Overexpression of Ref-1 Inhibits Hypoxia and Tumor Necrosis Factor–Induced Endothelial Cell Apoptosis Through Nuclear Factor-κB–Independent and –Dependent Pathways

Jennifer L. Hall, Xiaohong Wang, Van Adamson, Ying Zhao, Gary H. Gibbons

Abstract—We hypothesized that a redox-sensitive transcription factor, redox factor-1 (Ref-1) (HAP1, APE, and APEX), was critical in the regulation of endothelial cell survival in response to hypoxia and cytokines, including tumor necrosis factor (TNF)-α. Hypoxia resulted in a significant decrease in Ref-1 protein expression in both human umbilical vein endothelial cells and calf pulmonary artery endothelial cells. The hypoxia-induced decrease in Ref-1 expression was followed by a significant induction of apoptosis as measured by caspase 3 activity and nuclear morphology. Transient upregulation of Ref-1 significantly inhibited hypoxia-induced apoptosis. However, deletion of the redox-sensitive domain of Ref-1 abolished the antiapoptotic effect. We postulated that the antiapoptotic effects of Ref-1 were mediated through nuclear factor-κB (NF-κB). However, blockade of NF-κB with a dominant-negative IκB (S32A/S36A) expression vector had no effect on Ref-1–mediated survival under hypoxic conditions. The second aim of this study was to test the cytoprotective ability of Ref-1 upregulation in response to TNF-induced apoptosis. Ref-1 inhibition of TNF-induced death was associated with a significant potentiation of NF-κB activity. Deletion of the redox-sensitive domain of Ref-1 significantly inhibited TNF-induced NF-κB activation. Moreover, loss of the redox-sensitive domain also abolished the antiapoptotic effect of Ref-1 in response to TNF. To test whether Ref-1 induced activation of NF-κB was necessary to promote survival, we blocked NF-κB activity with a dominant-negative IκB (S32A/S36A). Indeed, blockade of NF-κB activity abolished the ability of Ref-1 to rescue TNF-induced apoptosis. In conclusion, upregulation of Ref-1 promotes endothelial cell survival in response to hypoxia and TNF through NF-κB–independent and NF-κB–dependent signaling cascades, respectively. Moreover, it seems that Ref-1 may act as a critical cofactor, mediating the TNF-induced NF-κB response in the vascular endothelium. (Circ Res. 2001;88:1247–1253.)

Key Words: endothelium • apoptosis • hypoxia • redox • nuclear factor-κB

Vascular endothelial cell apoptosis plays an important role in angiogenesis and vascular remodeling.1–8 Disruption of the endothelium in response to stroke, diabetes, ischemia, hypertension, and vascular injury is induced in part through activation of proapoptotic regulatory pathways.1–7 Moreover, recent evidence suggests that prevention of endothelial apoptosis may improve angiogenesis and endothelial function.6,8 Thus, defining novel genes expressed in the endothelium may lead to a better understanding of the signaling pathways mediating endothelial cell fate under conditions of angiogenesis and vascular remodeling.

Redox factor-1 (Ref-1) (also known as APE,9 HAP1,10 and APEX11) is a ubiquitous, multifactorial protein that is a redox-sensitive regulator of multiple transcription factors, including nuclear factor-κB (NF-κB), c-myc, AP-1, and HIF-1α9–11 as well as an apurinic/apyrimidinic endonuclease in the base excision repair pathway. The potential importance of Ref-1 gene expression as a critical determinant of cell fate is underscored by the fact that mice lacking a functional Ref-1 gene die during embryonic development, with embryos exhibiting a preponderance of pyknotic cells.15 In addition, cell lines expressing an antisense Ref-1 transcript exhibit a striking increase in sensitivity to oxidative stress.16 To our knowledge, the role of Ref-1 as a determinant of endothelial cell fate has not been described.

