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Protein kinase Akt/PKB phosphorylates heme oxygenase-1 in vitro and in vivo

Marta Salinas[a], Jinling Wang[b], María Rosa de Sagarra[a], Daniel Martín[a], Ana I. Rojo[a], Jorge Martin-Perez[a], Paul R. Ortiz de Montellano[b], Antonio Cuadrado[a,*]

[a] *Instituto de Investigaciones Biomedicas A. Sols UAM-CSIC and Departamento de Bioquímica, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, Madrid 28029, Spain*
[b] *Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-2280, USA*

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Abstract Heme oxygenase-1 (HO-1) is a stress response protein that protects cells against diverse noxious stimuli. Although regulation of HO-1 occurs mainly at the transcriptional level, its posttranslational modifications remain unexplored. We have identified a putative consensus sequence for phosphorylation by Akt/PKB of HO-1 at Ser188. Recombinant human and rat HO-1, but not mutant HO-1(S188A), are phosphorylated in vitro by Akt/PKB. Isotopic ^{32}P-labeling of HEK293T cells confirmed that HO-1 is a phosphoprotein and that the basal HO-1 phosphorylation is increased by Akt1 activation. HO-1(S188D), a single point mutant equivalent to the phosphorylated protein, exhibited over 1.6-fold higher activity than wild type HO-1. Fluorescence resonance energy transfer (FRET) studies indicated that HO-1(S188D) bound to cytochrome P450 reductase (CPR) and biliverdin reductase (BVR) with a slightly lower K_d than wild-type HO-1. Although the changes in activity are small, this study provides the first evidence for a role of the survival kinase Akt in the regulation of HO-1.

Keywords: PKB/Akt; Heme oxygenase; Oxidative stress

1. Introduction

The serine/threonine protein kinase Akt/PKB exerts a variety of cell functions that include regulation of glucose metabolism and cytoprotection against stress factors, such as oxidant injury [1]. The mechanisms used by this kinase to prevent oxidant-induced cell death remain poorly defined but probably involve transcription-dependent and -independent mechanisms [2]. We have recently reported the transcriptional regulation of heme oxygenase-1 (HO-1) by the PI3K/Akt pathway in response to nerve growth factor and to the antioxidant polyphenol, carnosol [3,4].

The heme oxygenase family is composed of at least two well-characterized isoenzymes: inducible HO-1 and constitutive HO-2 [5]. Both isoforms catalyze a common step-wise degradation of heme to release free iron and equimolar concentrations of carbon monoxide (CO) and the linear tetrapyrrole biliverdin, which in turn is converted to bilirubin by the enzyme biliverdin reductase (BVR). HO-1 is highly expressed in reticuloendothelial cells of the spleen and liver, where it participates in the processing of senescent or damaged erythrocytes and in protection against oxidative damage caused by free porphyrins [6]. In addition, stimuli that produce oxidative stress (UV light, heavy metals, glutathione depletion, and H_2O_2) dramatically enhance HO-1 expression in practically all tissues and cells tested [7]. Due to the potent antioxidant activity of biliverdin and bilirubin and to the cytoprotective actions of CO on vascular endothelium and nerve cells, it is widely accepted that activation of HO-1 represents an adaptive, and ultimately protective, response to oxidative injury [8].

The nitric oxide synthase (NOS) and HO systems share several functions, including the release of gaseous molecules, NO or CO, which activate cGMP cyclase, and may act as neurotransmitters [9]. Moreover, both systems utilize NADPH as the electron donor, both exhibit inducible and constitutive isoenzymes, and both exert cytoprotective functions in neurons and other tissues [10]. The NO synthase and HO families are comprised of isoenzymes that are subject to transcriptional regulation and phosphorylation. Thus, endothelial NOS is phosphorylated and activated by Akt/PKB [11,12] and calcium-calmodulin [13], while HO-2 but not HO-1 is phosphorylated and activated by caseine kinase 2 (CK2) [14] and calcium-calmodulin [15]. Therefore, in this study we analyzed the possibility that HO-1 could be regulated by Akt-mediated phosphorylation. Our results identify HO-1 as a new substrate of Akt and provide an additional mechanism for Akt-mediated cytoprotection.

