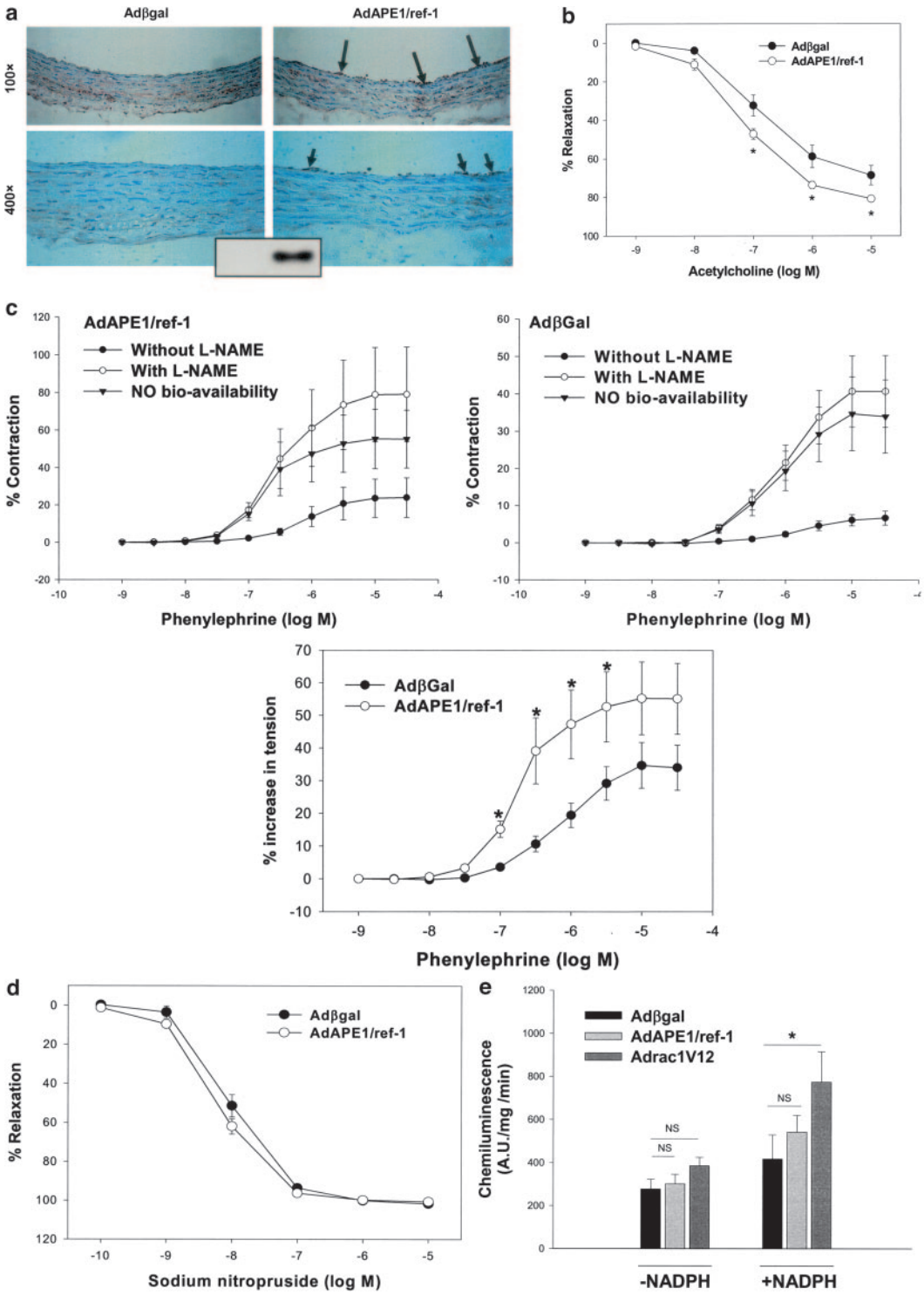

Figure 2. Endothelial overexpression of APE1/ref-1 promotes vasorelaxation. a, Top, Endothelial-specific adenoviral overexpression of APE1/ref-1 in rat aorta. Rat thoracic aortic rings were incubated ex vivo with the adenovirus Adβgal or AdAPE1/ref-1 and immunostained for APE1/ref-1 with peroxidase-labeled secondary antibody. Photographs are ×100 and ×400 magnification. Arrows show brown peroxidase-positive cells in the endothelium. Bottom inset, Western blot for APE1/ref-1 in protein extracts from aortic rings. b, Acetylcholine-induced endothelium-dependent relaxation in rat aortic rings infected with Adβgal or AdAPE1/ref-1 (n=4). *$P<0.05$ compared with Adβgal. c, bottom, Percent increase in PE-induced contraction by addition of L-NAME (100 μmol/L) in rat aortic rings infected with Adβgal and AdAPE1/ref-1 (n=4). *$P<0.05$ compared with Adβgal. c, top, The contractile dose response to PE in the presence and absence of L-NAME (100 μmol/L) of rat aortic rings infected with Adβgal and AdAPE1/ref-1. The NO bioavailability curve is the difference in contraction in the presence and absence of L-NAME. d, Sodium nitroprusside–induced endothelium-independent relaxation in rat aortic rings infected with Adβgal and AdAPE1/ref-1 (n=4). e, Lucigenin-enhanced chemiluminescence, reflective of vascular superoxide levels, in whole rat aortic rings infected with Adβgal, AdAPE1/ref-1, or AdRac1V12 (n=3). Luminescence was recorded for 10 minutes in the absence or presence of NADPH (100 μmol/L). *$P<0.05$ compared with Adβgal and AdAPE1/ref-1.