The endothelium plays a central role in modulating the tissue response to ischemic injury. In the context of ischemic injury, endothelial cell viability may be compromised by hypoxia and the release of cytokines by inflammatory cells. We hypothesized that Ref-1 plays a critical role in the regulation of endothelial cell fate in response to pathophysiological stimuli, such as hypoxia and tumor necrosis factor (TNF). The present study demonstrates that the transient upregulation of Ref-1 significantly inhibits both hypoxia and TNF-mediated endothelial cell apoptosis. Moreover, NF-κB activation seems to be a critical yet context-specific down-
stream signal of Ref-1–mediated survival. Deletion of the redox-sensitive domain of Ref-1 significantly inhibits NF-κB activity and abolishes this antiapoptotic effect. Taken together, our data suggest an important role for Ref-1 in the vascular endothelium.

Materials and Methods

Cells
Human umbilical vein endothelial cells (HUVECs) (Clonetics) were grown in endothelial growth media (Clonetics) that contains bovine brain extract (12 μg/mL), FBS (2%), hydrocortisone (1 μg/mL), human epidermal growth factor (10 μg/mL), gentamicin (100 μg/mL), and amphotericin-B (0.05 μg/mL) and used between passages 2 and 5.

Calf pulmonary artery endothelial (CPAE) cells (ATCC) were grown in DMEM, 10% FBS, and 1% penicillin/streptomycin (Gibco, Life Technologies) and used between passages 18 and 26.

Materials
The following materials were used: human recombinant TNF-α (Genzyme), BCA protein assay (Biorad), Hoechst 33342 (Molecular Probes) enhanced green fluorescent protein expression vector (pEGFP-C1) (Clontech), Mito tracker red expression vector (pDsRed1-Mito) (Clontech), pCB6-Ref-1 (kind gift from T. Curran, St. Jude Children’s Research Hospital, Memphis, Tenn), IκB dominant-negative expression vector (S32A/S36A) (Upstate Biotechnology), pDNA3.1 expression vector (Invitrogen), a previously described Ref-1 deletion mutant that abolishes all redox activity,17 pNF-κB–luciferase vector and thymidine-kine-luciferase vector (negative control) (Clontech), Effectene (Qiagen), Mito tracker red expression vector (Clontech), protease inhibitor cocktail (Roche Molecular Biochemicals), anti-Ref-1 (Santa Cruz Technologies), goat anti-rabbit HRP-linked secondary antibodies (Transduction Labs), vimentin monoclonal antibody (Sigma), chemiluminescent detection reagent (Amersham Pharmacia Biotech) caspase 3 cleavage activity assay (Biovision), and DEVD caspase 3 peptide blocker (DEVD-FMK) (Biovision).

Transfection
All transfections were carried out in CPAE cells using a lipid-based transfection strategy (Effectene). Transfection efficiency was ~25% to 30%. The previously described Ref-1 deletion mutant lacking the N-terminal 116 amino acids that abolishes all redox activity17 was constructed by amplifying 591 bp of Ref-1 with the following primers: 9'-GGTACAGTGCTAGGTATAGGGTGATAGG-3' and 9'-GTAAAGCTTATGGATCAATACTGGTCAGCTCCTTCGG-3'. The product was digested with HindIII and EcoRI, sequenced, and cloned into pDNA3.1.

CPAE cells were cotransfected with Effectene in the presence of growth media with one of the following expression vectors: pDsRed1-Mito or pEGFP-C1 (fluorescent markers) and pDNA3.1 (control), pCB6-Ref-1, or a previously described Ref-1 deletion mutant.17

A subset of experiments involved cotransfection of pCB6-Ref-1 or the control expression vector (pDNA3.1) with an IκB (S32A/S36A) dominant-negative expression vector.18 Twenty-four hours after transfection, CPAE cells were exposed to hypoxic (2% oxygen) or normoxic (21% oxygen) conditions while in the presence of growth media (DMEM+10% FBS) for 24 hours. Alternatively, CPAE cells were treated with human TNF in DMEM under normoxic conditions for 24 hours. Apoptotic nuclei were assessed with Hoechst 33342 staining in the transfected (pDsRed1-Mito or pEGFP-C1) population as previously described.19

