

# Increase of SUMO-1 expression in response to hypoxia: direct interaction with HIF-1 $\alpha$ in adult mouse brain and heart in vivo

Ruijin Shao<sup>a</sup>, Fu-Ping Zhang<sup>b</sup>, Fei Tian<sup>c</sup>, P. Anders Friberg<sup>a</sup>, Xiaoyang Wang<sup>d</sup>, Helen Sjöland<sup>c</sup>, Håkan Billig<sup>a,\*</sup>

<sup>a</sup>Division of Endocrinology, Department of Physiology and Pharmacology, Göteborg University, SE-40530 Göteborg, Sweden

<sup>b</sup>Department of Physiology, Institute of Biomedicine, University of Helsinki, FIN-00014 Helsinki, Finland

<sup>c</sup>Wallenberg Laboratory for Cardiovascular Research, Department of Heart and Lung Diseases, Sahlgrenska University Hospital, SE-41345 Göteborg, Sweden

<sup>d</sup>Perinatal Center, Department of Physiology and Pharmacology, Göteborg University, SE-40530 Göteborg, Sweden

Received 16 April 2004; revised 25 May 2004; accepted 30 May 2004

Available online 15 June 2004

Edited by Robert Barouki

**Abstract** The present study investigates the regulation of small ubiquitin-related modifier-1 (SUMO-1) expression in response to hypoxia in adult mouse brain and heart. We observed a significant increase in SUMO-1 mRNAs and proteins after hypoxic stimulation in vivo. Because SUMO-1 interacts with various transcription factors, including hypoxia-inducible factor-1 $\beta$  (HIF-1 $\beta$ ) in vitro, we not only demonstrated that the HIF-1 $\alpha$  expression is increased by hypoxia in brain and heart, but also provided evidence that SUMO-1 co-localizes in vivo with HIF-1 $\alpha$  in response to hypoxia by demonstrating the co-expression of these two proteins in neurons and cardiomyocytes. The specific interaction between SUMO-1 and HIF-1 $\alpha$  was additionally demonstrated with co-immunoprecipitation. These results indicate that the increased levels of SUMO-1 participate in the modulation of HIF-1 $\alpha$  function through sumoylation in brain and heart.

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**Keywords:** Small ubiquitin-related modifier-1; Hypoxia-inducible factor-1 $\alpha$ ; Hypoxia; Brain; Heart; Mouse

## 1. Introduction

The ubiquitin system has been implicated as a key intracellular signaling pathway for posttranslational modification of proteins [1]. The small ubiquitin-related modifier-1 (SUMO-1) is a 11.5 kDa ubiquitin-related protein that is 18% identical to ubiquitin [2–4]. The expression of SUMO-1 was found in both the nuclear pore complex and within nuclei in a wide range of tissues and cell types [2,5,6]. Previous studies have demonstrated that the SUMO-1 gene is highly conserved between human and mouse [6,7], and its mRNA and protein are expressed in the brain and heart in humans and mice [7–11]. The primary function of SUMO-1 has been suggested to prevent proteasome-mediated degradation of proteins, in opposition to ubiquitin [1,12]. Recent experiment has shown that SUMO-1,

via sumoylation, stabilizes nuclear proteins [13]. Although SUMO-1 expression patterns and its localization may provide some hints as to the functional involvement of sumoylation, no experimental evidence for the effect of hypoxia on regulation of SUMO-1 expression in mouse brain and heart in vivo has been obtained to date.

Hypoxia-inducible factor-1 (HIF-1) is a heterodimer, composed of  $\alpha$  and  $\beta$  subunits (also called the aryl hydrocarbon receptor nuclear translocator (ARNT)) with molecular masses of 120 and 91–94 kDa, which belongs to the basic helix–loop–helix (bHLH)/Per-Arnt-Sim (PAS) family [14,15] and acts as a functional transcription factor which contributes to a variety of developmental and postnatal physiological events, as well as human diseases [16–18]. The two subunits contain bHLH/PAS domains which mediate dimerization and DNA binding [16,19,20]. However, the cellular level of HIF-1 $\alpha$  mainly regulates the activities of HIF-1. Moreover, both subunits display distinct regulation patterns. The expression of HIF-1 $\beta$  is generally constitutive [21,22], whereas that of HIF-1 $\alpha$  mRNA and protein levels is upregulated in response to hypoxic stimulation in vivo [23–25]. It is well documented in the literature that the mechanism of HIF-1 $\alpha$  protein degradation under normoxic conditions is through ubiquitin–proteasome pathway [26–29], but HIF-1 $\alpha$  is stabilized under hypoxia and its degradation is prevented by hypoxic stimulation [30]. In addition to the oxygen-dependent degradation of HIF-1 $\alpha$  that occurs via the von Hippel Lindau (VHL)-mediated ubiquitination, oxygen-independent degradation is also mediated by the p53–Mdm2 complex [31] and by the chaperone Hsp90 inhibitor geldanamycin [32]. Recently, several studies have shown that SUMO-1 interacts with various transcription factors and further modulates their activities [2–4,6]. For instance, SUMO-1 modified HIF-1 $\beta$  and further regulated its transcription activity in vitro [33]. However, we are not aware of any previous reports in which the association of SUMO-1 and HIF-1 $\alpha$  has been shown either in vivo or in vitro. We propose that another posttranslational modification other than ubiquitylation may serve as a regulatory mediator to protect HIF-1 $\alpha$  protein against degradation.

In this study, we examined whether SUMO-1 expression is altered after hypoxic stimulation in adult mouse brain and heart in parallel to increased levels of HIF-1 $\alpha$  protein.

\* Corresponding author. Fax: +46-31-7733531.

E-mail address: [hakan.billig@fysiologi.gu.se](mailto:hakan.billig@fysiologi.gu.se) (H. Billig).

URL: <http://www.physiology.gu.se/endo/staff.htm>.

Furthermore, we investigated whether SUMO-1 interacts with HIF-1 $\alpha$  in vivo.