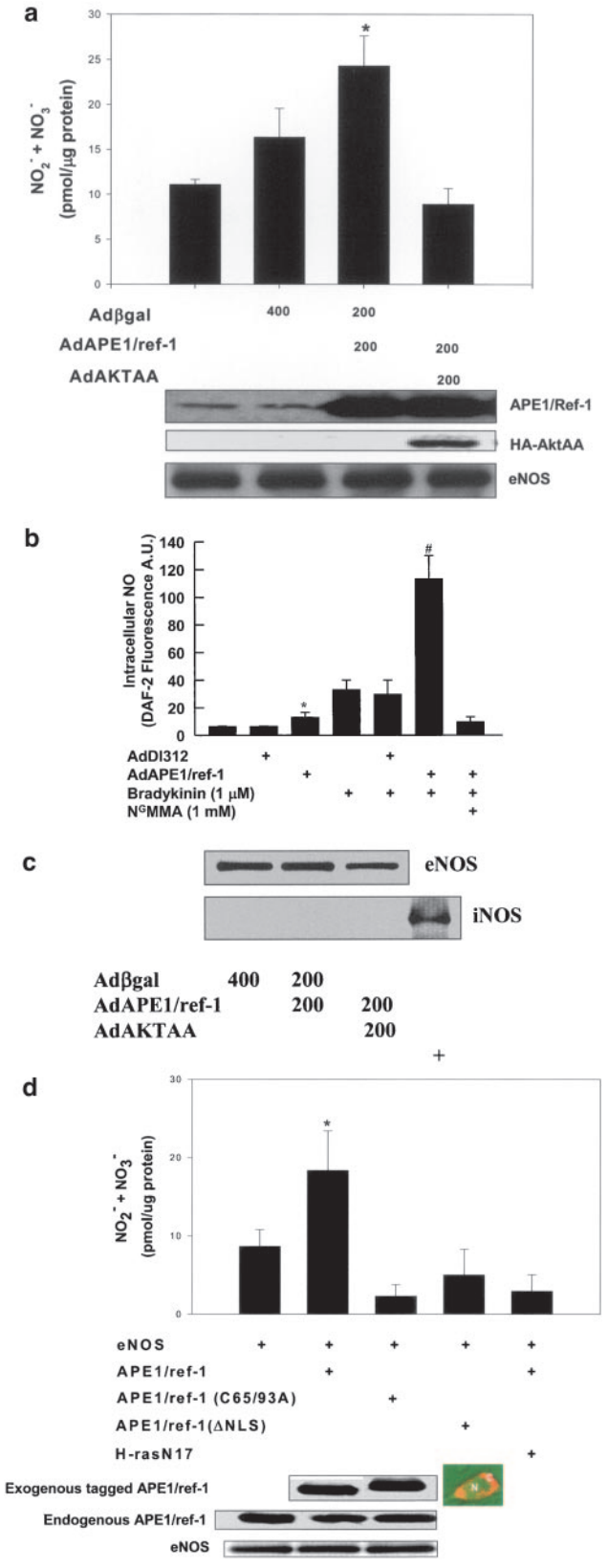

Figure 3. WT APE1/ref-1 but not the redox-deficient APE1/ref-1 promotes basal- and agonist-stimulated Akt kinase–dependent NO production. a, Basal accumulated $NO_2^- + NO_3^-$ in the growth media of uninfected BAECs and BAECs infected with Adβgal, AdAPE1/ref-1, and AdAktAA. Numbers indicate moi of each virus used. Overexpression of APE1/ref-1 and HA-tagged AktAA in cell lysates is shown at bottom. b, Basal and bradykinin-stimulated intracellular NO levels (measured by the NO-sensitive intracellular fluorophore DAF-2DA) in BAECs infected with AdDl312 and AdAPE1/ref-1. Viruses were used at 400 moi. *$P<0.05$ compared with AdDl312. c, Western blot for eNOS and iNOS in HUVECs infected with AdAPE1/ref-1, Adβgal, and AdAktAA. Lysates of lipopolysaccharide-stimulated RAW cells were used as a positive control for iNOS (+). d, Accumulated $NO_{2-} + NO_{3-}$ in the growth media of COS-7 cells cotransfected with eNOS and (1) APE1/ref-1, (2) redox-deficient APE1/ref-1 (C65/93A), (3) cytoplasmic APE1/ref-1(ΔNLS), or (4) APE1/ref-1+dominant-negative H-ras (H-rasN17). *$P<0.05$ compared with all other conditions. Western blots for APE1/ref-1 and eNOS and photomicrograph showing cytoplasmic localization of APE1/ref-1(ΔNLS) are shown at bottom. N indicates nucleus; C, cytoplasm.

(Figure 2d). The vascular NAD(P)H-dependent oxidase was functional in these aortic rings because adenoviral expression of active rac1 (rac1V12) resulted in an increase in superoxide levels. These data show that overexpression of APE1/ref-1 promotes the endothelium-dependent vasodilatory response of intact blood vessels. Moreover, the lack of effect of APE1/ref-1 overexpression on sodium nitroprusside–induced vasodilatation and on vascular superoxide levels indicates that it is not functioning as an antioxidant.

