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Redox factor-1: an extra-nuclear role in the regulation of endothelial oxidative stress and apoptosis

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Abstract

The rac1 GTPase promotes oxidative stress through reactive oxygen species (ROS) production, whereas the DNA repair enzyme and transcriptional regulator redox factor-1 (ref-1) protects against cell death due to oxidative stimuli. However, the function of ref-1 in regulating intracellular oxidative stress, particularly that induced by rac1, has not been defined. We examined the role of ref-1 in vascular endothelial cell oxidative stress and apoptosis. Ref-1 was expressed in both the cytoplasm and nuclei of resting endothelial cells. Cytoplasmic ref-1 translocated to the nucleus with the oxidative trigger hypoxia/reoxygenation (H/R). Forced cytoplasmic overexpression of ref-1 suppressed H/R-induced oxidative stress (H_2O_2 production), NF-κB activation, and apoptosis, and also mitigated rac1-regulated H_2O_2 production and NF-κB transcriptional activity. We conclude that inhibition of oxidative stress is another mechanism by which ref-1 protects against apoptosis, and that this is achieved through modulation of cytoplasmic rac1-regulated ROS generation. This suggests a novel extra-nuclear function of ref-1.
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Keywords: redox factor-1 (ref-1); rac1; reactive oxygen species (ROS); NAD(P)H oxidase; hypoxia/reoxygenation (H/R)

Abbreviations: HUVECs, human umbilical vein endothelial cells; H/R, hypoxia/reoxygenation; NF-KB, nuclear factor-kappa B; ROS, reactive oxygen species; ref-1, redox factor-1; redox, reduction-oxidation

Introduction

Exposure of cells or tissue to oxidative stimuli such as hypoxia/reoxygenation (H/R), ischemia/reperfusion (I/R), and inflammatory cytokines induces the generation of reactive oxygen species (ROS) resulting in alterations in intracellular reduction/oxidation (redox) state. Cellular redox changes can have profound effects on the activities of transcription factors, and such regulation of transcriptional activation has emerged as one of the important mechanisms that prokaryotic and eukaryotic cells employ for the expression of specific genes important in inflammation, death and survival.[1]

Stimuli that result in oxidative stress employ a number of cellular ROS generating systems. Among these is the NAD(P)H oxidase regulated by the ubiquitous small GTPase rac1. Many components of the phagocyte NADPH oxidase are expressed in non-pahgocytic cells, including cardiovascular cell types.[2] The functional significance of this oxidase is evident by the fact that rac1-regulates the production of ROS in a variety of cell types in response to a many oxidative stimuli such as tumor necrosis factor (TNF)[3] and H/R.[4]

Redox factor-1 (ref-1) is a ubiquitous 37 kD bi-functional protein. Ref-1 stimulates the DNA binding activities of the AP-1 family of transcription factors by a redox-dependent mechanism.[5,6] This effect is mediated through reduction of a conserved cysteine residue located at the DNA-binding domains of c-*fos* and c-*jun*.[7] Through a similar reducing action, ref-1 is also capable of modulating or activating other classes of transcription factors that regulate cell growth, differentiation, survival, and death including NF-κB, p53, Egr-1, c-Myb, HLF, and Pax-8.[6,8–12] Ref-1 also has DNA repair activity. It is an apurinic/apyrimidinic endonuclease (APE), an important enzyme in the base excision repair (BER) pathway.[13] Apurinic/Apyrimidinic (AP) damage of DNA occurs with diverse oxidative stimuli, is highly mutagenic, and AP repair is indispensable for the genomic integrity and viability of cells. Although the DNA repair and transcription factor reducing properties of ref-1 are well known, other fundamental mechanisms via which it may regulate redox-sensitive transcription, and influence cell death are yet to be elucidated.

Ref-1 has been implicated in protection against cell death resulting from oxidative stimuli. Depletion of ref-1 renders cells more sensitive to hyperoxia,[14] and a decrease in ref-1 expression is associated with apoptosis in ischemic neuronal tissue.[15] However, the role of ref-1 and the cellular phenotypes that it regulates in cardiovascular tissue is not known. The vascular endothelium is the first line of defense against a variety of oxidative stimuli, and redox-regulated activation and death of endothelial cells have been implicated in the pathogenesis of vascular disorders. We therefore investigated the role of ref-1 in regulating the response of vascular endothelial cells to the oxidative stimulus hypoxia/reoxygenation (H/R).

Here, we report that ref-1 protects endothelial cells from apoptosis, and prevents endothelial cell activation of the inflammatory transcription factor NF-κB in response to H/R through a novel mechanism of action: suppression of intracellular H_2O_2 regulated by the small GTPase rac1.

Although our experiments are limited to endothelial cells, the ubiquitous expression of ref-1 and rac1, and their importance in the regulation and response of cells to oxidative stimuli, suggests that the functional interaction between these two proteins described herein may also be relevant to the redox biology of other cell types.

Results

Expression of ref-1 in the cytoplasm and nucleus of endothelial cells: translocation to the nucleus with H/R

We first determined the sub-cellular expression pattern of ref-1 under basal normoxic conditions, and after hypoxia/reoxygenation (H/R) (Figure 1A,B). Under normoxic conditions, endogenous ref-1 was present in both the cytoplasm and nuclei of uninfected endothelial cells, and cells infected with the control Adβgal virus. Hypoxia/reoxygenation (H/R) of uninfected, and Adβgal-infected cells led to a significant increase in nuclear/cytoplasmic ref-1. H/R or hypoxia alone for 6 h resulted in no significant change in total expression of ref-1 (not shown).

