THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 20, Issue of May 17, pp. 17950–17961, 2002

Printed in U.S.A.

Heme Oxygenase-1-derived Carbon Monoxide Requires the Activation of Transcription Factor NF-κB to Protect Endothelial Cells from Tumor Necrosis Factor-α-mediated Apoptosis*

Received for publication, August 29, 2001, and in revised form, March 1, 2002
Published, JBC Papers in Press, March 5, 2002, DOI 10.1074/jbc.M108317200

Sophie Brouard‡§¶, Pascal O. Berberat‡§‖, Edda Tobiasch‡, Mark P. Seldon‡‡, Fritz H. Bach‡§§, and Miguel P. Soares‡**¶¶**

*From the ‡Immunobiology Research Center, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, 02115 and the **Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal*

We have shown that carbon monoxide (CO) generated by heme oxygenase-1 (HO-1) protects endothelial cells (EC) from tumor necrosis α (TNF-α)-mediated apoptosis. This effect relies on the activation of p38 MAPK. We now demonstrate that HO-1/CO requires the activation of the transcription factor NF-κB to exert this anti-apoptotic effect. Our data suggest that EC have basal levels of NF-κB activity that sustain the expression of NF-κB-dependent anti-apoptotic genes required to support the anti-apoptotic effect of HO-1/CO. Over-expression of the inhibitor of NF-κB α (IκBα) suppresses the anti-apoptotic action of HO-1/CO. Reconstitution of NF-κB activity, by co-expression of IκBα with different members of the NF-κB family, *i.e.* p65/RelA or p65/RelA plus c-Rel, restores the anti-apoptotic effect of HO-1/CO. Expression of the NF-κB family members p65/RelA or p65/RelA with p50 or c-Rel up-regulates the expression of the anti-apoptotic genes *A1, A20,* c-*IAP2*, and manganese superoxide dismutase (*MnSOD*). Inhibition of NF-κB activity by over-expression of IκBα suppresses the expression of some of these anti-apoptotic genes, *i.e.* c-*IAP2*. Under inhibition of NF-κB, co-expression of some of these anti-apoptotic genes, *i.e.* c-*IAP2* and *A1*, restores the anti-apoptotic action of HO-1/CO, whereas expression of *A20* or *MnSOD* cannot. The ability of c-IAP2 and/or A1 to restore the anti-apoptotic action of HO-1/CO is abolished when p38 MAPK activation is blocked by over-expression of a p38 MAPK dominant negative mutant. In conclusion, we demonstrate that HO-1/CO cooperates with NF-κB-dependent anti-apoptotic genes, *i.e.* c-*IAP2* and *A1*, to protect EC from TNF-α-mediated apoptosis. This effect is dependent on the ability of HO-1/CO to activate the p38 MAPK signal transduction pathway.

Signaling via "death receptors," such as the tumor necrosis factor-α (TNF-α)[1] receptor 1 (TNFR-1/CD120a), can trigger endothelial cell (EC) to undergo apoptosis. Cross-linking of TNFR-1 leads to the recruitment of intracytoplasmic signal transduction molecules, *e.g.* TRADD (TNF receptor-associated death domain), FADD (Fas-associated death domain), and RIP (receptor-interacting protein) (1, 2). These molecules form the death-inducing signaling complex (DISC; reviewed in Refs. 1 and 2), which activates serine proteases, referred to as caspases (3). Caspase activation by the death-inducing signaling complex occurs via FADD-dependent recruitment and proximal catalytic cleavage/activation of pro-caspase-8 into the active form of caspase-8 (4, 5), which activates additional pro-caspases into active caspases, *e.g.* caspase-3, that execute the terminal phase of apoptosis (reviewed in Ref. 3).

Under physiologic conditions, signaling via the TNFR-1 does not lead to EC apoptosis because TNFR-1 triggers the expression of early responsive anti-apoptotic genes such as the zinc finger *A20* (6), the *bcl-2* family member *A1* (7), the antioxidant manganese superoxide dismutase (*MnSOD*) (8), several members of the inhibitor of apoptosis (IAP) family (9), *IEXL-1* (10), and *PAI-2* (plasminogen activator inhibitor type-2) (11). These anti-apoptotic genes prevail over the pro-apoptotic signals thus preventing TNF-α-mediated EC apoptosis. Expression of these anti-apoptotic genes is dependent on the activation of the transcription factor nuclear factor κB (NF-κB) (12). Inhibition of NF-κB activity prevents the expression of these anti-apoptotic genes and thus sensitizes most cell types (13–15), including EC (12), to undergo TNF-α-mediated apoptosis.

The NF-κB family of transcription factors consists of several homo- or heterodimeric complexes of the Rel family, *i.e.* p50/NF-κB1, p65/RelA, c-Rel (Rel), p52/NF-κB2, and RelB (reviewed in Refs. 16 and 17). In quiescent EC, NF-κB is thought to be retained in the cytoplasm by a series of inhibitory proteins referred to as inhibitor of κB (IκB) (reviewed in Ref. 16). Binding of NF-κB to IκB molecules masks the nuclear localization signal in the NF-κB dimers, thereby preventing NF-κB nuclear translocation and transcription activity (18). Signaling via TNFR-1 triggers the release of NF-κB dimers from IκB molecules via site-specific phosphorylation, ubiquitination, and sub-

* This work was supported in part by Grant 998521355 from the Roche Organ Transplantation Research Foundation and by National Institutes of Health Grants HL67040 (to M. P. S.) and HL58688 (to F. H. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

¶ Supported by a grant from Institut National de la Santé et de la Recherche Médicale (INSERM), France.

‖ Supported by a fellowship of the Swiss National Science Foundation.

‡‡ Supported by Grant BD2990/00 from the "Fundaçao para a Ciencia e Tecnologia" Portugal.

§§ The Lewis Thomas Professor at the Harvard Medical School and a paid consultant for Novartis Pharma.

¶¶ To whom correspondence should be addressed: Immunobiology Research Center, Dept. of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, 99 Brookline Ave., Boston, MA 02215. Tel.: 617-632-0885; Fax: 617–632-0880; E-mail: msoares@caregroup.harvard.edu.

[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; TNFR-1, tumor necrosis factor-α receptor 1; Act.D, actinomycin-D; BAEC, bovine aortic endothelial cells; CMV, cytomegalovirus; EC, endothelial cell; EMSA, electrophoretic mobility shift assay; HO-1, heme oxygenase-1; HUVEC, human umbilical vein endothelial cell; IκBa, inhibitor of nuclear factor-κBα; MAPK, mitogen-activated protein kinase(s); MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor-κB; PAEC, porcine aortic endothelial cells; IAP, inhibitor of apoptosis; HA, hemagglutinin; LPS, lipopolysaccharide; RT, reverse transcriptase.