## 2. Materials and methods

### 2.1. Antibodies and reagents

Rabbit polyclonal anti-SUMO-1, mouse monoclonal anti-myosin heavy chain (MHC), rabbit polyclonal anti-VEGF, goat polyclonal anti-lamin B, and normal mouse IgG were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA); mouse monoclonal anti-HIF-1 $\alpha$  was purchased from Novus Biologicals, Inc. (Littleton, CO); mouse monoclonal anti-neuronal nuclear protein (NeuN) was purchased from Chemicon International, Inc. (Temecula, CA); alkaline phosphatase conjugated goat-anti-rabbit immunoglobulin were purchased from Tropic (Bedford, MA); mouse monoclonal anti- $\beta$ -actin, alkaline phosphatase conjugated goat-anti-mouse immunoglobulin, and alkaline phosphatase conjugated mouse-anti-goat immunoglobulin were purchased from Sigma (St. Louis, MO); normal rabbit serum and normal goat serum were obtained from DAKO Corp. (Carpinteria, CA); biotin-SP-conjugated donkey anti-rabbit IgG, fluorescein (FITC)-conjugated streptavidin and Cy 3-conjugated donkey anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Other reagents not mentioned in the text were purchased from Sigma or Merck AG (Darmstadt, Germany) and were of the highest purity grade available.

### 2.2. Animal and tissue collections

Eight to 10-week-old male and female mice (C57BL/6) were provided by Taconic M&B, Denmark. Mice were randomized and housed in a temperature-controlled animal room ( $21 \pm 2$  °C) under a constant 12:12-h light:dark cycle. They were fed with standard laboratory food and water ad libitum for at least one week before the experiments. For studies involving hypoxic conditions, mice were subjected to systemic hypoxia in a closed Perspex chamber supplied with a gas mixture consisting of 10% O<sub>2</sub> and 90% N<sub>2</sub> using an oxygen (atmospheric and dissolved) sensor (Model 600, Engineered Systems & Designs Inc., USA) for monitoring the O<sub>2</sub>% concentration, which gradually decreases from an ambient 20.9% to 10% during an adaptation time of 30 min. Mice were kept in room air to serve as control. The animals were killed at day 4 after hypoxic exposure. To prevent protein degradation, tissues (brain, heart, liver and lung) were either immediately frozen in liquid nitrogen and stored at  $-135$  °C until assayed, or fixed in 4% formaldehyde neutral buffered solution for 24 h at 4 °C and embedded in paraffin. In addition, hearts isolated from mice were quickly rinsed twice in ice-cold HEPES buffer (10 mM HEPES, pH 6.95, 115 mM NaCl, 5 mM KCl, 35 mM sucrose, 10 mM glucose and 4 mM taurine) at 4 °C to remove residual blood before processing. All animal studies described in this study were reviewed and approved by the ethics committee at Göteborg University, Sweden.

### 2.3. Preparation of whole cell, cytosolic and nuclear protein extracts

The preparation of whole cell protein lysates was performed as described recently [34]. Briefly, tissues were extracted with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.5% Nonidet-P40, 50 mM NaF, 0.5 mM Na<sub>3</sub>VO<sub>4</sub> and 20 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>·10H<sub>2</sub>O, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin and 1 mM DTT). Cytosolic and nuclear extracts were performed by following a minor modified procedure based on previously described protocol [35]. Tissues were gently homogenized using a pellet pestle mixer (Merck KGaA, Darmstadt, Germany) in ice-cold TEDG buffer (25 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT and 10% glycerol) in the presence of protease inhibitors (10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin and 1 mM PMSF). After centrifugation at  $800 \times g$ , the supernatant was removed and centrifuged with glycerol at  $100\,000 \times g$  for 1 h at 4 °C to obtain cytosolic extracts. The nuclear pellets (presence and purity of the nuclei were checked under a microscope) were resuspended with ice-cold STMG buffer (20 mM Tris, pH 7.8, 250 mM sucrose, 1.1 mM MgCl<sub>2</sub>, 8.5% glycerol and 5% Triton X-100). After centrifugation at  $1500 \times g$  for 10 min at 4 °C, the pellets were washed one time with ice-cold STMG buffer without 5% Triton X-100 and then centrifuged at  $1500 \times g$  for 10 min at 4 °C. The pellets were homogenized in ice-cold lysis buffer (20 mM HEPES, pH 7.9, 420 mM

NaCl, 1 mM EDTA, 0.1 mM EGTA and 25% glycerol) in the presence of protease inhibitors and incubated for 30 min at 4 °C. The nuclei were pelleted by centrifugation at  $13\,000 \times g$  for 30 min at 4 °C. The supernatants were collected as nuclear extracts. Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as standard.

### 2.4. Immunoblotting and co-immunoprecipitation

The immunoblotting and co-immunoprecipitation experiments was performed according to a procedure previously described [11]. Briefly, equal amounts of cellular extract were separated by 10% SDS-polyacrylamide gels (Novex) and transferred to polyvinylidene difluoride membranes (Amersham International, Buckinghamshire, UK). The specific antibodies against SUMO-1 or HIF-1 $\alpha$  diluted to 1:1000 or 1:500, respectively, were used to probe the blots. Binding was detected with alkaline phosphatase-linked secondary antibody (polyclonal secondary antibody at a dilution of 1:40 000 or monoclonal secondary antibody at a dilution of 1:80 000, respectively). Blots were re probed with a monoclonal anti- $\beta$ -actin antibody for loading normalization without stripping membranes.

For co-immunoprecipitation experiments, tissues were extracted with ice-cold lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1% SDS, 200 mM sodium deoxycholate, 1 mM DTT, 5 mM EDTA, 0.5 mM PMSF, 10 mM *N*-ethylmaleimide (NEM), 10 mM iodoacetamide, and a cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany)). Either anti-SUMO-1 or anti-HIF-1 $\alpha$  antibodies were added to 500  $\mu$ g protein extracts and incubated for 4 h at room temperature. Immune complexes were obtained by the addition of 50  $\mu$ l Pansorbin cells (Calbiochem, San Diego, CA). The resulting immobilized immune complexes were washed in RIRA buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 15 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40, 0.3% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 1 mM DTT and a cocktail of protease inhibitors), which was supplemented with 10 mM NEM and 10 mM iodoacetamide. The bound protein was eluted by boiling in 30  $\mu$ l SDS sample reducing loading buffer (Novex) for 5 min. Immunoprecipitated complexes for either SUMO-1 or HIF-1 $\alpha$  were loaded in the same gels which were run at the same time within the same electrophoresis unit and examined by immunoblotting as described above.