We then turned our attention to elucidating the mechanism by which APE1/ref-1 promotes endothelium-dependent relaxation. Endothelium-dependent vasorelaxation and resting vascular tone is, in large part, a function of basal endothelial NO production. Therefore, we first determined the effect of APE1/ref-1 on NO production in BAECs. APE1/ref-1 was adenovirally overexpressed in BAECs, and accumulation of NO_2^- and NO_3^- was measured in the medium, or intracellular NO detected with the NO-sensitive fluorophore DAF2-DA. Overexpression of APE1/ref-1 stimulates basal steady-state endothelial NO production compared with control uninfected or Adβgal-infected cells (Figure 3a and 3b). Similar results were obtained in HUVECs (supplemental Figure I). Moreover, NO production in response to the agonist bradykinin was also higher in cells overexpressing APE1/ref-1 (Figure 3b). However, APE1/ref-1 overexpression does not increase eNOS protein levels, nor does it lead to expression of iNOS in endothelial cells (Figure 3c). Identical results were obtained in COS-7 cells (which do not express eNOS) that were transfected with an eNOS expression vector (Figure 3d).

Because APE1/ref-1 has two functional domains, we also asked whether the endonuclease domain, the reducing domain, or both, are important in regulating endothelial NO production. In contrast to WT APE1/ref-1, expression of the redox-deficient mutant of APE1/ref-1 (cysteine to alanine mutations at codons 65 and 93), which has intact endonuclease activity, does not result in an increase in eNOS-catalyzed NO production (Figure 3d). Equal levels of eNOS, and tagged WT or redox-deficient exogenous APE1/ref-1, were expressed in COS-7 cell lysates, suggesting that APE1/ref-1 modulates eNOS catalytic activity through its reducing property.