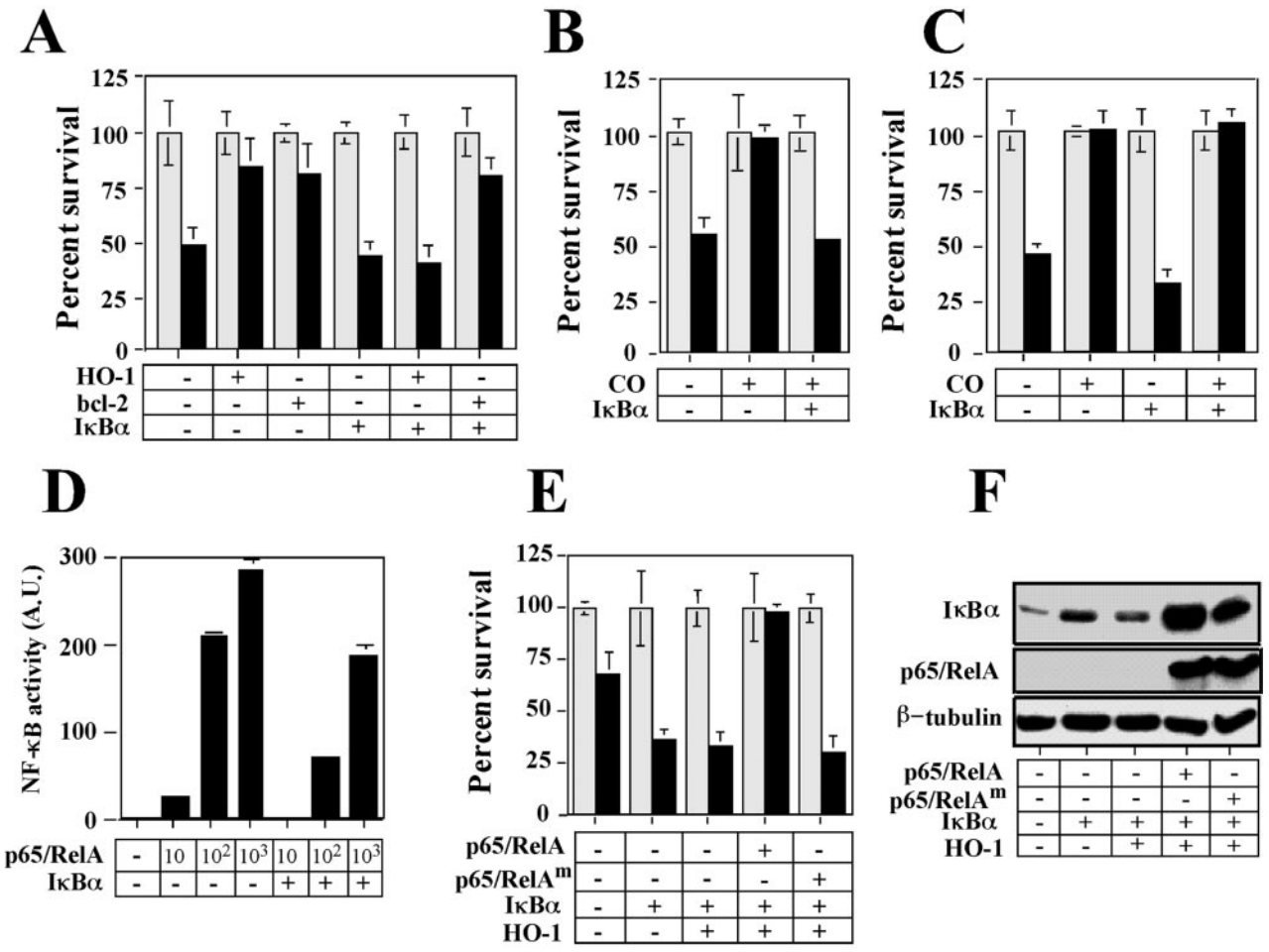

FIG. 1. **HO-1-derived CO requires NF-κB transcriptional activity to suppress endothelial cell apoptosis.** *A*, BAEC were transiently transfected with β-galactosidase (300 ng/3 × 10^5 cells) and when indicated (+) with HO-1 (β-actin/HO-1; 700 ng/3 × 10^5 cells), *bcl-2* (700 ng/3 × 10^5 cells), and/or IκBα (500 ng/3 × 10^5 cells) expression vectors. *Gray* and *black histograms* represent EC treated with Act.D or Act.D plus TNF-α, respectively. Act.D (10 μg/ml) and TNF-α (50 units/ml) were added to the culture medium 24 h after transfection as described under "Experimental Procedures." Percent survival of transfected EC was calculated 8 h after the addition of Act.D or Act.D plus TNF-α as described under "Experimental Procedures." Results shown are the mean ± standard deviation from duplicate wells in two independent experiments ($n = 4$). The percentage of cell survival in HO-1-transfected EC was increased in a very significant manner as compared with control EC transfected with pcDNA3 ($p = 0.0038$, unpaired *t* test). There was no significant increase in the percentage of survival of EC co-transfected with HO-1 and IκBα *versus* EC transfected with pcDNA3 ($p = 0.4970$, unpaired *t* test) or IκBα ($p = 0.4759$, unpaired *t* test) *B,* mouse 2F-2B EC were co-transfected with IκBα (500 ng/3 × 10^5 cells) plus β-galactosidase (300 ng/3 × 10^5 cells) expression vectors and when indicated (+) were exposed to exogenous CO (10,000 ppm), as described under "Experimental Procedures." *Gray* and *black histograms* represent EC treated with Act.D and Act.D plus TNF-α, respectively, as described in *A*, percent survival was calculated as in *A*. Results shown are the mean ± standard deviation from duplicate wells from one of three similar experiments. *C*, mouse 2F-2B EC were transfected as described in *B*. When indicated (+) EC where exposed to exogenous CO (10,000 ppm) as described under "Experimental Procedures." Twenty-four hours after transfection, EC were exposed to either 10% (*gray histograms*) or 1% (*black histograms*) fetal calf serum for 30 h. Percent survival was calculated as in *A* and *B*. Results shown are the mean ± standard deviation from duplicate wells in two independent experiments ($n = 4$). Percent of cell survival increased in a highly significant manner in EC exposed to CO (+) *versus* EC not exposed to CO (−) whether or not EC were transfected with IκBα ($p < 0.0001$, unpaired *t* test). *D*, BAEC were transiently transfected with a NF-κB-dependent luciferase (300 ng/3 × 10^5 cells) and β-galactosidase (300 ng/3 × 10^5 cells) reporters plus or minus IκBα (500 ng/3 × 10^5 cells). NF-κB activity was induced by transient over-expression of a p65/RelA (10, 10^2 or 10^3ng/3 × 10^5 cells), as described before (12). Results shown are mean ± standard deviation from one of three similar experiments and are expressed in arbitrary luciferase units (*A.U.*) normalized for β-galactosidase expression. *E*, mouse 2F-2B EC were transiently co-transfected with β-galactosidase (300 ng/3 × 10^5 cells). When indicated (+) EC were co-transfected HO-1 (β-actin/HO-1, 700 ng/3 × 10^5 cells), IκBα (500 ng/3 × 10^5 cells), c-Myc-tagged p65/RelA (1000 ng/3 × 10^5 cells) and/or a c-Myc-tagged DNA binding deficient mutant of p65/RelA^m (1000 ng/3 × 10^5 cells) expression vectors. *Gray* and *black histograms* represent EC exposed to medium or TNF-α (24 h after transfection; 50 units/ml, 16 h), respectively. Percent survival of transfected EC was evaluated as in *A–C*. Results shown are the mean ± standard deviation from duplicate wells in two independent experiments ($n = 4$). Percent of cell survival was very significantly increased in IκBα plus p65 *versus* IκBα transfected EC ($p = 0.0036$, unpaired *t* test). *F*, endogenous and over-expressed IκBα was detected by Western blot using an anti-human N-terminal IκBα polyclonal antibody (C21, Santa Cruz). Over expressed c-Myc-tagged p65/RelA and p65/RelA^m were detected using an anti-c-Myc monoclonal antibody.

sequent proteolytic IκB degradation through the 26 S proteasome pathway (19, 20). Once released from IκB molecules, NF-κB dimers translocate into the nucleus to bind specific decameric recognition motifs in the promoter region of NF-κB-dependent genes such as the anti-apoptotic genes *A1* (21), *A20* (22), *MnSOD* (23), and c-*IAP2* (24).

Other anti-apoptotic genes are expressed by EC independently of NF-κB. These include heme oxygenase-1 (*HO-1*) (reviewed in Refs. 25 and 26), a stress-responsive gene encoding a 32-kDa enzyme that degrades heme into biliverdin, iron, and the gas carbon monoxide (27). Although present only at basal levels in quiescent EC, HO-1 expression is rapidly up-regulated under oxidative stress conditions (reviewed in Refs. 25 and 26). We have previously shown that HO-1 protects EC from undergoing apoptosis (28). The three end products of HO-1 enzymatic activity can potentially act as antioxidants and thus can exert anti-

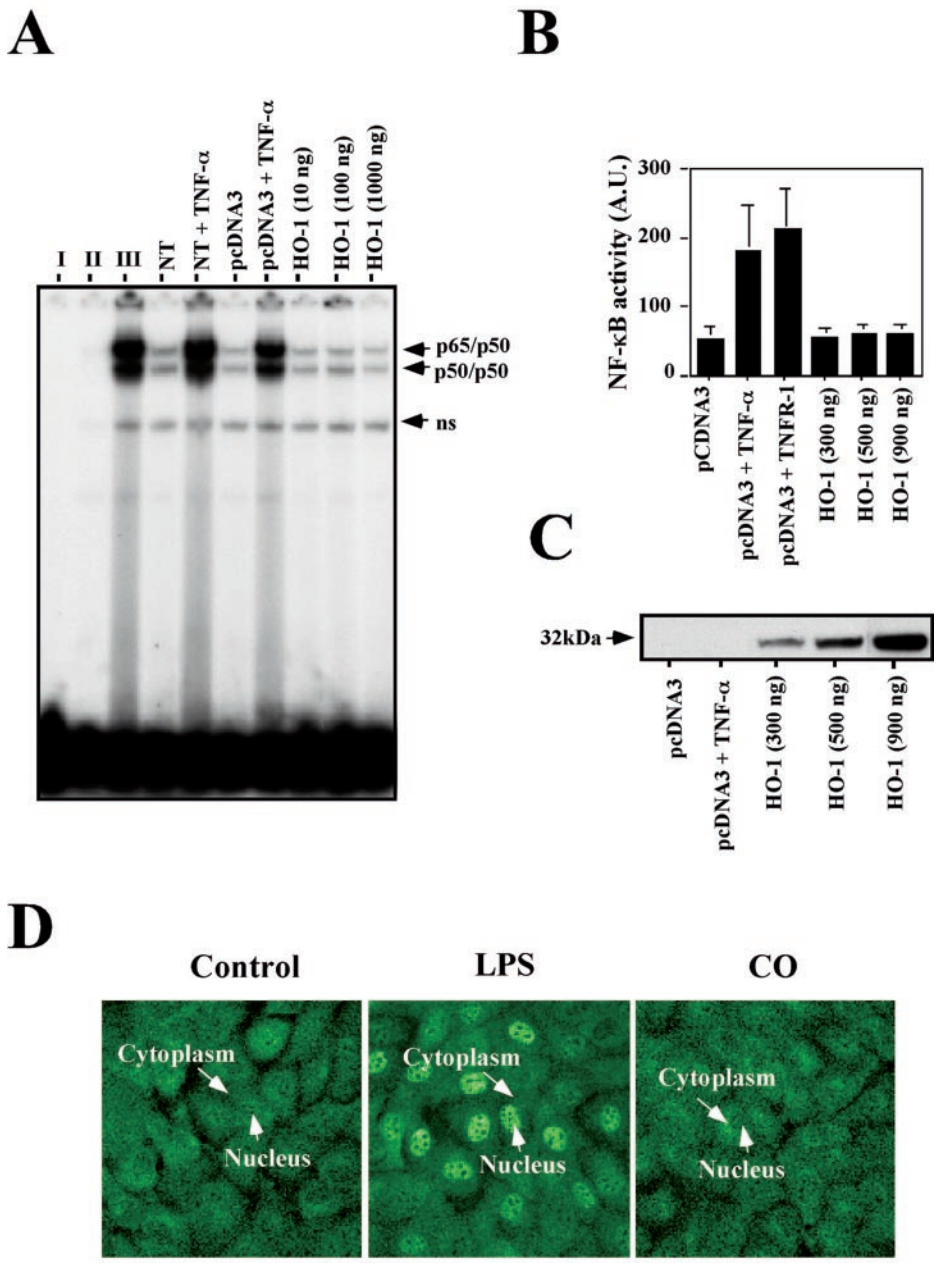

FIG. 2. **Transient over-expression of HO-1 does not modulate NF-κB activation.** *A*, specific binding of NF-κB dimers to the NF-κB consensus DNA binding motif, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, was detected by EMSA using nuclear extracts from nontransfected (*NT*), control (*pcDNA3*), or HO-1-transfected 2F-2B EC. Values in HO-1-transfected extracts indicate the amount of plasmid used/3 × 10^5 cells. EC were either not stimulated (−) or stimulated with TNF-α (+) (10 ng/ml; 50 units/ml) 20 min before nuclear extraction. Specificity of the assay was tested using free radiolabeled consensus oligonucleotide in absence of nuclear extracts (*lane I*) as well as by DNA binding competition with 50 ng of nonradiolabeled oligonucleotide (*lane II*) or by competition with 50 ng of non-radiolabeled irrelevant oligonucleotide (*lane III*). Competition controls were carried out using nuclear extracts from TNF-α stimulated (50 units/ml, 30 min) EC. Identification of the different NF-κB dimeric proteins was carried out using an antibody specific for p65/RelA as described under "Experimental Procedures." *B*, BAEC were transiently transfected with a NF-κB-dependent luciferase (300 ng/3 × 10^5 cells) plus a CMV-driven β-galactosidase (300 ng/3 × 10^5 cells) reporter. When indicated, EC were co-transfected with TNFR-1 (*pcDNA3 + TNFR-1*) or HO-1 (*pcDNA3 + HO-1*) expression vectors. Untreated and TNF-α (50 units/ml, 6 h)-treated control EC (pcDNA3) were used as negative and positive controls, respectively. Results shown are the mean ± standard deviation from duplicate wells taken from one of three similar experiments and are expressed in arbitrary luciferase units (*A.U.*) normalized for β-galactosidase expression. *C*, HO-1 expression was detected by Western blot using a polyclonal rabbit antibody directed against human HO-1. Values indicated the amount of pcDNA3/HO-1 expression vector used per well. *D*, BAEC were untreated (control), treated with LPS (1 μg/ml; 20 min), or exposed to exogenous CO (10,000 ppm, 2 h) and stained with an anti-p65/RelA antibody as described under "Experimental Procedures." Fluorescent staining was analyzed by confocal microscopy as described under "Experimental Procedures." Notice the cytoplasmic and nuclear staining (*arrows*). All magnification is 100×.

apoptotic effects (29, 30). However, CO seems to act in a dominant manner to mediate the anti-apoptotic effect of HO-1 (31). This effect requires the activation of the p38 mitogen-activated protein kinase (MAPK) signal transduction pathway (31).

Given that signaling transduction pathways leading to NF-κB activation are critical in modulating EC apoptosis, we questioned whether HO-1-derived CO cooperates with one or more NF-κB-dependent anti-apoptotic genes to prevent apoptosis. We found that HO-1 requires NF-κB activity and

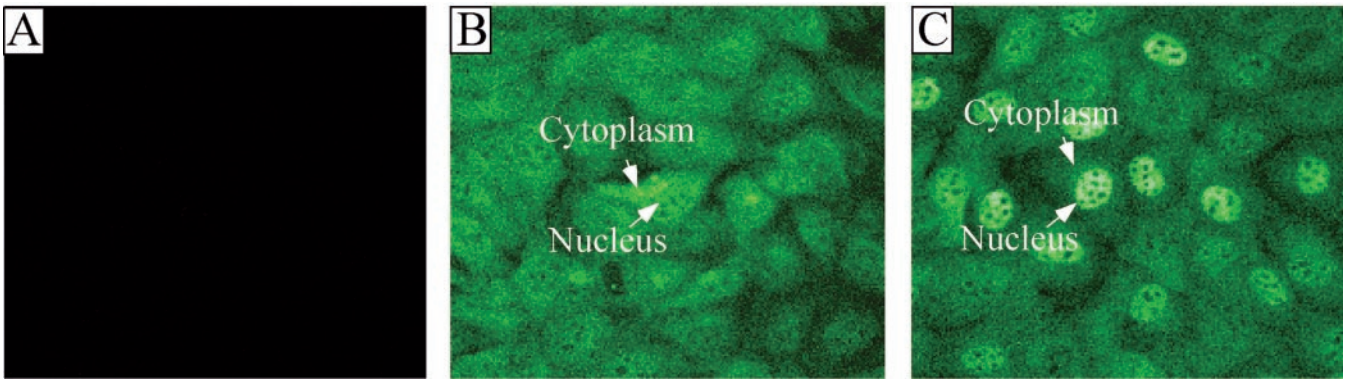

FIG. 3. **Quiescent EC have basal levels of nuclear NF-κB dimers containing p65/relA.** BAEC were either untreated (*A* and *B*) or treated with LPS (*C*) (1 μg/ml; 20 min) and stained with rabbit IgG (*A*) or an anti-p65/RelA rabbit IgG (*B* and *C*) as described under "Experimental Procedures." Fluorescent staining was analyzed by confocal microscopy as described under "Experimental Procedures." Notice cytoplasmic and nuclear localization (*white arrows*) of p65/RelA in quiescent BAEC. All magnification is 100×.

the expression of A1 or c-IAP2 to exert its anti-apoptotic effect in EC.