NF-κB Activity
NF-κB activity was determined by transiently transfecting CPAE cells with the pNF-κB–luciferase vector driven by a herpes simplex virus thymidine kinase promoter or the thymidine kinase–luciferase vector (negative control) along with pEGFP-C1 and pCB6-Ref-1, the Ref-1 redox-sensitive deletion mutant, or a control (pCDNA3.1) vector. The pNF-κB–luciferase vector contains the κ enhancer element within the promoter region. Once activated, endogenous NF-κB binds to the κ enhancer element and activates luciferase expression. At 24 hours after transfection, CPAE cells were exposed to hypoxia for 2, 4, 8, and 24 hours under conditions of DMEM plus 10% FBS or treated with TNF in DMEM (serum-free media) for 5 hours. Luciferase activity was measured with a luminometer (Victor II, Wallac) and normalized by assessing EGFP fluorescence (Victor II, Wallac). All data are expressed as fold activation of luciferase activity/EGFP fluorescence over control-transfected cells under baseline conditions.

Initial experiments confirmed that transfection with a control vector containing the herpes simplex virus thymidine kinase promoter along with luciferase in the absence of the κ enhancer elements had no significant effect on luciferase activity.

Western Blotting
CPAE cells and HUVECs were lysed with RIPA buffer (containing, in mmol/L, NaCl 150, Tris 10, EDTA 1, and PMSF 1 and 1% Triton X-100 and 1% deoxycholic acid) containing a protease-inhibitor cocktail, centrifuged, and assayed for total protein. Equal amounts of cell lysates were loaded on 12% SDS gels, transferred to nitrocellulose membranes, and probed as previously described.19 As an additional control, blots were reprobed with vimentin to confirm equal loading as well as to verify that the decrease in Ref-1 protein expression was not attributable to an overall decrease in protein synthesis. Finally, all membranes were stained with Ponceau Red to verify equal protein loading and equal transfer of all protein to the membrane.

Apoptosis
Both CPAE cells and HUVECs were grown on 6-well plates to near confluence for all apoptosis experiments. For the hypoxia experiments, CPAE cells were placed in DMEM plus 10% FBS and HUVECs were placed in endothelial basal media supplemented with bovine brain extract. These media conditions were chosen to minimize basal apoptosis. Both CPAE cells and HUVECs were then placed in an incubator containing 2% oxygen (hypoxia) or 21% oxygen (normoxia) for 24 hours. It is noted that a separate series of experiments were completed in which HUVECs were also placed in DMEM plus 10% FBS (similar to CPAE cells) and subjected to hypoxia (data not shown). Results were similar under both media conditions.

For TNF-induced experiments, CPAE cells were placed in DMEM in the absence of FBS and HUVECs in endothelial basal media supplemented with bovine brain extract and treated with TNF or PBS for 24 hours. Apoptosis was assessed by staining with the nuclear chromatin dye H33342 and quantitating the percentage of apoptotic nuclei in each sample (400 cells counted per sample). As an additional assay of apoptotic cell death, caspase 3 activity was assessed using a fluorometric caspase 3 cleavage activity assay according to the manufacturer’s directions.19 Caspase 3 cleavage activity is expressed as a normalized ratio of arbitrary fluorophore units integrable by DEVD-FMK in treated cells divided by caspase activity in untreated cells. Our laboratory has performed extensive validations of these assays in vivo and in vitro with other techniques for assessing apoptosis.19–21

Statistics
Comparisons between two groups were analyzed via Student’s t test (P<0.05), and comparisons between three groups were analyzed by ANOVA with a Student-Newman-Keuls posthoc test (P<0.05). n indicates the total number of replicates in multiple experiments. Data are presented as mean±SE.
hypoxia.