2. Materials and methods

2.1. Cell culture and transfections

Human embryonic kidney (HEK) 293T and mouse NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 80 μg/ml gentamycin. Transient transfection of HEK293T was performed with calcium phosphate, yielding up to 95% transfected cells.

*Corresponding author.
E-mail address: antonio.cuadrado@uam.es (A. Cuadrado).

Abbreviations: HO; heme oxygenase; CRP; cytochrome P450 reductase; BVR; biliverdin reductase; NOS; nitric oxide synthase; CPM; 7-Diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin; PDGF; platelet-derived growth factor; FRET; fluorescence resonance energy transfer

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2.2. Plasmids, expression and purification of human His-tagged HO-1 proteins

Expression vectors for HA-Akt1, myr-Akt1-HA, HA-Akt1(K179M) and platelet-derived growth factor (PDGF) beta-receptor have been reported previously [16]. The human HO-1 cDNA was amplified by PCR from pCEP-HO-1 (kindly provided by Dr. R. Petersen, Case Western Reserve University, Cleveland, Ohio) and subcloned into the *Hind*III/*Eco*RI sites of the pFlagCMV2 expression vector to generate an N-terminal, Flag-tagged HO-1 protein. The mutants HO-1(S188A) and HO-1(S188D) were generated by PCR-directed mutagenesis (details available upon request) and confirmed by sequence analysis. These eukaryotic expression vectors were used as templates to amplify truncated forms of HO-1 lacking the last 23 C-terminal amino acids. This deletion generates a soluble and catalytically active protein [17]. The amplified fragments were subcloned into the *Xho*I/*BamH*I sites of pET15b with an amino-terminal tag of 6 His. The truncated proteins were expressed in BL-21 bacteria and isolated using the ProBond™ purification system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For fluorescence resonance energy transfer (FRET) and HO-1 activity assays, proteins were purified and reconstituted with hemin as described before [18].

2.3. Immunoblotting

The antibodies used were: anti-HO-1 and anti-Akt from BD-Transduction Lab (Lexington, KY) and Stressgen (Victoria, BC, Canada), anti-HA from Roche (Indianapolis, IN), anti-phosphoAkt(T308) from New England Biolabs (Beverly, MA), anti-Flag from Sigma–Aldrich Corp. (St. Louis, MO) and anti-phospho-Ser from Zymed Laboratories (San Francisco, CA). Cells were washed once with cold phosphate-buffered saline and lysed on ice with 200 μl of lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4). Lysates were precleared by centrifugation, resolved by SDS–PAGE, and transferred to Immobilon-P membranes (Millipore Billerica, MA). Blots were analyzed with the appropriate primary antibodies (1:1000). Peroxidase-conjugated secondary antibodies (1:10000) were used to detect the proteins of interest by enhanced chemiluminescence.

2.4. In vitro kinase assays

In vitro HO-1 phosphorylation was performed in immunocomplexes with HA-tagged Akt1 that was immunoprecipitated with anti-HA specific antibodies from NIH3T3 cells stably transfected with pcDNA3-HA-Akt1 [16]. Substrates of the kinase reactions included 2 μg of rat recombinant HO-1 (Calbiochem, La Jolla, CA), histone 2B (Roche) or human, recombinant, His-tagged, HO-1, HO-1(S188A) and BAD (from pET30b-BAD, kindly provided by Dr. L. del Peso, Universidad Autónoma of Madrid, Madrid) in 20 μl of kinase buffer (20 μM ATP, 1 mM DTT 1–6 μCi [γ-^{32}P]-ATP). Kinase assays with recombinant Akt were performed using 100 ng of bacterially expressed Akt1 (Upstate Biotechnology, Lake Placid, NY) per reaction according to the manufacturer's instructions. Briefly, 2 μg of substrate was incubated with the kinase and 5 μCi [γ-^{32}P]ATP in 25 μl of reaction buffer (30 mM $MgCl_2$, 200 μM ATP in 8 mM MOPS, pH 7.2, 2 mM EGTA, 0.4 sodium orthovanadate and 0.4 mM dithiothreitol) for 40 min at 30 °C with continuous shaking. Kinase reactions were resolved in SDS/PAGE, blotted to Immobilon-P membranes (Millipore) and exposed to autoradiography or immunoblotted.

