Domain Mapping of Heat Shock Protein 70 Reveals That Glutamic Acid 446 and Arginine 447 Are Critical for Regulating Superoxide Dismutase 2 Function*

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Stress-inducible heat shock protein 70 (hsp70) interacts with superoxide dismutase 2 (SOD2) in the cytosol after synthesis to transfer the enzyme to the mitochondria for subsequent activation. However, the structural basis for this interaction remains to be defined. To map the SOD2-binding site in hsp70, mutants of hsp70 were made and tested for their ability to bind SOD2. These studies showed that SOD2 binds in the amino acid 393–537 region of the chaperone. To map the hsp70-binding site in SOD2, we used a series of pulldown assays and showed that hsp70 binds to the amino-terminal domain of SOD2. To better define the binding site, we used a series of decoy peptides derived from the primary amino acid sequence in the SOD2-binding site in hsp70. This study shows that SOD2 specifically binds to hsp70 at 445GERAMT450. Small peptides containing GERAMT inhibited the transfer of SOD2 to the mitochondria and decreased SOD2 activity in vitro and in vivo. To determine the amino acid residues in hsp70 that are critical for SOD2 interactions, we substituted each amino acid residue for alanine or more conservative residues, glutamine or asparagine, in the GERAMT-binding site. Substitutions of E446A/Q and R447A/Q inhibited the ability of the GERAMT peptide to bind SOD2 and preserved SOD2 function more than other substitutions. Together, these findings indicate that the GERAMT sequence is critical for hsp70-mediated regulation of SOD2 and that Glu446 and Arg447 cooperate with other amino acid residues in the GERAMT-binding site for proper chaperone-dependent regulation of SOD2 antioxidant function.

Mitochondrion-localized superoxide dismutase (SOD2, Mn-SOD), a member of the SOD family of antioxidants, plays an essential role in maintaining normal mitochondrial redox hemostosis, critical for the survival of eukaryotic cells. The SOD2 gene resides in the nucleus and is synthesized in the cytosol, and its protein product must be imported into the mitochondria. Further, as incorporation of Mn2+ into SOD2 also occurs inside the mitochondria (1), effective SOD2 catalytic activity can only be achieved when SOD2 is imported into the mitochondria (2). Hence, transfer of SOD2 to mitochondria after synthesis represents a critical step in the mechanisms regulating SOD2 function.

Previously, it has been reported that the cytosolic stress-inducible hsp70, a molecular chaperone, associates with SOD2 after synthesis and that the chaperone maintains the enzyme in an import-competent, unfolded conformation in an ATP-dependent manner (3–8). Mice homozygous for hsp70 gene disruption exhibit a marked reduction in SOD2 activity, providing in vivo evidence for the role of hsp70 in regulating SOD2 (9). Although the roles and mechanisms by which hsp70 binds, unfolds, and localizes client proteins to respective subcellular destinations are well studied (10–15), the structural basis of hsp70 interactions with SOD2 remains unknown.

Previous studies revealed that a small peptide, 2-phenylethylsulfonamide (PES), inhibits the hsp70 chaperone function by directly binding to the substrate-binding domain (SBD) in the chaperone (16–19). In line with this observation, we showed that PES caused a significant reduction in SOD2 import and activity in cultured endothelial cells (3), suggesting that SOD2 binds to a region within the hsp70 SBD. Because hsp70 is known to physically interact with an array of regulatory proteins, each client protein or group of proteins likely bind to a specific amino acid sequence within the hsp70 SBD. Therefore, the interaction between hsp70 and SOD2 likely requires proper alignment between the chaperone and antioxidant. Further, structural features of SOD2 also facilitate binding to hsp70, and these may include a region of hydrophobicity in the N-terminal domain, intermolecular hydrogen bonds, and van der Waal interactions between adjacent residues in SOD2 and hsp70 (20, 21).
The purpose of this study was to identify the location where SOD2 binds in hsp70 and define which amino acid sequence within the hsp70 SBD is responsible for the SOD2 interactions. Such knowledge will increase our understanding of the posttranslational regulation of SOD2 and the effects of posttranslational modifications of hsp70 on SOD2-mitochondrion signaling.

Here we identify the location where SOD2 binds in hsp70 and show that amino acid residues 445–450, GERAMT, are important for SOD2 interactions. Mutations of all amino acid residues in the GERAMT-binding site inhibited chaperone-dependent regulation of SOD2.