B, Identical blot as in panel A, reprobed for the structural protein vimentin to demonstrate specific decrease of Ref-1 expression in relation to another endogenous protein. C, Ref-1 protein in CPAE cells at 6 hours of hypoxia.

The hypoxia-induced decrease in Ref-1 protein expression was followed by a significant induction of apoptosis in CPAE cells at 24 hours as measured by assessing nuclear chromatin morphology and determining caspase 3 activity. In CPAE cells, hypoxia resulted in a significant increase in the percentage of apoptotic nuclei (normoxia, 1.00±0.01; n=6, P<0.001). These data suggest that endogenous Ref-1 likely plays a critical protective role in the endothelium. Moreover, they suggest that this Ref-1 redox deletion mutant may act as a dominant-negative construct, competing with and blocking endogenous Ref-1. Future studies will need to be completed to clarify this.

Figure 1. Representative Western blots for Ref-1 and vimentin after hypoxia in CPAE cells and HUVECs. A, Ref-1 protein in CPAE cells is significantly decreased after 6 hours of hypoxia. B, Identical blot as in panel A, reprobed for the structural protein vimentin to demonstrate specific decrease of Ref-1 expression in relation to another endogenous protein. C, Ref-1 protein in CPAE cells at 6 hours of normoxia and at 6 and 18 hours of hypoxia.

Results

Hypoxic Injury: Modulators of Endothelial Cell Fate

Hypoxia induced a decrease in Ref-1 protein expression in both CPAE cells and HUVECs (Figure 1). Ref-1 protein expression was significantly decreased after 6 hours of hypoxia in CPAE cells (Figure 1A) and at 18 hours in HUVECs (Figure 1C). The differences in the time course of decay in Ref-1 protein expression may likely be attributed to the different origin of the cell types: pulmonary aortic (CPAE cells) or umbilical vein (HUVECs). The loss of Ref-1 protein expression in response to hypoxia was not attributable to a general decrease in protein synthesis, because vimentin expression was unaffected (Figure 1B).

The hypoxia-induced decrease in Ref-1 protein expression was followed by a significant induction of apoptosis in CPAE cells and HUVECs at 24 hours as measured by assessing nuclear chromatin morphology and determining caspase 3 activity. In CPAE cells, hypoxia resulted in a significant increase in the percentage of apoptotic nuclei (normoxia, 5±1%; hypoxia, 15±1%; n=6, P<0.001). In accord with the significant increase in the condensed and coalesced nuclei, caspase 3 activity was significantly upregulated in CPAE cells after hypoxia (normoxia, 1.00±0.01 normalized ratio of arbitrary fluorophore units inhibitable by the DEVD caspase 3 blocker DEVD-FMK; hypoxia, 1.60±0.02; n=12, P<0.001). HUVECs also underwent apoptosis in response to hypoxia as measured by caspase 3 activity (normoxia 1.02±0.02; hypoxia, 1.13±0.01; n=12, P<0.001). We saw no significant change in the percentage of apoptotic nuclei at 8 or 16 hours in either the CPAE cells or HUVECs, suggesting that the decrease in Ref-1 protein expression precedes the induction of apoptosis.

We hypothesized that downregulation of Ref-1 may be permissive in promoting apoptosis in response to hypoxia. Accordingly, we examined whether upregulation of Ref-1 would modulate endothelial cell fate. The efficacy of the transfection of Ref-1 is demonstrated by the significant increase in Ref-1 protein expression (Figure 2). In accord with our hypothesis, augmentation of intracellular Ref-1 concentrations significantly inhibited hypoxia-induced apoptosis in CPAE cells (control-transfected, 26±2% versus Ref-1, 16±1%; n=8, P<0.001) (Figure 3).