EXPERIMENTAL PROCEDURES

Cell Cultures—The murine 2F-2B EC line (American Type Culture Collection (ATCC), Manassas, VA), primary bovine aortic EC (BAEC), porcine aortic EC (PAEC), and human umbilical vein EC (HUVEC) were isolated and cultured as described previously (12, 31, 32).

Expression Plasmids—The β-galactosidase expression vector has been described elsewhere (12). Two vectors encoding rat HO-1 cDNA were used, one under the control of the β-actin enhancer/promoter (β−actin/HO-1) (33) and the other under the control of the cytomegalovirus (CMV) enhancer/promoter (pcDNA3/HO-1) (31). The murine Bcl-2 expression vector (kind gift of R. Gerard, University of Texas Southwest Medical Center, Dallas, TX) has been described elsewhere (34). The porcine IκBα cDNA (ECI-6) was expressed in the pcDNA3/HA vector-derived from the pcDNA3 vector (Invitrogen) as described previously (12). The human A20 cDNA, cloned into the pAC expression vector was originally obtained from V. Dixit (Genentech, Inc., South San Francisco, CA) (35) and was expressed in the HA-tagged pcDNA3 expression vector (kind gift from C. Ferran in our Immunobiology Research Center) as described elsewhere (36). Human p65/RelA, c-Rel, and p50 cDNAs were cloned into the pcDNA3 expression vector (kind gift from Dr. J. Anrather, Cornell University, New York). The RelA (p65m) DNA binding-deficient mutant (37) has been described elsewhere (12). P38/CSBP1 MAPK was amplified from HeLa cDNA by PCR and cloned into the pcDNA3/HA vector derived from pcDNA3 (Invitrogen) by inserting a DNA fragment coding for an epitope derived from the hemagglutinin protein of the human influenza virus hemagglutinin (HA; MYPYDVPDYASL). A dominant negative mutant of p38/CSBP1 harboring a T180A and a Y182F substitution, was generated by overlap extension mutagenesis a described previously (31). This constructed was provided by Dr. J. Anrather (Cornell University, New York) The N-terminally HA-tagged human A1 expression vector (HA-A1) was provided by Dr. C. Ferran in our Immunobiology Research Center and has been described elsewhere (38). The human c-IAP2 cDNA (kind gift from D. Vaux, Australia) was expressed in the prCMV expression plasmid (kind gift from Dr. D. W. Ballard Vanderbilt University, Nashville, TN) (39). The human MnSOD cDNA was obtained from ATCC and cloned (*Eco*RI) into the pcDNA3 expression vector (Invitrogen). The human TNF-R1/pcDNA3 expression vector was obtained from D. V. Goeedel (Tularik, Inc., South San Francisco, CA).

Transient Transfections—BAEC and 2F-2B EC were transiently transfected as described elsewhere (12, 31, 40). β-Galactosidase-transfected cells were detected as described elsewhere (23, 28). Briefly, the number of random fields counted was determined to have a minimum of 200 viable transfected cells/control well (without apoptosis-inducing treatment). The number of viable cells was assessed by evaluating β-galactosidase-expressing cells that retained normal morphology under the apoptosis-inducing treatment, *i.e.* TNF-α or serum deprivation and control treatment (12, 31, 40). The percent survival was calculated for each DNA preparation by normalizing the number of viable β-galactosidase-expressing cells counted after the apoptosis-inducing treatment to that counted in the absence of the treatment (100% viability). All experiments were performed in duplicate two to four times.

Recombinant Adenoviruses—The recombinant β-galactosidase adenovirus was a kind gift of Dr. Robert Gerard (University of Texas Southwest Medical Center, Dallas, TX). The recombinant IκBα adenovirus expressing the porcine IκBα gene (*ECI-6*) has been described elsewhere (41). All recombinant adenoviruses were produced in 293 cells (ATCC), extracted, and purified through two cesium chloride gradient ultracentrifugations, and their titer was determined by limiting dilution in 293 cells as described before (41). Confluent BAEC were infected with a multiplicity of infection of 200 plaque-forming units/cell as described elsewhere (31).

Cell Extracts and Western Blot Analysis—Cell extracts were prepared and subjected to electrophoresis as described elsewhere (12). HO-1 was detected using a rabbit antihuman HO-1 polyclonal antibody (StressGen, Biotechnologies Corp., Victoria, CA). p65/RelA, p50, and c-Rel were detected using rabbit anti-mouse p50, c-Rel, and RelA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). c-Myc-tagged p65/RelA was detected using an anti-c-Myc monoclonal antibody (9E10 clone, ATCC). HA-tagged A1, A20, and IκBα were detected using a mouse or rat anti-HA monoclonal antibody (Roche Molecular Biochemicals). c-IAP2-Flag was detected using a anti-Flag M1 monoclonal antibody (Sigma). Human MnSOD was detected using a rabbit anti-human MnSOD antibody (Stressgen). β-Tubulin was detected using anti-human β-tubulin monoclonal antibody (Roche Molecular Biochemicals). Primary antibodies were detected using horseradish peroxidase-conjugated donkey anti-rabbit or goat anti-mouse IgG secondary antibodies (Pierce). Peroxidase enzymatic activity was visualized using the Enhanced Chemiluminescence assay (Amersham Biosciences), according to the manufacturer's instructions and stored in the form of photoradiographs (Biomax™MS, Eastman Kodak, Rochester, NY).

Cell Treatment and Reagents—Water soluble Actinomycin-D (Act.D, Sigma) was dissolved in phosphate-buffered saline and added to the culture medium, 24 h after EC transfection. The concentration of Act.D required to sensitize EC to TNF-α-mediated apoptosis was 10 μg/ml for 2F-2B EC and 0.1 μg/ml for BAEC. Human recombinant TNF-α (R&D Systems, Minneapolis, MN) was dissolved in phosphate-buffered saline, 0.1% bovine serum albumin and added to the culture medium (10 ng/ml, 50 units/ml) 24 h after EC transfection. When used in combination with Act.D, EC were exposed to TNF-α for a period of 8 h. When used in the absence of Act.D, EC (*i.e.* overexpressing IκBα) were exposed to TNF-α for a period of 16 h. Serum deprivation was carried out by exposing EC to 1% fetal calf serum during 30 h.

CO Exposure—Briefly, CO at a concentration of 1% (10,000 ppm) in compressed air was mixed with balanced air (21% O_2) in a stainless steel mixing cylinder before entering the exposure chamber. CO concentrations were controlled by varying the flow rates of CO in a mixing cylinder before delivering it to the chamber. Because the flow rate is primarily determined by the O_2 flow, only the CO flow was changed to deliver the final concentration to the exposure chamber. A CO analyzer (Interscan Corp., Chatsworth, CA) was used to measure CO levels in the chamber. Cells were exposed to CO for 1 h before stimulation with TNF-α or serum deprivation and continuously thereafter.

Reporter Assays—Cellular extracts were assayed for β-galactosidase activity using the Galacto-Light protocol (Applied Biosystems, Tropix Inc., Bedford, MA). Luciferase activity was assayed by adding 10 μl of cellular extract to 90 μl of a solution containing 24 mM glycl-glycine (pH 7.8), 2 mM ATP (pH 7.5), and 10 mM $MgSO_4$. Samples were read on the Microlumat LB 96P luminometer (EG&G Berthold, Wildbad, Germany)