2.5. Isotopic [^{32}P]-orthophosphate labeling and phosphoamino acid analyses

HEK293T cells were transiently transfected with pFlag-HO1 or pFlag-HO1(S188A) constructs. After 16 h, cells were serum-starved for 16 h, washed three times and incubated for 1 h in phosphate- and serum-free medium. Metabolic labeling was performed with 1 mCi/ml [^{32}P]-orthophosphate at 37 °C for 3 h. Cells were then stimulated with 20 ng/ml PDGF for 10 min. Ectopically expressed HO-1 was immunoprecipitated with anti-Flag antibodies, resolved by SDS–PAGE and analyzed by autoradiography. Phosphoamino acid analysis was carried out using two-dimensional thin layer electrophoresis [19]. Briefly, the band corresponding to labeled Flag-HO-1 was excised and incubated in 100 μl of 6 N HCl at 110 °C for 1 h for acid hydrolysis. The samples were lyophilized and the resultant pellets were resuspended in H_2O containing phosphoamino acid standards and applied on a cellulose-coated glass plate. First dimension electrophoresis was done in 1D running buffer (2.2% formic acid, 7.8% glacial acetic acid, pH 1.9) for 1.3 h at 1.5 kV. Second dimension was done in 2D running buffer (5% glacial acetic acid, 0.5% pyridine, pH 3.5) for 1 h at 1 KV. The plates were air-dried and sprayed with a ninhydrin solution (0.3 g ninhydrin in 100 ml acetone and 1 ml acetic acid) to visualize standard amino acids. Phosphoamino acids were detected by autoradiography.

2.6. Fluorescence resonance energy transfer assays

The binding affinities of human HO-1 and HO-1(S188D) to BVR and to cytochrome P450 reductase (CPR) were analyzed by FRET, according to the methods of Wang and Montellano [20]. Rat BVR and human CPR were expressed, labeled with the fluorescent probe 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin (CPM) (Molecular Probes, Eugene, OR) and purified as previously described [20]. The UV–Vis spectrum of HO-1 overlaps with the fluorescence emission of CPM and the association between HO-1 and either CPM-BVR or CPM-CPR results in fluorescence quenching. The optimal excitation wavelength for CPM in these assays was determined to be 350 nm. The percentage of fluorescence quenching at 455 nm was monitored in a SPECTRAmaxTM GEMINIX spectrofluorimeter and referred to the HO-1 concentration (ranging from 12.5 to 800 nM) to calculate K_d values. The binding assay was performed in the presence of a constant concentration of BVR or CPR (0.05 μM) in 0.1 M potassium-phosphate buffer, pH 7.4, at 25 °C.

2.7. HO-1 activity

The activities of wild-type HO-1 and the HO-1(S188D) mutant were determined as the rate of bilirubin formation in an HO-1/BVR coupled assay [17]. Briefly, the 100 μl reaction mixture contained human cytochrome P450 reductase (0.4 μM), rat BVR (4 μM), hemin (30 μM) and the appropriate recombinant HO-1 protein (1 μM) in 0.1 M potassium-phosphate buffer, pH 7.4. The reaction was initiated by NADPH (500 μM) addition. The initial rate of bilirubin formation was monitored at 468 nm for 0–90 s by UV–Vis spectroscopy at 25 °C. Bilirubin concentration was determined using an ε468 of bilirubin of 43.5 $mM^{-1}\,cm^{-1}$. For determination of HO activity in HEK293T, cells were lysed in 0.1 M potassium-phosphate buffer, pH 7.4, containing 0.5% Nonidet P40. HO activity was determined as described above except that 2 mg/ml total precleared lysate was used as an enzyme source and the rate of bilirubin formation was monitored for 15 min.

3. Results

HO-1 contains two sites that conform to the consensus sequence for Akt phosphorylation (RXRXXS/T, where X is any residue). As shown in Fig. 1, one of these sites (residues 233–246) is found in all vertebrates except humans, who have N instead of S or T, and was not analyzed further. The other site (179–193) is present from fish to humans with conservative changes to either S or T. Moreover, all HO-2 isoenzymes have a non-phosphorylatable A, instead of S or T, within this conserved sequence. According to the algorithm developed by Yaffe et al. [21] (online tool at http://scansite.mit.edu), this sequence is likely to be phosphorylated by Akt in vivo, because it has a score (0.2891) similar to that of other Akt substrates such as eNOS (0.2032). Moreover, the crystal structure of HO-1 [22] further indicates that this S is exposed on the surface of this enzyme, therefore being accessible to Akt.