APE1/ref-1 is localized mainly in the nuclei of the majority of cells. We wondered whether this nuclear localization is important for its effect on NO production. To answer this question, we constructed a deletion mutant of APE1/ref-1: APE1/ref-1(ΔNLS), which lacks the putative N-terminal nuclear localization signal (NLS). Expression of APE1/ref-1(ΔNLS) in COS-7

cells does not augment eNOS-catalyzed NO production (Figure 3d). This finding shows that nuclear localization of APE1/ref-1 is critical for stimulating eNOS activity. That nuclear localization and intact reducing function of APE1/ref-1 are essential for stimulating NO production implies that APE1/ref-1 governs NO production by modulating redox-sensitive transcription of proteins that regulate eNOS activity.

Endothelial NOS activity is regulated at multiple levels. Endothelium-dependent vasodilatory agonists lead to an increase in eNOS activity primarily through calcium influx. eNOS activity is also regulated by post-translational modifications. Activation of the phosphoinositide-3 kinase (PI3-K)/Akt kinase pathway results in calcium sensitization of eNOS by phosphorylating it at serine 1177 (S1179 on bovine eNOS).[10–12] This constitutes an important mechanism for increasing NO production by certain stimuli such as insulin, vascular endothelial growth factor (VEGF), bradykinin, and laminar shear stress. To test whether APE1/ref-1 requires the PI3-K/Akt pathway for augmenting basal NO production, we first determined whether Akt kinase mediates the APE1/ref-1–stimulated change in endothelial NO production and eNOS catalytic activity. APE1/ref-1 overexpression in BAECs leads to a significant upregulation of basal eNOS activity (Figure 4a). To inhibit Akt kinase, the dominant-negative form of the enzyme (AktAA) was adenovirally coexpressed with APE1/ref-1 (Figure 3a). Inhibiting Akt suppresses APE1/ref-1–induced stimulation of basal eNOS activity (Figure 4a) and results in a corresponding inhibition of basal- and bradykinin-stimulated NO production (Figure 3a and 3b). Consistent with these results, APE1/ref-1 overexpression in HUVECs stimulates phosphorylation of Akt on serine 473, phosphorylation of eNOS on serine 1177, and Akt kinase activity (measured by phosphorylation of its substrate GSK-3) without affecting Akt or eNOS expression (Figure 4b). Similar results were obtained in BAECs (supplemental Figure II). We also examined whether stimulation of Akt kinase and eNOS by APE1/ref-1 is dependent on the activation of PI3-K. Inhibiting PI3-K by adenoviral expression of the dominant-inhibitory mutant of the p85 regulatory subunit of PI3-K abrogates APE1/ref-1–stimulated phosphorylation of Akt and eNOS (Figure 4b).

Next, we assessed the effect of APE1/ref-1 overexpression on the calcium sensitivity of eNOS. NOS activity, measured at specified free calcium (Ca^{2+}) concentrations, in lysates of eNOS-transfected COS-7 cells overexpressing WT APE1/ref-1 demonstrates that the sensitivity of the enzyme to activation by calcium increases relative to control cells (Figure 4c). Moreover, treatment of cells with the PI3-K inhibitor wortmannin suppresses APE1/ref-1–stimulated calcium eNOS sensitization. In contrast, expression of the redox-deficient mutant of APE1/ref-1 (APE1/ref-1 C65/93A) results in desensitization of the enzyme to calcium (Figure 4d). Together, these results indicate that activation of the PI3-K/Akt axis, with resultant phosphorylation and calcium sensitization of eNOS, is responsible for the APE1/ref-1–stimulated increase in NO production.

Because the proto-oncogene H-ras lies upstream of PI3-K and Akt kinase in insulin and VEGF signaling, we wondered whether APE1/ref-1–stimulated eNOS activation is also dependent on H-ras. To investigate this possibility, we first determined the effect of APE1/ref-1 on endogenous H-ras activity and expression. We measured H-ras activity and expression in COS-7 cells transfected with APE1/ref-1. Compared with control transfected cells, those overexpressing APE1/ref-1 show a significant increase in total H-ras activity (Figure 4e). This increase is the result of an increase in total cellular H-ras expression (Figure 4e). In contrast to WT APE1/ref-1, expression of the redox-deficient APE1/ref-1(C65/93A) does not lead to upregulation of H-ras expression or activity. Moreover, in HUVECs, adenoviral overexpression of APE1/ref-1 results in upregulation of H-ras expression but does not change expression of the small GTPase rac1, which regulates oxidant (superoxide) production (Figure 4e). This specificity is consistent with the lack of effect of APE1/ref-1 on vascular superoxide levels.