Adenovirus-mediated cytoplasmic and nuclear overexpression of ref-1

With the goal of examining the role of ref-1 in oxidative stress and apoptosis, 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 HUVECs.[3] Infection of HUVECs with Adref-1 resulted in significant, and dose-dependent overexpression of ref-1 compared to cells infected with Adβgal (Figure 1B,C). Importantly, infection with Adref-1 led to an increase in both cytoplasmic and nuclear expression of ref-1 (Figure 1C). Moreover, when compared to Adβgal-infected cells, Adref-1-infected cells showed significantly higher cytoplasmic ref-1 expression even after H/R (Figure 1B). This demonstrates that infection with Adref-1 is able to significantly increase both cytoplasmic and nuclear ref-1 expression during normoxic conditions and after the H/R stimulus.

Overexpression of ref-1 suppresses H/R-induced apoptosis

Apoptosis plays an important part in post-hypoxic or post-ischemic cell death and tissue injury.[16,17] We therefore investigated the role of ref-1 in programmed death of endothelial cells induced by H/R (Figure 2A,B). Both uninfected and Adβgal-infected cells were susceptible to H/R-induced apoptosis. This reoxygenation-stimulated apoptosis was suppressed in Adref-1-infected cells in a dose-dependent fashion.

Oxidative stress is an important contributor to reoxygenation or reperfusion-induced apoptosis.[16,18,19] Therefore, one mechanism for the above-cited effect could be a change in the cellular anti-oxidant capacity induced by ref-1. To explore this possibility we examined the susceptibility

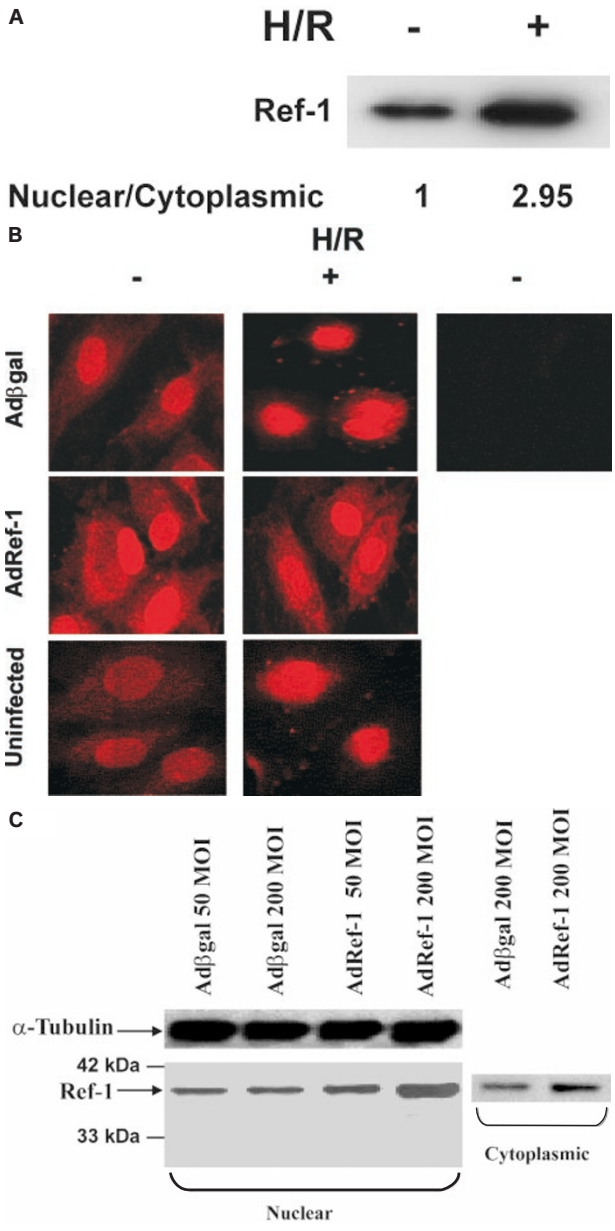

Figure 1 Basal and adenoviral-induced endothelial ref-1 expression and sub-cellular distribution during normoxia and with hypoxia/reoxgenation (H/R). (**A**) Western blot for ref-1 in nuclear fractions of endothelial cells under normoxic conditions, and after hypoxia 6 h/reoxygenation 2 h. Nuclear/cytoplasmic ratio is calculated after normalization for protein loading. (**B**) Immunostaining for ref-1 in uninfected, Adβgal- and Adref-1-infected cells, with and without H/R. Viruses used at 200 MOI, hypoxia 6 h, and reoxygenation 2 h. – ref-1 Ab indicates omission of primary ref-1 antibody, but inclusion of secondary antibody. (**C**) Western blot for ref-1 in cytoplasmic and nuclear fractions of cells infected with Adβgal and Adref-1. Protein loading of nuclear and cytoplasmic fractions is not comparable

of ref-1 overexpressing cells to H_2O_2-induced apoptosis, and compared it to cells infected with Adβgal (Figure 2C). A modest dose (100 μM) of H_2O_2 was used so as not to overwhelm the cell's anti-oxidant capacities. Forced overexpression of ref-1 did not protect against H_2O_2-induced apoptosis when compared to Adβgal-infected cells. Thus