Consequently, we analyzed the phosphorylation of recombinant HO-1 by Akt in in vitro kinase assays. As a source of active Akt, NIH3T3 cells, stably transfected with HA-tagged Akt1, were serum-starved overnight, stimulated with PDGF for 10 min, lysed and submitted to immunoprecipitation with anti-HA antibodies. Immunocomplex kinase assays were performed using commercially available recombinant rat HO-1

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Heme oxygenase-1

                    * *                              * *
Human      179-KQLYRSRMNSLEMTP-193    233-APGLRQRASNKVQD-246
Pig        179-KQLYRSRMNTLEMTP-193    233-ASDIRKRAGSRVQD-246
Rat        179-KQLYRARMNTLEMTP-193    233-TEFLRQRPASLVQD-246
Mouse      179-KQLYRARMNTLEMTP-193    233-MASLRQRPASLVQD-246
Xenopus    182-KQLYRSRMNSIETNT-196    237-ATELRSRGPKTENG-250
Torafugu   182-KRLYRSRMNSVELTE-196    233-VGTPRSRPATTLQV-246
Zebrafish  181-KQLYRSRMNSIEFTE-195

Heme oxygenase-2

                    * *
Human      199-KQLYRARMNALDLNM-213
Rabbit     195-KQFYRARMNALDLNL-209
Rat        198-KQFYRARMNALDLSM-212
Mouse      198-KQFYRARMNALDLNL-212
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Fig. 1. Amino acid alignment of HO-1 and HO-2 sequences around residues 179–193 and 233–246. Asterisks indicate the positions of the two arginines required to conform to the Akt phosphorylation site. The boxed residues correspond to the putative site of phosphorylation.

and two widely used substrates of Akt, recombinant BAD and histone 2B. As shown in Fig. 2A, HO-1 was phosphorylated in immunocomplexes from cells with PDGF-induced activation of HA-Akt1. The extent of phosphorylation was similar to that of BAD, a well-established substrate of Akt.

To further determine the role of S188 in Akt-mediated phosphorylation of human HO-1, we performed additional in vitro kinase assays using a wild-type version or a single point S188A mutant of recombinant His-tagged HO-1 as substrates. As shown in Fig. 2B, the wild-type enzyme but not the S188A mutant was phosphorylated by active myristoylated myr-Akt1-HA. In additional experiments, to exclude the possibility that it is a contaminating kinase present in the immunocomplex, rather than Akt itself, which is responsible for HO-1 phosphorylation, we performed these kinase reactions with bacterially expressed, purified Akt1. As shown in Fig. 2C, recombinant Akt1 phosphorylated wild type HO-1 but not the HO-1(S188A) mutant. These results indicate that Akt phosphorylates human HO-1 in vitro at residue S188.

Then, we analyzed the phosphorylation of HO-1 by Akt in vivo. HEK293T cells were co-transfected with expression vectors for PDGF-beta-receptor, HA-Akt1 and either Flag-HO-1(wild-type) or mutant Flag-HO-1(S188A). Cells were then serum-starved for 16 h, isotopically labeled with [^{32}P]-orthophosphate for 3 h, and finally stimulated with 20 ng/ml of PDGF for 10 min. Cell lysates were immunoprecipitated with anti-Flag antibodies, resolved by SDS–PAGE, transferred to Immobilon-P membranes, and exposed to autoradiography. The same membrane was also immunoblotted with anti-Flag antibodies. As shown in Fig. 3A, both HO-1(wild-type) and HO-1(S188A) exhibited a basal level of phosphorylation in non-stimulated cells, consistent with the notion that HO-1 is constitutively phosphorylated in a residue other than S188 [23]. Interestingly, PDGF increased ^{32}P-incorporation into both proteins but the phosphorylation of the wild-type enzyme was twofold higher than that of the S188A mutant. Moreover, as shown in Fig. 3B, phosphoamino acid analysis of both proteins further indicated that basal and induced phosphorylation takes place on serine residues. These results indicate that HO-1 is basally phosphorylated and that growth factor stimulation increases the phosphorylation of serine residues, including the Akt sensitive S188. To further determine if the observed phosphorylation of HO-1 could be attributed at least in part to the PI3K/Akt pathway, we analyzed the effect of the PI3K inhibitor, LY294002. NIH3T3 cells were serum-starved for 16 h and then submitted to 40 μM LY294002 or an equivalent amount of vehicle (DMSO) for 30 min. Under these conditions, LY294002 completely inhibited PI3K activity in our systems (data not shown and 4). Then, cells were stimulated with 20 ng/ml PDGF for 10 min. Endogenous HO-1 was immunoprecipitated with anti-HO-1 antibodies and immunoblotted with anti-phospho-Ser antibodies. As shown in Fig. 3C, we observed again a low level of HO-1 phosphorylation in non-stimulated cells, regardless of the presence of the PI3K inhibitor, suggesting that the basal phosphorylation of this protein is produced by an unknown serine protein kinase that is not related to PI3K signaling. On the other hand, PDGF induced an increase in phospho-HO-1 that was partially reduced in the presence of LY294002. Taken together, these results suggest that PDGF-induced phosphorylation of HO-1 involves, at least in part, the PI3K/Akt pathway.