Next, we determined the importance of H-ras upregulation in APE1/ref-1–stimulated calcium sensitization of eNOS and NO production. In COS-7 cells, endogenous H-ras activity was inhibited by expression of a dominant-negative allele, H-rasN17. In such cells, APE1/ref-1–stimulated NO production and calcium sensitization was blunted when compared with cells not expressing H-rasN17 (Figures 3d and 4c). To examine the governance of the H-ras–PI3-K/Akt kinase signaling pathway by APE1/ref-1 in a physiological setting, we measured expression of H-ras, phosphorylation (activity) of Akt, and expression and phosphorylation of eNOS in aortas of WT and APE1/ref-$1^{+/-}$ mice. When compared with their WT littermates, H-ras expression, phospho-S1177 eNOS, and phospho-S473 Akt are significantly lower in APE1/ref-$1^{+/-}$ animals (Figure 4f). Interestingly, total eNOS expression was higher in APE1/ref-$1^{+/-}$ mice compared with WT mice (Figure 4f). However, the proportion of eNOS that is phosphorylated (phospo-S1177/total eNOS) is markedly lower in the APE1/ref-$1^{+/-}$ mice when compared with WT mice.

Collectively, these findings show that APE1/ref-1 regulates H-ras expression through its reducing action. Moreover, they place H-ras upstream of PI3-K and Akt in calcium sensitization of eNOS by APE1/ref-1. Finally, they suggest that downregulation of the H-ras–PI3-K/Akt axis may be responsible for impairment of endothelium-dependent relaxation in APE1/ref-$1^{+/-}$ mice.

Discussion

Regulation of vascular reactivity by the redox status of the vascular wall is well described. Reducing agents typically promote vascular relaxation. Many of these reducing factors change the redox status of the vascular wall by themselves, acting as ROS scavengers or by augmenting the synthesis or function of endogenous ROS scavengers or antioxidant enzymes. Although we did not examine whether APE1/ref-1 regulates the overall redox state of the cell, our findings suggest this not to be the case. This conclusion is supported by the observation that APE1/ref-1 does not suppress superoxide levels in aortic rings. Moreover, previous reports have demonstrated that APE1/ref-1 does not alter the capacity of the cell to scavenge or catalyze hydrogen peroxide.[13] Rather, in concert with its nuclear reducing function, which is targeted toward promoting the DNA-binding activity of specific nuclear transcription factors, our findings point toward a redox-sensitive transcriptional mechanism by which