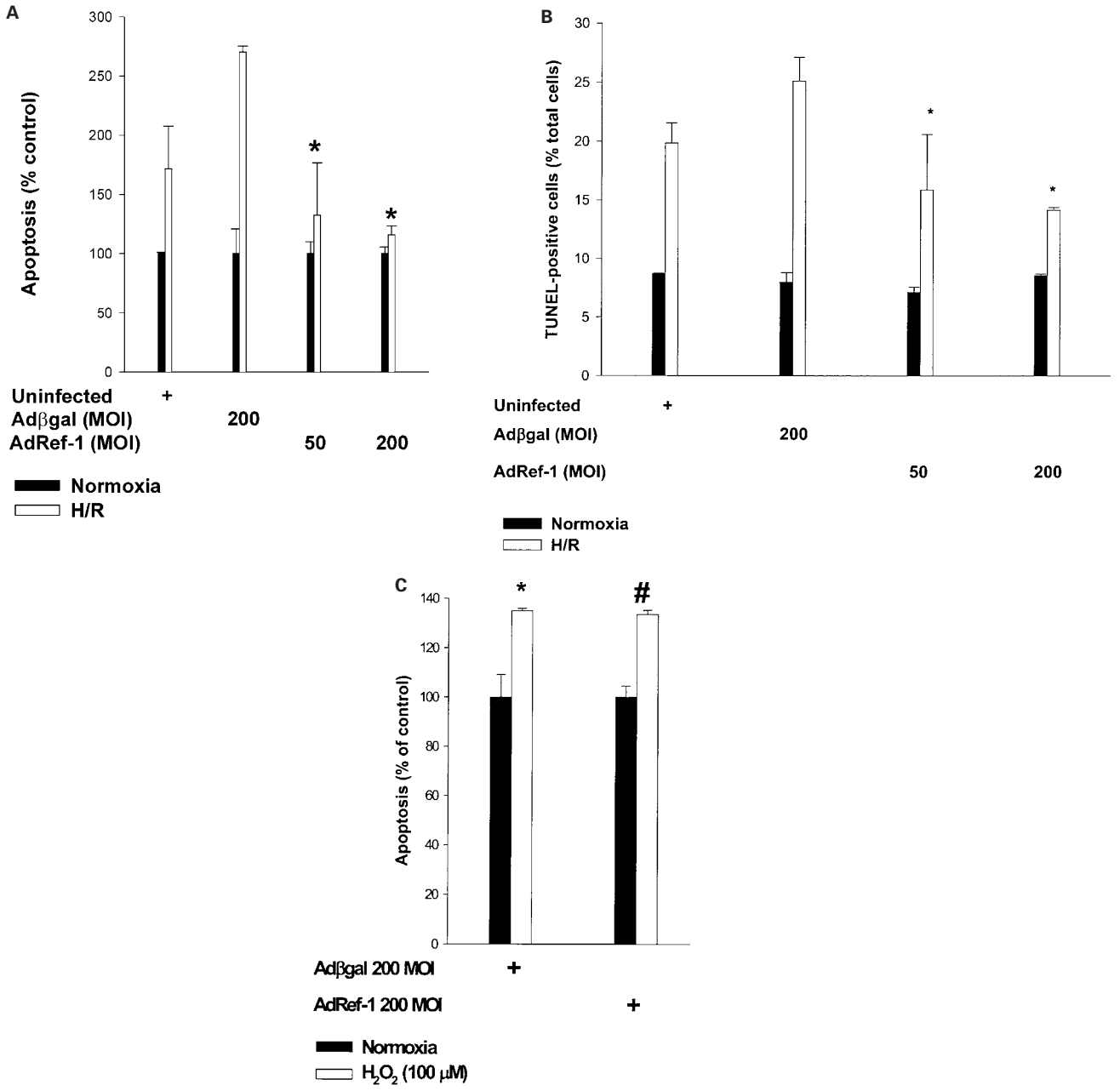

Figure 2 Ref-1 suppresses H/R-induced apoptosis. (**A**) Quantification of cell death (ELISA for cytoplasmic histone-DNA fragments) in uninfected, Adβgal-, and Adref-1-infected cells under normoxia and with H/R. Values are expressed as percent apoptosis compared to normoxic cells. Hypoxia 6 h, reoxygenation 16 h. *$P<0.05$ compared with Adβgal. (**B**) Quantification of apoptosis by TUNEL staining in uninfected, Adβgal-, and Adref-1-infected cells under normoxia and with H/R. *$P<0.05$ compared with Adβgal. (**C**) Quantification by ELISA of apoptosis induced by exogenous H_2O_2 in Adβgal, and Adref-1-infected. cells. Values are expressed as per cent apoptosis in the absence of H_2O_2. *$P<0.05$ compared with Adβgal without H_2O_2. #P=NS compared with Adβgal+H_2O_2. The figures are representative of two separate experiments

ref-1 does not protect cells from apoptosis resulting from a direct increase in intracellular oxidative stress through addition of exogenous H_2O_2. This suggests that ref-1 does not affect H_2O_2 elimination by increasing the expression of cellular anti-oxidant defenses against H_2O_2 such as catalase and glutathione peroxidase, but rather protects against H/R-induced apoptosis through suppression of intracellular H_2O_2 production.