Results

Domain Mapping of the SOD2 and hsp70 Interaction—We demonstrated previously, using in vitro immunoprecipitation assays, that SOD2 interacts with the cystolic stress-inducible hsp70 in ovine pulmonary artery endothelial cells (PAECs) (3). Given the importance of SOD2 in mitochondrial functions and cell survival, we sought to determine the specific location where SOD2 bound to hsp70. For this, we made GST-tagged mutants of hsp70 that correspond to the three published domains in hsp70 (Fig. 1A) and immunoprecipitated the mutants using an anti-GST epitope monoclonal antibody. Western blotting (WB) analysis of hsp70 immunoprecipitates showed that SOD2 preferentially bound to the hsp70 mutant corresponding to the amino acid 393–537 region in hsp70. SOD2 did not bind to the other regions tested (Fig. 1C). To verify this newly identified SOD2 domain in hsp70-mediated binding, we used bimolecular fluorescence complementation (BiFC) analysis to directly visualize SOD2/hsp70 interactions in live cells (22–24). The plasmid constructs were co-transfected with the GST-tagged 393–537 mutant (the region in hsp70 where SOD2 binds) into PAECs. GST pulldown assays revealed that hsp70 bound to the N-terminal region between amino acid residues 1–100 and 100–180, and 180–222 and GST-tagged 393–537 mutant, where SOD2 was suspected to bind in hsp70. WB analysis shows that each construct expressed protein of the expected size (Fig. 1D, top panel). The GST epitope was immunoprecipitated using anti-GST epitope monoclonal antibodies (Fig. 1D, center panel) and blotted for SOD2 using an anti-SOD2 polyclonal antibodies (Fig. 1D, bottom panel) (n = 4). D, representative immunoblots showing the molecular weight profiles of the SOD2 mutants and hsp70-binding domain in SOD2 in a pulldown assay. PAECs were co-transfected with plasmids expressing EGFP-tagged SOD2 (amino acid residues 1–100, 100–180, and 180–222) and GST-tagged 393–537 mutant, where SOD2 was suspected to bind in hsp70. WB analysis shows that each construct expressed protein of the expected size (Fig. 1D, top panel). The GST epitope was immunoprecipitated using anti-GST epitope monoclonal antibodies (Fig. 1D, center panel) and blotted for SOD2 using an anti-SOD2 polyclonal antibodies (Fig. 1D, bottom panel) (n = 4).

FIGURE 1. Domain mapping of hsp70 and SOD2. A and B, schematics of the hsp70 and SOD2 construct designs. C, representative immunoblots showing molecular weight profiles of the hsp70 mutants and the hsp70 mutant that associates with WT-SOD2 in a pulldown assay. Plasmids expressing GST-tagged hsp70 (amino acid residues 1–400, 393–537, and 537–641) and WT-SOD2 were co-transfected into PAECs. Lysates were Western-blotted to determine the molecular weight (MW) of each mutant (Fig. 1C, top panel). The GST epitope was immunoprecipitated (IP) (Fig. 1C, center panel) and blotted for SOD2 in the cell lysates (Fig. 1C, bottom panel) (n = 4). D, representative immunoblots showing the molecular weight profiles of the SOD2 mutants and hsp70-binding domain in SOD2 in a pulldown assay. PAECs were co-transfected with plasmids expressing EGFP-tagged SOD2 (amino acid residues 1–100, 100–180, and 180–222) and GST-tagged 393–537 mutant, where SOD2 was suspected to bind in hsp70. WB analysis shows that each construct expressed protein of the expected size (Fig. 1D, top panel). The GST epitope was immunoprecipitated using anti-GST epitope monoclonal antibodies (Fig. 1D, center panel) and blotted for SOD2 using an anti-SOD2 polyclonal antibodies (Fig. 1D, bottom panel) (n = 4). E, schematic of the Venus fluorescent proteins, SOD2, and hsp70 construct designs for the BiFC interaction partner. The left panel shows bright-field images of the cell, and the images shown in the right panel are imaged in the FITC channel. The green fluorescent signals are produced by SOD2/hsp70 associations) equivalent to signals produced by the control. Three independent experiments serving as biological replicates of each transfection were performed, with a minimum analysis of at least 5 cells/well/transfection.
bind SOD2. Plasmids encoding WT-SOD2 and WT-hsp70 or hsp70/H9004393–537 were spliced to either half of Venus fluorescent fragments (VN-1–155 or VC-155–238) at the amino or carboxyl terminus, as illustrated in Fig. 1E. The plasmids were transfected into HEK293T cells along with an internal reference encoding intact Venus protein. WB analysis revealed that the fusion proteins were expressed equally in transfected cells (data not shown). Of the eight different combinations tested, only cells co-expressing WT-SOD2 spliced to VN-1–155 (WT-SOD2-VN-1–155) and WT-hsp70 spliced to VC-155–238 (WT-hsp70-VC-155–238) produced green fluorescence signals that were equivalent to that produced by intact Venus fluorescent proteins (Fig. 2F). Cells expressing WT-SOD2-VN-155 alone or WT-hsp70-VC155–238 did not yield fluorescent signals. These findings demonstrate that SOD2 and hsp70 fusion proteins interact. As fluorescent signals could not be produced in cells co-transfected with the mutant hsp70Δ393–537-VC-155–238, we conclude that the 393hsp70537 region mediates SOD2 interaction.