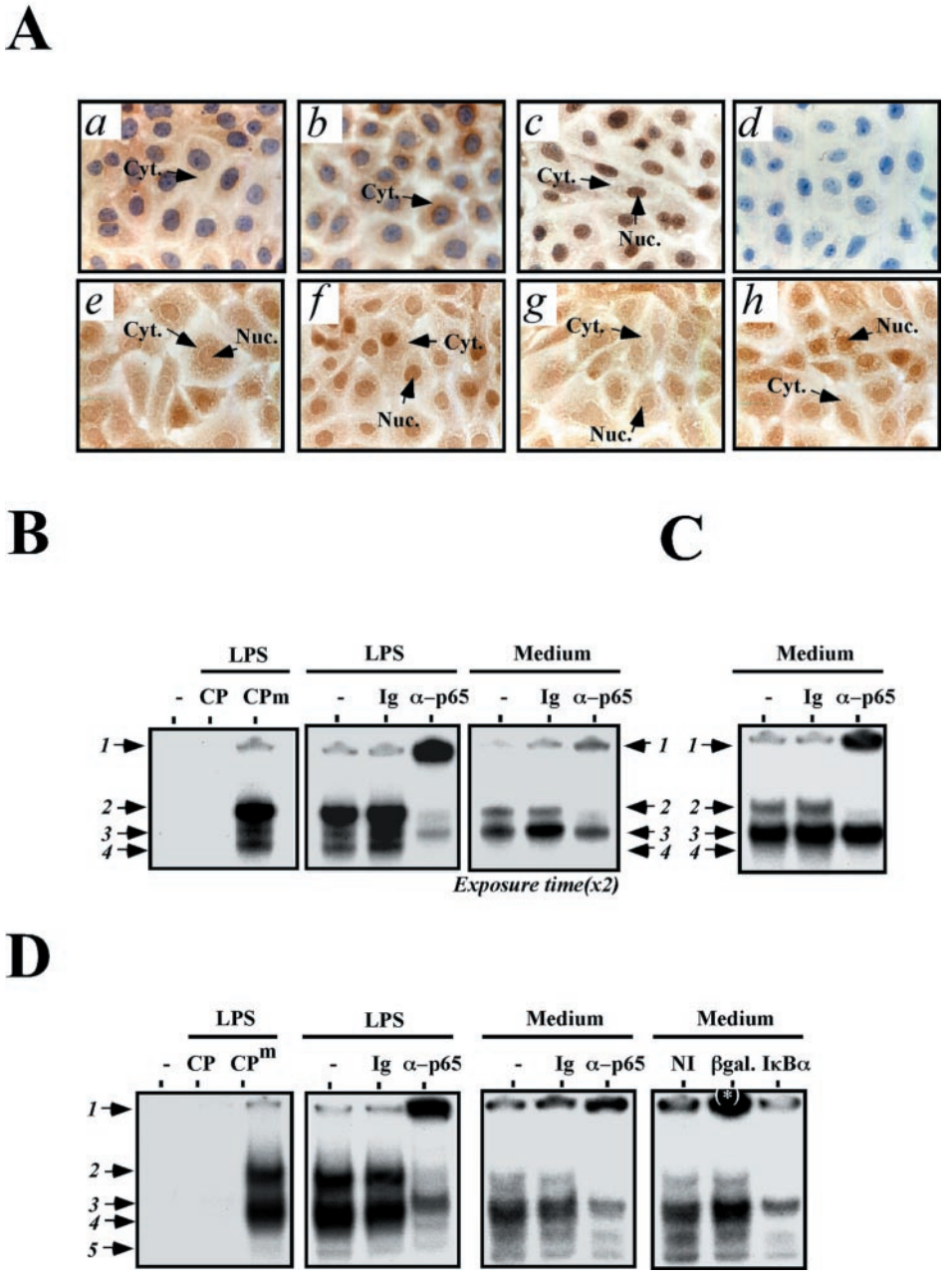

FIG. 4. **Quiescent EC have basal levels of free nuclear p65/RelA.** *A*, untreated 2F-2B EC were stained with anti-paxillin antibody and counter-stained with hematoxylin (*a*). Untreated (*b*) or TNF-α treated (10 μg/ml, 30 min) (*c*) 2F-2B EC were stained with an anti-p65/RelA purified rabbit antibody (IgG) and counter-stained with hematoxylin. Untreated 2F-2B EC were stained with a nonspecific rabbit IgG and counter-stained with hematoxylin (*d*). Untreated (*e*) or TNF-α treated (10 μg/ml; 30 min) (*f*) 2F-2B EC were stained with an anti-p65/RelA rabbit purified antibody (IgG) without hematoxylin counter-staining. Untreated (*g*) or TNF-α-treated (10 μg/ml, 30 min) (*h*) 2F-2B EC were stained with an anti-p65/RelA antibody recognizing the nuclear localization sequence exposed in non-IκB-bound p65/RelA without hematoxylin counter-staining. Notice the cytoplasmic (*Cyt.*) and nuclear (*Nuc.*) staining (*black arrows*). All magnification is 80×. *B*, specific binding of NF-κB dimers to the consensus DNA binding motif, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, was detected by EMSA using nuclear extracts from untreated (*Medium*) or LPS (5 μg/ml, 30 min) 2F-2B. Specificity of the assay for the consensus analyzed was tested using free radiolabeled consensus oligonucleotide in the absence of nuclear extracts (−) as well as by DNA binding competition with 50 ng of nonradiolabeled oligonucleotide (*CP*) and by competition with 50 ng of nonradiolabeled irrelevant oligonucleotide (*CPm*). Identification of the different NF-κB heterodimeric proteins was carried out using an antibody specific for the N-terminal region of human p65 (α-p65), as described under "Experimental Procedures." *C*, specific binding of NF-κB dimers to the consensus DNA binding motif, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, was detected by EMSA as described in *B* using nuclear extracts from untreated (*Medium*) PAEC. *D*, specific binding of NF-κB dimers to the consensus DNA binding motif, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, was detected by EMSA, as described in *B* and *C*, using nuclear extracts from untreated (*Medium*) or LPS (5 μg/ml; 30 min)-treated BAEC. The specificity of the assay for the consensus analyzed was tested as described in *B* using nuclear extracts from LPS-treated BAEC. Identification of the different NF-κB heterodimeric proteins was carried out as described in *B*. When indicated BAEC were infected with a β-galactosidase (*βgal.*) or an IκBα recombinant adenoviruses as described under "Experimental Procedures." *NI* indicates noninfected EC; *, indicates nonspecific labeling; *1*, indicates p65/RelA-containing dimer bound to anti-p65 antibody. Based on their p65/RelA content and relative molecular weights, we predict that dimers denominated as *2*, *3*, *4*, and *5* correspond to p65/RelA (65 kDa) + c-Rel (75 kDa), RelB (68kDa), and p52 and p50 (49 kDa), respectively.

using an injection mix consisting of 24 mM glycl-glycine and 0.1 mM luciferin (Sigma). Luciferase activity was normalized for β-galactosidase as follows: luciferase activity/β-galactosidase activity × 1000. Normalized luciferase activity is shown in arbitrary units.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared as described elsewhere (12, 42). All buffers were supple-

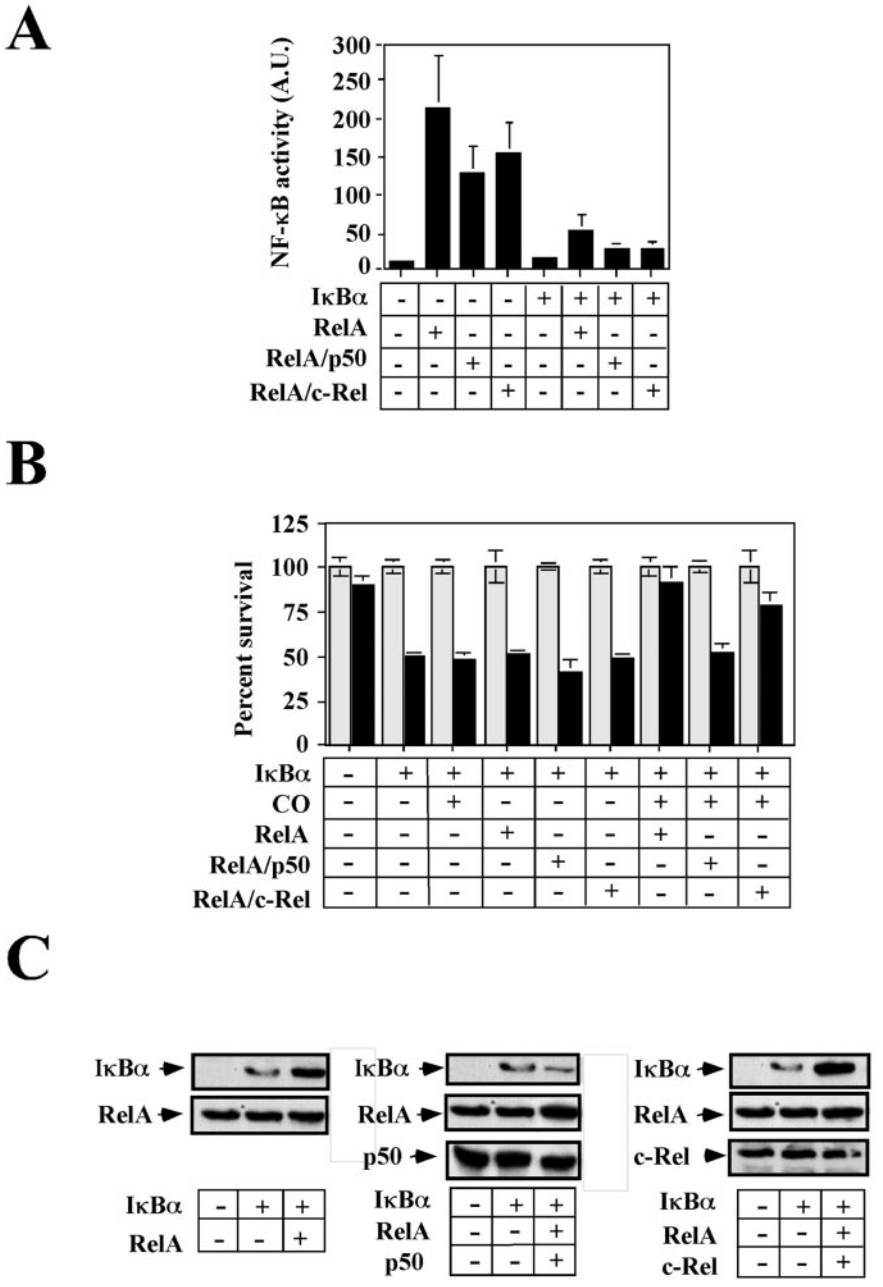

FIG. 5. **NF-κB activation is required to sustain the anti-apoptotic effect of HO-1-derived CO.** *A*, BAEC were transiently transfected with an NF-κB-dependent luciferase (300 ng/3 × 10^5 cells) plus a CMV-driven β-galactosidase (300 ng/3 × 10^5 cells) reporter. When indicated (+) EC were transiently co-transfected with p65/RelA (200 ng/3 × 10^5 cells), p65/RelA (100 ng/3 × 10^5 cells) plus p50 (100 ng/3 × 10^5 cells), or p65/RelA (100 ng/3 × 10^5 cells) plus c-Rel (100 ng/3 × 10^5 cells) with (+) or without (−) IκBα (300 ng/3 × 10^5 cells). Results shown are the mean ± standard deviation from duplicate wells in one representative experiment of three similar ones and are expressed in arbitrary luciferase units (*A.U.*) normalized for β-galactosidase expression. *B*, 2F-2B EC were transfected as described in *A*, without the NF-κB-dependent reporter. When indicated (+) EC were exposed to exogenous CO (10,000 ppm) as described under "Experimental Procedures." *Gray* and *black histograms* represent EC exposed to medium or TNF-α (24 h after transfection; 50 units/ml, 16 h), respectively. Percent survival of transfected EC was calculated as described under "Experimental Procedures." Results shown are the mean ± standard deviation from duplicate wells in three independent experiments ($n = 6$). Percent of cell survival was increased in a highly significant manner in IκBα plus p65 *versus* IκBα-transfected EC exposed to CO ($p = 0.0001$, unpaired t test). There was no significant increase in the percentage of survival of IκBα plus p65/p50 *versus* IκBα-transfected EC exposed to CO ($p = 0.4752$, unpaired t test). Percent of cell survival was increased in a highly significant manner in IκBα plus p65/c-Rel *versus* IκBα-transfected EC exposed to CO ($p = 0.0002$, unpaired t test). *C*, transfected HA-tagged IκBα was detected by Western blot using an anti-HA epitope monoclonal antibody as described under "Experimental Procedures." p65/RelA, p50, and c-Rel were detected using polyclonal antibodies directed against the human sequence of these NF-κB family members as described under "Experimental Procedures."

mented with 0.1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 0.1 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Equal amounts of nuclear extracts (5 μg) were incubated (30 min at room temperature) with 100,000 cpm of double-stranded, [γ-^{32}P]ATP-radiolabeled NF-κB consensus oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega), and the resulting DNA-protein complexes were separated on a 6% polyacrylamide gel in Tris-glycine-EDTA buffer at pH 8.5. The mass of the probes was ~20 fmol. Supershift assays were carried out in the same manner, but nuclear extracts were incubated with 2 μg of anti-human p65/RelA N-terminal antibody (Santa Cruz Biotechnology) for an additional hour before electrophoresis.

RT-PCR—RNA was extracted using TRIzol (Invitrogen) and reverse-transcribed into cDNA with the RNA PCR Kit (TaKaRa, PanVera, Madison, WI). A total of 2 μl of cDNA was amplified in a 50-μl reaction mix containing 10 mM dNTPs, 50 pg of 5′-prime and 3′-prime oligonucleotides, 2.5 units of LA-*Taq* polymerase (TaKaRa) and $MgCl_2$, specific to each primer pair used. The primers for murine and human *A20* (620 bp) (5′-AAT ATG CGG AAA GCT GTG AAG, 3′-GAT TCC AAA CTT CTT AGC ATT), *A1* (257 bp) (5′-AAA GAA TCT GAAGTC AT, 3′-ATA GGT AAG AGG ACA C), *bcl-x_L* (540 bp) (5′-GCC AGT GAG CTT CCC GTT CAG C, 3′-CAG AGC AAC CGG GAG CTG GT), *MnSOD* (511 bp) (5′-AAC GCG CAG ATC ATG CAG CTG C, 3′-ACA TTC TCC CAG TTG ATT CAC T), c-*IAP2* (567 bp) (5′-TGG GCT TCA GTA GGA GCC TGG T, 3′-ACT ACT AGA TGA CCA CAC GGA A), E-selectin (1157 bp) (5′-GGA TTG GAA TCA GAA AAG TCA A, 3′-GGA CTT GTA GGT GAA TTC TCC A), α-actin (525 bp) (5′-GCC ATC CTG CGT CTG GAC CTG G, 3′-TAC TCC TGC TTG CTG ATC CAC A), and IκBα (942 bp) (5′-TGG ACG ACC GCC ACG ACA GCG GC, 3′-CAG TCG ACC GGG TCG ACG ACG ACA TAG GCC CA) were obtained from Invitrogen). PCR reactions were performed after a 4-min denaturation at 94 °C a repeating the cycle 94 °C, 55 °C, and 72 °C each for 1 min for the number of cycles specific for each primer pair in a Peltier Thermal Cycler PTC-200 (MJ Research, Las Vegas, NV). PCR products (10–20 μl) were analyzed in an ethidium bromide-stained 1% agarose gel.

