S-nitrosation of Cys-800 of HIF-1α protein activates its interaction with p300 and stimulates its transcriptional activity

Inna M. Yasinska, Vadim V. Sumbayev*

Department of Biochemistry, Mechnikov Odessa National University, Apt. 208, Glushko prospect 16, Odessa 65104, Ukraine

Received 1 April 2003; revised 10 July 2003; accepted 10 July 2003

First published online 24 July 2003

Edited by Veli-Pekka Lehto

Abstract Hypoxia inducible factor 1 (HIF-1) is a heterodimeric transcriptional complex that plays pivotal role in the regulation of cellular utilization of oxygen as well as glucose and is an essential regulator of angiogenesis in solid tumor and ischemic disorders. Recently HIF-1α, a subunit of HIF-1 complex, was characterized as a potential target for *S*-nitrosation, providing no information about the impact of this posttranslational modification on the protein transactivation. Cys-800 of HIF-1α protein has reactive SH-group, which is critical for the recruitment of p300 co-activator that is necessary for transcriptional activity of HIF-1 complex. Here we report that *S*-nitrosation of Cys-800 activates HIF-1α–p300 interaction and stimulates protein transactivation. We have found that *S*-nitrosation of the HIF-1α C-terminal domain by nitric oxide derived from donors and nitric oxide synthase increases protein transcriptional activity. The increase of HIF-1 transcriptional activity was not observed in the case of Cys-800 substitution to Ala, though other protein thiol groups were nitrosated. Experiments with GST pull-down assay suggest that *S*-nitrosation of Cys-800 stimulates the recruitment of p300 co-activator protein to the HIF-1α C-terminal domain.

1. Introduction

Hypoxia inducible factor 1 (HIF-1) is a heterodimeric transcriptional activator, which consists of inducible α and constitutive β subunits. HIF-1α was found to be a limiting factor for HIF-1 transcriptional activity [1].

HIF-1 functions as a master regulator of cellular oxygen homeostasis. It plays central role in development of and in adaptation to hypoxia by directing the expression of genes that promote angiogenesis, embryogenesis, erythropoiesis, tumorogenesis and vasodilatation. HIF-1α degradation is mediated via its hydroxylation on Pro-402 and -564; proline-hydroxylation of HIF-1α allows it to bind the von Hippel–Lindau tumor suppressor protein (pVHL), the recognition component of a multiprotein E3 ubiquitin ligase complex [2–4]. Complex formed from HIF-1α and pVHL recruits also factor inhibiting HIF, which has recently been found to hydroxylate Asn-803 preventing the HIF-1 transcriptional activation through interaction with p300/CBP [5].

HIF-1α protein is stabilized by hypoxia as well as by the major growth factor-stimulated signal transduction pathways and redox-dependent mechanisms [6–8]. HIF-1α accumulating effect was also reported for the nitric oxide derived from different donors as well as enzymatically produced under normoxic conditions [9–12]. Recently we found that nitrosation of HIF-1α SH-groups correlates with the protein stabilization in different cell lines [13]. Cys-800 of HIF-1α is known to be critical for HIF-1 protein transactivation [14]. Substitution of Cys-800 to the hydrophobic amino acid (valine) increases protein transcriptional activity. In the present work we report that *S*-nitrosation of Cys-800 of HIF-1α protein leads to the increase in HIF-1 transcriptional activity [15]. We found that *S*-nitrosation of the HIF-1α transactivation domain by the nitric oxide derived from donors and inducible nitric oxide synthase (iNOS) increases protein transcriptional activity. The increase of HIF-1 transcriptional activity was not observed in the case of Cys-800 substitution to Ala, despite that other protein SH-groups were nitrosated. Experiments with a glutathione *S*-transferase (GST) pull-down assay suggest that *S*-nitrosation of Cys-800 supports the recruitment of p300 co-activator protein to the HIF-1α C-terminal domain.