The 393hsp70537 Region Is Important for Mitochondrial Localization of SOD2—To determine the effect of hsp70/SOD2 interactions on the localization and import of SOD2 into the mitochondria, PAECs were treated with siRNA targeting native hsp70 and then transfected with either WT-hsp70 or hsp70Δ393–537. WB analysis revealed that the siRNA decreased hsp70 protein in the cytosolic fractions by ~80% (Fig. 2A) and that this decrease led to a significant reduction in SOD2 protein in the mitochondria (0.38 versus 0.12; S.E. ± 0.024; p < 0.001; Fig. 2, A and B). Re-expression of WT-hsp70 in siRNA knockdown cells restored the mitochondrial SOD2 protein levels (0.396 versus 0.12, S.E. ± 0.016, p < 0.05). On the other hand, overexpression of hsp70/H9004393–537 failed to restore the mitochondrial SOD2 levels (Fig. 2A). An increase in mitochondrial SOD2 protein levels does not guarantee that the newly imported SOD2 will be functional. Accordingly, we determined whether the observed increases in SOD2 protein resulted in increased SOD2 activity by quantifying the rates of H2O2 formation, the catalytic product of superoxide (O2·−) dismutation. As expected, re-expression of WT-hsp70 in the hsp70 knockdown cells increased the specific activity of SOD2 by more than 3-fold (Fig. 2B). These changes led to an increase in the formation of H2O2 in the mitochondria, as determined by a plasmid encoding pHyper-dMito, a mitochondrially targeted H2O2 sensor. 48 h after transfection, mitochondria were stained and visualized with MitoTracker Deep Red. The strength of the green fluorescence signals correlates with rates of H2O2 produced in mitochondria (n = 3).
(eNOS) could acts as a decoy peptide to inhibit eNOS/hsp90 interactions and, consequently, NO generation in endothelial cell cultures (25). On the basis of these published findings, we reasoned that small peptides derived from the primary amino acid sequence of hsp70 could be used to determine where SOD2 binds to hsp70. Accordingly, nine overlapping decoy peptides (p1–p9) were made, spanning the entire length of the hsp70 region, where SOD2 is expected to bind to hsp70. The peptides were screened to determine which one could inhibit SOD2/hsp70 interactions. Our studies showed that, of the nine decoy peptides tested, p4 (YSDNOPGVLIQVYEGERAMT, amino acid residues 423–443) and p5 (GERAMTRDNNLLGREFELSGIP, amino acid residues 439–459) significantly disrupted SOD2 association with hsp70 in endothelial cell lysates (Fig. 3A). Adding the peptides to PAEC lysates led to a profound reduction in SOD2 activity compared with the enzyme activity in HBSS-treated controls (Fig. 3B). Because GERAMT is common to both the p4 and p5 peptides, we speculated that this sequence may represent the minimal portion responsible for disrupting SOD2 interactions with hsp70. To test this hypothesis, we added the GERAMT peptide alone or GERAMT-less peptides to aliquots of cell lysates. Colorimetry analysis revealed that GERAMT-treated rats (data not shown). The lungs of GERAMT-
treated SD rats exhibited an ~60–70% reduction in SOD2 activity (0.21 versus 0.07 ± S.E. 0.023 units/mg of protein, p < 0.001, Fig. 5A) and increased mitochondrial O$_2^-$ levels, as determined by HPLC quantification of 2-OH-Mito-E$^+$ (1.5 × 10$^7$ versus 8 × 10$^6$ mmol/mg of protein, ± S.E. 0.54, p < 0.005, Fig. 5B). This suggests that the GERAMT peptide effectively inhibits SOD2 function in vivo.