Immunocytochemistry—2F-2B EC were cultured on gelatinized glass slides (PerkinElmer Life Sciences), fixed in 75% acetone, and stained with anti-paxillin antibody (Upstate Biotechnology, Lake Placid, NY), anti-N-terminal p65/RelA antibody (2 μg/ml, Santa Cruz Biotechnology, SC 372), or anti-p65/RelA nuclear localization domain sequence-specific antibody (Roche Molecular Biochemicals). Primary antibodies were detected using biotinylated secondary antibodies and biotinylated-horseradish peroxidase-coupled streptavidin reaction (Pierce). Nonspecific

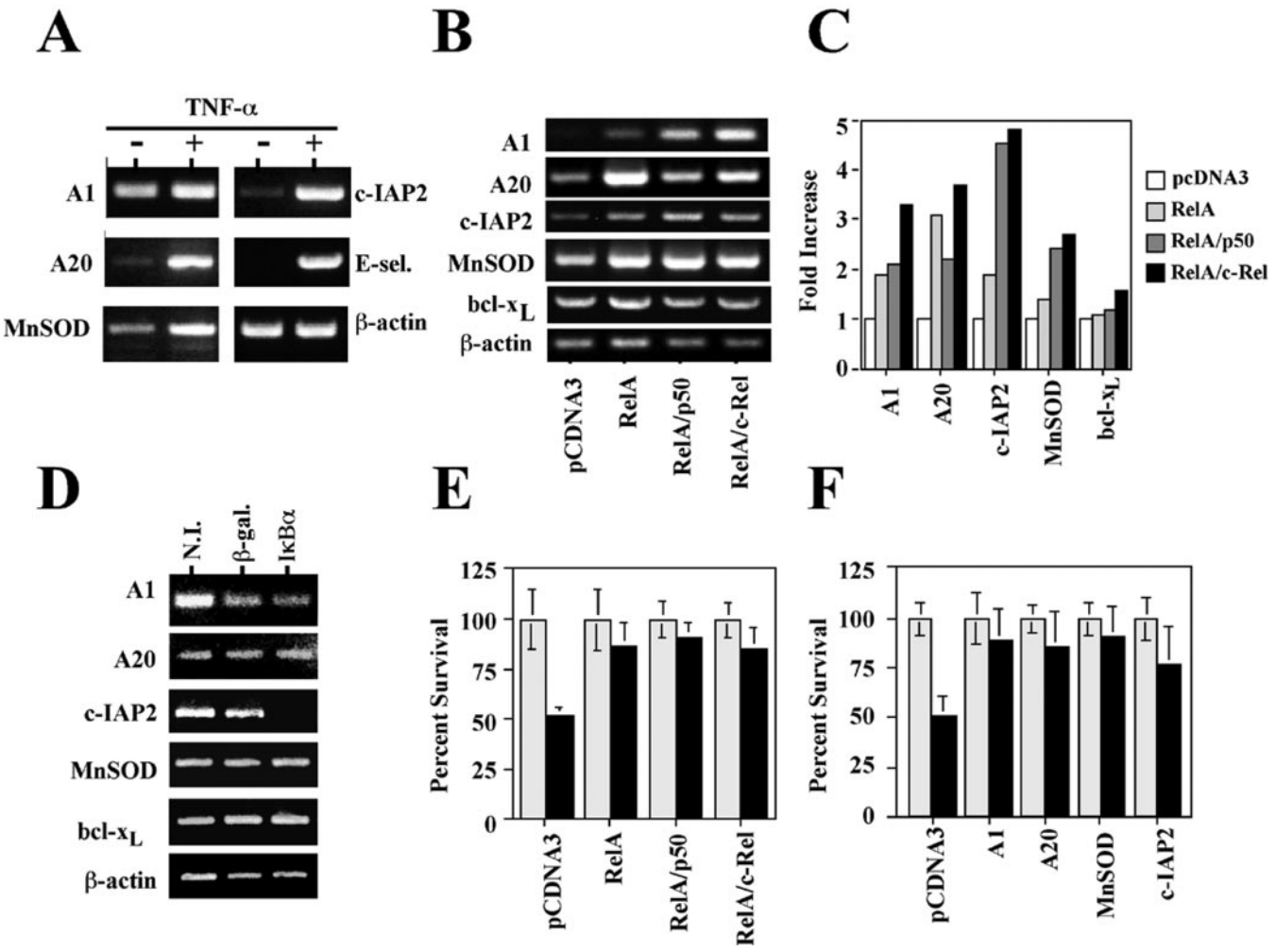

FIG. 6. ***A1, A20, MnSOD,* and c-*IAP2* are NF-κB-dependent anti-apoptotic genes that protect EC from TNF-α-mediated apoptosis.** *A*, HUVEC were either untreated (−) or treated (+) with TNF-α (50 units/ml, 2 h) before RNA extraction. Expression of *A1*, *A20*, *MnSOD*, c-*IAP2*, E-selectin, and β-actin mRNA was detected by RT-PCR as described under "Experimental Procedures." Notice that in the absence of TNF-α stimulation, HUVEC express basal levels of *A1*, *A20*, *MnSOD*, and c-*IAP2* but not E-selectin. *B*, 2F-2B EC were transiently transfected with p65/RelA (200 ng/3 × 10^5 cells), p65/RelA (100 ng/3 × 10^5 cells) plus p50 (100 ng/3 × 10^5 cells), or p65/RelA (100 ng/3 × 10^5 cells) plus c-Rel (100 ng/3 × 10^5 cells). Control EC were transfected with pcDNA3. Expression of different anti-apoptotic genes was analyzed by RT-PCR as described under "Experimental Procedures." *C*, the results shown in *B* were normalized to β-actin expression and expressed as fold increase as compared with control EC transfected with pcDNA3. *D*, HUVEC were either not infected or infected with β-galactosidase (*β-gal.*) or IκBα recombinant adenoviruses as described under "Experimental Procedures." DNA was extracted 24 h after adenoviral infections, and the expression of anti-apoptotic genes was analyzed by RT-PCR as described in *A*. Notice that IκBα over-expression suppressed the expression of c-*IAP2* but not that of the other anti-apoptotic genes analyzed. The results shown are representative of two independent experiments. *E*, 2F-2B EC were transiently transfected with a β-galactosidase reporter (300 ng/3 × 10^5 cells) plus p65/RelA (1000 ng/3 × 10^5 cells), p65/RelA (500 ng/3 × 10^5 cells) plus p50 (500 ng/3 × 10^5 cells), or p65/RelA (500 ng/3 × 10^5 cells) plus c-Rel (500 ng/3 × 10^5 cells). *Gray* and *black histograms* represent EC exposed to Act.D (10 μg/ml) or Act.D (10 μg/ml) plus TNF-α (50 units/ml) at 24 h after transfection for a period of 8 h. Percent survival of transfected EC was calculated as described under "Experimental Procedures." The results shown are the mean ± standard deviation from duplicate wells in four independent experiments ($n = 8$). Notice the highly significant increase in the percent survival of EC transfected with p65/RelA, p65/RelA plus p50, or p65/RelA plus p50 *versus* control EC transfected with pcDNA3 ($p < 0.0001$, unpaired *t* test). *F*, 2F-2B EC were transiently transfected with a β-galactosidase reporter (300 ng/3 × 10^5 cells) and *A1*, *A20*, *MnSOD*, or c-*IAP2* (1000 ng/3 × 10^5 cells). *Gray* and *black histograms* represent EC exposed to Act.D (10 μg/ml) or Act.D plus TNF-α (50 units/ml) at 24 h after transfection for a period of 8 h. Percent survival of transfected EC was calculated as described under "Experimental Procedures." The results shown are the mean ± standard deviation from duplicate wells in three independent experiments ($n = 6$). Notice the significant increase in the percent survival of EC transfected with A1 ($p = 0.0006$, unpaired *t* test), A20 ($p = 0.0022$, unpaired *t* test), MnSOD ($p = 0.0004$, unpaired *t* test), and c-IAP2 ($p = 0.019$, unpaired *t* test) *versus* control EC transfected with pcDNA3.

purified Ig isotype was used as negative controls.

Confocal Microscopy—BAEC were cultured on gelatinized glass slides (PerkinElmer Life Sciences), fixed in 3.7% paraformaldehyde (Sigma), and stained with an anti-N-terminal p65/RelA antibody (2 μg/ml, Santa Cruz Biotechnology, SC 372). Primary antibody was detected using fluorescein isothiocyanate-labeled goat anti-rabbit antibody (F-9262, Pierce). Fluorescent labeling was detected ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 518$ nm) using a multiphoton confocal microscope (BioRad, MRC 1024) equipped with LaserSharp, version 3.2 software (BioRad).

RESULTS

HO-1-derived CO Requires NF-κB Transcriptional Activity to Suppress EC Apoptosis—Inhibition of transcription by Act.D sensitized control EC transfected with pcDNA3 to undergo TNF-α-mediated apoptosis (60–70% apoptosis) (Fig. 1*A*). Over-expression of HO-1 or Bcl-2-protected EC from apoptosis (5–10% apoptosis) (Fig. 1*A*) (12, 31). When EC over-expressed IκBα, a gene that suppresses NF-κB activation (41), HO-1 was no longer able to prevent TNF-α-mediated EC apoptosis (60–70% apoptosis) (Fig. 1*A*). In contrast, Bcl-2 protected EC from TNF-α-mediated apoptosis when IκBα was over-expressed (5–15% apoptosis) (Fig. 1*A*). As previously shown (12, 31) exogenous CO protected EC cells from TNF-α-mediated apoptosis (Fig. 1*B*). Exogenous CO also protected EC from serum depri-

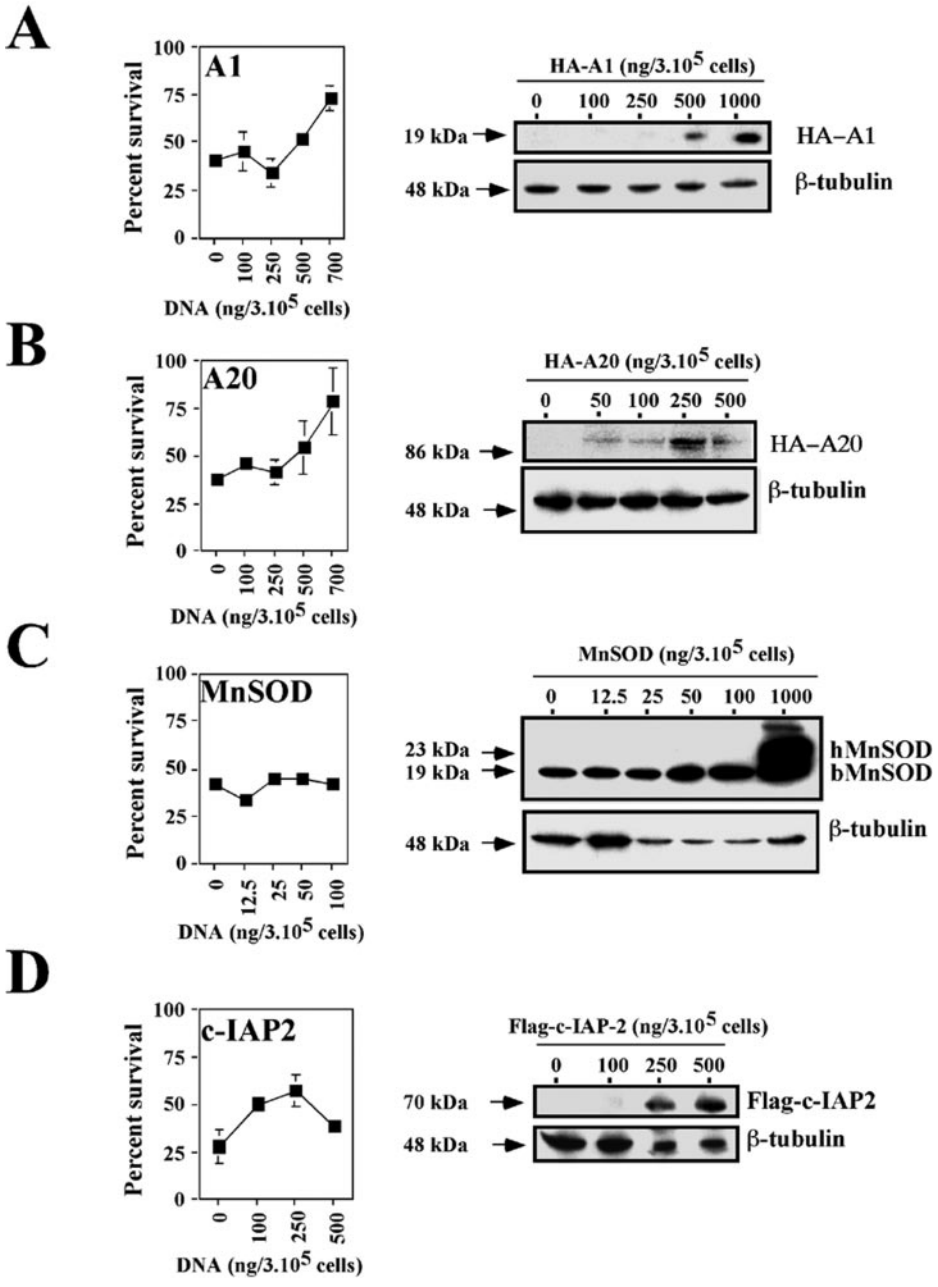

FIG. 7. **Titration of the anti-apoptotic effect of NF-κB-dependent anti-apoptotic genes in EC.** 2F-2B EC were transiently transfected with a β-galactosidase reporter (300 ng/3 × 10^5 cells), IκBα (500 ng/3 × 10^5 cells), *A1* (*A*), *A20* (*B*), *MnSOD* (*C*), or c-*IAP2* (*D*) expression vectors used at the amounts indicated. Control EC were transfected with β-galactosidase plus pcDNA3 expression vectors. Apoptosis was induced by TNF-α (24 h after transfection; 50 units/ml, 16 h). Percent survival of transfected EC was calculated as described under "Experimental Procedures." The results shown are mean ± standard deviation from duplicate wells in one of three similar experiments. Expression of HA-tagged A1, HA-tagged A20, MnSOD, and Flag-tagged c-IAP2 was detected in BAEC by Western blot using a rat anti-HA, mouse anti-Flag, or rabbit anti-MnSOD antibodies as described under "Experimental Procedures." Notice that transfected human MnSOD (*hMnSOD*, 23 kDa) can be distinguished from endogenous BAEC (*bMnSOD*, 19 kDa) based on the higher molecular mass of the human form of MnSOD (23 kDa).

vation-induced apoptosis (Fig. 1*C*). CO no longer protected EC from TNF-α-mediated apoptosis when IκBα was over-expressed (50–60% apoptosis) (Fig. 1, *A* and *B*). However, CO was still able to protect EC from serum deprivation-mediated apoptosis when IκBα was over-expressed (5–10% apoptosis) (Fig. 1*C*). These data indicate that CO requires the activation of NF-κB to protect EC from TNF-α but not from serum deprivation-mediated apoptosis.