To reprobe the blot with another antibody, the blot was rehydrated in methanol, rinsed and incubated with stripping buffer (62.5 mM Tris-HCl, 2% SDS and 100 mM  $\beta$ -mercaptoethanol, pH 6.8) at 50 °C for 30 min.

### 2.5. Histology and immunohistochemistry

Serial 5  $\mu$ m sections were cut from the paraffin-embedded blocks and allowed to dry overnight at 37 °C, and stained with hematoxylin and eosin (H&E) using standard protocols. Immunohistochemical analysis was performed as described previously [11,34]. In brief, after deparaffinization and rehydration, adjacent paraffin-embedded sections were pretreated with 0.01 M sodium citrate buffer (pH 6.0), 3% hydrogen peroxide in Tris-buffered saline (TBS, 50 mM Tris and 0.9% NaCl, pH 7.5), and blocked with 3% BSA and 5% normal goat serum in TBS with 0.3% Triton X-100. Sections were incubated with the primary antibody against SUMO-1 diluted to 1:500 in TBS containing 3% BSA overnight at 4 °C. Bound antibodies were detected using the avidin-biotinylated-peroxidase complex detection system (ABC Kit, Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. Immunostaining was then visualized using 3,3'-diaminobenzidine tetrahydrochloride for 5–10 min. Sections were washed and mounted with Histo Mounting Medium (Mountex, HistoLab, Sweden). Slides were viewed on a Nikon E-1000 microscope (Japan) and photomicrographed.

For double-fluorescence immunohistochemistry of SUMO-1 and HIF-1 $\alpha$ , sections were incubated with a mixture of antibodies against SUMO-1 and HIF-1 $\alpha$  diluted to 1:500 and 1:200, respectively, in PBS containing 1% BSA and 3% fat-free milk. Each primary antibody was serially diluted to optimize the concentration used to achieve maximum sensitivity and specificity. Immunodetection was accomplished using the species-specific secondary antibodies, either a biotin-conjugated anti-rabbit antibody together with streptavidin conjugated with FITC or Cy 3-conjugated anti-mouse antibody. Sections were washed and mounted with Fluorescent Mounting Media (DAKO, Carpinteria, CA). Slides were viewed on a ZEISS Axioskop II microscope (Germany) and photomicrographed. The immunohistochemical findings

illustrated are representative of those observed in numerous sections from multiple animals. The resulting stain was evaluated by two blinded observers.

To assess specificity of SUMO-1 and HIF-1 $\alpha$  antibodies, separate control sections were processed with non-immune rabbit and mouse IgG in place of the primary antibodies. Additional control sections were processed as above, but with the omission of either primary or secondary antibody. These control sections showed no labeling.

#### 2.6. RNA preparation, RT-PCR and Northern hybridization analyses

Total RNA was isolated from individual tissues using Trizol Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. The integrity of extracted RNA was determined by visualizing ethidium bromide-stained 28S and 18S rRNA bands after migration on 1.2% denatured agarose gel and concentration was determined by spectrophotometry. Any contaminating genomic DNA was digested by RNase-free DNase.

For amplification of the targets, RT and PCR were run in two separate steps. Briefly, equal amounts of total RNA (2  $\mu$ g/each) were heat denatured and reverse transcribed by incubation at 42 °C for 60 min and at 52 °C for another 60 min with 12.5 U AMV-RT (Promega Corp., Madison, WI), 20 U ribonuclease inhibitor (RNasin) (Promega), 200 nM deoxy-NTP mixtures and 1 nM random hexamers (Promega) in a final volume of 30  $\mu$ l of 1 $\times$  AMV-RT buffer. The reaction was terminated at 97 °C for 5 min and then chilled on ice, followed by dilution of RT cDNA samples with nuclease-free H<sub>2</sub>O (final volume 100  $\mu$ l). For PCR analysis, 10  $\mu$ l aliquots of the cDNA samples were amplified in 50  $\mu$ l of 1 $\times$  PCR buffer in the presence of 2.5 U Taq-DNA polymerase (Promega), 200 nM deoxy-NTP mixtures and 1 nM of each primer for SUMO-1 gene (5'-GAGAAGGGACGGATTTGTAACCTCA-3' (forward) and 5'-TGACCCCCGTTTGTTCCTGA-3' (reverse)) (Sigma-Genosys Ltd, Cambridgeshire, UK). PCRs consisted of a denaturing step at 97 °C for 3 min, the cycles of amplification defined by denaturation at 96 °C for 0.5 min, annealing at 57 °C for 1 min and extension at 72 °C for 2 min. A final extension cycle of 72 °C for 10 min was included. The amount of cDNA and the number of cycles were optimized to ensure amplification in the exponential phase of PCR. Different numbers of cycles were tested for SUMO-1 (ranging between 25 and 40). Based on the analysis of cycle dependency of the intensity of the generated PCR signals, 30 cycles were chosen for further analysis of SUMO-1. PCR products were electrophoresed in 1.5% agarose gels along with a 100 bp DNA ladder (Fermentas, Hanover, MD) and visualized by ethidium bromide staining. The predicted size of the PCR-amplified products was 389 bp for SUMO-1. The intensity of bands was digitally photographed. The specificity of amplified products was confirmed by including the control reactions containing water alone or RNA in the absence of RT. No PCR products were obtained in these experiments for any of the pairs of primers.

Northern hybridization analyses were performed as previously described [36]. RNA samples (12  $\mu$ g/each) were resolved onto 1.2% denaturing agarose gels and transferred onto Hybond-XL nylon membranes (Amersham International, Aylesbury, UK) using the capillary method. The membranes were cross-linked by short-wave UV irradiation and prehybridized at 65 °C for at least 4 h in a solution containing 50% deionized formamide, 3 $\times$  SSC, 5 $\times$  Denhardt's solution, 0.1 g/l heat-denatured calf-thymus DNA, 1% SDS, and 0.1 g/l yeast transfer RNA. For generation of cRNA probe, DNA fragment generated by RT-PCR corresponding to 225–938 of SUMO-1 cDNA [7] was subcloned into T-vector (Promega) and used as template. [<sup>32</sup>P]-labeled complementary RNA (cRNA) probes specific for the SUMO-1 gene was generated using the Riboprobe System II Kit (Promega, Madison, WI) before use. Hybridization was carried out at 66 °C (cRNA probe) overnight in the same pre-hybridization solution after adding the probe. After hybridization, the membranes were washed in 2 $\times$  SSC, 0.1% SDS for 20 min at room temperature, in 0.5 $\times$  SSC, 0.1% SDS twice for 30 min at 65 °C, and twice in 0.1 $\times$  SSC, 0.1% SDS for 1 h at 65 °C. The filters were exposed to Kodak X-ray films (Kodak XAR-5, Eastman Kodak, Rochester, NY) at –70 °C for 8–24 h.