To begin to identify the region of Ref-1 responsible for this antiapoptotic effect, we used a previously described Ref-1 deletion mutant in which the well-defined redox-sensitive domain pertaining to the N-terminal 116 amino acids had been deleted.17 Interestingly, the redox deletion mutant abolished the antiapoptotic effect of Ref-1 under hypoxic conditions and, in fact, potentiated the death response (control-transfected, 26±2%; Ref-1, 16±1%; Ref-1 redox deletion mutant, 42±2%; n=8, P<0.001). Upregulation of the redox deletion mutant also resulted in significant potentiation of death under normoxic conditions (control-transfected, 17±1%; Ref-1, 12±2%; Ref-1 redox deletion mutant, 42±4%; n=8, P<0.001). These data suggest that endogenous Ref-1 likely plays a critical protective role in the endothelium. Moreover, we suggest that this Ref-1 redox deletion mutant may act as a dominant-negative construct, competing with and blocking endogenous Ref-1. Future studies will need to be completed to clarify this.

Ref-1 has been shown to activate NF-κB12,13; thus, we hypothesized that the ability of Ref-1 to inhibit hypoxia-mediated apoptosis was mediated through potentiation of NF-κB activity. This hypothesis was based in part on previous studies demonstrating activation of NF-κB in response to hypoxia in endothelial cells.22–24 In accord with our postulate, Ref-1 significantly potentiated NF-κB transactivation in CPAE cells with a luciferase-based reporter system under hypoxic conditions (results expressed as fold activation over control cells of luciferase activity normalized to EGFP fluorescence) (control-transfected, 1.00±0.05; Ref-1–transfected, 1.27±0.02; n=6, P<0.001). However, under our
conditions of 2% hypoxia, NF-κB was not significantly activated above baseline in CPAE cells at multiple time points (2, 6, 8 and 24 hours of hypoxia) (data not shown). In line with this data, blockade of NF-κB with a dominant-negative IκB construct did not have any effect on hypoxia-induced apoptosis. Moreover, it had no significant effect on the ability of Ref-1 to protect CPAE cells from apoptosis induced by hypoxia; Ref-1 upregulation resulted in a 28±7% reduction in apoptotic nuclei, whereas Ref-1 in the presence of the dominant-negative IκB construct resulted in a 44±10% reduction. Taken together, our data suggest that NF-κB does not play a major role in promoting endothelial cell survival in response to 2% hypoxia and suggest that the survival-promoting effects of Ref-1 in the setting of hypoxia are mediated through an NF-κB–independent pathway.

Cytokine Injury: Modulations of Endothelial Cell Fate

To determine if Ref-1 was able to promote endothelial cell survival in response to other pathobiological stimuli present in the context of ischemic injury, we exposed HUVECs and CPAE cells to human TNF for 24 hours. TNF induced a dose-dependent apoptotic response in HUVECs as measured by H33342 (Figure 4). TNF induced a similar dose-dependent effect in CPAE cells (vehicle, 7±1%; 0.25 ng/mL TNF, 16±1%; 0.4 ng/mL TNF, 18±2%; 1 ng/mL TNF, 23±1%; 40 ng/mL TNF, 27±1%; n=10, P<0.001). As another means of confirming the apoptotic response, we measured caspase 3 activity. Indeed, caspase 3 activity was significantly enhanced in CPAE cells treated with TNF (control, 1.00±0.02 versus 1.32±0.01; n=5, P<0.001).

Ref-1 gene expression was transiently upregulated in CPAE cells to test our postulate that Ref-1 was an important mediator of endothelial cell fate in response to TNF. Augmentation of intracellular Ref-1 concentrations significantly inhibited apoptosis induced by TNF (control-transfected, 13%; Ref-1-transfected, 22%; n=13, P<0.001) (Figure 5).

