THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 45, Issue of November 10, pp. 35377–35383, 2000

Printed in U.S.A.

Rac1 Regulates Stress-induced, Redox-dependent Heat Shock Factor Activation*

Received for publication, June 18, 2000, and in revised form, August 15, 2000
Published, JBC Papers in Press, August 21, 2000, DOI 10.1074/jbc.M005287200

Michitaka Ozaki‡§, Shailesh S. Deshpande‡, Piamsook Angkeow‡, Seiichi Suzuki§, and Kaikobad Irani‡¶

From the ‡Division of Cardiology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, §Division of Experimental Surgery, National Children's Medical Research Center, Tokyo 154-8509, Japan

The signaling pathway by which environmental stresses activate heat shock factors (HSFs) is not completely understood. We show that the small GTPase rac1, and Rac1-regulated reactive oxygen species (ROS) play an important role in stress-stimulated heat shock response. A dominant-negative allele of Rac1 (Rac1N17) inhibits the hypoxia/reoxygenation and sodium arsenite-induced transcriptional activity of HSF-1 and the transcription of heat shock protein 70. Rac1N17 also suppresses the production of intracellular ROS induced by hypoxia/reoxygenation or sodium arsenite. Moreover, direct suppression of intracellular ROS levels by antioxidants decreases stress-stimulated HSF activity. However, expression of a constitutively active mutant of Rac1 (Rac1V12) in the absence of extracellular stresses does not increase intracellular ROS levels or induce the heat shock response. These results show that Rac1 is a necessary but insufficient component of the stress-induced signaling pathway that leads to ROS production, activation of HSFs, and transcription of heat shock proteins.

Induction of heat shock genes in response to cellular stresses is transcriptionally mediated by heat shock factors (HSFs)[1] (1). Mammalian HSFs are activated by a variety of stresses, including exposure to heat, cytokines, genotoxic agents, heavy metals, and hypoxia/reoxygenation (1, 2). In unstimulated cells, HSFs exist in the cytoplasm as inactive monomers that oligomerize as homotrimers with high DNA binding affinity after exposure to cellular stresses (3–5). Previous studies have linked the induction of the heat shock response *in vitro* and *in vivo* to redox changes (6, 7). In particular, millimolar doses of H_2O_2 can stimulate binding of HSFs to the heat shock element (HSE) and result in heat shock protein synthesis through a thioredoxin-dependent mechanism (8). In addition, thiol-reducing agents inhibit the heat shock response (7). Furthermore, many cellular stresses, such as hypoxia/reoxygenation, cytokines, and heat shock, that lead to HSF activation are capable of increasing the intracellular production of reactive oxygen species (ROS) (9–11). Nonetheless, the signaling pathway through which such extracellular stresses lead to the production of ROS, activation of heat shock factors, and transcription of heat shock genes is not fully characterized.

Rho proteins participate in a variety of cellular functions including cytoskeletal reorganization, growth, and transformation (12). The Rac1 GTPase belongs to the rho family of small GTP binding proteins, and its role in the production of reactive oxygen species in phagocytic cells such as neutrophils is well established (13). In such cells, Rac proteins are essential for the assembly of the plasma membrane NADPH oxidase that is responsible for the transfer of electrons to molecular oxygen leading to the production of superoxide anions. Rac proteins, in particular Rac1, serve a similar function in nonphagocytic cells (14, 15). Rac1 regulates cell growth, migration, and cellular transformation by controlling the intracellular production of ROS (11, 14). Furthermore, Rac1-regulated ROS can lead to the induction of redox-sensitive transcription factors such as nuclear factor κB (16). In this report, we investigated the possibility that Rac1, through the production of reactive oxygen species, may also modulate the transcriptional activity of heat shock factors and thereby participate in the stress-stimulated heat shock response.

EXPERIMENTAL PROCEDURES

Cells, Transfection, and CAT and Luciferase Reporter Assays—HepG2 cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells were seeded at a density of $1.5 \times 10^4/cm^2$ the day prior to transfection. LipofectAMINE (Life Technologies, Inc.) was used as the transfectant according to the manufacturer's recommendations. 48 h after transfection, the cells were exposed to the indicated stress, and chloramphenicol acetyltransferase (CAT) activity was measured by the phase extraction method as described previously (16). Luciferase activity was assessed using a luciferase reporter kit (Promega), according to the manufacturer's recommendation. CAT activity was normalized against protein content and luciferase activity, to correct for variations in transfection efficiencies. The normalized CAT activity is expressed relative to that from cells transfected with pEXV under normoxic conditions. Results of each CAT reporter assay were reproducible in three separate experiments.

Plasmids and Adenoviruses—The empty expression vector pEXV, the expression vectors encoding the constitutively active (*rac1V12*) and dominant negative (*rac1N17*) Myc epitope-tagged cDNAs of Rac1, and the expression plasmid for dominant negative c-*ras* (*rasN17*), have been previously described (14, 17). The wild-type (Δ5N/−105) and mutant (LSPN) HSF reporter plasmids, expression vectors for SOD1, catalase, and SEK(KR) have also been previously described (14, 18–20). Δ5N/

* This work was supported by the Johns Hopkins University Clinician Scientist Award (to K. I.), the American Heart Association (K. I.), the W. W. Smith Charitable Trust (to K. I.), the American Liver Foundation (to M. O.), the CardioFellows Foundation (P. A.), the Bernard Foundation, and an endowment from Abraham and Virginia Weiss. 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.

¶ To whom correspondence should be addressed: Kaikobad Irani, The Johns Hopkins University School of Medicine, Ross 1023, 720 Rutland Ave., Baltimore, MD 21205. Tel.: 410-502-6902; Fax: 410-614-6481; E-mail: kirani@mail.jhmi.edu.

[1] The abbreviations used are: HSF, heat shock factor; HSE, heat shock element; ROS, reactive oxygen species; CAT, chloramphenicol acetyltransferase; DPI, diphenylene iodonium; DCF, 7′-dichlorodihydrofluorescein; JNK, c-Jun N-terminal kinase.

−105 is a CAT reporter plasmid that has 105 base pairs, containing two HSE binding sites, of the inducible hsp70 promoter upstream of the initiation codon. pRSV-Luc is a constitutive expression plasmid encoding firefly luciferase. The adenovirus Adβgal, encoding the inert *Escherichia coli lacZ* gene and the adenovirus AdRac1N17, encoding the Myc-tagged dominant negative *rac1* cDNA, which was constructed in our laboratory, have been previously described (21). Both viruses were amplified in HEK 293 cells and purified on double cesium gradients. HepG2 cells were infected with adenoviruses at a multiplicity of infection of 50 for 18 h, after which the infection medium was aspirated and replaced with fresh medium for another 48 h.