#### 2.7. Data analysis and statistics

All experiments were repeated three times if not otherwise stated. Results are expressed as means  $\pm$  S.E.M. The data were analyzed using Analyse-It program (Analyse-It Software, Ltd., UK). One-way ANOVA with post hoc Tukey's test was used for multiple comparisons. A *P* value <0.05 was considered significant.

### 3. Results

#### 3.1. Effect of hypoxic stimulation on SUMO-1 gene expression *in vivo*

To investigate whether the expression of SUMO-1 could be affected by hypoxia in adult mice *in vivo*, we performed immunoblotting and immunohistochemical analyses using both brain and heart tissues. When the nuclear and cytosolic extracts were subjected to immunoblotting analyses, we observed that free SUMO-1 protein with a molecular mass of approximately 18.7 kDa [37] was present in the nuclear fraction, but not in the cytosolic fraction (data not shown). This is in line with previous studies demonstrating that SUMO-1 is present in the cell nucleus [2,5,6]. Immunoblotting analyses revealed that SUMO-1 protein expression was significantly increased in brain and heart from both female and male mice after hypoxic stimulation compared to normoxic conditions, which served as controls (Figs. 1A and 2A). Moreover, immunohistochemical labeling revealed that the number of SUMO-1-positive cells was also significantly increased in brain and heart after hypoxic stimulation compared to normoxic conditions (Figs. 1B and 2B), which is in agreement with immunoblotting analyses. In addition, double-immunofluorescence analysis demonstrated SUMO-1-positive cells in brain sections from hypoxia mice corresponded to NeuN protein positive-cells, a marker for neuron-specific nuclear protein (data not shown) [38,39]. In heart tissues, SUMO-1 was expressed in cardiomyocytes because immunohistochemical analyses demonstrated that immunolabeling of myosin heavy chain (MHC) specific for cardiomyocytes was predominantly associated with SUMO-1-positive cell cytoplasm within the same cells in heart sections [40]. Omission of the primary antibody eliminated the signal (data not shown).

Northern analysis was demonstrated that one major RNA band hybridized to a mouse SUMO-1 cDNA probe in both brain and heart. The increase of SUMO-1 protein is paralleled by a rapid increase of SUMO-1 mRNA in both brain (Fig. 3A) and heart (Fig. 3B) after hypoxic stimulation. Using specific RT-PCR primer pairs for mouse SUMO-1, we demonstrated that a single band in both brain and heart corresponded to the predicted size based on the mouse SUMO-1 gene sequence. RT-PCR of SUMO-1 mRNA expression confirmed upregulated by hypoxia (data not shown).

#### 3.2. Increase of HIF-1 $\alpha$ protein expression occurs predominantly in nuclear fraction

HIF-1 $\alpha$  is required to regulate the response to hypoxia in almost every tissue in humans and rodents [23–25,41–44]. Because HIF-1 $\alpha$  has a short half-life in a normoxic environment [28], we added CoCl<sub>2</sub>, which inhibits HIF-1 $\alpha$  degradation [45], to tissue lysis buffer during protein preparation. To confirm the analyses of HIF-1 $\alpha$  expression, both nuclear and cytosolic extracts from brain and heart tissues were analyzed by immunoblotting analyses. We found that HIF-1 $\alpha$  protein was present in the nuclear fraction from hypoxia, but not from normoxia in brain and heart of both female and male mice (Fig. 4A). This is well in accordance with the immunohistochemical results obtained by us and others [25]. In contrast, we did not observe HIF-1 $\alpha$  protein expression in either the nuclear or the cytosolic fractions under normoxic condition nor the cytosolic fraction under hypoxic condition

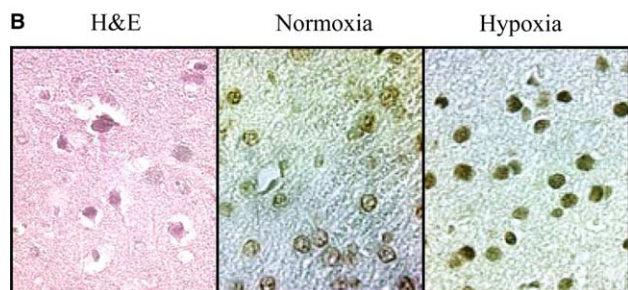
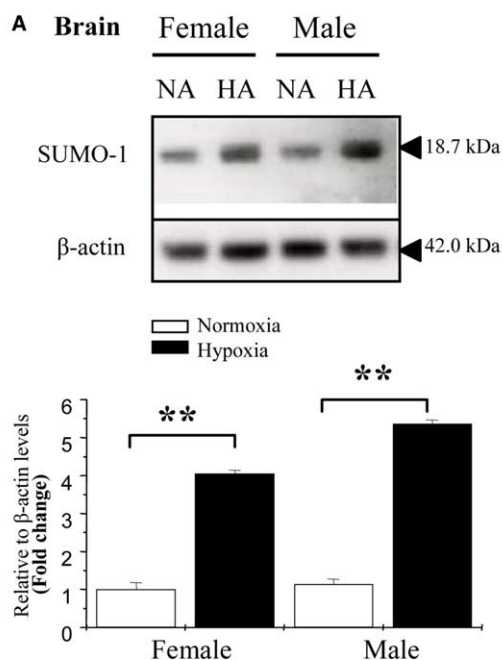