Ref-1 inhibits H/R and TNF-induced oxidative stress

Our next goal was to further explore the mechanism behind ref-1's ability to protect against post-hypoxic death. Therefore, we examined the role of ref-1 in regulating the increase in intracellular oxidative stress in response to H/R (Figure 3A). There was no significant difference in intracellular H_2O_2 levels

between uninfected, Adβgal-infected, and Adref-1-infected cells under basal normoxic conditions. H/R resulted in a marked increase in intracellular H_2O_2 in Adβgal-infected cells. In comparison, cells infected with Adref-1 showed significant reductions in H/R-stimulated intracellular H_2O_2 levels. This demonstrates that overexpression of ref-1 in endothelial cells suppresses H/R-induced intracellular oxidative stress.

Next, we determined whether ref-1's ability to suppress the rise in intracellular H_2O_2 was stimulus-specific. To this end, we examined the effect of ref-1 overexpression on tumor necrosis factor-α (TNF)-induced oxidative stress (Figure 3A). In Adβgal-infected cells TNF resulted in a predictable increase in intracellular H_2O_2 levels. In comparison, cells infected with Adref-1 demonstrated a significant reduction in TNF-induced rise in H_2O_2. Thus, ref-1 inhibits a mechanism that leads to an increase in oxidative stress shared by both H/R, and TNF.

Since overexpression of ref-1 did not protect against apoptosis induced by addition of exogenous H_2O_2, we also determined whether ref-1 had any effect on the elimination of intracellular H_2O_2 (Figure 3B). Addition of exogenous H_2O_2 resulted in a dose-dependent increase in intracellular peroxide in control Adβgal-infected cells. Overexpression of ref-1 did not suppress this rise in intracellular H_2O_2. This further suggests that ref-1 suppresses H/R-induced increase in intracellular H_2O_2 by decreasing production, and not by altering the cell's capacity to eliminate peroxide.

Both TNF and H/R activate an intracellular oxidase regulated by the ubiquitous small GTPase rac1.[3,20] All the components of this multi-molecular NAD(P)H oxidase are expressed in vascular endothelial cells.[2] We therefore determined the role of ref-1 in regulating the production of H_2O_2 by rac1 (Figure 3C). To accomplish this we co-infected cells with Adref-1 and Adrac1V12, an adenovirus encoding the activated allele of rac1. Co-infection with the two viruses resulted in co-expression of ref-1 and rac1V12. Expression of rac1V12 led to an increase in steady state intracellular H_2O_2 levels that was partly mitigated by overexpression of ref-1. This shows that overexpression of ref-1 suppresses rac1V12-stimulated oxidative stress.

Ref-1 overexpression suppresses H/R-induced, rac1-regulated NF-κB activation

The transcription factor nuclear factor-kappa B (NF-κB) is activated in response to oxidative stimuli in general,[21] and H/R or I/R in particular.[22–24] NF-κB activation has been implicated in a variety of inflammatory conditions including vascular inflammation seen with reperfusion injury.[25] We therefore examined the effect of ref-1 overexpression on NF-κB activity (Figure 4A). H/R led to an approximately threefold induction of NF-κB transcriptional activity in both uninfected and Adβgal-infected cells. Infection with Adref-1 resulted in a significant and dose-dependent inhibition of basal, and H/R-induced NF-κB transcriptional activity.

The findings from the transcriptional reporter assay were confirmed with an electrophoretic mobility shift assay (Figure 4B). H/R led to an increase in NF-κB DNA binding activity in control Adβgal-infected cells. In comparison, cells infected with Adref-1 showed significant suppression of