The observation that IκBα abrogates the ability of HO-1 and/or CO to prevent TNF-α-mediated apoptosis (Fig. 1, *A* and *B*) could be attributed to an intrinsic pro-apoptotic effect of IκBα that would act independently of its ability to suppress NF-κB activity. To test this hypothesis, we analyzed whether reconstitution of NF-κB activity, under IκBα over-expression, would restore the anti-apoptotic effect of HO-1. Over-expression of p65/RelA resulted in high levels of NF-κB transcriptional activity in EC (Fig. 1*D*) in a dose-dependent manner, in that increasing amounts of p65/relA resulted in increasing levels of NF-κB transcriptional activity (Fig. 1*D*). Over-expression of IκBα inhibited p65/RelA transcriptional activity only when p65/RelA was expressed at low levels (10 ng/3 × 10^5 cells) (Fig. 1*D*). When the amount of p65/RelA was increased to levels higher then 100 ng/3 × 10^5 cells, IκBα over-expression was no longer fully able to suppress p65/RelA transcriptional activity (Fig. 1*D*), a result consistent with our previous observations (12). Over-expression of IκBα sensitized EC to TNF-α-mediated apoptosis in the absence of Act.D (65–75% apoptotic EC) (Fig. 1*E*) (12). Co-expression of p65RelA with HO-1 restored the anti-apoptotic effect of HO-1 and prevented TNF-α-mediated apoptosis of IκBα-expressing EC (2–5% apoptotic EC) (Fig. 1*E*). This protective effect was not observed when EC were co-transfected with HO-1 plus a DNA binding-deficient mutant of p65/RelA, which has no transcriptional activity (37) (Fig. 1*E*). Expression of HO-1, IκBα, and p65/RelA proteins was confirmed by Western blot (Fig. 1*F*).

Over-expression of HO-1 or Exposure to Exogenous CO Does Not Activate NF-κB in EC—Given that HO-1/CO requires NF-κB activity to exert its anti-apoptotic effect, we tested

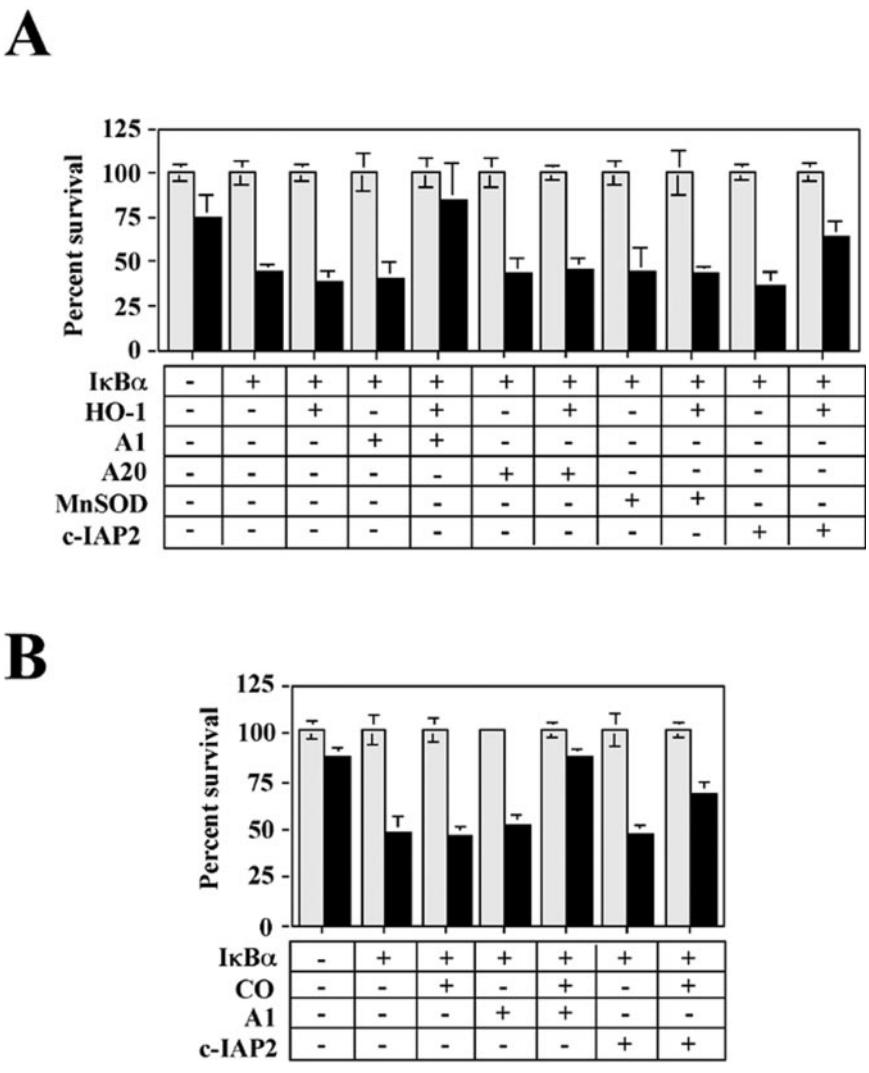

FIG. 8. **Functional interaction between NF-κB-dependent anti-apoptotic genes and HO-1-derived CO in protecting EC from apoptosis.** *A*, 2F-2B EC were transiently transfected with a β-galactosidase reporter (300 ng/3 × 10^5 cells) and when indicated (+) with IκBα (500 ng/3 × 10^5 cells), HO-1 (700 ng/3 × 10^5 cells) and/or A1 (250 ng/3 × 10^5 cells), A20 (250 ng/3 × 10^5 cells), MnSOD (50 ng/3 × 10^5 cells), or c-IAP2 (50 ng/3 × 10^5 cells) expression vectors. A1, A20, MnSOD, and c-IAP2 were used at suboptimal amounts, which *per se* do not prevent EC apoptosis (see Fig. 7). *Gray* and *black histograms* represent EC exposed to medium or TNF-α (24 h after transfection; 50 units/ml; 16 h), respectively. Percent survival of transfected EC was calculated as described under "Experimental Procedures." The results shown are the mean ± standard deviation from duplicate wells in three independent experiments ($n = 6$). Notice the highly significant increase in the percent survival of EC transfected with HO-1 plus A1 ($p = 0.0008$, unpaired t test) or c-IAP2 ($p = 0.0002$, unpaired t test) *versus* EC that do not express HO-1 but express A1 or c-IAP2, respectively. *B*, 2F-2B EC were transiently transfected with a CMV-driven β-galactosidase reporter (300 ng/3 × 10^5 cells) and when indicated (+) with IκBα (500 ng/3 × 10^5 cells) and/or A1 (250 ng/3 × 10^5 cells), or c-IAP2 (50 ng/3 × 10^5 cells) as described in *A*. When indicated EC were exposed to CO (10,000 ppm) as described under "Experimental Procedures." *Gray* and *black histograms* represent EC exposed to medium or TNF-α (24 h after transfection; 50 units/ml, 16 h), respectively. Percent survival of transfected EC was calculated as described in *A*. The results shown are mean ± standard deviation from duplicate wells in three independent experiments ($n = 6$). Notice the highly significant increase in the percent survival of EC transfected with A1 or c-IAP2 and exposed to CO *versus* EC not exposed to CO ($p < 0.0001$, unpaired t test).

whether HO-1 induced NF-κB nuclear translocation/activity in EC. Transient HO-1 over-expression in EC did not induce a detectable increase in NF-κB nuclear translocation and/or NF-κB binding to NF-κB-specific DNA binding consensus sequences as compared with quiescent EC (Fig. 2*A*). In a similar manner, over-expression of HO-1 was not associated with detectable increase in NF-κB transcriptional activity (Fig. 2*B*). Over-expression of HO-1 protein was confirmed by Western blot (Fig. 2*C*). Exposure of EC to exogenous CO (10,000 ppm, 2 h) did not cause an significant increase in the nuclear translocation of NF-κB (p65/RelA) as analyzed by confocal microscopy (Fig. 2*D*) and EMSA (data not shown).

Quiescent EC Have Basal Levels of Nuclear NF-κB—The data illustrated in Fig. 3 suggest that quiescent BAEC have basal levels of nuclear NF-κB (p65/RelA), evidenced by confocal microscopy using an antibody that recognizes the N-terminal region of p65/RelA (Fig. 3). Nuclear p65/RelA was also seen in quiescent 2F-2B EC by immunocytochemistry using antibodies that recognizes either the N-terminal region of p65/RelA or the nuclear localization signal region of p65/RelA, which is rendered accessible when p65/RelA is released from IκB molecules (Fig. 4*A*). The presence of nuclear p65/RelA in quiescent EC (Figs. 3 and 4*A*) correlated with the detection of p65/RelA containing NF-κB dimers that bound to NF-κB-specific DNA binding sequences in EMSA (Fig. 4*B*). Quiescent 2F-2B EC had two types of nuclear NF-κB dimers containing p65/RelA (referred to as dimers *2* and *3*; Fig. 4*B*). Stimulation of 2F-2B EC with lipopolysaccharide (LPS) resulted in increased nuclear translocation of dimers 2 and 3 as well as in nuclear translocation of an additional NF-κB dimer containing p65/RelA (referred to as dimer *4*; Fig. 4*B*). NF-κB dimers 1 and 2 were also detected in quiescent primary PAEC (Fig. 4*C*) and BAEC,