To determine the relevance of phosphorylation in the regulation of HO-1, we attempted to analyze the activity of in vitro phosphorylated HO-1. However, we could not detect any significant production of bilirubin, either in the phosphorylated enzyme or in the control unphosphorylated enzyme that was submitted to the same treatment. We speculate that some constituent of the kinase reaction must irreversibly inhibit HO-1 activity. To circumvent this problem, we compared the catalytic activity of wild-type HO-1 with that of the single point mutant, HO-1(S188D), in which S188 was replaced with an Asp, a residue that mimics the negative charge introduced by phosphorylation. Additionally, the sequences coding for the last 23 C-terminal amino acids were removed to generate soluble but catalytically active recombinant enzymes [16] and a His-tag was added at the amino-terminus for protein purification. Bacterially expressed His-HO-1(wild-type) and His-HO-1(S188D) were incubated with the substrate heme in the presence of purified BVR and CPR, and the production of bilirubin was monitored at 468 nm. As shown in Table 1, the single point mutant S188D exhibited a slightly higher activity (over 1.6-fold) than wild-type HO-1. As an alternative approach to determine the effect of Akt on HO-1, we analyzed HO activity in HEK293T cells cotransfected with expression vectors for Flag-HO-1 and either active Akt (myr-Akt1-HA) or control inactive Akt (HA-Akt1(K179M)). As shown in Table 2, lysates from cells expressing active Akt exhibited a moderate 20% increase in total HO activity.