Fig. 1. Effect of hypoxic stimulation on expression of SUMO-1 protein in mouse brain. (A) Protein samples were isolated from brain of female and male mice treated with either normoxia or hypoxia. Total protein (30  $\mu$ g per lane) was subjected to immunoblotting analysis of SUMO-1 expression. The representative blots show data from five animals in each group. The level of  $\beta$ -actin was used as an internal standard to verify equal loading of proteins. The migrations of SUMO-1 and  $\beta$ -actin proteins are designated by arrowheads. The SUMO-1/ $\beta$ -actin ratio was calculated for each sample and the values were the relative SUMO-1 expression (the average value for the normoxia female animals was set to 1). Results are presented as means  $\pm$  S.E.M. from five mice/group. \*\*,  $P < 0.01$ . NA, normoxia; HA, hypoxia. (B) Cellular localization of SUMO-1 protein expression in female mouse brain. H&E stains demonstrating the neurons of mouse brain (left panel). Immunoreactivity of SUMO-1 in the brain sections showing increased immunolabeling intensity in hypoxia (right panel) compared with normoxia (middle panel). Random sections from three female and male mice provided similar results. All micrographs are at 40 $\times$  magnifications.

in brain and heart tissues (Fig. 4A). The expression of lamin B, a representative protein specifically located in the cell nucleus, was examined in order to confirm the proper extraction of cytoplasmic and nuclear fractions in both brain and heart (Fig. 4A), and confirmed that the cytosolic extracts of both brain and heart tissue samples were not contaminated with nuclear fractions. Liver and lung tissue from hypoxic mice served as positive and negative controls (data not shown) [25].

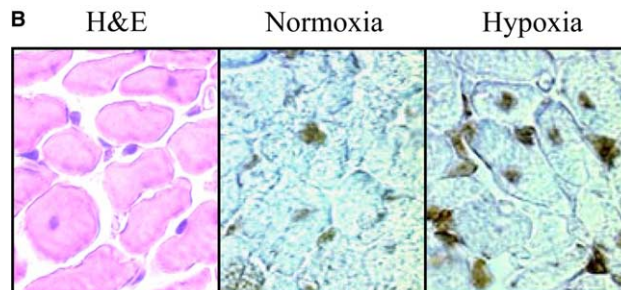
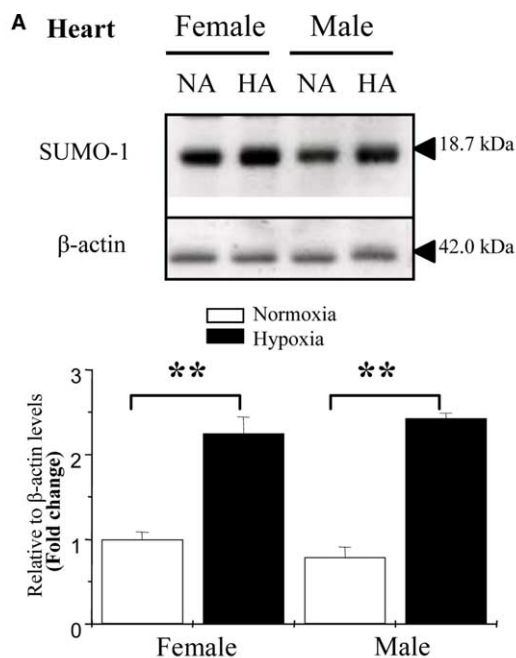


Fig. 2. Effect of hypoxic stimulation on expression of SUMO-1 protein in mouse heart. (A) Protein samples were isolated from heart of female and male mice treated with either normoxia or hypoxia. Total protein (30  $\mu$ g per lane) was subjected to immunoblotting analysis of SUMO-1 expression. The representative blots show data from five animals in each group. The level of  $\beta$ -actin was used as an internal standard to verify equal loading of proteins. The migrations of SUMO-1 and  $\beta$ -actin proteins are designated by arrowheads. The SUMO-1/ $\beta$ -actin ratio was calculated for each sample and the values were the relative SUMO-1 expression (the average value for the normoxia female animals was set to 1). Results are presented as means  $\pm$  S.E.M. from five mice/group. \*\*,  $P < 0.01$ . NA, normoxia; HA, hypoxia. (B) Cellular localization of SUMO-1 protein expression in female mouse heart. H&E stains demonstrating the cardiomyocytes of mouse heart (left panel). Immunoreactivity of SUMO-1 in the heart sections showing increased immunolabeling intensity in hypoxia (right panel) compared with normoxia (middle panel). Random sections from three female and male mice provided similar results. All micrographs are at 40 $\times$  magnifications.

### 3.3. Nuclear co-localization of SUMO-1 and HIF-1 $\alpha$ under hypoxic conditions

Because the expression of SUMO-1 and HIF-1 $\alpha$  significantly increased in a similar pattern in brain and heart under hypoxic conditions, it was of interest to determine if these two molecules were also co-expressed. We carried out double-labeled immunofluorescence experiments on the same brain and heart tissue sections from mice after hypoxic stimulation using antibodies that recognize each of these proteins. Simultaneous labeling with SUMO-1 and HIF-1 $\alpha$  antibodies showed that