FIGURE 4. Effects of GERAMT peptides on mitochondrial O$_2^-$ formation. A, representative confocal microscopy images showing co-localization of SOD2 with mitochondria. ACh stimulation of PAECs induces SOD2 (green) localization to mitochondria (red). Treatment of PAECs with 10 µM GERAMT peptides reduces localization of SOD2 to the mitochondria at baseline and following ACh stimulation (n = 3). B, mitochondrial O$_2^-$ was assessed in intact PAECs using MitoSOX (mito-HE epifluorescence), as shown in the representative photomicrographs. 10 µM ACh reduced mito-HE fluorescence (I) compared with baseline (II). 1 µM antimycin A (AA, a complex III inhibitor) increased mito-HE fluorescence (positive control, III) compared with an untreated control (I). However, 100 units/ml PEG-SOD decreased mito-HE fluorescence in antimycin A-treated cells (*, p < 0.05 from AA-treated cells, n = 4, IV), verifying that fluorescence is due to increased O$_2^-$. Treatment of PAECs with 10 µM phorbol 12-myristate 13-acetate (an NADPH oxidase activator) has no effect on MitoSOX fluorescence signals (O$_2^-$ location control, V). Incubation of 10 µM GERAMT-TAT increased mito-HE fluorescence signals in intact PAECs (VI) and abolished the ACh-mediated reduction in fluorescence signals (VII), whereas scramble peptides have no such effect (VIII) (*, **, and ***; p < 0.001 from controls, AA, and scramble peptides). Epifluorescence signals were quantified using MetaView software from three biological replicates obtained from three independent experiments with minimum analysis of four topographic locations in each well (*, p < 0.001, n = 3).

FIGURE 5. Effects of GERAMT peptides on SOD2 activity and mitochondrial O$_2^-$ formation in vivo. A, scatterplot showing the in vivo effect of GREAMT peptides on SOD2 activity. An Alzet mini-osmotic pump containing either 1 mg/kg/day GERAMT peptides or scramble sequence peptides was implanted in the interscapular region of 6-weeks-old SD rats of either gender. 10 days after treatment, rats were euthanized, and lungs were harvested. Lungs tissues were homogenized in HEPES buffer and prepared for SOD2 activity quantification using a Cayman activity assay kit and instructions (*, p < 0.001 from scramble peptide-treated SD rats, n = 7). B, scatterplot of mitochondrial O$_2^-$ levels in PAs isolated from GERAMT peptide- or scramble sequence peptide-treated SD rats. PA segments were then incubated with 50 µM MitoSOX at 37 °C for 30 min in the presence or absence of Mito-Q, a mitochondrially targeted antioxidant. Protein was extracted in methanol, and levels of 2-OH-Mito-E$^+$, the O$_2^-$ specific product of Mito-HE, were quantified using HPLC (* and **, p < 0.001 from scramble- and Mito-Q-treated rats, n = 4).

Substitution of Glu$^{446}$ and Arg$^{447}$ in hsp70 Disrupts SOD2/hsp70 Interactions—To determine which amino acid residue(s) in the GERAMT-binding site is/are critical for SOD2 interactions, we substituted each amino acid residue with alanine or more conservative residues, glutamine and asparagine, in a stepwise fashion and tested the effects of mutations on SOD2 interactions, taking into consideration their potential effect on
the stability of the β sheet sandwich that might affect the affinity of SOD2/hsp70 interactions and, thus, SOD2 function (20, 21). Substituting alanine for glutamic acid (E446A) and arginine (R447A) attenuated the ability of the GERAMT peptide to reduce SOD2 activity more than other substitutions (Fig. 6A). Similarly, substituting glutamine and asparagine for glutamic acid (E446Q) and arginine (R447N) also attenuated the ability of the GERAMT peptide to inhibit SOD2 activity (Fig. 6B). This finding implies that Glu⁴⁴⁶ and Arg⁴⁴⁷ are critical for chaperone-dependent regulation of SOD2 antioxidant function.