Extracellular Stresses—Hypoxia was attained in a modular incubator chamber (Billups-Rothenberg) by flushing the chamber with a 95% N_2, 5% CO_2 gas mixture for 15 min and sealing the chamber. This method has been shown to achieve a pO_2 of 10 ± 5 torr (22). Cells were reoxygenated by opening the chamber and replacing the hypoxic medium with oxygenated medium. Sodium arsenite, diphenylene iodonium (DPI), and *N*-acetylcysteine were obtained from Sigma.

Immunoblot Analysis—48 h after infection with adenoviruses or transfection with expression plasmids, 20 μg of whole cell extracts were resolved by SDS-polyacrylamide gel electrophoresis on a 4–20% gel and transferred to nitrocellulose membrane. The Myc epitope-tagged constitutively active and dominant negative forms of Rac1 were detected using an antibody against Myc (9E10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibodies to HSF-1 and HSF-2 were a generous gift from R. Morimoto (Northwestern University). GST-tagged SEK(KR) expression was confirmed with an antibody to GST. In some cases detection of tubulin with a monoclonal antibody (DM 1A; Sigma) was used as an internal control to verify equal protein loading. Immunocomplexes were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as described previously (16). 5 μg of extract was incubated with 10^6 cpm of an end-labeled HSE consensus sequence oligonucleotide (5′-GCCTCGAATGTTCGCGAAGTT-3′) for 30 min at room temperature in binding buffer (10 mM Tris, pH 7.4, 80 mM KCl, 5% glycerol, 0.5 mM dithiothreitol, 2 μg of dIdC, and 10 μg of bovine serum albumin). HSF-1 and HSF-2 antibodies (αHSF1 and αHSF2) were preincubated with the nuclear extract for 15 min on ice prior to adding labeled oligonucleotide. Protein-DNA complexes were resolved on a 4% polyacrylamide gel and autoradiographed.

Measurements of Intracellular ROS—Intracellular fluorescence of the peroxide-sensitive fluorophore 2′-7′-dichlorodihydrofluorescein diacetate (DCF diacetate) was used to qualitatively assess ROS production (15). Briefly, cells were loaded with DCF diacetate (5 μg/ml; Molecular Probes, Inc., Eugene, OR) for 5 min and imaged immediately using a Zeiss confocal laser-scanning fluorescence microscope. A representative field is shown. Intracellular hydrogen peroxide concentration was quantified using the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes) according to the manufacturer's recommendations. The fluorescence of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a highly sensitive and stable probe for hydrogen peroxide (23), was measured with a Cytofluor 2300 (Millipore Corp.). A standard curve, generated using exogenous H_2O_2, was used to calculate hydrogen peroxide concentration. Values represent mean ± S.D. from a representative experiment that was reproduced twice.

Detection of Activated c-Jun N-terminal Kinases (JNKs)—30 μg of whole cell lysates were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Active JNKs were detected using an antibody directed specifically against dually phosphorylated (Thr^{183} and Tyr^{185}) JNK1 (46 kDa) and JNK2 (54 kDa) (Promega), according to the manufacturer's recommendations. Total JNKs were detected with an antibody to JNK1 and JNK2 (Cell Signaling). Immunocomplexes were visualized by ECL (Amersham Pharmacia Biotech).

Northern Blot Analysis—Total cellular RNA was extracted by a standard protocol (24). After electrophoresis on a 1.4% agarose gel, RNA was transferred to a nylon membrane, which was hybridized with 3×10^7 cpm of ^{32}P-labeled single-strand cDNA of human inducible hsp70. After washing under stringent conditions, the membrane was autoradiographed. The membrane was then stripped and rehybridized with a probe to 18 S rRNA and autoradiographed. Hsp70 mRNA normalized to 18 S rRNA was quantified using ImageQuant software (Molecular Dynamics). Results are from a single representative experiment that was reproduced once.

RESULTS

Rac1 Regulates Hypoxia/Reoxygenation-induced HSF Activation—Past studies have shown that exposure to hypoxia followed by reoxygenation (hypoxia/reoxygenation) results in a robust heat shock response (6, 25, 26). We examined the role of Rac1 in hypoxia/reoxygenation-induced HSF transcriptional activity. Hypoxia/reoxygenation but not hypoxia alone leads to a marked increase in CAT reporter activity in HepG2 cells transfected with the pEXV expression vector alone (Fig. 1*A*). The increase in CAT activity was specifically due to HSF activation, since cells transfected with a similar reporter plasmid (LSPN) bearing a 4-base mutation within the HSE sites show no comparable increase with hypoxia/reoxygenation. Expression of Rac1N17 results in reduction of basal and inhibition of hypoxia/reoxygenation-induced HSF activity without affecting steady state levels of HSF-1 or HSF-2 (Fig. 1*B*). Importantly, transfection with an expression plasmid for constitutively active Rac1 (Rac1V12) has no effect on HSF activity under unstressed conditions. Expression of the Rac1 mutants was confirmed by Western blotting (Fig. 1*C*). Thus, Rac1 is necessary but not sufficient for basal and hypoxia/reoxygenation-induced activation of HSFs.

Individual proteins of the Ras superfamily have specific cellular targets. We therefore examined the specificity of Rac1 in regulating hypoxia/reoxygenation-induced HSF activity. Expression of a dominant negative allele of c-*ras*, *rasN17*, is not effective at suppressing hypoxia/reoxygenation-induced HSF activation (Fig. 1*A*). Thus, the regulation of hypoxia/reoxygenation-induced HSF activation by Rac1 is specific for this small GTPase and was not shared by other small GTP-binding proteins of the Ras superfamily, in particular, c-Ras.

The heat shock response is a multistep process that involves the conversion of HSFs from nonbinding to DNA binding states and transcriptional competency. We therefore also examined the role of Rac1 in hypoxia/reoxygenation-induced HSF binding to its sequence-specific HSE using an electrophoretic mobility shift assay. HepG2 cells were infected with a recombinant replication-deficient adenovirus encoding Rac1N17 (AdRac1N17). Control cells were infected with an adenovirus encoding the inert *E. coli lacZ* gene (Adβgal). The cells were then maintained under normoxic conditions or exposed to hypoxia/reoxygenation. Nuclear extracts from these cells were subjected to an electrophoretic mobility shift assay using a sequence-specific HSE oligonucleotide. Hypoxia/reoxygenation results in a significant increase in specific HSF-HSE binding in Adβgal-infected cells (Fig. 2*A*). Infection with AdRac1N17 blunts this hypoxia/reoxygenation-induced increase in HSF-HSE binding. Preincubation of the nuclear extract with an antibody to HSF-1 suppresses hypoxia/reoxygenation-induced binding to HSE and results in a supershifted band implicating activation of HSF-1 by hypoxia/reoxygenation. In contrast, antibody to HSF-2 had little effect on DNA binding activity. Expression of the epitope-tagged Rac1N17 was evident in HepG2 cells infected with AdRac1N17 but not in Adβgal-infected cells (Fig. 2*B*). Thus, in addition to transcriptional competency, Rac1 also regulates hypoxia/reoxygenation-induced HSF-1 DNA binding.