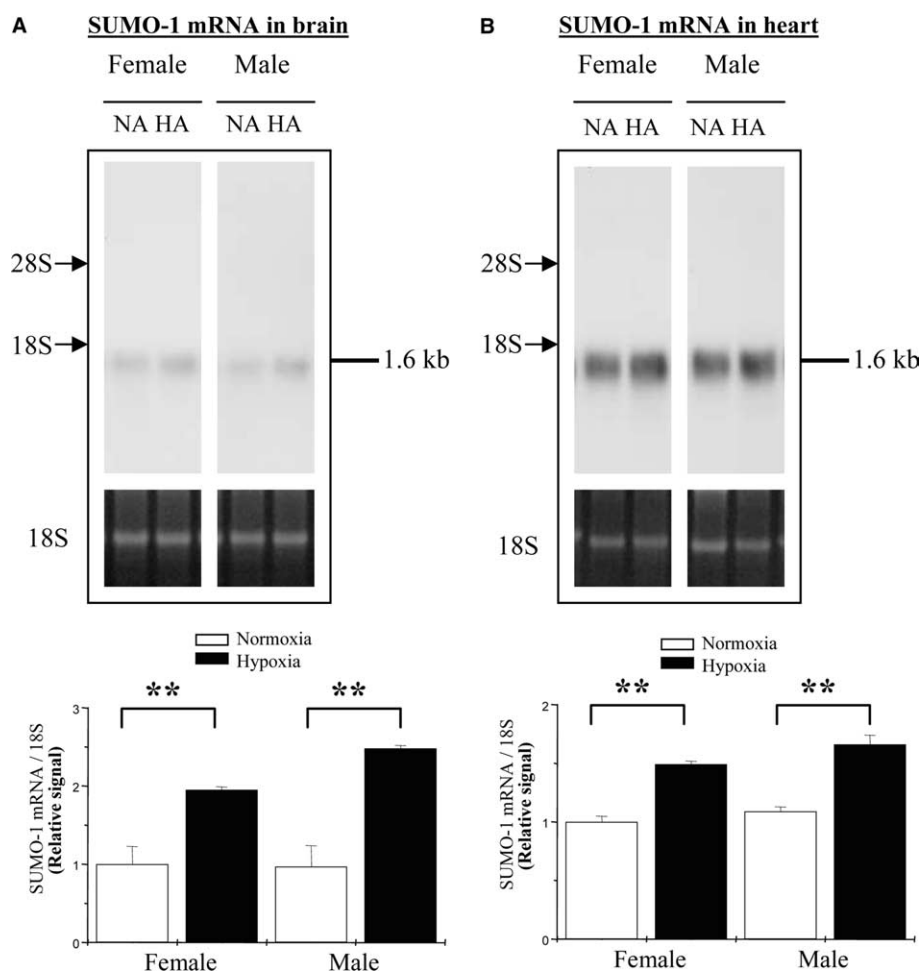


Fig. 3. Effect of hypoxic stimulation on expression of SUMO-1 mRNA in mouse brain (A) and heart (B). mRNA samples were isolated from brain (A) and heart (B) of female and male mice treated with either normoxia or hypoxia. Total mRNA (12  $\mu$ g per lane) was subjected to Northern hybridization analysis of SUMO-1 expression. The calculated size (kilobase pairs) of the specific transcript is indicated on the right. The representative blots show data from three animals in each group. The level of 18S and 28S was used as an internal standard to verify equal loading of mRNA. The intensity of the bands was quantitated by densitometry. The SUMO-1/18S ratio was calculated for each sample and the values were the relative SUMO-1 expression (the average value for the normoxia female animals was set to 1). Results are presented as means  $\pm$  S.E.M. from three mice/group. \*\*,  $P < 0.01$ . NA, normoxia; HA, hypoxia.

both SUMO-1 and HIF-1 $\alpha$  were strongly and prominently localized in the nucleus. The merged image clearly revealed that both SUMO-1 and HIF-1 $\alpha$  co-expressed in the neurons (Fig. 4B) and cardiomyocytes (Fig. 4C) under hypoxic conditions. No immunofluorescence staining was evident in control sections reacted in the absence of either anti-SUMO-1 or anti-HIF-1 $\alpha$  antibodies (data not shown).

#### 3.4. SUMO-1 interacts directly with HIF-1 $\alpha$ in response to hypoxic stimulation

In order to determine if SUMO-1 and HIF-1 $\alpha$  interact in the intracellular environment *in vivo*, we performed co-immunoprecipitation experiments. Because sumoylation is fully reversible protein modification [2–4], demonstration of SUMO-1 functions as a mediator of protein–protein interactions through isopeptide linked to target proteins could be technically challenging. Therefore, we used iodoacetamide as an isopeptidase inhibitor and NEM, to block specific SUMO-1 proteases *in vivo* [46], and to prevent SUMO-1 de-conjugation to proteins when cell lysates were prepared [47]. Total tissue lysates were subjected to immunoprecipitation with anti-HIF-

1 $\alpha$  or anti-SUMO-1 antibodies in order to obtain endogenous either SUMO-1 or HIF-1 $\alpha$  from normoxic and hypoxic brains and hearts. Subsequently, the immunocomplexes were analyzed by immunoblotting analyses with an anti-HIF-1 $\alpha$  antibody to detect the interacted complexes of SUMO-1/HIF-1 $\alpha$ . We observed that: (1) in the anti-HIF-1 $\alpha$  immunoprecipitates, a major single HIF-1 $\alpha$ -immunoreactive band was revealed in brain and heart tissues from mice after hypoxic stimulation (Fig. 5, left panel), consistent with the observation in direct immunoblotting analyses (Fig. 4A), (2) in the anti-SUMO-1 immunoprecipitates, three HIF-1 $\alpha$ -immunoreactive bands with apparent molecular masses of  $\sim$ 198,  $\sim$ 152 and  $\sim$ 105 kDa were detected in hypoxic brain and heart. In contrast, a protein of the expected HIF-1 $\alpha$  ( $\sim$ 120 kDa) was not recognized in the anti-SUMO-1 immunoprecipitates (Fig. 5, middle panel) and (3) two slowly migrating immunoreactive bands ( $\sim$ 198 and  $\sim$ 152 kDa) compared to predicted molecular weight of HIF-1 $\alpha$  ( $\sim$ 120 kDa) under reducing gel-running conditions, were also detected after stripped and reprobed same membranes with the anti-SUMO-1 antibody (Fig. 5, right panel), suggesting that these two high molecular bands are specific for HIF-1 $\alpha$