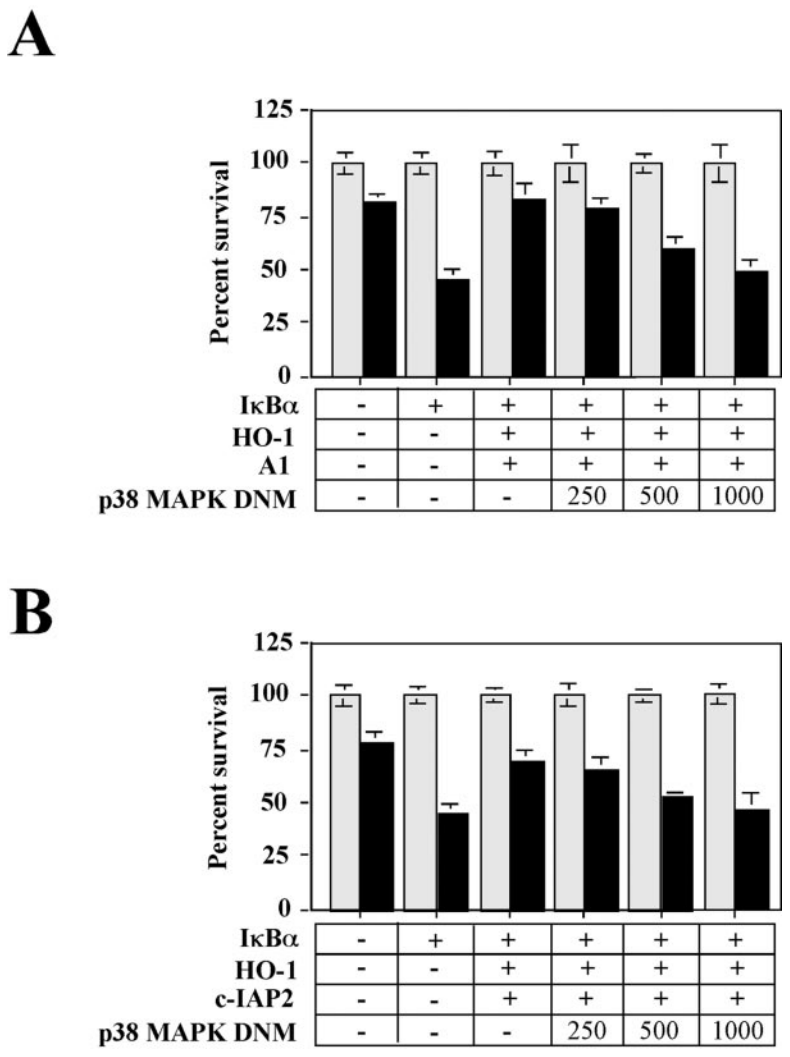

FIG. 9. **Functional interaction between NF-κB-dependent genes and HO-1 requires the activation of p38 MAPK.** 2F-2B EC were transiently transfected with a β-galactosidase reporter (300 ng/3 × 10^5 cells) and when indicated (+) with IκBα (500 ng/3 × 10^5 cells), HO-1 (700 ng/3 × 10^5 cells) and/or A1 (250 ng/3 × 10^5 cells) (*A*), or c-IAP2 (50 ng/3 × 10^5 cells) (*B*) expression vectors. When indicated EC were co-transfected with a p38/CSBP1 dominant negative mutant (*p38 MAPK DNM*; 250, 500, or 1000 ng/3 × 10^5 cells) expression vector. A1 and c-IAP2 expression vectors were used at suboptimal amounts, which *per se* do not prevent EC apoptosis (see Fig. 7). *Gray* and *black histograms* represent EC exposed to medium or TNF-α (24 h after transfection; 50 units/ml, 16 h), respectively. Percent survival of transfected EC was calculated as described under "Experimental Procedures." The results shown are the mean ± standard deviation from duplicate wells in three independent experiments ($n = 6$). *A*, notice that the increased percent of survival in EC expressing HO-1 and A1 is highly significant *versus* EC expressing HO-1 without A1 ($p < 0.0001$, unpaired *t* test). The decreased percent of survival in EC expressing the dominant negative mutant (*DNM*) of p38 MAPK is highly significant ($p < 0.0001$, unpaired *t* test) *versus* EC expressing HO-1 and A1 when the p38 MAPK dominant negative mutant was expressed at 500–1000 ng/3 × 10^5 cells. This decrease was not significant ($p = 0.2531$, unpaired *t* test) when the p38 MAPK dominant negative mutant was expressed at 250 ng/3 × 10^5 cells. *B*, increased percent of survival in EC expressing HO-1 and c-IAP2 is highly significant *versus* EC expressing HO-1 without c-IAP2 ($p < 0.0001$, unpaired *t* test). The decrease in percent survival in EC expressing the dominant negative mutant (*DNM*) of p38 MAPK is highly significant ($p < 0.0001$, unpaired *t* test) *versus* EC expressing c-IAP2, when the p38 MAPK dominant negative mutant was expressed at 500 ng/3 × 10^5 cells This decrease was very significant ($p = 0.022$, unpaired *t* test) when the p38 MAPK dominant negative mutant was expressed at 1000 ng/3 × 10^5 cells but was not significant at 250 ng/3 × 10^5 cells ($p = 0.2054$, unpaired *t* test).

which had an additional p65/RelA containing NF-κB dimer (referred to as dimer 5; Fig. 4*D*). Over-expression of IκBα in BAEC suppressed DNA binding of dimer 2 and significantly decreased that of dimers 3–5 (Fig. 4*D*).

NF-κB Activity Restores the Anti-apoptotic Effect of HO-1-derived CO—We tested whether basal level of NF-κB activity would support the anti-apoptotic effect of HO-1-derived CO. Transient over-expression of p65/RelA, p65/RelA plus p50, or p65/RelA plus c-Rel induced NF-κB transcriptional activity in EC (Fig. 5*A*). p65/RelA was more efficient in doing so than p65/RelA plus p50 or c-Rel (Fig. 5*A*). Co-expression of IκBα with these Rel family members inhibited NF-κB transcriptional activity (Fig. 5*A*). However, NF-κB activity was still 2–3 times higher in EC that co-expressed IκBα with the different Rel proteins compared with EC that expressed IκBα alone (Fig. 5*A*). When co-expressed with IκBα at these levels, p65/RelA or p65/RelA plus p50 or c-Rel *per se* did not protect EC from TNF-α-mediated apoptosis (60–70% of EC apoptosis) (Fig. 5*B*). However, at the same level of expression both p65/RelA and p65/RelA plus c-Rel restored the anti-apoptotic effect of CO in EC that over-expressed IκBα (Fig. 5*B*). Co-expression of p65/RelA with p50 did not restore the anti-apoptotic effect of CO (Fig. 5*B*), which was not correlated with a lower transcriptional activity of p65/RelA plus p50 *versus* p65/RelA plus c-Rel (Fig. 5*A*). Similarly, when EC were co-transfected with HO-1 and IκBα, p65/RelA or p65/RelA plus c-Rel with IκBα restored the anti-apoptotic effect of HO-1 (data not shown). Expression of p65/RelA, p50, and c-Rel was detected by Western blot using

antibodies that recognize both the endogenous and over-expressed forms of these NF-κB family members (Fig. 5*C*). Over-expressed IκBα was detected by Western blot using an anti-HA antibody (Fig. 5*C*).

Quiescent EC Have Basal Expression of NF-κB-dependent Anti-apoptotic Genes—We hypothesized that the anti-apoptotic effect associated with basal NF-κB activity acted via the expression of NF-κB-dependent anti-apoptotic genes. Consistent with this hypothesis, quiescent EC expressed basal levels of mRNA encoding the NF-κB-dependent anti-apoptotic genes *A1, A20*, *MnSOD*, and c-*IAP2* as detected by RT-PCR (Fig. 6, *A* and *D*). These cells did not express mRNA encoding the pro-inflammatory gene E-selectin, illustrating their "quiescent" state (Fig. 6*A*). The following set of observations demonstrated that expression of *A1, A20, MnSOD*, and c-*IAP2* is NF-κB-dependent in these cells: i) up-regulation of these genes by TNF-α was abrogated when NF-κB activation was blocked by IκBα over-expression (data not shown); ii) over-expression of p65/RelA, p65/RelA with p50, or p65/RelA with c-Rel up-regulated the expression of these genes but not that of the non-NF-κB-dependent anti-apoptotic gene *bcl-x*$_L$ (Fig. 6, *B* and *C*); and iii) over-expression of IκBα suppressed the basal level of expression of some of these genes, *i.e.* c-*IAP2* but not *A1, A20*, or *MnSOD* (Fig. 6*D*). Up-regulation of the expression of these anti-apoptotic genes correlated with the ability of p65/RelA, p65/RelA plus p50 or p65/RelA plus c-Rel to protect EC from TNF-α plus Act.D-mediated apoptosis (Fig. 6*E*). That the anti-apoptotic effect of these Rel family members is mediated through the up-regulation of A1, A20, MnSOD, and c-IAP2 is supported by the observation that over-expression of these anti-apoptotic genes was sufficient *per se* to protect EC from TNF-α plus Act.D-mediated apoptosis (Fig. 6*F*).

The Anti-apoptotic Effect of HO-1 Requires the Expression of NF-κB-dependent Anti-apoptotic Genes—To mimic basal level of expression of *A1, A20, MnSOD,* and c-*IAP2* in quiescent EC the expression/function of these genes was analyzed in a dose-responsive manner under inhibition of endogenous NF-κB activity by IκBα. Co-expression of A1 or A20 with IκBα protected EC from TNF-α-mediated apoptosis (Fig. 7, *A* and *B*). This effect was dose-dependent in that higher levels of A1 or A20 expression increased protection (Fig. 7, *A* and *B*). Co-expression of MnSOD with IκBα did not prevent EC apoptosis, a situation that mimics that of HO-1 (Fig. 7*C*). Co-expression of c-IAP2 with IκBα also protected EC from apoptosis (Fig. 7*D*). However, the protective effect of c-IAP2 was not strictly dose-dependent in that protection was lost when c-IAP2 was expressed above a certain threshold level (Fig. 7*D*). This effect was not altered when TNF receptor-associated receptor 2 was co-expressed with c-IAP2 (data not shown), a phenomenon reported to occur in other cell types (43). We tested whether expression of these anti-apoptotic genes at a level that, *per se*, would not prevent EC from undergoing apoptosis (*i.e.* attempting to mimic the situation found in quiescent EC) would reconstitute the anti-apoptotic effect of HO-1. When co-expressed with IκBα, A1 (250 ng/3 × 10^5 cells), A20 (250 ng/3 × 10^5 cells), MnSOD (25 ng/3 × 10^5 cells), or c-IAP2 (25 ng/3 × 10^5 cells) did not prevent EC apoptosis (Fig. 8*A*). However, when co-expressed with IκBα, A1 and c-IAP2 but not A20 or MnSOD restored the anti-apoptotic effect of HO-1 (Fig. 8*A*). We tested whether CO would act in a similar manner to protect EC from TNF-α-mediated apoptosis. Co-expression of suboptimal levels of A1 or c-IAP2 with IκBα supported the anti-apoptotic function of exogenous CO (10,000 ppm), but A20 and MnSOD did not (Fig. 8*B*).

Given that HO-1-derived CO activates the p38 MAPK signal transduction pathway (31) and that the anti-apoptotic effect of HO-1-derived CO is dependent on the activation of p38 MAPK (31), we tested whether the "functional interaction" between HO-1/CO and A1 or c-IAP2 (Fig. 8*A*) required the activation of p38 MAPK. The ability of A1 and c-IAP2 to restore the anti-apoptotic effect of HO-1 was abrogated when p38 MAPK activation was inhibited by co-expression of a p38α dominant negative mutant (Fig. 9).

DISCUSSION

In EC the expression of HO-1 can be induced by a multitude of pro-oxidant stimuli including free heme released from hemoproteins such as hemoglobin and/or myoglobin when these are oxidized during tissue injury and necrosis. Free heme intercalates into EC membranes to reach the intracellular compartment and exert potent cytotoxic effects (29, 44). The only known mechanism by which EC can clear high levels of intracellular heme is through the up-regulation of HO-1 expression. Under these circumstances HO-1 becomes the rate-limiting enzyme in the catabolism of heme into free iron, bilirubin, and CO (25, 26). We have previously shown that expression of HO-1 is part of a physiological response to injury by which EC are protected from undergoing apoptosis (28, 31). We have also shown, along with others, that the anti-apoptotic effect of HO-1 acts via the generation of CO (31, 45) and that in EC this anti-apoptotic action depends on the activation of the p38 MAPK a signal transduction pathway (31).

In the present manuscript we demonstrate that in addition to the p38 MAPK signal transduction pathway, the anti-apoptotic action of HO-1 is also dependent on the activation of the transcription factor NF-κB. Once NF-κB activation is inhibited, as by overexpression of its natural inhibitor IκBα, HO-1 and/or CO can no longer protect EC cells from undergoing TNF-α-mediated apoptosis (Fig. 1). The need for NF-κB activation seems specific to the TNF-α signal transduction pathway because CO can protect EC from serum deprivation-induced apoptosis even if NF-κB activation is inhibited (Fig. 1). Given that we cannot detect significant levels of NF-κB activation by HO-1/CO in EC (Fig. 2), we reasoned that quiescent EC must have basal levels of NF-κB activity that are required to sustain the anti-apoptotic action of CO. In fact we found that quiescent EC have significant levels of nuclear NF-κB dimers that contain "free" p65/RelA that can bind to NF-κB DNA consensus sequences (Figs. 3 and 4). This finding is keeping with the observation by others that NF-κB dimers, containing p65/RelA, can "shuttle" between the cytoplasm and the nuclei of quiescent cells as a result of their permanent dissociation from IκBα molecules (46). Our present data suggest that when these NF-κB dimers are no longer able to bind to NF-κB DNA consensus sequences in the regulatory region of NF-κB-dependent anti-apoptotic genes, the cytoprotective action of CO is impaired (Fig. 4*D*).