Discussion

In this study, we showed that, as newly synthesized precursor (inactive) SOD2 emerges from the cytosolic ribosomes, it binds to the hsp70 SBD at amino acid residues 445–450, GERAMT, in hsp70. The ability of small-molecule peptides containing the GERAMT sequence to inhibit hsp70/SOD2 interaction in intact cells and whole animals provides strong evidence for the importance of this sequence in hsp70-dependent regulation of SOD2 function.

According to the recently published 3D structure of the human stress-inducible hsp70 SBD (Fig. 7A), the GERAMT sequence is part of the predicted peptide-binding pocket in the hsp70 SBDβ (29). It is located in one of the β sheets/turn motifs and linked to the rest of hsp70 SBDβ by a series of evolutionarily conserved connecting loops, L₃ and L₄ (PDB code 4P02).

Given its role in cell survival, GERAMT is highly conserved among different hsp70 isoforms. Protein sequence alignment analysis by ClustalW revealed that GERAMT is identical in seven of the 13 human hsp70 isoforms we tested (Fig. 7C). This high level of conservation, particularly among the cytosolic stress-inducible hsp70 isoforms, indicates the importance of this sequence to hsp70 chaperone functions. Notably, the “GER” residues (Gly⁴⁴⁵, Glu⁴⁴⁶, and Arg⁴⁴⁷) are even more conserved among key hsp70 isoforms compared with the “AMT” residues (Ala⁴⁴⁸, Met⁴⁴⁹, and Thr⁴⁵⁰), which show considerable variations, particularly in the “organellar” hsp70 isoforms (endoplasmic reticulum Grp75 and mortalin-mitochondrial hsp70). This variation likely determines the functional specialization of the hsp70 isoforms. Whether the GERAMT-binding site in hsp70 is exclusively for SOD2 interactions remains to be defined and requires further studies.

Observations that substitutions of alanine or even more conservative residues, glutamine or asparagine, for glutamic acid (E446A/Q) and arginine (R447A/N) in the GERAMT peptide prevented inhibition of the hsp70/SOD2 interaction demonstrate the importance of Glu⁴⁴⁶ and Arg⁴⁴⁷ in the protein–protein interaction (Fig. 6A and B). These findings not only show where the SOD2-binding site is located on hsp70 but also identify which amino acid residues in hsp70 are critical for SOD2 interactions. Although the E446A/Q and R447A/N mutations exert a greater impact on SOD2 activity, all GERAMT residues must cooperate with each other for proper chaperone-dependent regulation of SOD2 function.

The specific domain of SOD2 that binds hsp70 also remains to be determined in detail. According to the 3D structure of SOD2 dimers (PDB code 2ADQ) and our data in Fig. 2D, it evidently involves the amino-terminal residue 1–100 portion of SOD2 (Fig. 7B), which consists of stretches of leucine-rich hydrophobic residues. Site-directed mutagenesis followed by molecular modeling could further help clarify the mechanisms of SOD2 interactions with its interaction partner hsp70.

Given the importance of GERAMT in orchestrating hsp70-mediated targeting of SOD2 to the mitochondria, it is anticipated that synthetic peptides that preferentially bind GERAMT might negatively influence SOD2 function. Expectedly, decoy peptides containing the GERAMT sequence effectively inhibit the hsp70/SOD2 interactions and reduced SOD2 activity in cultured endothelial cells and SD rats (Figs. 3C and 5A). Relative SOD2 deficiency has been implicated in the pathogenesis of many cardiovascular diseases as well as in metastasis of certain breast cancers (30–41). However, direct evidence linking SOD2 to these diseases is lacking because of a lack of specific inhibitors of SOD2 and embryonic lethality of SOD2 gene deletion in animal models. The GERAMT decoy peptide, therefore, provides an important biologic tool to induce relative SOD2 deficiency in vivo and study the role of SOD2 signaling in diseases pathogenesis.