Rac1 Regulates Arsenite-induced HSF Activation—Previous studies have shown that heavy metals such as arsenic and mercury induce many heat shock proteins (27). Therefore, we examined the role of Rac1 in sodium arsenite-induced HSF activation. Sodium arsenite leads to induction of HSF transcriptional activity in HepG2 cells transfected with the pEXV vector alone (Fig. 3). Transfection with the expression plasmid for Rac1N17 results in a significant inhibition of both absolute and relative increase of sodium arsenite-induced HSF activation. Thus, sodium arsenite, another known activator of the

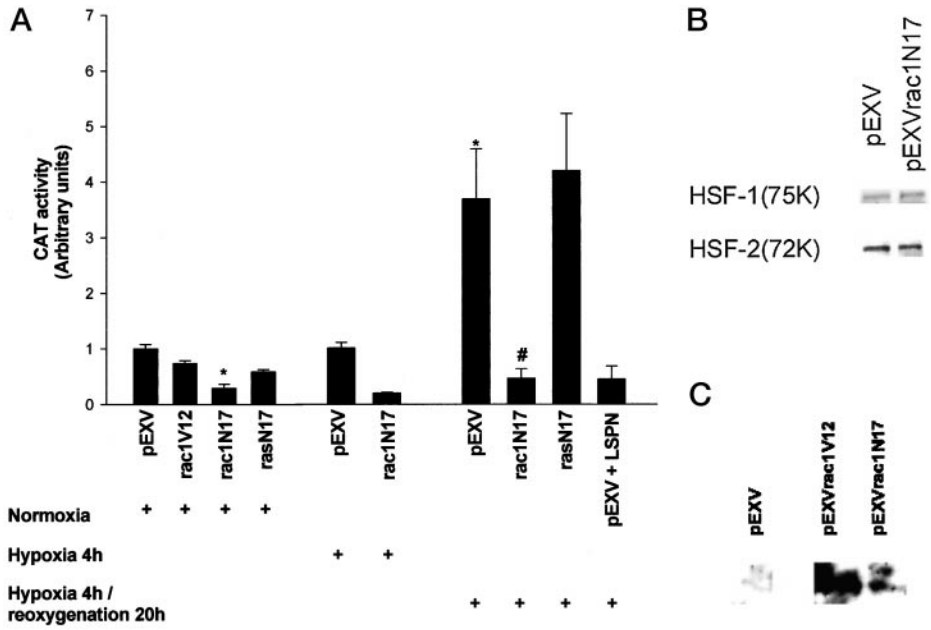

FIG. 1. **Expression of Rac1N17 suppresses hypoxia/reoxygenation-induced HSF activation.** *A*, HepG2 cells were co-transfected with 0.5 μg of the HSF reporter plasmid (Δ5N/$-$105) and 0.2 μg of the constitutively active pRSV-Luc construct. Where indicated, the mutant reporter plasmid LSPN was used instead of Δ5N/$-$105. In addition, cells were transfected with 3.0 μg of either the empty plasmid (pEXV) or expression plasmids encoding *rac1N17*, *rac1V12*, or *rasN17*. 48 h after transfection, the cells were exposed to hypoxia or hypoxia/reoxygenation or maintained under normoxia. CAT activity was measured 16 h later and normalized against luciferase activity. Results are expressed as mean $\pm$ SD of triplicate determinations from a single representative experiment. *, $p < 0.05$ compared with pEXV normoxia; #, $p < 0.05$ compared with pEXV hypoxia/reoxygenation (Student's *t* test). *B*, Western blot analysis showing no effect of Rac1N17 on HSF-1 or HSF-2 expression. Lysates from cells transfected with 3.0 μg of the empty plasmid pEXV or expression plasmid for Rac1N17 were used for immunoblotting with anti-HSF-1 or anti-HSF-2 antibodies. *C*, Western blot analysis showing expression of Rac1 mutants. Lysates from cells transfected with 3.0 μg of the empty plasmid pEXV or the expression plasmids for Myc-tagged Rac1N17 and Rac1V12 were used. An antibody to the Myc epitope was used to detect the Rac1 mutants.

heat shock response, is also dependent upon Rac1-regulated pathways for this response.

Rac1N17 Inhibits Hypoxia/Reoxygenation and Sodium Arsenite-stimulated Production of ROS—Many of the diverse cellular stresses, including hypoxia/reoxygenation and heavy metals, that induce the expression of heat shock protein genes, lead to an increase in intracellular oxidative stress. We therefore assessed the role of Rac1 in regulating hypoxia/reoxygenation and sodium arsenite-induced intracellular production of ROS. ROS levels were measured using two intracellular peroxide-sensitive fluorophores: DCF and Amplex Red. DCF fluorescence has been used extensively in the past to detect intracellular ROS, and Amplex Red is a new, highly sensitive and specific fluorophore that fluoresces upon interaction with H_2O_2. Hypoxia/reoxygenation results in a profound increase in DCF fluorescence in HepG2 cells transfected with the control plasmid (Fig. 4*A*). Cells transfected with the *rac1N17* expression plasmid show a significant decrease in hypoxia/reoxygenation-stimulated rise in DCF fluorescence. This finding was corroborated and quantified using the Amplex Red probe (Fig. 4*B*). The 2–3-fold rise in hypoxia/reoxygenation-induced intracellular H_2O_2 concentration seen in cells transfected with the control pEXV vector is almost completely blunted in cells transfected with the *rac1N17* expression plasmid. Similarly, sodium arsenite-induced H_2O_2 accumulation within HepG2 cells is also significantly inhibited by Rac1N17. In contrast, hypoxia alone, which does not activate the heat shock response in HepG2 cells, does not result in an increase in intracellular H_2O_2. Thus, induction of the heat shock response by two independent extracellular stresses correlates with the intracellular Rac1-dependent generation of H_2O_2 by these stresses.

In further support of a role for ROS in Rac1-regulated signaling pathway(s) responsible for stress-induced HSF activation, we found that expression of Rac1V12 alone, in the absence of extracellular stresses, does not activate HSFs (Fig. 1*A*) and has no effect on intracellular H_2O_2 production (Fig. 4*B*). Moreover, since c-Ras appeared not to be involved in stress-stimulated HSF activity, we also examined the role of this small GTPase in hypoxia/reoxygenation and sodium arsenite-stimulated production of intracellular ROS. Transfection of HepG2 cells with an expression vector encoding the dominant negative allele of c-*ras*, *rasN17*, does not affect H_2O_2 production by either stimulus (Fig. 4*B*). Thus, the inability of Rac1V12 and RasN17 to regulate ROS production under nonstressed and stress conditions, respectively, correlates with their ineffectiveness in modulating HSF activity.