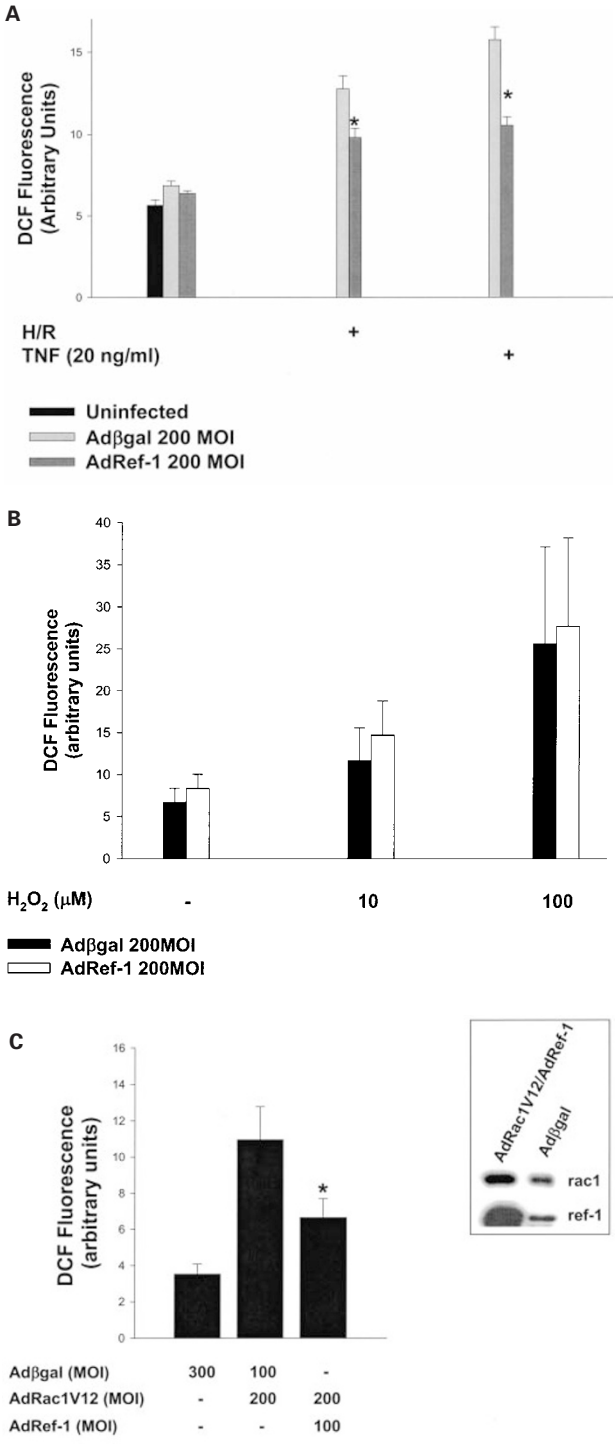

Figure 3 Ref-1 inhibits H/R-induced, and rac1-regulated oxidative stress. (**A**) Intracellular H_2O_2 levels in uninfected, Adβgal-, and Adref-1-infected cells, under normoxia, and with H/R and TNF. *$P<0.05$ compared with Adβgal. Reoxygenation was for 5 min, and TNF 15 min. (**B**) Intracellular H_2O_2 levels 5 min after addition of exogenous H_2O_2 in Adβgal-, and Adref-1-infected cells. (**C**) Intracellular H_2O_2 levels in cells co-infected with Adβgal-, Adrac1V12-, and Adref-1. Inset shows adenoviral-induced co-overexpression of ref-1 and rac1V12. The figures are representative of two separate experiments