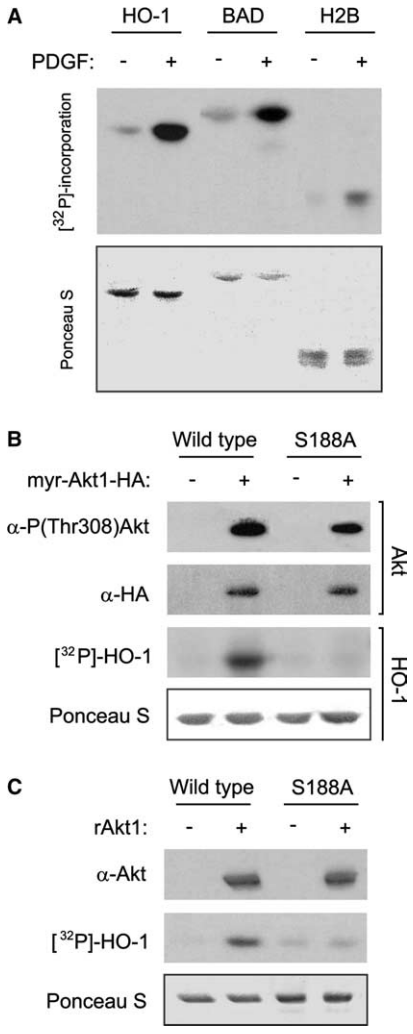

Fig. 2. Akt phosphorylates HO-1 in vitro. (A) NIH3T3 cells stably overexpressing HA-Akt were serum-starved for 16 h, stimulated with 20 ng/ml PDGF and used as a source of active kinase. Immunocomplex kinase assays were performed with rat recombinant HO-1 and human recombinant BAD or purified histone 2B as controls. The kinase reactions were resolved by SDS–PAGE and transferred to Immobilon-P membranes. *Upper panel*, autoradiography showing ^{32}P incorporation into these substrates. *Lower panel*, same blot stained with Ponceau S showing the substrates used in the kinase reaction. (B) NIH3T3 cells, transfected with vector alone or overexpressing active myr-Akt1-HA, were used as a negative control and as a source of active Akt. Immunocomplex kinase assays were performed as in (A), but using as substrates human, C-terminal truncated, soluble, His-tagged, HO-1 wild-type or single point mutant S188A. *Upper panel*, demonstration of active Akt in the immunocomplexes with activation-specific anti-phosphoAkt(T308). *Upper middle panel*, immunoblot with anti-HA antibodies showing specific immunoprecipitation of HA-tagged, myristoylated Akt. *Lower middle panel*, the kinase reactions were resolved in SDS–PAGE, transferred to Immobilon-P membranes and submitted to autoradiography. *Lower panel*, same blot stained with Ponceau S showing the substrates used in the kinase reaction. (C) Kinase assays were performed with bacterially expressed Akt1. *Upper panel*, immunoblot with anti-Akt antibodies showing recombinant Akt in the kinase reactions. *Middle panel*, autoradiography showing ^{32}P incorporation into recombinant HO-1. *Lower panel*, same blot stained with Ponceau S showing similar amount of HO-1 protein in each kinase reaction.

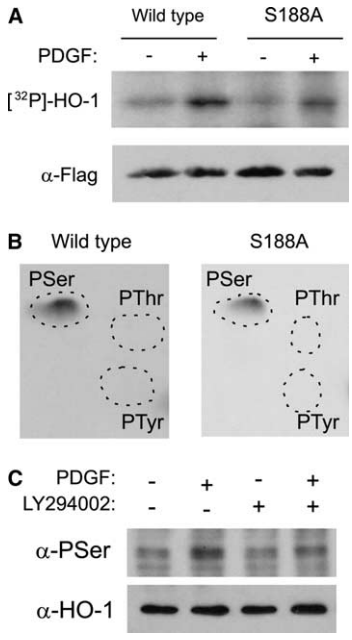

Fig. 3. Akt phosphorylates HO-1 in vivo. (A) HEK293T cells were cotransfected with expression vectors for the PDGF beta-receptor, HA-tagged Akt and either Flag-tagged wild-type HO-1 or Flag-tagged HO-1(S188A) single point mutant. Then, cells were treated as detailed in Methods and stimulated with 20 ng/ml PDGF for 10 min as indicated. *Upper panel*, cell lysates were immunoprecipitated with anti-Flag antibodies, resolved by SDS–PAGE, transferred to Immobilon P-membrane and exposed to autoradiography. *Lower panel*, immunoblot of the same blot with anti-Flag antibodies showing similar load of Flag-HO1 per lane. (B) Phosphoamino acid analysis of wild type and S188A Flag-HO-1 from the corresponding PDGF-stimulated bands in (A). Punctuated areas indicate the positions of phosphoamino acid standards as determined by ninhydrin staining. (C) HO-1 phosphorylation is partially blocked by the PI3K inhibitor, LY294002. Serum-starved NIH3T3 cells were preincubated with vehicle or 40 μM LY294002 for 30 min and then stimulated with 20 ng/ml PDGF for 10 min. Endogenous HO-1 was immunoprecipitated with anti-HO-1 specific antibodies, resolved in PAGE/SDS, and immunoblotted with anti-phospho-Ser antibodies. *Upper panel*, detection of phospho-Ser in the HO-1 immunocomplexes. *Lower panel*, immunoblot of the same blot with anti-HO-1 antibodies showing even amount of immunoprecipitated HO-1 per lane.

Table 1
Activity of His-tagged HO-1 proteins determined as the rate of bilirubin formation in the presence of BVR and CPR

Enzyme	BR formation rate (mM min^{-1} ± S.D.)	*N*	Activity (%)
Experiment 1			
His-HO-1(wild-type)	0.273 ± 0.057	7	100
His-HO-1(S188D)	0.481 ± 0.086	9	176
Experiment 2			
His-HO-1(wild-type)	0.208 ± 0.045	9	100
His-HO-1(S188D)	0.336 ± 0.077	9	161

N indicates number of samples per experiment.