Finally, we also assessed the effects of transfecting HepG2 cells with a plasmid encoding catalase or treating them with the thiol agent *N*-acetylcysteine (NAC) on intracellular H_2O_2 levels. Transfection with catalase or treatment with NAC both result in significant inhibition of hypoxia/reoxygenation and sodium arsenite-induced increase in H_2O_2 concentrations (Fig. 4*B*). Notably, NAC is more effective than catalase at reducing hypoxia/reoxygenation-induced H_2O_2 levels. Thus, catalase and NAC are both potent scavengers of H_2O_2 in HepG2 cells.

Enzymatic and Chemical Antioxidants Suppress Hypoxia/Reoxygenation-induced HSF Activation—Previous studies have demonstrated that both the addition of exogenous oxidants and modulation of the intracellular redox state influences the heat shock response (8). Having shown that Rac1 mediates hypoxia/reoxygenation and sodium arsenite-induced intracellular accumulation of ROS and activation of HSF, we next assessed the role of intracellular ROS in stress-induced HSF activation. Levels of intracellular ROS-1 were directly manipulated by either expression of superoxide dismutase and catalase or by treatment with NAC. HepG2 cells transfected with expression plasmids for superoxide dismutase or catalase or pretreated with NAC show a significant decrease in hypoxia/

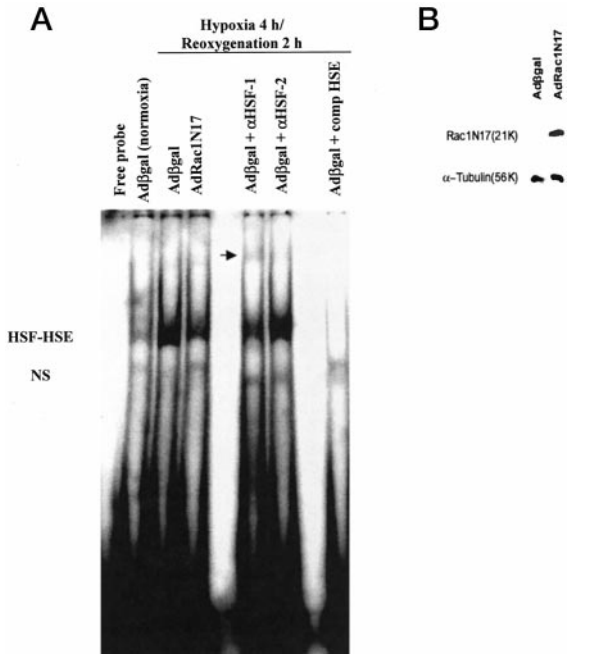

FIG. 2. **Infection with AdRac1N17 inhibits hypoxia/reoxygenation-induced HSF DNA binding.** *A*, nuclear extracts from HepG2 cells infected with Adβgal or AdRac1N17 at a multiplicity of infection of 50 and maintained under normoxia or subjected to hypoxia/reoxygenation were incubated with a ^{32}P-labeled consensus HSE oligonucleotide and analyzed by electrophoretic mobility shift assay. Where indicated, antibodies to HSF-1 and HSF-2 (αHSF1 and αHSF2) or a 100-fold excess of unlabeled consensus HSE oligonucleotide (comp HSE) were added to the binding mixture. The *arrow* indicates a supershifted band. *NS*, nonspecific binding. *B*, whole cell lysates from HepG2 cells infected with Adβgal or AdRac1N17 at a multiplicity of infection of 50 were analyzed by immunoblotting with an antibody to Myc. Equal protein loading was confirmed by immunoblotting with an antibody to α-tubulin.

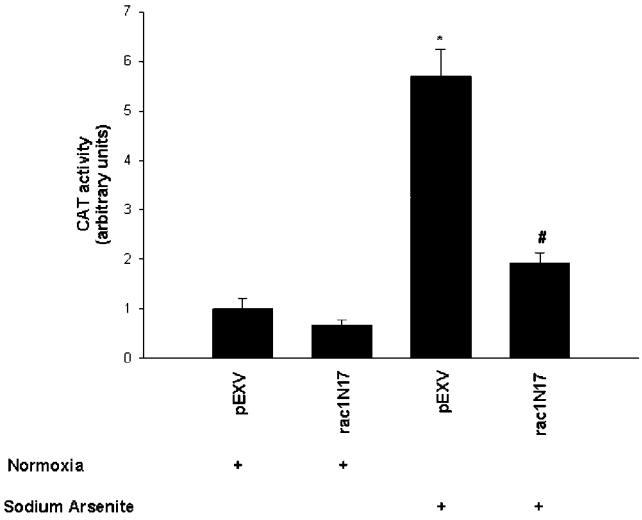

FIG. 3. **Rac1N17 suppresses sodium arsenite-induced HSF activation.** HepG2 cells were co-transfected with Δ5N/$-$105. pRSV-Luc, and either pEXV or *rac1N17*. Cells were treated with 100 μM sodium arsenite for 4 h, and normalized CAT activity was measured. Results are expressed as mean $\pm$ S.D. of triplicate determinations from a single representative experiment. *, $p < 0.05$ compared with pEXV normoxia; #, $p < 0.05$ compared with pEXV sodium arsenite (Student's *t* test).

reoxygenation-induced HSF activation (Fig. 5*A*). Of the three antioxidants examined, NAC has the greatest effect, with HSF activity approaching basal levels. However, none of the antioxidants is as effective in suppressing hypoxia/reoxygenation-induced HSF activation as Rac1N17. Qualitatively similar results were obtained with an electrophoretic mobility shift assay (data not shown).

Rac1 regulates ROS production by a NAD(P)H-dependent flavoprotein oxidase (14). We therefore examined whether DPI, a flavoprotein antagonist, has any effect on stress-stimulated HSF activity. Treatment of cells with DPI dose-dependently suppresses H/R-induced HSF transcriptional activity (Fig. 5*B*).

Effect of Rac1 on Stress-induced HSF Activation Is Mediated through JNKs—Many environmental stresses including hypoxia/reoxygenation and heavy metals lead to activation of the JNK family of stress-activated protein kinases (28, 29). JNKs are one of the many downstream targets of the rho family of small GTP-binding proteins (30). It has recently been shown that JNKs can mediate the transcriptional activation and post-translational modification of certain heat shock proteins (31). We therefore investigated the possibility that regulation of stress-induced HSF activity by Rac1 was mediated via the activation of JNKs. JNK activation was inhibited by transfection of HepG2 cells with a plasmid encoding for dominant-negative stress-activated protein kinase/extracellular signal-regulated kinase kinase-1 (SEK1). SEK1 is a kinase that lies immediately upstream of JNKs and is necessary for their activation, and SEK(KR) is a dominant inhibitory mutant of this kinase (18). Similar to *rac1N17*, transfection with the SEK(KR) expression plasmid inhibits hypoxia/reoxygenation-induced HSF activation (Fig. 6). Therefore, activation of JNKs is necessary for the hypoxia/reoxygenation-induced heat shock response.