2. Materials and methods

2.1. Materials

Medium and supplements were purchased from Biochrom (Berlin, Germany). Fetal calf serum was bought from Life Technologies (Berlin, Germany). For plasmid purification we used HiSpeed plasmid purification Qiagen kit (Valencia, USA). To make substitutions in the plasmid we used QuickChange® site-directed mutagenesis kit. Enzyme-labeled streptavidin was bought from Rockland (Gilbertsville, USA). Methyl-methane-thiosulfonate (MMTS) and *N*-(6-[biotinamido])hexyl)-3′-(2′-pyridyldithio) propionamide (biotin-HPDP) were purchased from Pierce (Rockville, USA). GSNO was synthesized as previously described [16]. All other chemicals were of the highest grade of purity and commercially available.

2.2. Cell culture

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml) [17]. Renal carcinoma cells (RCC) were grown in DMEM supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml) [18].

2.3. Plasmids

We used the plasmid, which encodes the chimeric protein containing C-terminal transactivation domain of HIF-1α fused to the Gal4 DNA-binding domain (Gal4DBD/HIF-1α 727-826). Also we used the same plasmid with Asn-803 substituted to Ala [19]. Both plasmids were provided by Dr. M. Whitelaw, Adelaide, Australia. The last construct was also used for further substitutions. Cys-800 in its struc-

*Corresponding author.
E-mail address: sumbayev@yahoo.co.uk (V.V. Sumbayev).

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00807-X

ture was substituted to Ala using QuickChange® site-directed mutagenesis kit according to the manufacturer's protocol.

2.4. Transfections

HEK-293 as well as RCC cells were plated at 2–3×10^5 cells/60-mm dish 1 day before co-transfection with luciferase containing Gal4-reporter plasmid and Gal4DBD/HIF-1α 727-826 expression plasmids using calcium-phosphate precipitation [10].

2.5. Western blot detection of the HIF-1α C-terminal fragment

HIF-1α was measured by Western blot [10,19]. Briefly, cells were incubated for the times indicated, washed two times with ice-cold phosphate-buffered saline (PBS), scraped off, lysed in 200 μl of lysis buffer (50 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0) and sonicated. After centrifugation (17 000×*g*, 15 min) the protein content in the supernatants was analyzed. Finally, 100 μg of protein was added to the same volume of 2× sample buffer (125 mM Tris–HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerine, 1 mM dithiothreitol (DTT), 0.002% bromphenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 7.5% SDS–polyacrylamide gels and blotted to nitrocellulose membrane. Molecular weights were calibrated in proportion to the running distance of rainbow markers. Transblots were washed twice with TBS (50 mM Tris–HCl, 140 mM NaCl, pH 7.3) containing 0.1% Tween 20 before blocking unspecific binding with TBS plus 5% skim milk for 1 h. The HIF-1α (1:1000 in TBS plus 5% milk) polyclonal antibody acting against the C-terminal domain of the protein was added and incubated for 60 min at room temperature. Afterwards, nitrocellulose membranes were washed five times for 15 min with TBS containing 0.1% Tween 20. For protein detection, blots were incubated with goat secondary antibodies conjugated with horse radish peroxidase (1:1000 in TBS plus 5% milk) for 60 min, followed by ECL detection.

2.6. Detection of S*-nitrosothiols by Western analysis*

Western blot analysis of protein *S*-nitrosothiols was performed as previously described [13,20]. In brief, cells were washed two times with ice-cold PBS. Lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and 0.5% NP-40) was added, cells were scraped off and centrifuged (10 000×*g*, 10 min). Four volumes of blocking buffer (9 volumes of HEN buffer plus 1 volume 25% SDS, 20 mM MMTS (from a 2 M stock prepared in dimethylformamide) was added to the 1 volume of the 10 000×*g* supernatant and incubated for 20 min at 50°C with frequent vortexing. MMTS was then removed by protein precipitation with acetone. Biotin-HPDP (final concentration of 2 mM) and sodium ascorbate (1 μl to reach a final concentration of 1 mM) were added. After incubation for 1 h at 25°C, SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer was added and the samples were resolved by SDS–PAGE and transferred for immunoblotting. Considering that cysteine biotinylation is reversible, SDS–PAGE sample buffer was prepared without reducing agents. Furthermore, to prevent non-specific reactions of biotin-HPDP, samples were not boiled prior to electrophoresis. All steps preceding SDS–PAGE were carried out in the dark. Immunoblots were washed twice with TBS (140 mM NaCl, 50 mM Tris–HCl, pH 7.3) containing 0.1% Tween 20, blocked to avoid unspecific binding with TBS plus 5% skim milk for 1 h and incubated with enzyme (horse radish peroxidase)-labeled streptavidin (1:500 in TBS plus 5% milk) for 1 h at room temperature. Nitrocellulose membrane was washed five times for 5 min each with TBS containing 0.1% Tween 20 prior to the detection of *S*-nitrosated proteins by ECL development.