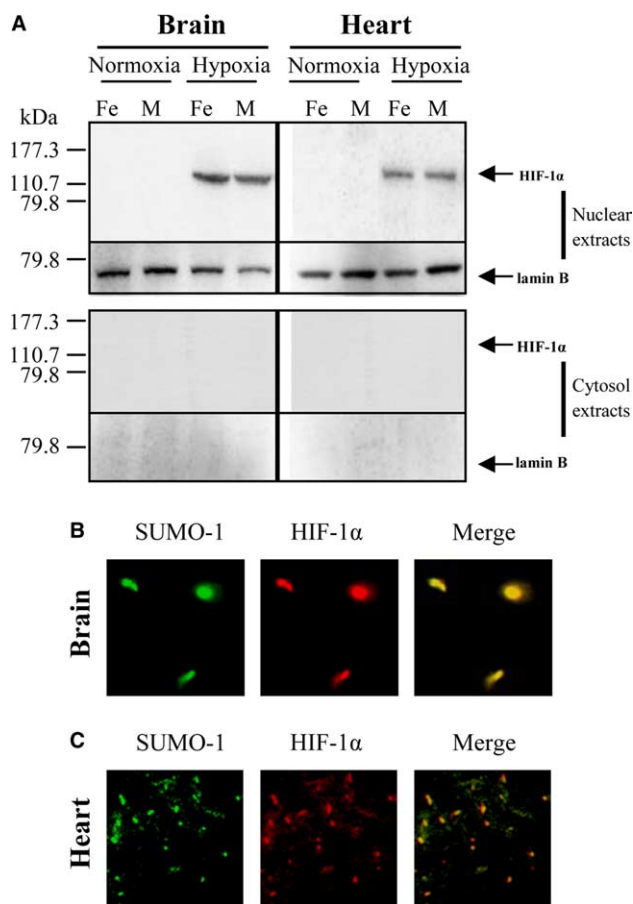


Fig. 4. Effect of hypoxic stimulation on expression of HIF-1 $\alpha$  protein in mouse brain and heart (A). Nuclear and cytosol extracts were isolated from brain or heart of female and male mice treated with either normoxia or hypoxia. Total protein (30  $\mu$ g per lane) was subjected to immunoblotting analysis of SUMO-1 expression. The expression of lamin B was examined in order to confirm the proper extraction of nuclear and cytoplasmic fractions. The representative blots show data from five animals in each group. The molecular mass markers (kDa, kilodaltons) are indicated on the left. Fe: female mouse, M: male mouse. Representative micrographs of brain (B) and heart (C) tissue sections from mice treated with hypoxia were subjected to double-immunofluorescence staining. The green signal was obtained with FITC-conjugated streptavidin and the red signal with an anti-mouse Cy 3 antibody. Superimposing the two colors (merged images) results in a yellow signal, when both proteins co-localize. Nuclear co-localization of SUMO-1 and HIF-1 $\alpha$  is seen in neurons and cardiomyocytes under hypoxic condition. Each set of images is representative of staining patterns seen in specimens from three female and male mice examined in the respective groups. All micrographs are at 63 $\times$  magnifications in B and at 10 $\times$  magnifications in C.

modified forms. Although only two modified forms were detected with anti-SUMO-1 antibody in the same membrane. This could be due to reduced sensitivity after stripping off anti-HIF-1 $\alpha$  antibody. To determine whether we could detect those HIF-1 $\alpha$ -reactive bands again, we performed immunoblotting on the same blot using anti-HIF-1 $\alpha$  antibody. This time, we also detected three HIF-1 $\alpha$ -reactive bands that had same mobility to those detected in the previous experiment (data not shown). Thus, this experiment provides strong evidence that slower migrating bands are immunoreactive for SUMO-1/HIF-1 $\alpha$  conjugates.

#### 4. Discussion

In the present study, we have demonstrated that SUMO-1 gene expression is enhanced by hypoxic stimulation in adult mouse brain and heart *in vivo*. Furthermore, SUMO-1 not only co-localizes with HIF-1 $\alpha$  under hypoxic conditions in neurons and cardiomyocytes, but also physically interacts with HIF-1 $\alpha$  in response to hypoxic stimulation *in vivo*, suggesting sumoylation of HIF-1 $\alpha$  may be of importance to stabilize HIF-1 $\alpha$  protein and its action.

Recently, it has been reported that both SUMO-1 mRNA and protein levels are increased after hypoxic stimulation in the number of cultured cell lines [48], which is in line with the present *in vivo* data. The significant increases in SUMO-1 mRNAs and proteins after hypoxic stimulation in brain and heart from both adult female and male mice suggested, to us, a role for SUMO-1 in the hypoxic response of those tissues. Previous studies have been demonstrated that SUMO-1 conjugates to a number of functional diverse proteins involved in a multi-enzyme ligase process, so-called sumoylation, a post-translational processes for protein modification [2,3,5,6]. Initiation of SUMO-1 activation requires the conversion of its precursor to a mature form by specific proteases [3]. These SUMO proteases needed for maturation of SUMO-1 have been identified in the bovine and human brain [46,49]. Subsequently, mature SUMO-1 can be covalently conjugated to target proteins by specific enzymatic cascades and that further regulates cellular functions [2,3,5,6]. On the other hand, SUMO proteases play a dual role because they can also cleave the isopeptide bond between SUMO-1 and the target proteins to release SUMO-1 in unmodified forms [3,47]. Indeed, no immunoreactive bands for SUMO-1/HIF-1 $\alpha$  conjugates were detectable in the absence of the NEM and iodoacetamide in lysis buffer for brain and heart protein preparations (data not shown), suggesting that SUMO-1 conjugates to target proteins is a reverting process.

HIF-1, a heterodimer composed of HIF-1 $\alpha$  and HIF-1 $\beta$  [14,15] that is an ubiquitously expressed transcriptional master regulator of several genes involved in mammalian oxygen homeostasis in many tissues. The formation of HIF-1 $\alpha$  with HIF-1 $\beta$  complex via their bHLH/PAS domains [20] is required for its transcriptional activity. However, the expression and activity of HIF-1 $\alpha$  subunit are oxygen-regulated [23–25]. Previous studies have been shown that the expression of HIF-1 $\alpha$  is normally low and upregulated after hypoxic conditions in the brain and heart of the rodents [25,41,42]. Similarly, there are no detectable levels of HIF-1 $\alpha$  protein in normal human brain and heart tissues, except for its protein overexpression associated with human tumorigenesis, including brain tumors [43,44]. Using *in vivo* hypoxia-induced model, we confirmed that specific expression of HIF-1 $\alpha$  protein in mouse brain and heart after hypoxic stimulation is significantly increased, which is in line with previous studies [25,41,42]. Gene targeting studies in mice have shown that HIF-1 $\alpha$  null mice exhibits abnormal neural development and impaired cardiovascular responses to chronic hypoxia [50,51], indicating that HIF-1 $\alpha$  plays a critical role in brain and heart. Furthermore, several studies have demonstrated that in normoxic cells HIF-1 $\alpha$  is targeted for rapid ubiquitination by the proteasomal degradation, and that accumulation in hypoxia involves a reduction in this degradation, mostly due to stabilization of HIF-1 $\alpha$  protein [26–29]. In contrast, SUMO-1 modification has been