We also examined the effect of Rac1N17 on stress-induced JNK activation. Hypoxia/reoxygenation and sodium arsenite led to an induction of JNKs, which is inhibited by both Rac1N17 and SEK(KR) (Fig. 7). Moreover, treatment with NAC suppressed hypoxia/reoxygenation-induced JNK activation. This suggests that the stress-induced activation of JNKs is governed by Rac1-regulated ROS and that this activation is at least partly responsible for the stress-induced heat shock response.

Rac1 and ROS Regulate Hypoxia/Reoxygenation-induced hsp70 Transcription—Activated HSFs bind to upstream sequence-specific motifs present in the promoters of all stress-inducible heat shock protein genes, promoting their transcription. Hsp70 is a prototypical example of a heat shock protein that is induced by stresses that change the cellular redox status, such as hypoxia/reoxygenation. We therefore looked at the role of Rac1 in hsp70 gene transcription. Hypoxia/reoxygenation strongly induces hsp70 mRNA in HepG2 cells (Fig. 8). Expression of Rac1N17 significantly suppresses the induction of hsp70. Expression of catalase or treatment with NAC leads to a partial inhibition of hsp70 transcription. Quantification of the Northern blot demonstrates a correlation between the efficacy of the two antioxidants, catalase and NAC, to suppress hypoxia/reoxygenation-induced intracellular ROS (Fig. 5) and hsp70 transcription. Finally, expression of the constitutively active Rac1V12, which does not lead to HSF activation, also does not result in induction of hsp70 transcription.

DISCUSSION

HSF activation and hsp transcription occurs in response to myriad stresses. Many such stresses result in an increase in intracellular ROS. In this study we found that sodium arsenite and hypoxia/reoxygenation depend upon Rac1 for the induction of the heat shock response through activation of HSF-1, the HSF best known to be responsive to extracellular stresses. Moreover, Rac1 also regulates the production of intracellular H_2O_2 induced by these stresses. In contrast, hypoxia does not induce the heat shock response in HepG2 cells and correspondingly does not increase intracellular H_2O_2 in these cells. This

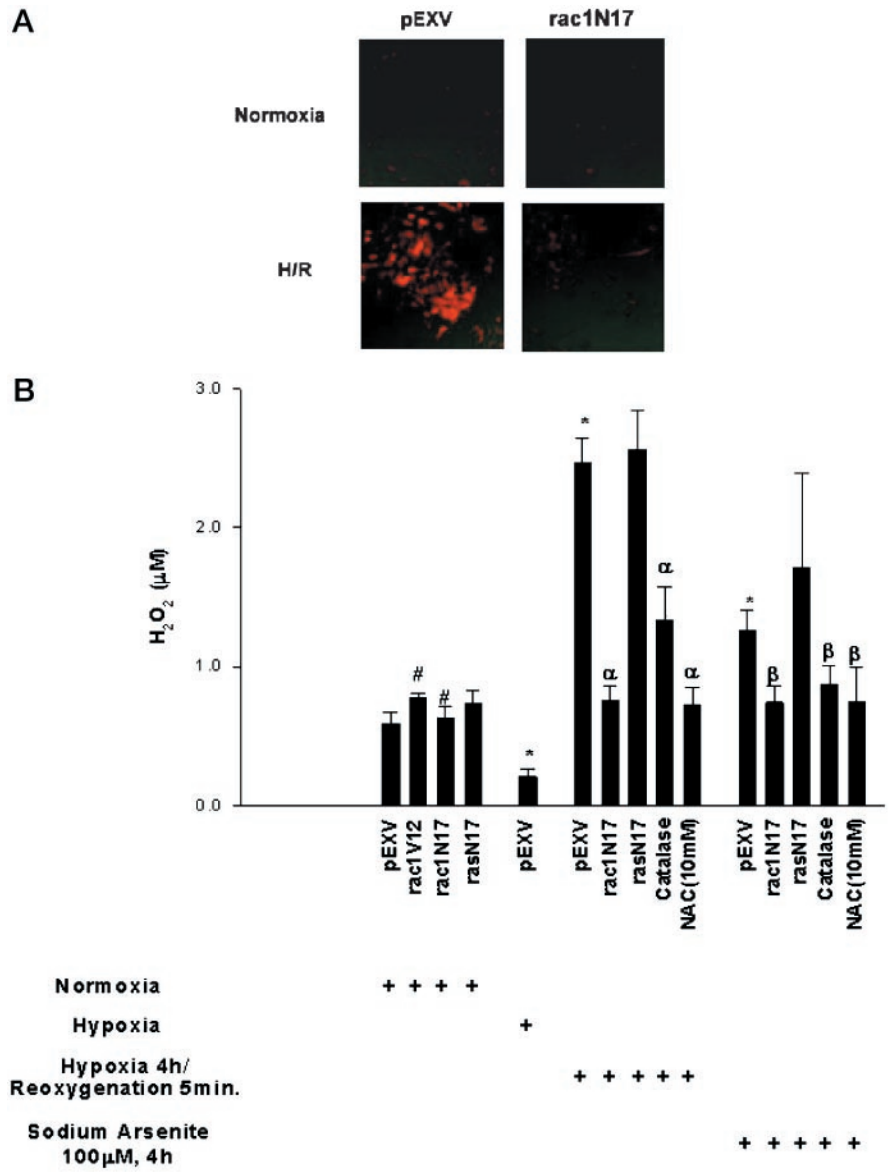

FIG. 4. **Expression of Rac1N17 suppresses stress-induced intracellular ROS production.** *A*, HepG2 cells transfected with 3.0 μg of either the empty plasmid pEXV or the Rac1N17 expression plasmid were subjected to hypoxia/reoxygenation (*H*/*R*) or maintained under normoxia. Intracellular ROS generation was assessed 5 min after reoxygenation using the peroxide-sensitive fluorophore DCF, and images were obtained with a confocal laser-scanning fluorescence microscope. *B*, intracellular H_2O_2 concentration was quantified using the H_2O_2-sensitive fluorophore Amplex Red. 3×10^5 HepG2 cells in six-well plates were transfected with 3.0 μg of the empty plasmid pEXV or with expression plasmids for Rac1N17, Rac1V12, RasN17, or catalase, or they were treated with NAC. Cells were subjected to hypoxia/reoxygenation or sodium arsenite or maintained under normoxia. Red fluorescence intensity was measured on a Cytofluor, and H_2O_2 concentration was calculated using a standard curve. Results are the means ± S.D. of triplicate measurements from a representative experiment. #, p not significant compared with pEXV normoxia; *, $p < 0.05$ compared with pEXV normoxia; α, $p < 0.05$ compared with pEXV hypoxia/reoxygenation; β, $p < 0.05$ compared with pEXV sodium arsenite (Student's t test).

finding is in contrast to some other studies, which report that hypoxia alone can up-regulate HSF binding and transcription (32). The apparent discrepancy between these findings and ours might reflect differences in cell types, or they could be due to variations in hypoxic conditions. In the present study, HSF transcriptional activity was measured immediately after hypoxia and therefore is a true reflection of hypoxia and not hypoxia/reoxygenation-induced HSF activity.