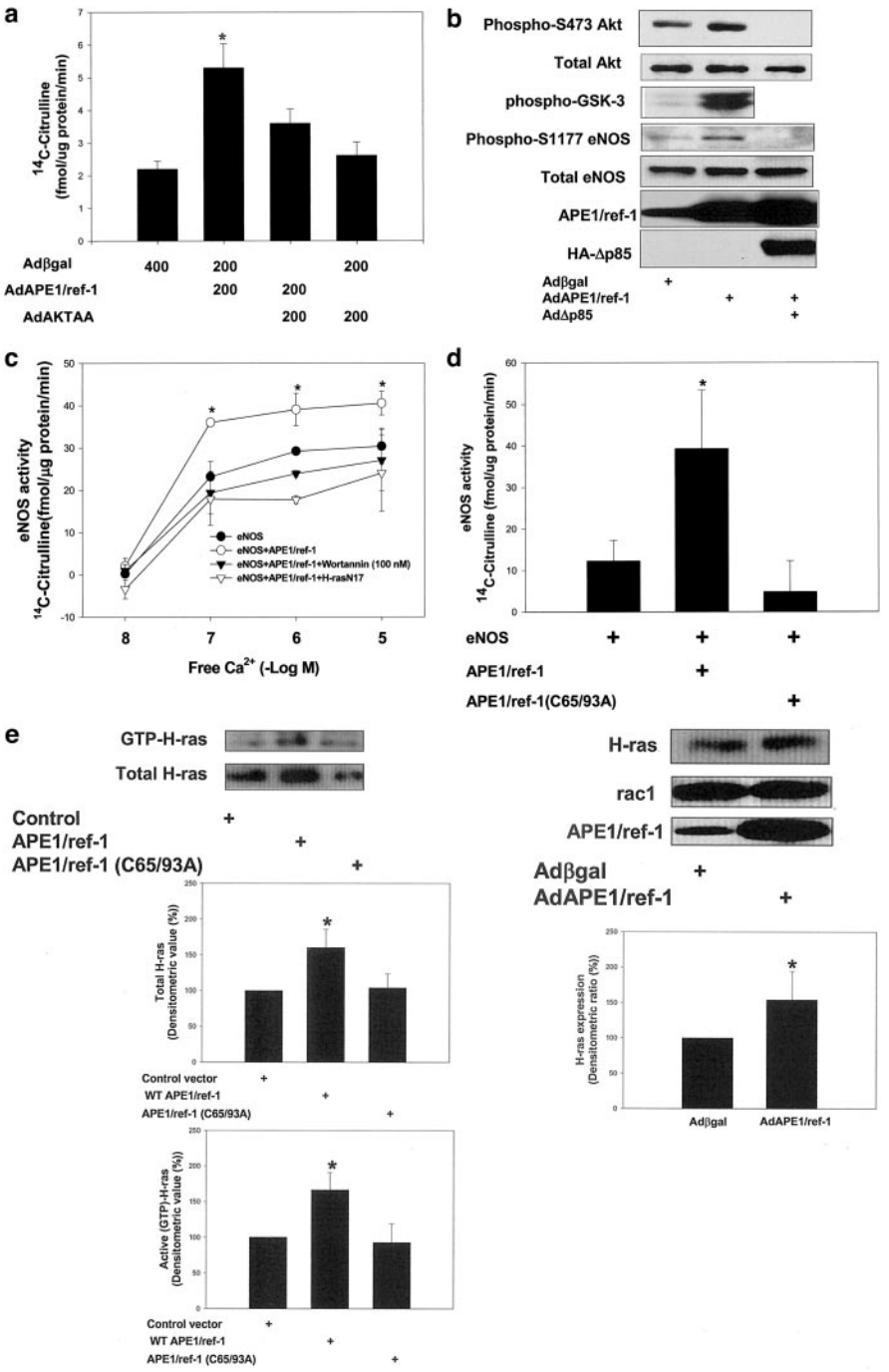

Figure 4. APE1/ref-1 stimulates eNOS catalytic activity through a PI3-K/Akt kinase–dependent mechanism by upregulating H-ras expression. a, In vitro NOS catalytic activity at a Ca^{2+} of 0.6 mmol/L measured in lysates of BAECs infected with Adβgal, AdAPE1/ref-1, and AdAktAA. Numbers indicate moi of each virus. *$P<0.05$ compared with all other conditions. b, Representative Western blots for total Akt, P-S473-Akt, P-S1177-eNOS, APE1/ref-1, and HA-Δp85 in lysates of HUVECs infected with Adβgal, AdAPE1/ref-1, and AdΔp85 (adenovirus encoding an HA-tagged dominant-inhibitory form of the p85 regulatory subunit of PI3-K). Phospho-GSK-3 blot indicates in vitro phosphorylation of glycogen synthase kinase-3 by immunoprecipitated active Akt from whole-cell lysates. All viruses were used at 200 moi. c, In vitro NOS catalytic activity at titrated concentrations of free Ca^{2+} (pCa = Log-free Ca^{2+}) in lysates of COS-7 cells cotransfected with eNOS and (1) APE1/ref-1, or (2) APE1/ref-1+H-rasN17. *$P<0.05$ compared with all other conditions d, In vitro NOS catalytic activity in lysates of COS-7 cells cotransfected with eNOS and (1) APE1/ref-1, or (2) APE1/ref-1 (C65/93A). Free Ca^{2+} concentration in reaction buffer was titrated to 10^{-6} mol/L (n=3). *$P<0.05$ compared with all other conditions. e, left, Total and GTP-bound H-ras in lysates of COS-7 cells transfected with control plasmid, APE1/ref-1, or APE1/ref-1(C65/93A). Densitometric quantification of total and GTP–H-ras is shown at bottom. Right, H-ras, rac1, and APE1/ref-1 expression in HUVECs infected with Adβgal or AdAPE1/ref-1 at 200 moi each. Comparison of densitometric values is shown at bottom. *$P<0.05$ compared with Adβgal. f, Expression of total H-ras, total Akt, P-S473-Akt, total eNOS, and P-S1177-eNOS in extracts of homogenized whole aortas from APE1/ref-1$^{+/-}$ and WT mice. Bottom, Comparison of densitometric values of H-ras and P-S1177–eNOS is shown at bottom. *$P<0.05$ compared with WT.