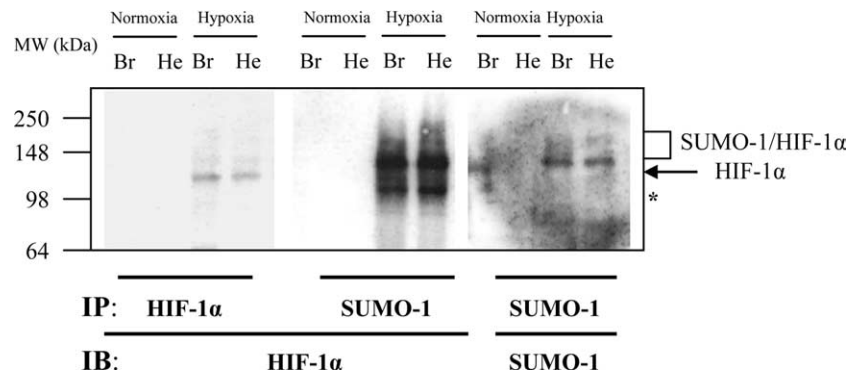


Fig. 5. SUMO-1 interacts directly with HIF-1 $\alpha$  in response to hypoxia in vivo. Whole tissue extracts isolated from brain or heart of mice treated with either normoxia or hypoxia, were immunoprecipitated with either anti-HIF-1 $\alpha$  or anti-SUMO-1 antibodies. Subsequently, each immunoprecipitate was immunoblotted with anti-HIF-1 $\alpha$  (left and middle panels). The immunoreactive bands corresponding to unconjugated HIF-1 $\alpha$  (arrow) and SUMO-1-conjugated HIF-1 $\alpha$  (a square bracket) and the molecular weight markers (kDa, kilodaltons) are indicated. Unidentical band was marked by an asterisk. After enhanced chemiluminescence development, the blot used in left and middle panels was stripped and reprobed with the anti-SUMO-1 antibody (right panel). The blot shown is a representative one of two independent experiments with a different tissue sample in each lane without sex difference. IP: immunoprecipitation, IB: immunoblotting, Br: brain, He: heart.

shown to act antagonistically to ubiquitinylation by enhancing the stability of nuclear protein [13]. Therefore, a proposed function for hypoxia-induced SUMO-1 expression is to conjugate and stabilize HIF-1 $\alpha$  protein under hypoxia in brain and heart. Interestingly, HIF-1 $\alpha$  and HIF-1 $\beta$  has same dimerization and DNA binding domains, including bHLH and PAS. In particular, the sumoylation site of HIF-1 $\beta$  is localized within PAS motif [33]. One of the novel findings of the present study is the demonstration of the interaction of SUMO-1 with HIF-1 $\alpha$  in response to hypoxia in adult mouse brain and heart in vivo. Our results clearly demonstrated that SUMO-1 not only co-localizes with HIF-1 $\alpha$  in the neurons and cardiomyocytes, but also conjugates to HIF-1 $\alpha$  in response to hypoxic stimulation. Although immunoprecipitation assays may not allow quantitative analysis of the distribution of various SUMO-1/HIF-1 $\alpha$  conjugates that are immunoprecipitated, these data support the hypothesis that SUMO-1 may modify HIF-1 $\alpha$  through sumoylation in response to hypoxia. Based upon the observations that: (1) when SUMO-1 is expressed, the interaction of SUMO-1 with HIF-1 $\alpha$  is absent in the brain and heart under normoxic condition and (2) no SUMO-1 proteins are detected in the cytosolic fractions of brain and heart under normoxic or hypoxic conditions. Although an in vitro study demonstrated that HIF-1 $\alpha$  may be translocated from the cytoplasm to the nucleus in response to hypoxic stimulation [27], it appears that HIF-1 $\alpha$  undergoing hypoxia-induced SUMO-1 modification takes place in the cell nucleus of both brain and heart [33,37]. If true, further investigation into the details of sumoylation of HIF-1 $\alpha$  during hypoxia responses will be required. HIF-1 $\alpha$  is critical in regulating a variety of hypoxia-inducible genes in several systems. SUMO modification of proteins has recently been shown to act as a stabilizing factor by counteracting ubiquitin-mediated degradation [13]. Therefore, an increase in SUMO-1 may contribute to HIF-1 $\alpha$  stabilization during hypoxic conditions, which is of importance for HIF-1 $\alpha$ -dependent gene activation (i.e., the activation of vascular endothelial growth factor (VEGF), erythropoietin, glucose transporters and endothelial cell proliferation) involved in vascular biology, cellular metabolism and tissue tolerance to hypoxia [17]. VEGF has been shown in vivo and in vitro to be the principal mediator of hypoxia-

induced angiogenesis [17,18,31,52]. In support of previous findings, we demonstrated that, using immunoblotting experiments with the same brain and heart tissues, which had been used for the detection of SUMO-1 and HIF-1 $\alpha$  expression, indeed, the induction of HIF-1 $\alpha$  protein is accompanied with increased production of VEGF protein by hypoxia in both brain and heart, albeit to various degrees in vivo (data not shown). Such downstream targets of HIF-1 $\alpha$  may be of considerable clinical importance in obstructive ischemic diseases of the brain, heart and lower extremities. Thus, modulation of HIF-1 $\alpha$  level and its activity through manipulation of SUMO-1 expression could be one of the attractive approaches for the effective treatment of ischemic disease.

In summary, this is the first report investigating the effect of hypoxia on SUMO-1 gene expression in adult mouse brain and heart in vivo. The hypoxia-induced increase in SUMO-1 expression is correlated with the induction of HIF-1 $\alpha$  protein as a transcription regulatory mediator in response to hypoxia. Moreover, we have shown that SUMO-1 not only co-localizes but also interacts with HIF-1 $\alpha$ . Our results provide support to the notions that the regulation of endogenous SUMO-1 may modify HIF-1 $\alpha$  and further regulate HIF-1 $\alpha$  function by sumoylation pathway, which remain to be identified.

**Acknowledgements:** We are grateful to reviewers for critical commentary on the manuscript. This work was supported by Grants 10380 and 13550 from the Swedish Medical Research Council, the Assar Gabrielssons Forsknings Foundation, the Scientific Foundation of Eva och Oscar Ahrens, Wilhelm och Martina Lundgrens, Rudolf och Ellen Maria Mörnes, as well as Hjalmar Svensson and Adlerbertska Research Foundation.

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