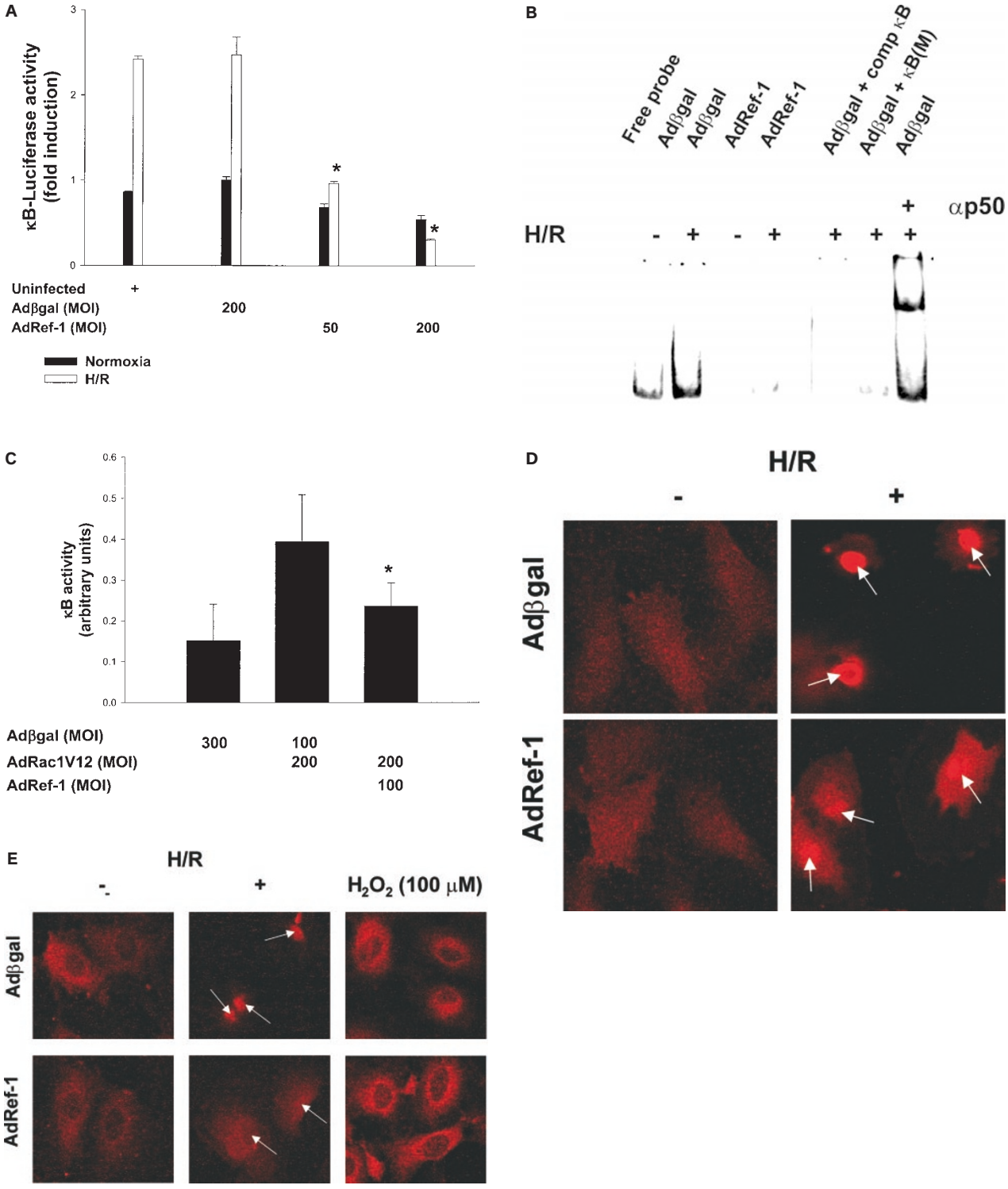

Figure 4 Ref-1 inhibits H/R-induced, rac1-regulated NF-κB activity by inhibiting its nuclear translocation. (**A**) NF-κB transcriptional activity measured by reporter assay in uninfected, Adβgal-, and Adref-1-infected cells. Values are expressed as fold induction compared with Adβgal-infected cells. $^*P<0.05$ compared with Adβgal. The data is representative of three separate experiments. (**B**) EMSA for NF-κB DNA binding in Adβgal-, and Adref-1-infected cells. Where indicated, 100× excess unlabelled κB oligonucleotide (comp κB), mutant κB oligonucleotide (κB (M)), or antibody to the p50 subunit of NF-κB (αp50) were added to the binding mixture. The figure is representative of two separate experiments. (**C**) NF-κB reporter assay in cells co-infected with Adβgal-, Adrac1V12-, and Adref-1. $^*P<0.05$ compared with AdRac1v12/Adβgal. Immunostaining for the p50 (**D**) and p65 (**E**) subunits of NF-κB in Adβgal-, and Adref-1-infected cells under normoxia (**D**,**E**), after H/R (**D**,**E**), and after exogenous H_2O_2 addition (**E**). Reoxgenation and H_2O_2 treatment were for 1 h. Arrows indicate nuclei. The figures are representative of two separate experiments

both basal, and H/R-induced NF-κB DNA-binding activity. Supershift analysis demonstrated that the H/R-stimulated increase in DNA binding activity was primarily due to p50 subunit containing dimers. This shows that overexpression of ref-1 inhibits H/R-induced NF-κB activation in endothelial cells.

Activation of rac1 in response to stimuli including cytokines and I/R is essential for full induction of NF-κB transcriptional activity.[16,26] We therefore also examined whether ref-1 modulates NF-κB activation specifically induced by rac1 (Figure 4C). Expression of the activated rac1V12 allele resulted in significant increase in NF-κB transcriptional activity that was partly abrogated overexpression of ref-1. This, taken together with the inhibitory effect of ref-1 on rac1-stimulated H_2O_2 production, further implies that ref-1 specifically inhibits a component(s) of the rac1-regulated machinery that leads to oxidative stress and NF-κB activation.

We also determined the stage in NF-κB activation affected by ref-1. Basal nuclear and cytoplasmic levels of the p50 and p65 subunits of NF-κB and their re-distribution with H/R were assessed by immunostaining (Figure 4D,E). H/R led to nuclear translocation of both p50 and p65 in control Adβgal-infected cells. In contrast, overexpression of ref-1 suppressed the H/R-stimulated nuclear translocation of p50 and p65, when compared to Adβgal-infected cells. This suggests that ref-1 inhibits the H/R-stimulated signaling mechanism(s) that leads to nuclear translocation of NF-κB.

Finally, we determined the effect of ref-1 overexpression on H_2O_2-stimulated NF-κB re-distribution (Figure 4E). Unlike H/R, addition of exogenous H_2O_2 to endothelial cells did not lead to nuclear translocation of the p65 subunit. However, H_2O_2 did result in strong peri-nuclear localization. In comparison with control Adβgal-infected cells, cells overexpressing ref-1 did not show any difference in this H_2O_2-stimulated re-distribution of NF-κB. This shows that similar to its inability to inhibit exogenous H_2O_2-induced apoptosis, ref-1 is also unable to affect cellular re-distribution of NF-κB in response to addition of exogenous H_2O_2.

Discussion

Reports to date have shown that nuclear ref-1 operates via two mechanisms. Through its N-terminus cysteine residues it modulates transcription by reduction of nuclear transcription factors, and its C-terminus functions as an AP endonuclease. In our efforts to examine the role of ref-1 in endothelial cell apoptosis we uncovered that ref-1 is also capable of inhibiting oxidative stress, most likely by modulating H_2O_2 production, a mechanism hitherto unappreciated. This conclusion is supported by the following findings: (1) overexpression of ref-1 resulted in suppression of H/R and TNF-stimulated, and rac1-induced rise in intracellular H_2O_2, and (2) overexpression of ref-1 did not affect the elimination of exogenously supplied H_2O_2.

The ability of ref-1 to inhibit stimulus-induced oxidative stress provides an additional mechanism for its protective effect against various pro-apoptotic oxidative stimuli. However, it is also possible that the DNA repair and transcriptional-enhancing properties of ref-1 play a role in suppressing cell death. Regardless of the precise mechanism(s) through which ref-1 inhibits apoptosis, its ability to regulate the production of cytoplasmic H_2O_2 can only be explained by an extra-nuclear function.

Ref-1 was first isolated from nuclear fractions of HeLa cells.[27] Although ref-1 does have a nuclear localizing signal at its N-terminus, *in vitro* studies have also demonstrated localization to the cytoplasm.[13] Moreover, numerous studies show that *in vivo*, ref-1 has a differential cellular and subcellular expression pattern[28–31] suggesting a potential physiologic extra-nuclear role. Using an overexpression strategy we were able to uncover one such extra-nuclear function. In endothelial cells, the oxidative stimulus H/R 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 cytoplasmic to nuclear translocation of ref-1.[32] In contrast, adenoviral-driven change in nuclear/cytoplasmic ref-1 under normoxia, and particularly after H/R, was associated with suppression of oxidative stress, and NF-κB translocation. It is worth pointing out that Adref-1 did lead to a marked increase in nuclear ref-1. However, this did not translate to an increase in H/R-induced NF-κB DNA binding or transcriptional activity. This is explained by the temporal sequence of NF-κB activation. DNA binding and transcription occur after, and are dependent upon, nuclear translocation. In addition, although this report focuses on ref-1's regulation of oxidative stress as a mechanism for it's effect on NF-κB activation, it is worth considering that cytoplasmic ref-1 may also, directly or indirectly, affect other steps in the NF-κB activation sequence such as the activity of IκB kinases, or binding affinity to IκB.