APE1/ref-1 increases endothelial NO production. In this respect, the mechanism of APE1/ref-1 is different from most reducing agents.

APE1/ref-1 has a hydrophobic, basic amino acid–rich stretch of amino acids at its N terminus, suggestive of a nuclear localization signal. Consistent with this, APE1/ref-1 is mostly, although not exclusively, localized to the nuclei of many cells, including endothelial cells.[13] The APE1/ref-1 construct with a deletion of the N-terminal nuclear localization signal (APE1/ref-1 ΔNLS) is localized strictly to the cytoplasm of cells (Figure 3d) but retains the cysteines at positions 65 and 93, which are critical to its reducing function. The finding that APE1/ref-1 ΔNLS was incapable of increasing NO production suggests that in addition to its reducing property, nuclear localization of APE1/ref-1 is also crucial for stimulating eNOS activity. Together, these observations strongly suggest that the modulation of transcriptional activity, which requires redox-competent APE1/ref-1 to be present in the nucleus, may be the key to the effect of APE1/ref-1 on H-ras expression and thus eNOS activity (Figure 5). Although we did not examine the precise mechanism(s) by which APE1/ref-1 governs H-ras expression, it is noteworthy that the 5′ noncoding region of the H-ras gene does have several guanine cytosine (GC)-rich regions that govern its basal expression.[14–16] Interestingly, these GC-rich *cis* elements in the H-ras gene are binding sites for the Sp1 transcription factor,[17] the DNA-binding activity of which is stimulated in a

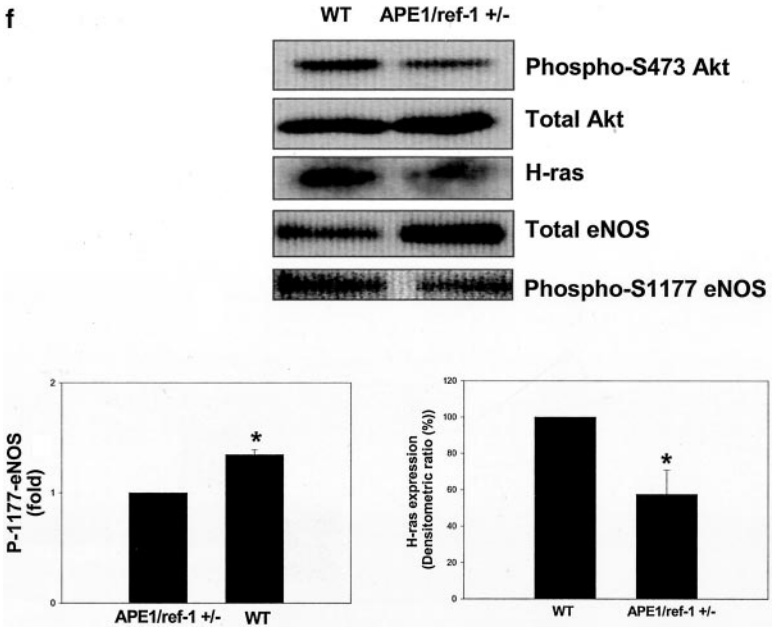

Figure 4 (continued).

redox fashion by the APE1/ref-1–associated reducing protein thioredoxin.[18]

Local production of endothelial NO plays a pivotal role in governing the overall redox state of the vascular wall. A reduction in endothelial NO production would be expected to result in an increase in vascular oxidative stress. Arachidonic acid is a prime target of oxidants and is peroxidized to yield metabolites including F2-isoprostanes. APE1/ref-$1^{+/-}$ mice have been reported to have elevations in circulating markers of oxidative stress such as F2-isoprostanes.[19] F2-isoprostanes themselves lead to vasoconstriction by direct action on vascular smooth muscle cells via thromboxane receptors.[20] Therefore, in addition to a decrease in NO-mediated smooth muscle relaxation, the hypertension seen in APE1/ref-$1^{+/-}$ mice may also be attributable to the paracrine vasoconstricting effects of F2-isoprostanes and other oxidative stress–induced metabolites of arachidonic acid.