2.7. Preparation of the GST-p300CH1 construct for a pull-down assay

The construct was prepared as previously described [19]. In brief, the GST-p300CH1 domain (amino acids 300 to 528) fusion protein was expressed from pGEX-4T3/p300CH1 and purified from bacterial extract by glutathione agarose. A whole cell extract (200 μg) from transfected cells was mixed with 2 μg of glutathione-bound GST-CH1 in binding 20 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl, 0.1% NP40, 1 mM DTT, 2 μM EDTA, 20 μM $ZnCl_2$ as well as protease inhibitors and incubated during 2 h at 4°C with gentle rotation. The beads were pelleted, washed three times with binding buffer, and bound proteins were eluted for SDS–PAGE with SDS sample buffer.

2.8. Quantification of NO release from GSNO

NO production was detected using the oxyhaemoglobin assay [21].

2.9. Nitrite measurements

The concentration of nitrite was determined by the Griess assay [22].

2.10. Protein quantification

The amount of protein was measured by the Bradford method [23].

2.11. Statistical analysis

Each experiment was performed at least three times and statistical analysis was done using the two-tailed Student's *t*-test. The statistical probability (*P*) was expressed as $*P < 0.01$. The normal distribution of data was checked.

3. Results and discussion

3.1. S-nitrosation of the HIF-1α Cys-800 leads to the increase in protein transcriptional activity

S-nitrosation of protein thiol groups by nitric oxide is a widely recognized protein modification [24]. Only few intracellular *S*-nitrosated proteins have been identified and it has been reported that *S*-nitrosation can serve as a regulatory process in signal transduction pathways [25]. As we recently reported HIF-1α protein can be considered as a target for *S*-nitrosation, which correlates with protein stabilization in a straight manner [13]. Cys-800 of HIF-1α protein has the reactive thiol group, which is of major importance for the p300

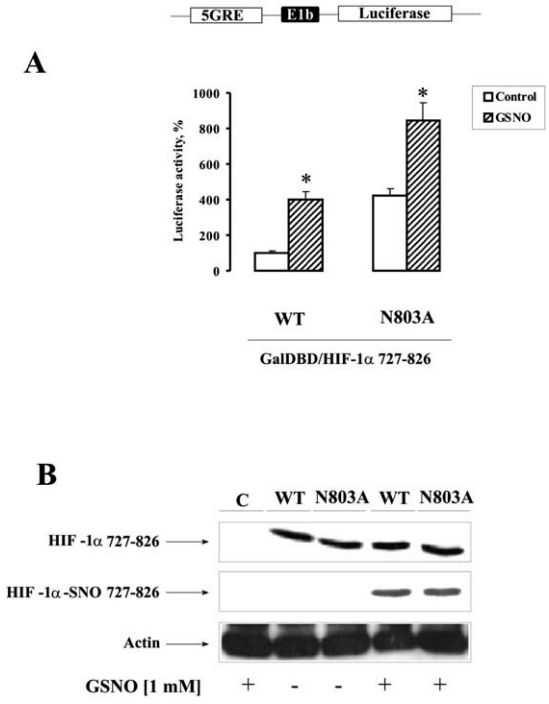

Fig. 1. GSNO treatment of HEK293 cells leads to *S*-nitrosation of the HIF-1α C-terminal domain, increasing its transcriptional activity. HEK293 cells were co-transfected with WT and N803A Gal4DBD/HIF-1α 727-826 as well as Gal4 response element-driven luciferase reporter gene using calcium phosphate precipitation. After transfection cells were incubated for 24 h followed by 8 h of stimulation with 1 mM GSNO. In 8 h cells were harvested and the luciferase activity was assayed by the luminometric method (A). We also used parallel cell treatments to detect the HIF-1α C-terminal fragment as well as its *S*-nitrosation by Western analysis as outlined in Section 2. To verify equal loading of the protein we used actin staining (B). Digital data are mean values ± S.D. of at least five individual experiments. $*P < 0.01$ vs. control. All Western blot data are from one experiment representative of three that gave similar results.