In summary, using molecular, cell, and in vivo models and high-throughput assays, we characterized the specific amino acid sequence within the peptide-binding pocket of the hsp70 SBDβ that regulates SOD2 interactions. We speculate that impaired SOD2/hsp70 interaction, as we reported previously, plays a key role in the pathogenesis of mitochondrial oxidative stress underlying neonatal pulmonary hypertension and other
cardiovascular disorders. The GERAMT sequence identified in this study is a new biological tool that can be used to inhibit SOD2 activity and study the role of SOD2 deficiency in cardiovascular diseases in vivo as well as in cancers, in which SOD2 promotes invasiveness of tumors.

**Experimental Procedures**

**Cell Isolation and Culture**—All animal (sheep and rat) protocols were performed in compliance with NIH guidelines and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. PAECs were isolated from near-term lambs of either gender as described previously (33, 42). The PAEC identity was confirmed by CD31 (550274, BD Biosciences) staining for each experiment. PAECs (passage 4 or less) and HEK293T cells were cultured in DMEM (Invitrogen) containing 10% (v/v) FCS and penicillin (100 units/ml), gentamicin, and 1% antifungal in a humidified incubator at 37 °C in 21% O2 with 5% CO2 and used for experiments.

**Domain Mapping of hsp70 and SOD2 in PAECs**—Three GST-tagged hsp70 (ovine) deletion mutants were made (1–400, 393–537, and 537–641), as well as EGFP-tagged WT-SOD2 (TOPGene, ON, Canada). To normalize all transfections, the expression vector pET21 was used. PAECs were grown to 80% confluence in antibiotic-free culture medium in 100-mm dishes, and 3–5 μg of GST-tagged hsp70 mutants and 3–5 μg of EGFP-tagged SOD2 were mixed with transfection reagent, FuGENE 6 (E2691, Promega, Madison, WI) at a 2:1 dilution ratio in Gibco Opti-MEM® (17985-088, Life Technologies). After incubation at 25 °C for 30 min, the plasmids/cationic complexes were applied directly to the cultured cells. In vitro translation studies confirmed that each construct expressed proteins of the expected size (Fig. 1, C and D). 48 h after transfection, the GST epitope was immunoprecipitated in cell lysates using 1 μg/100 μg of anti-GST monoclonal antibody (GXT70197, GeneTex, Irvine, CA), and the immunoprecipitates were blotted for SOD2 using an anti-GFP epitope polyclonal antibody (GTX628528, GeneTex). To identify the corresponding interaction domain in SOD2, three deletion constructs of EGFP-tagged SOD2 were made (1–100, 100–180, and 180–222). 5 μg of plasmids expressing the EGFP-tagged SOD2 and GST-tagged 393–537 mutants, where SOD2 binds to hsp70, was co-transfected into PAECs, and the SOD2/hsp70 associations were assessed by immunoprecipitation.

**Bimolecular Fluorescent Complementation Assay**—To verify this newly identified SOD2 domain in hsp70-mediated binding, we used a BiFC assay to directly visualize SOD2/hsp70 interactions in live cells. cDNAs encoding hybrids of Venus fluorescent protein fragments (amino acid residues 1–155 of the N terminus of Venus protein, i.e. VN–1–155, and amino acid residues 155–238 of the C terminus of Venus protein, i.e. VC–155–238), spliced to WT-SOD2, WT-hsp70, or the hsp70Δ393–537 sequence alignment of human hsp70 proteins. A, crystalline structure of the hsp70 SBD adopted from PDB code 4PO2. The protein structure is shown in ribbon style. The green and yellow ribbons represent the domains and secondary structure elements, and the β sheets are shown in green and blue. The predicted SOD2-binding domain (GERAMT) is shown in molecular surface view. B, crystalline structure of the SOD2 dimer showing the hsp70-binding domain (PDB code 2ADQ). The protein structure is shown in ribbon style, whereas the Mn2⁺ in SOD2 is shown in atoms and bonds. The hsp70 interaction domain in SOD2 is shown in yellow. C, sequence alignment of human hsp70 isoforms. Human hspA1A, hspA1B, hspA1L, hspA2, hspA5, hsp26, hspA7, hspA8, hspA9, hspA12A, hspA12B, hspA13, and hsp70A14 sequences are used for this alignment. The conserved and variable residues are shown in red and green, respectively.
Posttranslational Regulation of SOD2