In contrast to the response to hypoxia, it is noteworthy that TNF did not alter Ref-1 protein expression in either CPAE cells or HUVECs in a detailed time-course analysis (3 to 24 hours) (Figure 6). This suggests that endogenous Ref-1 expression may be sufficient to maintain cell viability under normal conditions but insufficient in response to the potent cytokine TNF.

On the basis of recent studies, we postulated that the ability of Ref-1 to inhibit TNF-induced endothelial apoptosis was attributable in part to activation of NF-κB. Treatment of CPAE cells with TNF induced a significant upregulation of NF-κB activity (Figure 7). In accord with our hypothesis, augmentation of Ref-1 expression resulted in a significant additive increase in NF-κB activity at baseline and in response to TNF (Figure 7).

To additionally delineate the role of NF-κB as a distal downstream signaling element critical to Ref-1–mediated survival in response to TNF, we used a Ref-1 deletion mutant in which the well-defined redox-sensitive domain pertaining to the N-terminal 116 amino acids had been deleted.17 Loss of the redox-sensitive domain abolished Ref-1–induced NF-κB activation (Figure 7). These data suggest that Ref-1 may be a necessary cofactor mediating TNF-induced NF-κB activation. Moreover, the cytoprotective effect of Ref-1 in response to TNF was abolished in experiments involving the Ref-1 redox-sensitive deletion mutant (control-transfected cells, 37±1% apoptotic nuclei; Ref-1-transfected, 20±2%; Figure 6).

Figure 4. TNF induces a dose-dependent increase in the percentage of apoptotic nuclei in HUVECs (vehicle, 6±1%; 0.4 ng/mL TNF, 12±1%; 1 ng/mL TNF, 14±1%; 4 ng/mL TNF, 16±1%; 10 ng/mL TNF, 20±1%; 40 ng/mL TNF, 19±1%; n=8, P<0.001). Data are expressed as mean±SE.

Figure 5. Transient upregulation of Ref-1 inhibits apoptosis induced by TNF. CPAE cells were transiently cotransfected with the reporter gene pDSRed1-Mito along with either pcDNA3.1 (control) or Ref-1. The percentage of apoptotic nuclei in the pDSRed1-Mito–positive subset of transfected cells was quantitated (control-transfected, 43±2%; Ref-1–transfected, 22±2%; n=13, P<0.001). Data are expressed as mean±SE.

Figure 6. Western blot demonstrating endogenous Ref-1 protein expression is unchanged in response to TNF in HUVECs at 6 and 9 hours.

Figure 7. NF-κB activation (Figure 7). These data suggest that Ref-1 may be a necessary cofactor mediating TNF-induced NF-κB activation. Moreover, the cytoprotective effect of Ref-1 in response to TNF was abolished in experiments involving the Ref-1 redox-sensitive deletion mutant (control-transfected cells, 37±1% apoptotic nuclei; Ref-1-transfected, 20±2%; Ref-1
TNF, 2.69, which the N-terminal 116 amino acids corresponding to the gene (pcDNA3.1), pCB6-Ref-1, or a Ref-1 deletion mutant in with the reporter gene, pEGFP-C1, along with a control trans-

cates NF-κB–mediated endothelial cell survival is independent of NF-κB activation. In contrast, Ref-1 potentiation of an NF-κB–signaling cascade seems to be critical in mediating the antiapoptotic effect of Ref-1 in response to TNF. Thus, NF-κB activation seems to play an important yet context-

specific role as a downstream signaling target in the antiapoptotic function of Ref-1 in the vascular endothelium.