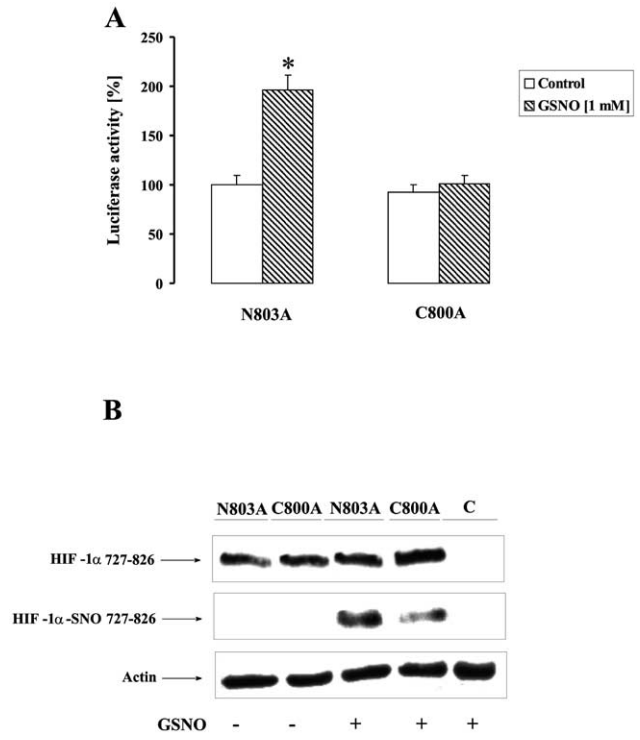

Fig. 2. Substitution of Cys-800 in the HIF-1α C-terminal domain to Ala attenuates *S*-nitrosation-dependent activation of the protein transcriptional activity. HEK293 cells were co-transfected with N803A and C800A Gal4DBD/HIF-1α 727-826 as well as Gal4 response element-driven luciferase reporter gene using calcium phosphate precipitation. After transfection cells were incubated for 24 h followed by 8 h of stimulation with 1 mM GSNO. In 8 h cells were harvested and the luciferase activity was assayed by the luminometric method (A). We also used parallel cell treatments to detect the HIF-1α C-terminal fragment as well as its *S*-nitrosation by Western analysis as described in Section 2. To verify equal loading of the protein we used actin staining (B). Digital data are mean values ± S.D. of at least five individual experiments. $*P < 0.01$ vs. control. All Western blot data are from one experiment representative of three that gave similar results.