Arguably, the most intriguing finding of this study is that ref-1 suppresses rac1-induced oxidative stress and NF-κB activation. It is important to emphasize that our data show a functional interaction between ref-1 and rac1, and do not suggest a direct association between these two proteins. Nonetheless, the observation that both TNF and H/R-induced oxidative stress was inhibited by ref-1 is also consistent with such a functional interaction, since both these oxidative stimuli lead to ROS production, at least partly through a rac1-regulated NAD(P)H oxidase.[3,20] Importantly, all the components of this oxidase are expressed in endothelial cells. In this regard, it is noteworthy that ref-1 physically binds with thioredoxin (TRX), another redox-regulatory protein.[33] TRX is capable of binding to p40phox, a component of the phagocyte cytoplasmic NADPH oxidase, and critical thiol residues on TRX are necessary for this interaction.[34] This raises the interesting possibility that cytoplasmic ref-1 via its critical cysteines, alone or in concert with TRX, may also modulate the activity, of one or more components of a rac1-regulated NAD(P)H-dependent oxidase expressed in endothelial cells. However, it would also be fair to say that the effect of ref-1 in modulating rac1-regulated H_2O_2 production does not exclude other mechanisms by which it may inhibit cytoplasmic oxidative stress. Notably, emerging evidence suggests that ref-1 is also localized to the mitochondria,[35,36] a major site of production of H_2O_2 and other ROS

that have been implicated in apoptosis. Furthermore, independent of oxidant production, extra-nuclear ref-1 may also participate in other important cellular functions and processes (reviewed in[37–42]).

Ref-1 activates p53 to bind to DNA,[10] and promotes the *trans*-activating and pro-apoptotic functions of p53.[43] At first, this seems to contradict our observation that it has a protective role in H/R-induced apoptosis. However, most *in vitro* and *in vivo* studies have shown that hypoxia, and H/R-induced apoptosis occur independent of p53 status.[17,44] Moreover, prior studies examining the role of ref-1 in apoptosis have not distinguished between cytoplasmic and nuclear expression. Thus the manner in which ref-1 modulates apoptotic death may depend on its cellular compartmentalization and be stimulus-specific.

A recent report examined the role of ref-1 in endothelial cell NF-κB activity and apoptosis.[45] This report showed that total cellular ref-1 decreased after severe (18 h) of hypoxia, and that overexpression of ref-1 (using a liposome-based transfection protocol) resulted in an increase in NF-κB activation, and suppression of and TNF/hypoxia-induced apoptosis. However, the subcellular distribution of exogenous and endogenous ref-1, with or without oxidative stimuli (hypoxia or TNF), was not examined by the authors. Nor did the authors attempt to determine whether the degree of ref-1 down-regulation or overexpression correlates with the extent of oxidative stress resulting from TNF or hypoxia. Our novel observations that forced adenoviral-mediated overexpression of ref-1 leads to (1) suppression of oxidative stress, and (2) inhibition of NF-κB activation add new insights into the role of ref-1 in endothelial cell biology, and are in contradiction with the findings of the above-mentioned report. The different conclusions of the two studies may be a result of differences in methodological approaches to overexpression coupled with differences in the degree of scrutiny regarding the sub-cellular compartmentalization of ref-1 in endothelial cells.

In summary, this report shows that ref-1 has an important function in suppressing intracellular oxidative stress and apoptosis in endothelial cells, through modulation of a rac1-regulated oxidase, implicating an extra-nuclear function of ref-1 (Figure 5). Similar observations in an animal model of hepatic ischemia/reperfusion injury (data not shown) suggest that this extra-nuclear function may not be limited to endothelial cells. Moreover, taken in conjunction with other reports, our data suggests a paradigm in which ref-1 plays dual and opposing roles in the regulation of NF-κB activation. Cytoplasmic ref-1, through the feedback mechanism resulting in decreased ROS (H_2O_2) production, inhibits rac1-stimulated, redox-regulated nuclear translocation of NF-κB. Once in the nucleus, by maintaining NF-κB in the reduced state, and thereby enhancing its ability to bind DNA, ref-1 promotes κB-driven transcription. Therefore levels of nuclear *versus* cytoplasmic ref-1, and the ability of the cell to regulate these levels in response to oxidative stimuli, may well determine its net effect on NF-κB activity. Importantly, a similar dual function of TRX in NF-κB and heat shock factor activation has been shown.[46,47] Thus, such spatial and temporal regulation of transcription factor activity by redox proteins may represent a common theme in the cellular response to oxidative stimuli.