We found that basal levels of nuclear NF-κB dimers promote the expression of some anti-apoptotic genes, *e.g.* c-*IAP2*, in quiescent EC that "interact functionally" with CO to prevent TNF-α-mediated apoptosis. Our evidence includes the observations that: i) IκBα over-expression suppresses the basal level of expression of some NF-κB-dependent anti-apoptotic genes such as c-*IAP2* (Fig. 6); ii) over-expression of NF-κB dimers in EC induces the expression of anti-apoptotic genes such as c-*IAP2*; and iii) under inhibition of NF-κB by IκBα, ectopic expression of anti-apoptotic genes such as c-*IAP2* partially restores the anti-apoptotic effect of HO-1 and/or CO (Fig. 8). Taken together these data would suggest that the ability of CO to protect EC from TNF-α-mediated apoptosis requires the expression of anti-apoptotic genes that are themselves dependent on NF-κB activation for their presence. c-IAP2 is one member of the family of NF-κB-dependent anti-apoptotic genes. Given that c-*IAP2* only partially restores the anti-apoptotic action of CO (Fig. 8) when NF-κB activity is inhibited, there must be addi-

tional NF-κB-dependent anti-apoptotic genes that interact functionally with the events stimulated by CO to prevent EC apoptosis. We found that genes such as *A1* also can restore the anti-apoptotic effect of CO under inhibition of NF-κB activity (Fig. 8). In contrast with c-*IAP2*, however, the basal level of *A1* expression in quiescent EC is not suppressed when NF-κB activation is inhibited by IκBα (Fig. 6). This observation would suggest that the events that are induced by HO-1-derived CO also interact functionally with NF-κB-dependent anti-apoptotic genes such as *A1* that are up-regulated only when NF-κB in EC is fully activated, as happens during EC activation.

The ability of CO-stimulated responses to interact functionally with NF-κB-dependent anti-apoptotic genes is specific to some of these genes, *i.e. A1* and c-*IAP2*, as this interaction does not occur with other well established NF-κB-dependent anti-apoptotic genes, *i.e. A20* and *MnSOD* (Fig. 7). This excludes the possibility that the functional interaction between CO and A1 or c-IAP2 results simply from a nonspecific change in the overall "apoptotic threshold" of these cells when additional anti-apoptotic genes are co-expressed with HO-1.

Not all subsets of NF-κB dimers seem to be able to support the anti-apoptotic effects of CO in that NF-κB dimers containing c-Rel do so, but those containing p50 do not (Fig. 5*B*). This functional difference should not be attributed directly to a quantitative difference between the quantitative ability of the different NF-κB dimers to induce NF-κB transcriptional activity (Fig. 5*A*). Our interpretation is that NF-κB dimers containing p65/RelA and c-Rel selectively up-regulate the expression of genes that can cooperate with CO to prevent EC apoptosis, an idea supported by the observation that NF-κB dimers containing c-Rel are more efficient then those containing p50 in up-regulating the expression of anti-apoptotic genes such as *A1* (Fig. 5*B*). This is also in keeping with the observation by others that c-Rel is required for *A1* expression in B cells (21, 47).

The mechanism by which A1 and/or c-IAP2 interact functionally with CO to promote EC survival is not clear. Presumably CO does not interact physically with the products of these genes but rather activates one or several signaling transduction pathways, *e.g.* p38 MAPK (31, 48), that modulate their anti-apoptotic function. This model is supported by the observation that the functional interaction of CO with A1 or c-IAP2 is suppressed under the inhibition of p38 MAPK activation (Fig. 9). This notion is also is in keeping with the observation that MAPK signal transduction pathways can interact directly with anti-apoptotic genes such as *bcl-2* to modulate their level of expression and thus regulate their ability to protect EC from undergoing apoptosis (49).

In conclusion we have identified a functional interaction between the NF-κB and the HO-1/CO signal transduction pathways that converge to suppress EC apoptosis. This set of findings highlights the biological significance of NF-κB-dependent anti-apoptotic genes in regulating the anti-apoptotic effect of other signaling transduction pathways, such as those involved in the expression of HO-1 and the generation of CO. Given the recent identification of the potent protective effects of CO in preventing inflammatory reactions such as those that lead to the rejection of transplanted organs (50), our present findings may have important implications not only for the understanding of the mechanisms regulating the protective effects of CO but also for the development of therapeutic approaches to suppress these inflammatory reactions.

Acknowledgments—We thank Eva Csizmadia (Beth Israel Deaconess Medical Center) for expert support with immunohistochemical staining and Josef Anrather (Cornell University) for critical review of the manuscript and providing constructs generated in his laboratory. We also thank Dr. Nuno Moreno (Instituto Gulbenkian de Ciência) for assistance in using the confocal microscope.

REFERENCES

1. Peter, M. E., and Krammer, P. H. (1998) *Curr. Opin. Immunol.* **10,** 545–551
2. Ashkenazi, A., and Dixit, V. M. (1998) *Science* **281,** 1305–1308
3. Thornberry, N., and Lazebnik, Y. (1998) *Science* **281,**
4. Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A., Mann, M., Krammer, P. H., and Peter, M. E. (1997) *EMBO J.* **16,** 2794–2804
5. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *Cell* **85,** 817–827
6. Lee, E. G., Boone, D. L., Chai, S., Libby, S. L., Chien, M., Lodolce, J. P., and Ma, A. (2000) *Science* **289,** 2350–2354
7. Ackermann, E. J., Taylor, J. K., Narayana, R., and Bennett, C. F. (1999) *J. Biol. Chem.* **274,** 11245–11252
8. Wong, G. H., Elwell, J. H., Oberley, L. W., and Goeddel, D. V. (1989) *Cell* **58,** 923–931
9. Deveraux, Q. L., and Reed, J. C. (1999) *Genes Dev.* **13,** 239–252
10. Wu, M. X., Ao, Z., Prasad, K. V., Wu, R., and Schlossman, S. F. (1998) *Science* **281,** 998–1001
11. Gan, H., Newman, G. W., and Remold, H. G. (1995) *J. Immunol.* **155,** 1304–1315
12. Soares, M. P., Muniappan, A., Kaczmarek, E., Koziak, K., Wrighton, C. J., Steinhauslin, F., Ferran, C., Winkler, H., Bach, F. H., and Anrather, J. (1998) *J. Immunol.* **161,** 4572–4582
13. Beg, A. A., and Baltimore, D. (1996) *Science* **274,** 782–784
14. Vanantwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) *Science* **274,** 787–789
15. Wang, C. Y., Mayo, M. W., and Baldwin, A. S. (1996) *Science* **274,** 784–787
16. Karin, M., and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* **18,** 621–663
17. Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16,** 225–260
18. Baeuerle, P. A., and Baltimore, D. (1988) *Science* **242,** 540–546
19. Traenckner, E. B., Wilk, S., and Baeuerle, P. A. (1994) *EMBO J.* **13,** 5433–5441
20. Traenckner, E. B., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1995) *EMBO J.* **14,** 2876–2883
21. Grumont, R. J., Rourke, I. J., and Gerondakis, S. (1999) *Genes Dev.* **13,** 400–411
22. Krikos, A., Laherty, C. D., and Dixit, V. M. (1992) *J. Biol. Chem.* **267,** 17971–17976
23. Jones, P. L., Ping, D., and Boss, J. M. (1997) *Mol. Cell. Biol.* **17,** 6970–6981
24. Hong, S. Y., Yoon, W. H., Park, J. H., Kang, S. G., Ahn, J. H., and Lee, T. H. (2000) *J. Biol. Chem.* **275,** 18022–18028
25. Choi, A. M., and Alam, J. (1996) *Am. J. Respir. Cell Mol. Biol.* **15,** 9–19
26. Maines, M. D. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37,** 517–554
27. Tenhunen, R., Marver, H. S., and Schmid, R. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **61,** 748–755
28. Soares, M. P., Lin, Y., Anrather, J., Csizmadia, E., Takigami, K., Sato, K., Grey, S. T., Colvin, R. B., Choi, A. M., Poss, K. D., and Bach, F. H. (1998) *Nat. Med.* **4,** 1073–1077
29. Balla, G., Jacob, H. S., Balla, J., Rosenberg, M., Nath, K., Apple, F., Eaton, J. W., and Vercellotti, G. M. (1992) *J. Biol. Chem.* **267,** 18148–18153
30. Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N., and Ames, B. N. (1987) *Science* **235,** 1043–1046
31. Brouard, S., Otterbein, L. E., Anrather, J., Tobiasch, E., Bach, F. H., Choi, A. M. K., and Soares, M. (2000) *J. Exp. Med.* **192,** 1015–1025
32. Anrather, J., Csizmadia, V., Brostjan, C., Soares, M. P., Bach, F. H., and Winkler, H. (1997) *J. Clin. Investig.* **99,** 763–772
33. Lee, P. J., Alam, J., Wiegand, G. W., and Choi, A. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10393–10398
34. Badrichani, A. Z., Stroka, D. M., Bilbao, G., Curiel, D. T., Bach, F. H., and Ferran, C. (1999) *J. Clin. Investig.* **103,** 543–553
35. Opipari, A. J., Boguski, M. S., and Dixit, V. M. (1990) *J. Biol. Chem.* **265,** 14705–14708
36. Cooper, J. T., Stroka, D. M., Brostjan, C., Palmetshofer, A., Bach, F. H., and Ferran, C. (1996) *J. Biol. Chem.* **271,** 18068–18073
37. Franzoso, G., Carlson, L., Brown, K., Daucher, M. B., Bressler, P., and Siebenlist, U. (1996) *EMBO J.* **15,** 3403–3412
38. Stroka, D. M., Badrichani, A. Z., Bach, F. H., and Ferran, C. (1999) *Blood* **93,** 3803–3810
39. Chu, Z. L., McKinsey, T. A., Liu, L., Gentry, J. J., Malim, M. H., and Ballard, D. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 10057–10062
40. Soares, M. P., Brouard, S., Smith, R. N., Otterbein, L., Choi, A. M., and Bach, F. H. (2000) *Emerg. Therap. Targets* **4,** 11–27
41. Wrighton, C. J., Hofer, W. R., Moll, T., Eytner, R., Bach, F. H., and De Martin, R. (1996) *J. Exp. Med.* **183**, 1013–1022
42. Anrather, J., Csizmadia, V., Soares, M. P., and Winkler, H. (1999) *J. Biol. Chem.* **274,** 13594–13603
43. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998) *Science* **281,** 1680–1683
44. Balla, J., Jacob, H. S., Balla, G., Nath, K., Eaton, J. W., and Vercellotti, G. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9285–9289
45. Petrache, I., Otterbein, L. E., Alam, J., Wiegand, G. W., and Choi, A. M. (2000) *Am. J. Physiol.* **278,** L312–L319
46. Carlotti, F., Dower, S. K., and Qwarnstrom, E. E. (2000) *J. Biol. Chem.* **275,** 41028–41034
47. Zong, W. X., Edelstein, L. C., Chen, C., Bash, J., and Gelinas, C. (1999) *Genes Dev.* **13,** 382–387
48. Otterbein, L. E., Bach, F. H., Alam, J., Soares, M. P., Tao, H. L., Wysk, M., Davis, R., Flavell, R., and Choi, A. M. K. (2000) *Nat. Med.* **6,** 422–428
49. Breitschopf, K., Haendeler, J., Malchow, P., Zeiher, A. M., and Dimmeler, S. (2000) *Mol. Cell. Biol.* **20,** 1886–1896
50. Sato, K., Balla, J., Otterbein, L., Smith, N. R., Brouard, S., Lin, Y., Czismadia, E., Sevigny, J., Robson, S. C., Vercellotti, G., Choi, A. M. K., Bach, F. H., and Soares, M. P. (2001) *J. Immunol.* **166,** 4185–4194