recruitment [15]. We asked whether *S*-nitrosation of the Cys-800 leads to the increase in HIF-1 transcriptional activity. In a first set of experiments we co-transfected HEK293 cells with Gal4DBD/HIF-1α 727-826 (wild-type or WT and mutated plasmid, where Asn-803 is substituted to Ala or N803A) encoding plasmid and luciferase containing Gal4 reporter plasmid [19]. Cells were incubated for 24 h after transfection followed by 8 h of stimulation with 1 mM GSNO. The rate of NO release from GSNO was 299 ± 1.5 based on the oxyhaemoglobin assay ($t_{1/2}$ of this compound at 37°C corresponds to 80 h) [26,27]. In 8 h cells were harvested and luciferase activity was detected by luminometric method. GSNO treatment caused the increase in the luciferase activity in the WT GalDBD/HIF-1α 727-826 plasmid containing cells compared to the co-transfected in the same manner but not stimulated cells (Fig. 1A). The activity of luciferase was significantly increased in non-treated N803A GalDBD/HIF-1α 727-826 plasmid containing cells compared to the non-stimulated WT GalDBD/HIF-1α 727-826 plasmid containing cells. Treatment of these cells with GSNO caused further increase in the luciferase activity (Fig. 1A). Asn-803 is the only target for degressive hydroxylation of the HIF-1α C-terminal fragment. The results obtained by luciferase assay are consistent with the Western blot data (Fig. 1B). Transfected construct is detectable in the transfected cells. Its *S*-nitrosation was observed in the transfected and GSNO-stimulated cells. Equal loading of the protein is verified by actin staining. If we assume that the increase in the luciferase activity is the result of the NO-dependent inhibition of HIF-1α proline hydroxylation, this hypothesis will work only for the WT plasmid, because the N803A construct has no targets to be hydroxylated [19]. Then we can hypothesize that protein *S*-nitrosation is the reason for the increase in its transcriptional activity. C-terminal fragment of HIF-1α protein has three SH-groups, however the thiol group of Cys-800 is qualified as the reactive one that is of major importance for the recruitment of co-activators. These data allow to predict that nitrosation of Cys-800 SH-group leads to the increase of the protein transcriptional activity. To confirm this hypothesis we substituted Cys-800 of the N803A plasmid to Ala using QuickChange® site-directed mutagenesis kit. In the experimental set-up we co-transfected N803A or C800A plasmid together with the luciferase containing Gal4 reporter plasmid as described in the beginning of this section. Stimulation of N803A containing cells with GSNO resulted in increase in the transcriptional activity (Fig. 2A), which is consistent with our previous data (see Fig. 1A). Eight hours of treatment of the C800A plasmid containing cells with GSNO resulted in the absence of transcriptional activity increase (Fig. 2A). Both N803A and C800A constructs were detectable in the cells by Western blot. However C800A construct was characterized by lower *S*-nitrosation compared to N803A (Fig. 2B). Equal loading of the protein validated by actin staining. These data suggest that *S*-nitrosation of the HIF-1α Cys-800 is critical for the increase in the HIF-1 transcriptional activity. However this effect is demonstrated only upon GSNO-dependent *S*-nitrosation, which is an artificial one.

3.2. *Cytokine-dependent induction of NOS causes* S*-nitrosation of the HIF-1α protein C-terminal fragment and increase in its transcriptional activity*

In the next set of experiments we examined the impact of *S*-nitrosation of HIF-1α by nitric oxide derived from iNOS on HIF-1 transactivation. We asked whether physiological increase of the NO production (activation of iNOS) leads to the increase in HIF-1 transcriptional activity. Our recent data show that the whole HIF-1α protein is *S*-nitrosated when the iNOS is activated by cytokines. However there is no indication about the impact on the protein transactivation. For these experiments we used RCC cells because it is impossible to induce NOS activity in HEK293 cells. We co-transfected the cells with WT or N803A GalDBD/HIF-1α 727-826 encoding plasmids together with the luciferase containing Gal4 reporter plasmid. Cells were incubated for 24 h after transfection followed by 24 h of stimulation with cytokine mix (75 U/ml IL-1β, 250 U/ml IFN-γ, 25 μg/ml LPS) [28]. To confirm the involvement of NOS we blocked NO formation by *N*-monomethyl-L-arginine (NMMA), which revealed no major impact by itself [13]. In 24 h nitrite content was detected in the cell culture medium, the cells were harvested for the luciferase activity assay. Cytokine treatment of the RCC cells caused an increase in nitrite content confirming NOS activation (Fig. 3). This resulted in the *S*-nitrosation of the HIF-1α C-terminal fragment correlating with the transcriptional activation of both WT and N803A proteins

(Fig. 3). Blockage of nitrite formation by the NMMA nullified *S*-nitrosation and attenuated the increase in transcriptional activity suggesting the involvement of a NOS, while NMMA by its own revealed no major impact (Fig. 3).

3.3. Nitrosation of Cys-800 of HIF-1α protein supports its interaction with p300 co-activator protein

Recent data suggest that substitution of HIF-1α Cys-800 to valine increases HIF-1 transcriptional activity by the activation of HIF-1α–p300 interactions [14]. *S*-NO group is characterized by the equal distribution of the electron density, which makes the group to develop hydrophobic mimicking activity and allows the assumption that *S*-nitrosation of Cys-800 supports HIF-1α–p300 interaction. The HIF-1α C-terminal domain functions by recruiting transcriptional co-activator p300/CBP, which directly interacts with it via its CH1 domain. To examine the impact of nitric oxide on the HIF-1α–p300 interaction we used recombinant GST-CH1 in GST pull-down experiments with the protein extracts from cells transiently transfected with the constructs expressing the HIF-1α C-terminal region (in this experimental set-up we used only N803A and C800A plasmids). Whole cell extracts obtained from non-stimulated cells as well as from cells exposed to 1 mM GSNO for 8 h were mixed with recombinant GST-p300CH1 (amino acids 300 to 528) in a pull-down assay.