In addition to eNOS-derived NO production, the governance of H-ras expression by APE1/ref-1 could also contribute to some of the phenotypic changes associated with APE1/ref-1. APE1/ref-1 confers protection against apoptosis induced by oxidative stimuli.[13] Moreover, H-ras, via the PI3-K/Akt–signaling pathway, has been implicated in mechanisms of DNA repair triggered by oxidative stimuli.[21] Therefore, it is tempting to speculate that in addition to NO production, there may be cross-talk between APE1/ref-1 and H-ras with respect to DNA repair and protection from oxidative apoptotic stimuli.

Although phosphorylation at S1177 of endogenous eNOS was significantly lower in the APE1/ref-$1^{+/-}$ mice compared with the WT mice, an unexpected finding was that total eNOS expression was upregulated in the APE1/ref-$1^{+/-}$ animals. Several mechanisms may be responsible for this upregulation. First, the increase in eNOS expression may be a homeostatic response to the elevated blood pressure in APE1/ref-$1^{+/-}$ mice. Upregulation of eNOS expression is evident in other animal models of systemic hypertension.[22,23] Second, eNOS expression is sensitive to ROS,[24] and therefore an increase in oxidative stress in the vasculature of APE1/ref-$1^{+/-}$ mice (attributable to a decrease in vascular NO) may also result in upregulation of eNOS expression. Third, and perhaps most interestingly, APE1/ref-1 may itself negatively regulate eNOS expression. APE1/ref-1 acts as a negative transcriptional regulator of several genes in response to physiological triggers, such as the parathyroid hormone gene,[25] and may act in a similar capacity to govern eNOS expression. If so, on the basis of our findings in cultured endothelial cells that overexpression of APE1/ref-1 does not change basal eNOS levels, we would expect APE1/ref-1 to mediate but not trigger eNOS downregulation.

In summary, our data point toward a previously unrecognized role for APE1/ref-1 in governing endothelial NO production via an H-ras–PI3-K/Akt-dependent mechanism. Importantly, the reducing property of APE1/ref-1 is essential to fulfill this role. Because this reducing property of APE1/ref-1 is responsible for the targeted reductive activation of many nuclear transcription factors, it is likely that H-ras

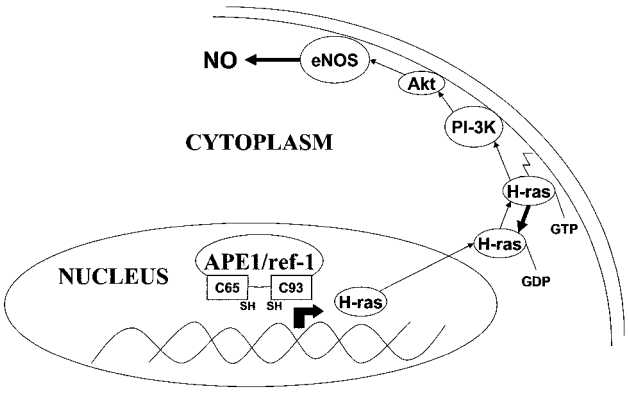

Figure 5. Scheme showing effect of APE1/ref-1 on the H-ras–PI-3K/Akt signaling pathway, leading to an increase in endothelial NO production.

upregulation by APE1/ref-1 occurs at the transcriptional level. Equally important, this reductive property of APE1/ref-1 does not suppress vascular oxidant levels; therefore, it is unlikely that APE1/ref-1 is promoting endothelium-dependent relaxation by simply acting as an antioxidant. Finally, although this work has focused on the redox property of APE1/ref-1 and its role in stimulating eNOS activity, the DNA repair function of APE1/ref-1 may also play an important role in maintaining vascular homeostasis.

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