The interaction of HO-1 with CPR and BVR is based primarily on electrostatic charge pairing on a surface binding site that includes residues close to S188 [20]. Therefore, phosphorylation of this residue might change the affinity among these molecules. For that reason, we determined the dissociation constants of CPR and BVR by FRET using recombinant His-HO-1(wild-type) and His-HO-1(S188D) and CPM-labeled BVR or CPR, as reported previously for other HO-1 point mutants [20]. As shown in Table 3, the mutant His-HO-1(S188D)

Table 2
Measurement of HO activity in HEK293T cells transfected with expression vectors for Flag-HO-1 and active (myr-Akt1-HA) or inactive (HA-Akt1(K179A)) versions of Akt protein kinase

Enzyme	BR formation rate (mM min^{-1} ± S.D.)[a]	Activity (%)
Experiment 1		
Flag-HO-1 + HA-Akt1(K179M)	102.57 ± 5.78	100
Flag-HO-1 + myr-Akt1-HA	122.72 ± 11.38	119.64
Experiment 2		
Flag-HO-1 + HA-Akt1(K179M)	94.58 ± 10.86	100
Flag-HO-1 + myr-Akt1-HA	112.63 ± 2.3	119.07

Even amounts of Flag-HO-1 for each experimental condition were confirmed in aliquots resolved by SDS–PAGE and stained with Coomassie blue (data not shown). HO activity was determined in cell lysates as the rate of bilirubin formation in the presence of BVR and CPR.
[a] Each value is the average of three samples. The basal rate of HO activity, corresponding to control cells transfected with empty vectors, was 6.89 ± 0 and 4.59 + 0.5 for experiments 1 and 2, respectively.

Table 3
Binding of CPR and BVR to HO-1 by FRET

Enzyme	K_d CPR (nM)[a]	K_d relative to wild type HO-1
His-HO-1(wild-type)	179.5 ± 45.5	
His-HO-1(S188D)	139.5 ± 50.36	0.77
Enzyme	K_d BVR (nM)[a]	K_d relative to wild type HO-1
His-HO-1(wild-type)	14.8 ± 9.195	
His-HO-1(S188D)	9.88 ± 5.365	0.66

[a] Each value is the average of two independent experiments with three samples each. The S.D. of each experiment was less than 30%. The indicated range ± is half of the difference between the two independent experiments.

exhibited a slightly reduced K_d for both BVR and CPR. Therefore, the negative charge introduced by phosphorylation of S188, which is mimicked by the S188D mutant, modestly increases the affinity of HO-1 for these molecular partners.

4. Discussion

HO-1, initially identified as Hsp32, appears to be involved in oxidative stress protection. The well-characterized transcriptional induction of this enzyme might provide long-term oxidant protection, but as in the case of NOS and HO-2, immediate protection might depend on fast activation of the pre-existing basal HO-1 levels [14,15]. Since Akt/PKB is rapidly activated in response to strong oxidants such as hydrogen peroxide [24], we reasoned that, similarly to NOS, HO-1 activity might be regulated through Akt-mediated phosphorylation. In this study, we have confirmed that HO-1 is phosphorylated by Akt in vitro and in vivo. Moreover, we have observed a moderate increase in in vitro HO-1 activity and in binding affinity for BVR and CPR and in the HO activity of cells expressing active Akt.

Ser 188 lies on the surface involved in electrostatic interactions between HO-1 and its well-characterized partners, BVR and CPR. Some ionic surface residues involved in these interactions include Lys18, Lys22, Lys179, Arg183, Arg198, Glu19, Glu127, and Glu190 for CPR and Lys18, Lys22, Lys179, Arg183, and Arg185 for BVR [20]. Therefore, S188 lies very close to this interface and phosphorylation may change the strength of binding. However, considering the large number of residues involved in the interaction, a large change in binding affinity is not to be expected for a single phosphorylation event. Indeed, we report that the change of Ser for Asp, a residue that mimics the negative charge of phosphorylation, results in a slightly lower K_d for both CPR and BVR and a slightly increased HO-1 activity.

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