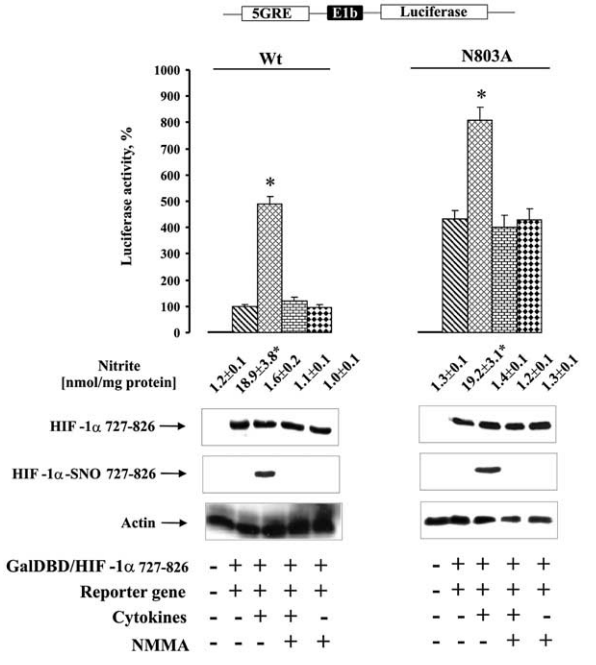

Fig. 3. NOS-derived nitric oxide *S*-nitrosates the HIF-1α C-terminal domain and stimulates its transactivation. RCC cells were co-transfected with WT and N803A Gal4DBD/HIF-1α 727-826 as well as Gal4 response element-driven luciferase reporter gene using calcium phosphate precipitation. After transfection cells were incubated for 24 h followed by 24 h of stimulation with 75 U/ml of IL-1β, 250 U/ml of IFNγ and 25 μg/ml of LPS for 24 h. NOS activity was blocked with 1 mM NMMA. Nitrite was determined by the Griess assay. Luciferase activity was assayed by the luminometric method, HIF-1α and *S*-nitrosated HIF-1α were determined by Western analysis as outlined in Section 2. Actin staining confirmed equal protein loading. Digital data are mean values ± S.D. of at least five individual experiments. $*P < 0.01$ vs. control. All Western blot data are from one experiment representative of three that gave similar results.

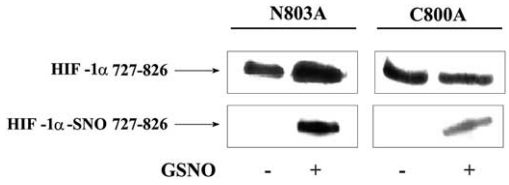

Fig. 4. Nitrosation of Cys-800 of HIF-1α protein supports its interaction with p300 co-activator protein. The whole extracts obtained from non-stimulated HEK293 cells as well as from cells exposed to 1 mM GSNO for 8 h were mixed with the recombinant GST-p300CH1 (amino acids 300 to 528, prepared as described in Section 2) in a pull-down assay. Aliquots of the whole cell extracts (20% of input) and 50% of the GST-CH1 pull-down were separated by SDS–PAGE and the HIF-1α C-terminal domain was detected by Western blot using polyclonal antibody directed against HIF-1α C-terminus. *S*-nitrosation of the HIF-1α C-terminal fragment was also determined by Western analysis. The data are from one experiment representative of three that gave similar results.

Aliquots of the whole cell extracts (20% of input) and 50% of the GST-CH1 pull-down were separated by SDS–PAGE and the HIF-1α C-terminal domain was detected by Western blot using polyclonal antibody directed against HIF-1α C-terminus [19]. *S*-nitrosation of the HIF-1α C-terminal fragment was also detected. We found that *S*-nitrosation of the N803A construct correlated with the increase in binding to p300CH1 (Fig. 4), however *S*-nitrosation of C800A protein resulted in no increase. These results suggest that *S*-nitrosation of Cys-800 of HIF-1α protein supports its interaction with p300 co-activator protein.