Ref-1 is a multifactorial protein involved in both DNA repair as well as redox-mediated transcriptional events, including the activation of transcription factors NF-κB, AP-1, c-myb, and members of the ATF/CREB family.12,13,17 The N-terminal 116 amino acid domain, partially absent in its bacterial homolog exonuclease III, has been shown to be responsible for its redox regulation, whereas the C-terminal domain is largely responsible for its DNA repair activity.17 Several studies have documented that reducing agents, including dithiothreitol, thioredoxin (upstream activator of Ref-1), and Ref-1, stimulate DNA-binding activity of the p50 subunit of NF-κB.12,13,25,26 Moreover, Walker et al27 have identified a single cysteine residue (cysteine 65) as being the redox-active site of Ref-1, thereby additionally substantiating that the region of Ref-1 required to activate NF-κB is located in the N-terminal region. Our data suggest that loss of the redox domain abolishes the antiapoptotic effects of Ref-1 in response to TNF and hypoxia. Moreover, upregulation of the Ref-1 redox deletion mutant actually potentiated death under these conditions as well as under control conditions. Al-

Figure 7. Transient upregulation of Ref-1 in CPAE cells potenti-
ates NF-κB activity. CPAE cells were transiently cotransfected with the reporter gene, pEGFP-C1, along with a control trans-
gen (pcDNA3.1), pCB6-Ref-1, or a Ref-1 deletion mutant in which the N-terminal 116 amino acids corresponding to the redox-sensitive domain have been deleted and the NF-κB–lucif-
erase reporter construct. CPAE cells were exposed to TNF for 5 hours in DMEM. Results are expressed as fold activation over control-transfected cells at baseline (control-transfected cells at baseline, 1 ± 0.06; control-transfected cells + TNF, 3.5 ± 0.41; Ref-
1–transfected cells at baseline, 2.13 ± 0.27; Ref-1–transfected + TNF, 6.89 ± 0.91; Ref-1 deletion mutant at baseline, 0.88 ± 0.06; Ref-1 deletion mutant + TNF, 1.66 ± 0.14; n = 12, P < 0.001). Data are expressed as mean ± SE.

Figure 8. Blockade of NF-κB activation with IκB (S32A/S36A) abolished the Ref-1–mediated inhibition of TNF-induced apoptosis. CPAE cells were transiently cotransfected with IκB (S32A/ S36A) or a control transgene (pcDNA3.1) and with pCB6–Ref-1. Cells were treated with TNF, and apoptosis was quantitated in the transfected subset (control-transfected, 45 ± 3% apoptotic nuclei; Ref-1–transfected, 19 ± 2%; Ref-1 + IκB (S32A/S36A), 36 ± 3%; control–IκB (S32A/S36A), 51 ± 2%; n = 6, P < 0.001). Data are expressed as mean ± SE.

Discussion

Increasing evidence points to a role for apoptotic regulatory pathways as critical determinants in the progression of angiogenesis and vascular remodeling. Moreover, prevention of endothelial apoptosis improves angiogenesis after ischemia and preserves endothelial dysfunction in disease states such as diabetes.6–8 We have demonstrated a critical antiapo-

potentiation of the activation of NF-κB.

Deletion mutant, 51 ± 4%; n = 10, P < 0.001). It is noteworthy that loss of the redox-sensitive domain potentiated the death response, suggesting that the Ref-1 redox-sensitive domain plays an integral protective role in the endothelium. These experiments lend additional support to our hypothesis that Ref-1–mediated survival in response to TNF was mediated in part through NF-κB activation.