The central role of ROS, in particular H_2O_2, as mediators of stress-induced, Rac1-regulated HSF activation is supported by two observations. First, RasN17, which is ineffective at suppressing the stress-induced heat shock response, is also incapable of inhibiting H_2O_2 production. Second, expression of Rac1V12 has no discernible effect on either H_2O_2 generation or HSF activation in HepG2 cells. This latter finding is in contrast to the effect of Rac1V12 on H_2O_2 production in HeLa cells (16). However, our findings agree with studies looking at the role of Rac1 in ROS generation, specifically in HepG2 cells (33). Such studies have shown only a very modest increase in intracellular ROS in HepG2 cells expressing Rac1V12, and only after NAD(P)H stimulation. Thus, the effect of expressing activated Rac1, in the absence of extracellular stimuli or stresses, on intracellular ROS production may be very cell type-specific. In this context, a previous report has shown that Rho proteins signal to the nucleus in cell type-specific manners (34).

Close analysis of our data shows that the peroxide scavengers catalase and NAC are more effective than superoxide dismutase in suppressing hypoxia/reoxygenation-induced HSF activity, implying that H_2O_2 is the main oxidant species responsible for HSF activation. This is in agreement with previous reports showing that the activation of HSF by exogenous oxidants is selective for the type of oxidant used, H_2O_2 being effective while superoxide was not (35). The fact that superoxide dismutase does suppress HSF activation to a modest degree suggests that a secondary oxidant such as the hydroxyl radical, the formation of which requires both H_2O_2 and superoxide, may also participate in HSF activation. Regardless of the oxidant(s) responsible for HSF activation, the molecular targets of this oxidant(s) are likely to include intracellular thiols such as GSH, since agents that modulate the intracellular GSSG/GSH ratio can profoundly influence the induction of heat shock proteins (7, 36, 37). Within this context, it is interesting to note that another oxidant species, nitric oxide, which leads to an increase in oxidized glutathione, induces hsp70 expression in hepatocytes (38). Therefore, it is probable that Rac1-regulated production of superoxide, hydrogen peroxide, or their deriva-

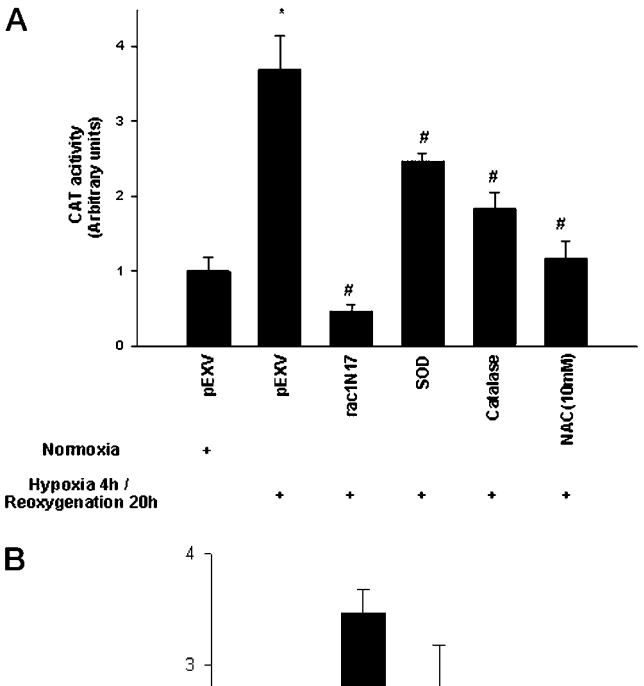

FIG. 5. **Antioxidants inhibit hypoxia/reoxygenation-induced HSF activity.** *A*, HepG2 cells were co-transfected with 0.5 μg of the reporter construct Δ5N/$-$105, 0.2 μg of pRSV-Luc, and 3.0 μg of the empty plasmid pEXV or expression plasmids for Rac1N17, superoxide dismutase, or catalase, or they were treated with NAC. *B*, cells were treated with DPI for 30 min. Cells were then subjected to hypoxia/reoxygenation or maintained under normoxia. CAT activity was measured 20 h later and normalized against luciferase activity. Results are expressed as mean ± S.D. of triplicate determinations from a single representative experiment. *, $p < 0.05$ compared with pEXV normoxia; #, $p < 0.05$ compared with pEXV hypoxia/reoxygenation (Student's *t* test).

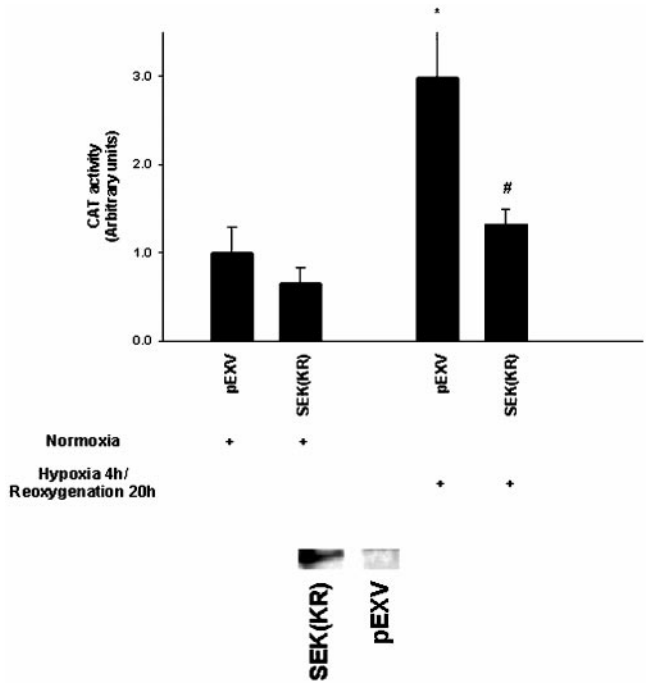

FIG. 6. **Inhibition of JNK activation suppresses hypoxia/reoxygenation-induced HSF activity.** HepG2 cells were co-transfected with 0.5 μg of Δ5N/$-$105, 0.2 μg of pRSV-Luc, 3.0 μg of the empty plasmid pEXV, or an expression plasmid for SEK(KR). Cells were subjected to hypoxia/reoxygenation, treated with sodium arsenite, or maintained under normoxia. CAT activity was measured 16 h later and normalized against luciferase activity. Results are expressed as mean ± S.D. of triplicate determinations from a single representative experiment. *, $p < 0.05$ compared with pEXV normoxia; #, $p < 0.05$ compared with pEXV hypoxia/reoxygenation (Student's *t* test). Expression of GST-tagged SEK(KR) in whole cell lysates was determined by Western blotting.