References

[1] Semenza, G.L. (2002) Trends Mol. Med. 8, S62–S67.
[2] Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001) EMBO J. 20, 5197–5206.
[3] Semenza, G. (2000) Genes Dev. 14, 1983–1991.
[4] Wenger, R.H. (2000) J. Exp. Biol. 203, 1253–1263.
[5] Lando, D., Peet, J.P., Gorman, J.J., Whelan, D.A., Whitelaw, M. and Bruick, R.K. (2002) Genes Dev. 16, 1466–1471.
[6] Zhong, H., Chiles, K., Felser, D., Laughner, E., Hanrahan, C., Georgescu, M.M., Simons, J.W. and Semenza, G.L. (2000) Cancer Res. 60, 1541–1545.
[7] Hellwig-Bürgel, T., Rutkowski, K., Metzen, E., Fandrey, J. and Jelkmann, W. (1999) Blood 94, 1561–1567.
[8] Richard, D.E., Berra, E. and Pouyssegur, J. (2000) J. Biol. Chem. 275, 26765–26771.
[9] Sandau, K.B., Fandrey, J. and Brüne, B. (2001) Blood 97, 1009–1015.
[10] Sandau, K.B., Zhou, J., Kietzmann, T. and Brüne, B. (2001) J. Biol. Chem. 276, 39805–39811.
[11] Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D'Acquisto, F., Addeo, R., Makuuchi, M. and Esumi, H. (2000) Blood 95, 189–197.
[12] Palmer, L.A., Gaston, B. and Johns, R.A. (2000) Mol. Pharmacol. 58, 1197–1203.
[13] Sumbayev, V.V., Budde, A., Zhou, J. and Brüne, B. (2003) FEBS Lett. 535, 106–112.
[14] Ruas, J.L., Poellinger, L. and Pereira, T. (2002) J. Biol. Chem. 277, 38723–38730.
[15] Semenza, G.L. (2001) Curr. Opin. Cell Biol. 13, 167–171.
[16] Hart, T.W. (1997) Tetrahedron Lett. 26, 2013–2016.
[17] Bae, M.-K., Ahn, M.-Y., Jeong, J.-W., Bae, M.-H., Lee, Y.M., Bae, S.-K., Park, Y.-W., Kim, K.-R. and Kim, K.-W. (2002) J. Biol. Chem. 277, 9–12.
[18] Wykoff, C.C., Pugh, C.W., Maxwell, P.H., Harris, A.L. and Ratcliffe, P.J. (2000) Oncogene 19, 6297–6305.
[19] Lando, D., Peet, J.P., Whelan, D.A., Gorman, J.J. and Whitelaw, M. (2002) Science 295, 858–861.

[20] Jaffrey, S.R., Erdjument-Bromage, H., Ferris, C.D., Tempst, P. and Snyder, S.H. (2001) Nat. Cell Biol. 3, 193–197.
[21] Feelisch, M., Kubitzek, D. and Werringloer, J. (1996) in: Methods in Nitric Oxide Research (Feelisch, M. and Stamler, J.S., Eds.), pp. 455–478, Wiley, Chichester.
[22] Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. 126, 131–138.
[23] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[24] Steffen, M., Sarkela, T., Gybina, A., Steele, T.W., Trasseth, N.J., Kuehl, D. and Giulivi, C. (2001) Biochem. J. 356, 395–402.
[25] Stamler, J.S. (1994) Cell 78, 931–936.
[26] Radomski, M.W., Rees, D.D., Dutra, A. and Moncada, S. (1992) Br. J. Pharmacol. 107, 745–749.
[27] Gibson, A., Babbedge, R., Brave, S.R., Hart, S.L., Hobbs, A.J., Tucker, J.F., Wallace, P. and Moore, P.K. (1992) Br. J. Pharmacol. 107, 715–721.
[28] Sandau, K., Pfeilschifter, J. and Brüne, B. (1997) J. Immunol. 158, 4938–4946.