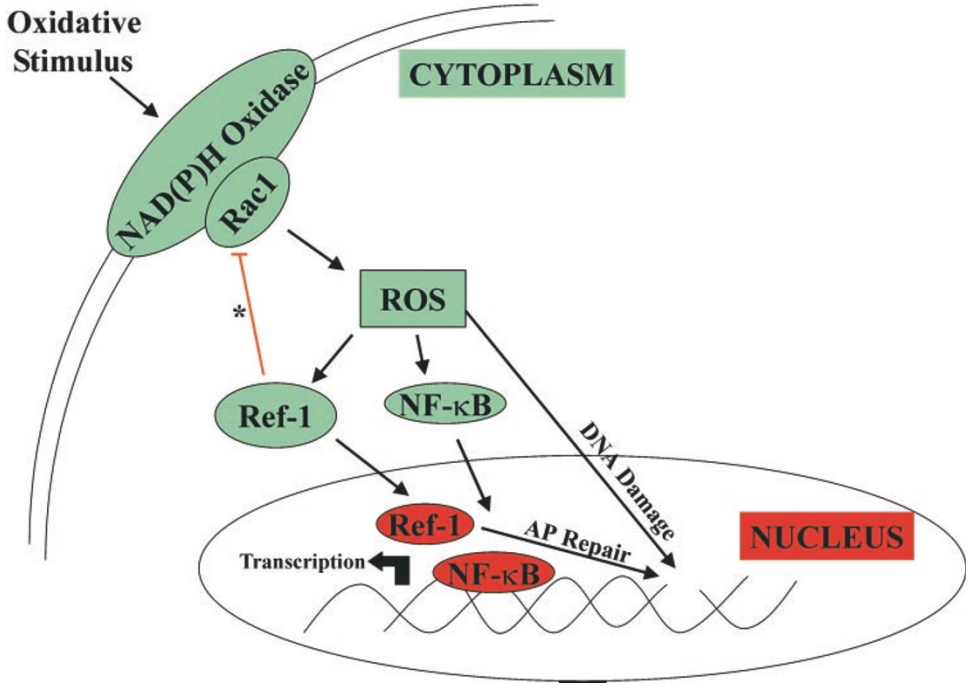

Figure 5 Nuclear and Cytoplasmic Roles of ref-1. Summary diagram illustrating the cytolasmic and nuclear effects of ref-1. *Signifies the role of ref-1 in suppressing rac1-regulated ROS production demonstrated in this study. Figure does not include other potential mechanisms by which ref-1 may suppress NF-κB activation, nor does it include other potential extra-nuclear actions of ref-1. AP: Apurinic/Apyrimidinic

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA, USA) and were grown and maintained in endothelial growth medium. Cells were passaged by standard trypsinization, and passages 2–7 were used in all experiments.

Hypoxia/reoxygenation and quantification of apoptosis

Hypoxia (6 h) and reoxygenation were attained as previously described[48] in a hypoxia chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA). Apoptosis was quantified according to a previously described method[3] using an ELISA that detects cytoplasmic histone-DNA fragments (Cell Death Detection ELISA, Boehringer Mannheim).

Adenoviruses

Recombinant replication-deficient adenoviruses were used in all experiments. Adref-1 encoding the ref-1 cDNA was constructed by homologous recombination in HEK293 cells. Adβgal, encoding the inert *E. Coli* lacZ gene, and Adrac1V12, encoding the constitutively active rac1 GTPase, have been described previously.[3] Adβgal-infected cells, or uninfected cells were used as controls in all experiments. Recombinant adenoviruses were propagated, purified, and tittered, and HUVECs were infected at the specified multiplicity of infection (MOI) as previously described.[3]

Western blotting and immunostaining

Nuclear and cytoplasmic protein was extracted and fractionated as previously described.[49] Western blots were performed with an antibody against ref-1 (SC-334, Santa Cruz Biotechnology), or rac1 (23A8, UBI) and bound immunocomplexes were visualized by enhanced chemiluminescence (Amersham). In some experiments, mouse monoclonal antibody against α-tubulin (DM1A, Sigma) was used to confirm equal protein loading. Immunostaining employed primary antibodies against ref-1, p50 (SC-7178, Santa Cruz Biotechnology), or p65 (SC-372, Santa Cruz Biotechnology), and secondary fluorescein-tagged antibody that were used according to the manufacturer's recommendations. Images were captured on a Zeiss confocal laser-scanning microscope.

Measurement of intracellular reactive oxygen species

Intracellular H_2O_2 was detected with the peroxide-sensitive fluorophore 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) as previously described.[3] The fluorescence was measured after 5 min of incubation with DCF using excitation and emission wavelengths of 488 and 515 nm, respectively. Absolute fluorescence of 20–25 random cells was quantified with MetaMorph™ software. Values are mean $\pm$ S.D.

NF-κB reporter assay

HUVECs were co-transfected with 1.5 μg of a luciferase or CAT reporter plasmid containing three κB binding sites, and 0.75 ug of constitutive CAT or luciferase expression plasmid, using LipofectAMINE™ reagent (Life Technologies) according to the manufacturer s instructions. After 16 h of reoxygenation luciferase and CAT activities in cell lysates were determined and normalized against each other to account for variations in transfection efficiency. All conditions were performed in triplicate and values are mean $\pm$ S.D.

Electrophoretic mobility shift assay (EMSA)

EMSA for NF-κB DNA-binding activity 2 h after reoxygenation were carried out as previously described[3] using a κB consensus sequence oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGGC-3′). In supershift experiments, antibody to p50 subunit was added to nuclear extracts for 30 min on ice prior to addition of labeled oligonucleotide.

Statistical analysis

Data are expressed as mean $\pm$ S.D. Differences in measured variables between experimental and control groups were determined using a two-tailed paired *t*-test. $P<0.05$ was considered to be statistically significant.

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