mutant (deletion of amino acid residues 393–537 in hsp70) at either the amino and carboxyl terminus, were made and subcloned into expression plasmids (TOPGene). The Venus fluorescent protein was a gift from Chang-Deng Hu (Purdue University). HEK293T cells were grown in glass-bottom 35-mm Petri dishes (P35G-1.5-20-C, Mat-Tek Corp., Ashland, MA). At 60% confluence, 2 μg of cDNA encoding the SOD2 and hsp70 fusions was co-transfected in eight distinct combinations into HEK293T cells, along with an internal reference plasmid encoding intact fluorescent proteins as positive controls. 24 h after transfection, we verified the expression of the appropriate proteins. Cells were then washed with Gibco Hanks’ balanced salt solution (HBSS, 1747266, Life Technologies) to remove non-viable cells and excess Venus fluorescent protein particles. Fluorescence was imaged on a TCS SP2 laser-scanning confocal microscope (Leica, Buffalo Grove, IL).

Immunoprecipitation and Immunoblotting—PAECs treated for the respective analyses were lysed in modified radioimmunoprecipitation assay buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA/EGTA/sodium orthovanadate, 10 mM β-glycerophosphate, and protease inhibitors. When indicated, decoy peptides were incubated with aliquots of cell lysates from PAECs for 2 h at 4 °C. Cell lysates were sonicated, and protein concentration was determined in the supernatants using a BCA assay and reagent (Pierce BCA protein assay kit, Qi223173). An aliquot of cell lysates was precleared with protein A/G (40 μl of a 50% slurry for 2 h at 4 °C). Hsp70 was then immunoprecipitated, and the immunoprecipitate was blotted for SOD2 as described previously (3).

Immunofluorescence Microscopy—Cells, grown on a Lab-Tek II four-well glass slide (154526, 03211640, Thermo Fisher Scientific, Rochester, NY) were treated as described above for individual studies. In some experiments, cells were incubated at 37 °C for 30 min with MitoTracker Deep Red with excitation/emission wavelengths of 568 nm. Cells were subsequently washed with PBS and fixed for 30 min in 4% paraformaldehyde at room temperature. Cells were blocked in 5% BSA in PBS and incubated with SOD2 polyclonal antibody or other antibodies as indicated. Then, the cells were incubated in a mixture of two fluorescence-conjugated secondary antibodies (FITC-conjugated horse anti-mouse and Texas Red-conjugated goat anti-rabbit) as indicated. Nuclei were stained and visualized with DAPI. Slides were cover slipped with fluorescent mounting medium and visualized under fluorescence microscopy for analysis. Fluorescence images were acquired with the 63×, 1.3 oil-immersion objectives, using a Leica DM5500B microscope programed with the Leica Application Suite, Advanced Fluorescence v 1.8 from Leica Microsystems, Inc.

Transient siRNA-mediated Knockdown of Naive hsp70—Transient knockdown of native hsp70 in PAECs was achieved via transfection with siRNA specific for ovine hsp70 (sc-29352, Santa Cruz Biotechnology) as described previously. Scramble siRNA (sc-37007) served as a control. PAECs were grown to 80% confluence in 60-mm dishes. Hsp70-targeting or scramble siRNA duplex was mixed with transfection medium (Opti-MEM™, 17985-088, Life Technologies) and FuGENE 6 transfection reagent (E2691). After incubation at 25 °C for 30 min, the siRNA/cationic complexes were applied directly to the growing cells. Following a 48-h transfection, hsp70 levels were verified by WB.

Transfection of PAEC with hsp70 Mutants and SOD2 Activity—Epitope-tagged cDNAs were made for WT-hsp70 and hsp70 mutant (hsp70A393–537) (TOPGene Laboratory). PAECs were plated in 60-mm dishes and transfected with 5 μg of WT-hsp70 and 5 μg of hsp70A393–537 cDNA. 24 h after transfection, the expression of appropriate proteins was confirmed. Lysates were prepared for immunoblotting and measurement of SOD2 specific activity by colorimetric assay.

Mitochondrial Isolation—Cell fractionation was performed as described previously (2). Briefly, PAECs, grown in 100-mm dishes, were pelleted, and the pellets were washed in 10 ml of cold 1× PBS, resuspended in mitochondrial buffer (20 mM HEPS buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose with EDTA), and homogenized on ice by passage in a 1-ml syringe with a 27-gauge needle 10 times. Next, lysates were treated as indicated and separated into cytosolic and mitochondrial fractions by differential centrifugations. The cytosolic and mitochondrial lysates were used for further studies.