Moreover, to directly test if potentiation of NF-κB activity was mediating the antiapoptotic signaling pathway induced by Ref-1, CPAE cells were transiently cotransfected with the dominant-negative IκB (S32A/S36A) construct or an empty expression vector along with Ref-1. Cells were treated with TNF, and apoptosis was quantitated in the transfected subset. Initial experiments confirmed blockade of TNF-induced NF-κB activity (control-transfected cells at baseline, 1.00 ± 0.03; IκB (S32A/S36A) at baseline, 0.84 ± 0.04; control-transfected + TNF, 2.69 ± 0.29; IκB (S32A/S36A)+TNF, 1.3 ± 0.07; n = 3, P < 0.02). In accord with our hypothesis, blockade of NF-κB activation with IκB (S32A/S36A) abolished the ability of Ref-1 to inhibit TNF-induced apoptosis (Figure 8). Thus, the data suggest that the ability of Ref-1 to inhibit apoptosis is attributable in part to its role in potentiating the activation of NF-κB.
though speculative, our data would suggest the likely possibility that this redox deletion mutant acts in a dominant-negative manner. The increased susceptibility of endothelial cells to both TNF- and hypoxia-induced apoptosis after deletion of the redox domain of Ref-1 suggests that endogenous Ref-1 plays a critical protective role in the vasculature. Hypoxia and TNF have both been shown to induce NF-κB activation in endothelial cells.22–24,28–30 However, the role of NF-κB under these conditions in promoting an antiapoptotic signaling cascade is controversial.24,28–31 We postulated that the ability of Ref-1 to protect endothelial cells from TNF-induced apoptosis is independent of NF-κB. As an additional critical test of our hypothesis that Ref-1 significantly potentiates hypoxia-induced expression of the Ref-1 redox-sensitive deletion mutant signifi-

In conclusion, we have demonstrated for the first time that Ref-1 is a critical intrinsic factor promoting endothelial cell survival in response to pathophysiologically relevant stimuli. Indeed, the regulation of Ref-1 may play an important role in regulating vascular endothelial cell fate and function.

Acknowledgments

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References

3. Li D, Yang B, Mehta JL. Tumor necrosis factor-α. Enhanced activation of AP-1 by Ref-1 has been described in several cell types.12,13,32 However, the role of AP-1 in regulating endothelial cell fate is not clear.33,34 Ref-1 has also been demonstrated to achieve p53 and induce apoptosis in human tumor cell lines.35 Given that we clearly document an antiapoptotic effect of Ref-1 on both TNF-α and hypoxia-induced apoptosis, it is unlikely that a proapoptotic effect via p53 is a major component of its effect in endothelial cells.

Inhibition of endothelial apoptosis in response to hypoxia may have several therapeutic advantages. Indeed, the regulation of a family of hypoxia-inducible factors (HIFs) that bind specifically to promoters and enhancers in genes important in adaptation to hypoxia, such as critical glycolytic enzymes, glucose transporters, and vascular endothelial growth factor, seems to play an important role in promoting endothelial cell survival.36,37 Interestingly, Ref-1 plays an important role in the regulation of HIF-1α expression.14 However, recent work by Carrero et al18 and Ema et al39 suggests that Ref-1 is critical in the linking of two coactivator proteins, CBP/p300 and SRC-1, to HIF-1α. Complete loss of HIF-1α expression results in vascular malformations that correlate with cell death and eventual embryonic lethality.37,40

A reporter construct containing the HIF-1α–binding site.14 Moreover, recent work by Carrero et al18 and Ema et al39 suggests that Ref-1 is critical in the linking of two coactivator proteins, CBP/p300 and SRC-1, to HIF-1α. Complete loss of HIF-1α expression results in vascular malformations that correlate with cell death and eventual embryonic lethality.37,40 However, the influence of HIF-1α on cell fate is cell type–specific and dependent on several factors, including growth factors, cell density, pH, and glucose concentrations.37 Thus, the possibility that Ref-1 may inhibit hypoxia-induced apoptosis through the regulation and stabilization of HIF-1α is intriguing. Our findings that deletion of the redox domain of Ref-1 abolished the antiapoptotic effect of Ref-1 additionally suggest that this N-terminal domain is likely responsible for biochemical interactions with several transcription factors mediating survival, including HIF-1α. Future studies will be needed to directly address the role of HIF-1α and other transcription factors as downstream mediators of Ref-1 signaling in hypoxic conditions.

In conclusion, we have demonstrated for the first time that Ref-1 is a critical intrinsic factor promoting endothelial cell survival in response to pathophysiologically relevant stimuli. Indeed, the regulation of Ref-1 may play an important role in regulating vascular endothelial cell fate and function.
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