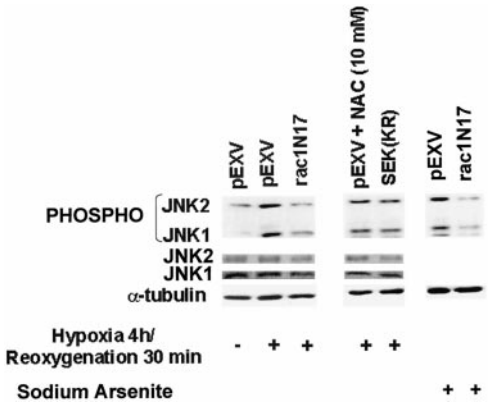

FIG. 7. **Rac1 regulates hypoxia/reoxygenation-induced JNK activation.** HepG2 cells were transfected with 3.0 μg of the empty plasmid pEXV or expression plasmids for Rac1N17 or SEK(KR), or they were treated with NAC. Transfected cells were exposed to hypoxia/reoxygenation or sodium arsenite, or they were maintained under normoxia. Whole cell extracts were assessed for the phosphorylated activated forms of JNKs and for total JNKs by Western blotting. Protein loading was assessed with an antibody to α-tubulin.

tives, results in an induction of the heat shock response by a similar mechanism.

Although ROS appear to have an important role, this study also implicates redox-independent mechanisms in regulating basal and stress-induced heat shock transcription. Rac1N17 results in a significant reduction of basal HSF transcriptional activity. However, under these same conditions there is no difference in intracellular H_2O_2 concentrations between control cells and those expressing Rac1N17, thus signifying the importance of Rac1-dependent, ROS-independent mechanisms in regulating HSF-mediated transcription in unstressed conditions. The role of redox-independent mechanisms in also mediating stress-induced heat shock response is suggested by the observation that NAC is very effective at inhibiting the hypoxia/reoxygenation-induced increase in intracellular H_2O_2 levels but only partly effective at suppressing hsp70 transcription.

The precise redox-sensitive mechanisms by which HSFs gain full transcriptional activity are likely to be multiple and complex. HSF-1 contains redox-sensitive cysteine residues, and oxidants have been shown to inhibit HSF-DNA binding (8). Thus, direct redox modifications of cysteine residues in HSF-1 is an unlikely mechanism for its activation. However, it is plausible that the stress-induced oxidation of critical cysteine

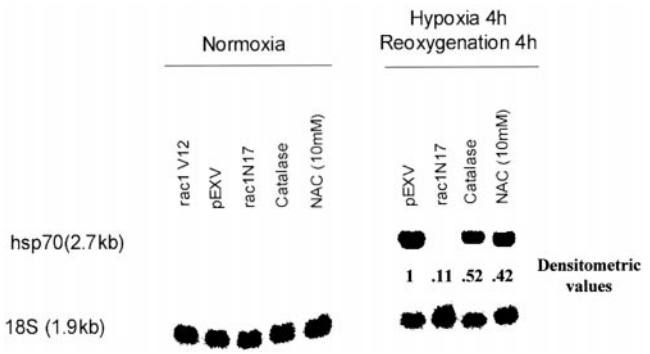

FIG. 8. **Rac1N17 and antioxidants inhibit hypoxia/reoxygenation-induced hsp70 transcription.** HepG2 cells were transfected with 3.0 μg of the empty plasmid pEXV or expression plasmids for Rac1N17, Rac1V12, catalase, or treated with NAC and subjected to hypoxia/reoxygenation or maintained under normoxia. RNA was hybridized with human inducible hsp70 cDNA and 18 S cDNA probes. Normalized densitometric values of hsp70 transcript are expressed relative to pEXV-transfected cells.

residues within HSFs is balanced by the up-regulation of endogenous oxidant-stimulated reducing agents such as thioredoxin and redox factor-1 (Ref-1), thereby permitting HSF activation (8). Extracellular stresses also lead to phosphorylation of HSFs (39), and therefore redox-modulated changes in protein phosphorylation may contribute to HSF-1 activation. In this regard, several studies have implicated JNK-regulated phosphorylation of HSFs in augmenting the heat shock response to specific stresses (31, 40). Our observation that SEK(KR) abrogates the hypoxia/reoxygenation-induced HSF activation is in concert with these previous reports. In contrast to JNKs, phosphorylation of HSF-1 via the Ras-extracellular signal-regulated kinase pathway has been shown to correlate with repression of its activity (41–43). This too is consistent with our finding that RasN17 is ineffective at inhibiting stress-induced HSF transcriptional activity. Thus, the activation of c-Ras and Rac1, with consequent induction of extracellular signal-regulated kinases and JNKs, respectively, has distinct and perhaps opposing effects on stress-induced HSF activation.

Induction of heat shock proteins is a universal cellular response to a variety of developmental cues and environmental stresses, and expression of many heat shock proteins conveys protection against such stresses. The physiological relevance of this adaptive response is amply demonstrated in animal studies in which forced overexpression of inducible heat shock proteins protect against hypoxic and post-hypoxic organ damage (44–46). This study implicates Rac1 and Rac1-regulated ROS production as an important step in the multistep process that results in the heat shock transcriptional response to two distinct environmental stresses. As such, it suggests that Rac1 may play an integral part in the cell's defense mechanisms against diverse extracellular stresses ranging from ischemia/reperfusion to heavy metals, and adds to the growing list of functions for this ubiquitous and versatile small GTPase.

Acknowledgments—We thank R. Morimoto for the wild-type and mutant HSF reporter plasmids, the hsp70 cDNA, and the HSF antibodies; D. Borchelt for the superoxide dismutase 1 expression plasmid; S. Erzurum for the catalase expression plasmid; L. Zon for the SEK(KR) expression plasmid; R. Crystal and T. Finkel for the Adβgal; K. Hirota and C. J. Lowenstein for critical review; and K. Baughman and S. Dirks for encouragement.