SOD2 Activity Assay—SOD2-specific activity was measured using a colorimetric assay kit (706002, Cayman Chemical, Ann Arbor, MI) as described previously (2, 3). Briefly, PAECs grown in 60-mm dishes to 80% confluence were pelleted, and the pellets were homogenized in cold 20 mM HEPS buffer containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, and cell debris was removed by centrifugation at 13,000 rpm at 4 °C for 10 min. 1 mM potassium cyanide (151-50-8, Sigma-Aldrich) was added to the lysates during the assay process to inhibit both the cytosolic CuZn-SOD and extracellular SOD (in tissue lysates only). Absorbance was read at 440–460 nm using a microplate reader (FLUOstar Omega, BMG Labtech). The protein concentration in each well was estimated by BCA method to calculate SOD2 activity per milligram of protein.

Assessment of Mitochondrial H2O2 Formation—PAECs were grown in Mat-Tex 10-mm glass-bottom plates. At 60% confluence, cells were treated as described above for the respective studies and then transfected with 5 μg of pHyper-dMito, a mitochondrially targeted ratiometric H2O2 sensor (FP942, Evrogen, Farmingdale, NY). After 48 h, mitochondria were stained and visualized with MitoTracker® Deep Red 633 (Molecular Probes, 23057W, Invitrogen). Fluorescence images were acquired in live cells using confocal microscopy.

Decoy Peptide Synthesis and Screening—Nine overlapping decoy peptides (~20-mers, p1-p9) were designed based on the amino acid sequence of the SOD2 domain, where SOD2 was expected to bind hsp70 (amino acid residues 393–535, ovine). These and other peptides, including a nine-amino acid TAT protein transduction peptide, were synthesized using Fmoc (N-(9-fluorenyl-methoxycarbonyl) chemistry (Biomatik, Wilmington, DE). All peptides were made without C- and N-terminal modifications and HPLC-purified, and the sequence and predicted molecular weights were confirmed by mass spectrometry.
Identification of Amino Acid Residues in hsp70 required for SOD2 Interaction—An aliquot of cell lysates (100 µg in 0.5 ml) was incubated with each of the individual decoy peptides (1-10 µg, final concentration 10 µM) for 2 h at 4 °C. Next, hsp70 was immunoprecipitated from the cell lysate using anti-hsp70 monoclonal antibody (1 µg/100 µg of cell lysates). SOD2 and its associated proteins were separated by SDS-PAGE (4–12% gel), transferred to nitrocellulose, and blotted for hsp70 and SOD2.

Quantification of Mitochondrial Superoxide (O$_2^-$) Radical—We assessed mitochondrial O$_2^-$ levels in intact cells and PAs in situ using mitochondrially targeted hydroethidine (MitoSOXTM, Molecular Probes, M36008, Invitrogen). PAECs were grown to 40–50% confluence in eight-well chamber slides, and cells in selected wells were treated with GERAMT-TAT or scrambled sequence peptides in the presence or absence of 10 µM ACh. Cells treated with 1 µM antimycin A (a complex III inhibitor, A8674, Sigma) served as a positive control. PEG-SOD (100 units/ml, S9549-1MG, Sigma) was used to quench the signal produced by antimycin A to verify the specificity of the fluorescent signals for O$_2^-$.

Animal Studies—Time-dated pregnant SD rats were obtained from Harlan Laboratories (Madison, WI), and 6-week-old pups were used for the studies. Litters from three pregnant rats were used for each experiment, and each experiment was performed at least three times. Alzet micro-osmotic pumps (model 1002, 10334-14, Durect Corp., Cupertino, CA) containing GERAMT-TAT or scramble-TAT were implanted subcutaneously into the interscapular region of 6-week-old rats of either gender kept at 21% oxygen for the duration of treatment. After 10 days of continuous infusion, rats were euthanized, either gender kept at 21% oxygen for the duration of treatment. Time-dated pregnant SD rats were obtained from Harlan Laboratories (Madison, WI), and 6-week-old rats were used for each experiment, and each experiment was performed with a minimum of three independent trials. Results are expressed as the mean ± S.E. Statistical analysis was performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Data were analyzed by Student’s t test or one-way ANOVA, followed by Tukey-Kramer post hoc test, as appropriate, based on the number of comparison groups. $p < 0.05$ was accepted as showing a significant difference between groups.

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