REFERENCES

1. Morimoto, R. I. (1993) *Science* **259,** 1409–1014
2. Wu, C. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 441–469
3. Westwood, J. T., and Wu, C. (1993) *Mol. Cell. Biol.* **13,** 3481–3486
4. Sarge, K. D., Murphy, S. P., and Morimoto, R. I. (1993) *Mol. Cell. Biol.* **13,** 1392–1407
5. Sorger, P. K. (1991) *Cell* **65,** 363–366
6. Schoeniger, L. O., Andreoni, K. A., Ott, G. R., Risby, T. H., Bulkley, G. B., Udelsman, R., Burdick, J. F., and Buchman, T. G. (1994) *Gastroenterology* **106,** 177–184
7. Huang, L. E., Zhang, H., Bae, S. W., and Liu, A. Y. (1994) *J. Biol. Chem.* **269,** 30718–30725
8. Jacquier-Sarlin, M. R., and Polla, B. S. (1996) *Biochem. J.* **318,** 187–193
9. Reddy, M. V., and Gangadharam, P. R. (1992) *Infect. Immun.* **60,** 2386–2390
10. Larrick, J. W., and Wright, S. C. (1990) *FASEB J.* **4,** 3215–3223
11. Kim, K. S., Takeda, K., Sethi, R., Pracyk, J. B., Tanaka, K., Zhou, Y. F., Yu, Z. X., Ferrans, V. J., Bruder, J. T., Kovesdi, I., Irani, K., Goldschmidt-Clermont, P., and Finkel, T. (1998) *J. Clin. Invest.* **101,** 1821–1826
12. Nobes, C., and Hall, A. (1994) *Curr. Opin. Genet. Dev.* **4,** 77–81
13. Heyworth, P. G., Knaus, U. G., Settleman, J., Curnutte, J. T., and Bokoch, G. M. (1993) *Mol. Biol. Cell* **4,** 1217–1223
14. Irani, K., Xia, Y., Zweier, J. L., Sollott, S. J., Der, C. J., Fearon, E. R., Sundaresan, M., Finkel, T., and Goldschmidt-Clermont, P. J. (1997) *Science* **275,** 1649–1652
15. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Sulciner, D. J., Gutkind, J. S., Irani, K., Goldschmidt-Clermont, P. J., and Finkel, T. (1996) *Biochem. J.* **318,** 379–382
16. Sulciner, D. J., Irani, K., Yu, Z. X., Ferrans, V. J., Goldschmidt-Clermont, P., and Finkel, T. (1996) *Mol. Cell. Biol.* **16,** 7115–7121
17. Irani, K., Herzlinger, S., and Finkel, T. (1994) *Biochem. Cell Biol.* **202,** 1252–1258
18. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) *Nature* **372,** 794–798
19. Williams, G. T., McClanahan, T. K., and Morimoto, R. I. (1989) *Mol. Cell. Biol.* **9,** 2574–2587
20. Borchelt, D. R., Lee, M. K., Slunt, H. S., Guarnieri, M., Xu, Z. S., Wong, P. C., Brown, R. H., Jr., Price, D. L., Sisodia, S. S., and Cleveland, D. W. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8292–8296
21. Pracyk, J. B., Tanaka, K., Hegland, D. D., Kim, K. S., Sethi, R., Rovira, I. I., Blazina, D. R., Lee, L., Bruder, J. T., Kovesdi, I., Goldschmidt-Clermont, P. J., Irani, K., and Finkel, T. (1998) *J. Clin. Invest.* **102,** 929–937
22. Crawford, L. E., Milliken, E. E., Irani, K., Zweier, J. L., Becker, L. C., Johnson, T. M., Eissa, N. T., Crystal, R. G., Finkel, T., and Goldschmidt-Clermont, P. J. (1996) *J. Biol. Chem.* **271,** 26863–26867
23. Mohanty, J. G., Jaffe, J. S., Schulman, E. S., and Raible, D. G. (1997) *J. Immunol. Methods* **202,** 133–141
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd. Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Das, D. K., Engelman, R. M., and Kimura, Y. (1993) *Cardiovasc. Res.* **27,** 578–584
26. Kukreja, R. C., Kontos, M. C., Loesser, K. E., Batra, S. K., Qian, Y. Z., Gbur, C. J., Jr., Naseem, S. A., Jesse, R. L., and Hess, M. L. (1994) *Am. J. Physiol.* **267,** H2213–H2219
27. Bauman, J. W., Liu, J., and Klaassen, C. D. (1993) *Fund. Appl. Toxicol.* **21,** 15–22
28. Pombo, C. M., Bonventre, J. V., Avruch, J., Woodgett, J. R., Kyriakis, J. M., and Force, T. (1994) *J. Biol. Chem.* **269,** 26546–26551
29. Liu, Y., Guyton, K. Z., Gorospe, M., Xu, Q., Lee, J. C., and Holbrook, N. J. (1996) *Free Radical Biol. Med.* **21,** 771–781
30. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* **81,** 1137–1146
31. Lee, Y. J., and Corry, P. M. (1998) *J. Biol. Chem.* **273,** 29857–29863
32. Benjamin, I. J., Kroger, B., and Williams, R. S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6263–6267
33. Cool, R. H., Merten, E., Theiss, C., and Acker, H. (1998) *Biochem. J.* **332,** 5–8
34. Teramoto, H., Crespo, P., Coso, O. A., Igishi, T., Xu, N., and Gutkind, J. S. (1996) *J. Biol. Chem.* **271,** 25731–25734
35. Jacquier-Sarlin, M. R., Jornot, L., and Polla, B. S. (1995) *J. Biol. Chem.* **270,** 14094–14099
36. Aucoin, M. M., Barhoumi, R., Kochevar, D. T., Granger, H. J., and Burghardt, R. C. (1995) *Am. J. Physiol.* **268,** H1651–H1658
37. Saunders, E. L., Maines, M. D., Meredith, M. J., and Freeman, M. L. (1991) *Arch. Biochem. Biophys.* **288,** 368–373
38. Kim, Y. M., de Vera, M. E., Watkins, S. C., and Billiar, T. R. (1997) *J. Biol. Chem.* **272,** 1402–1411
39. Mivechi, N. F., Koong, A. C., Giaccia, A. J., and Hahn, G. M. (1994) *Int. J. Hyperthermia* **10,** 371–379
40. Kim, S. H., Kim, D., Jung, G. S., Um, J. H., Chung, B. S., and Kang, C. D. (1999) *Biochem. Biophys. Res. Commun.* **262,** 516–522
41. Mivechi, N. F., and Giaccia, A. J. (1995) *Cancer Res.* **55,** 5512–5519
42. Knauf, U., Newton, E. M., Kyriakis, J., and Kingston, R. E. (1996) *Genes Dev.* **10,** 2782–2793
43. Chu, B., Soncin, F., Price, B. D., Stevenson, M. A., and Calderwood, S. K. (1996) *J. Biol. Chem.* **271,** 30847–30857
44. Otterbein, L. E., Kolls, J. K., Mantell, L. L., Cook, J. L., Alam, J., and Choi, A. M. (1999) *J. Clin. Invest.* **103,** 1047–1054
45. Hutter, J. J., Mestril, R., Tam, E. K., Sievers, R. E., Dillmann, W. H., and Wolfe, C. L. (1996) *Circulation* **94,** 1408–1411
46. Plumier, J. C., Ross, B. M., Currie, R. W., Angelidis, C. E., Kazlaris, H., Kollias, G., and Pagoulatos, G. N. (1995) *J. Clin. Invest.* **95,** 1854–1860