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Stabilization of hypoxia-inducible factor-1 α in buffer containing cobalt chloride for Western blot analysis

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Abstract

Hypoxia-inducible factor-1 α (HIF-1 α) is a widely studied protein with significant biomedical impact. Care is needed to stabilize HIF-1 α protein during sample preparation for Western blot analysis due to its rapid degradation in the presence of oxygen. Enzyme inhibitor cocktails can be complex and expensive. We present a protease inhibitor-free buffer, containing cobalt chloride, which is effective at stabilizing HIF-1 α , while being inexpensive, straightforward, convenient, and has potential for widespread application.

Keywords

Hypoxia-inducible factor-1 α ; Prolyl hydroxylase; Cobalt chloride; Hypoxia; Methods

Hypoxia inducible factor-1 α (HIF-1 α) is a central transcription factor regulating gene expression during hypoxia. In normoxic tissues, HIF-1 α is constitutively expressed and constantly degraded by the enzyme prolyl hydroxylase. HIF-1 α is stabilized under low oxygen conditions, which may occur during reduced oxygen supply (hypoxia), reduced blood flow (ischemia) or cancer development. Numerous reports suggest that HIF-1 α and its target genes could be important mediators of hypoxia-induced ischemic tolerance and an important target in therapeutics [1–5]. Because HIF-1 α plays an important role in many diseases, it has been a focal point of hypoxia research ever since the HIF-1 α gene was first characterized a decade ago [6]. To date, more than 6000 research articles have been published, demonstrating its importance in hypoxia research and has been termed the master regulator of gene expression during hypoxia [7,8]. This paper proposes a modification that simplifies the extraction and stabilization of HIF-1 α for *ex vivo* analysis.

Regulation of HIF-1 α is complex and involves multiple mechanisms of control at the level of protein degradation and stabilization [9]. Under normoxia, HIF-1 α subunit is rapidly degraded within minutes by the von Hippel-Lindau tumour suppressor gene product (pVHL)

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–ubiquitin-mediated proteasome pathway [10] aided by HIF-prolyl 4-hydroxylases [11,12]. The half-life ($t_{1/2}$) of HIF-1 α degradation is approximately 5 minutes [13]. This makes it necessary, to stabilize HIF-1 α in an effective manner during sample preparation.

Stabilization of HIF-1 α *in vivo* has been achieved through various mechanisms including inhibition of HIF-prolyl 4-hydroxylase by inactivation by metals such as cobalt, zinc and nickel [11]. Additional mechanisms, such as direct binding of these metals to HIF-1 α or the iron binding center of HIF- hydroxylases, thereby inhibiting its degradation by VHL-dependent and independent pathways [14,15] are also available.

To date, studies use an array of expensive protease inhibitors and/or use snap freezing in liquid nitrogen to stabilize HIF-1 α during sample preparation[1,13] which makes sample preparation challenging. While there are reports of stabilizing HIF-1 α *in vivo* and *in vitro* using metals like cobalt [14–16], there is no information on adding these to the homogenization buffer during sample preparation and processing for analysis such as Western blots.

In this report, we describe a simple but effective method to stabilize HIF-1 α and prevent its degradation during tissue processing for Western blot analysis by adding cobalt (in the form of cobalt chloride) to the homogenization buffer. We modified the HEPES homogenization buffer, which is widely used to study HIF-1 α in brain for Western blot analysis [17], by adding CoCl_2 with or without protease inhibitors to test the stabilization of HIF-1 α during processing of tissue.

Adult male Wistar Rats (250 ± 5 g body weight) were exposed to acute normobaric hypoxia (8 % O_2 + 92 % N_2 for 3 h) and brain tissue was rapidly removed after isoflurane anaesthesia (2% isoflurane + 8 % O_2 + 90 % N_2) and decapitation. Control rats were caged in the same area as hypoxic rats breathing room air. Control animals were decapitated under 2 % isoflurane + 21 % O_2 + 77 % N_2 .

Tissues from hypoxic and control animals were homogenized in each of the following homogenization buffers. Buffer I had ice cold HEPES [17] (20 mM HEPES, pH 7.5, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.45 M NaCl) and contained protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride and 0.5 mM sodium ortho vanadate, 0.2 mM DL-Dithiothreitol). Buffer II had 1 mM CoCl_2 without any protease inhibitors in HEPES buffer. Buffer III was Buffer I with two of the protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride and 0.5 mM sodium ortho vanadate and 1 mM CoCl_2). DL-Dithiothreitol was excluded as it forms a dark brown precipitate in the presence of CoCl_2 . All chemicals are from Sigma-Aldrich, Canada until unless specified. All experiments using animals were carried out under institutional and national guidelines.

Control and hypoxic brain tissues were homogenized in test buffers and homogenates were centrifuged at 10,000 g at 4 °C for 10 minutes. Supernatants were mixed with Laemmli sample buffer in 1: 1 (v/v) ratio which contains 250 mM Tris–HCl, pH 6.8, 10% SDS, 30% Glycerol, 5% b-mercaptoethanol, 0.02% bromophenol blue (Bio-Rad, Laboratories, Inc., Hercules, CA) and boiled in a hot water bath for 5 minutes.

Equal amount of proteins were loaded in each lane after determining protein concentrations using Bio-Rad Protein Assay Dye Reagent concentrate (Bio-Rad Laboratories, Inc., Hercules, CA), and subjected to electrophoresis using 8 % SDS-PAGE and transferred to PVDF membrane (Immobilon-P Transfer membrane, Millipore Corporation, Bedford, MA, USA). Hypoxia induced and uninduced COS-7 Nuclear Extract (NB800-PC26, Novus Biologicals, Littleton, CO, USA) were used as positive and negative controls for HIF-1 α . Molecular weight markers (161-0374, Precision Plus Protein Standards Dual color markers,

Bio-Rad Laboratories, Mississauga, ON, Canada), was loaded into a separate lane. The membrane was blocked using 5 % blotting grade blocker non-fat dry milk (Bio-Rad, Hercules, CA, USA) in Tris-Buffered Saline (pH 7.5) containing 0.1% Tween-20 (v/v) (TBST) overnight at 4 °C. The blocked membrane was incubated for 2h at room temperature (RT) with rabbit polyclonal rabbit anti-HIF-1 α antibody (1:1000), followed by incubation with secondary affinity purified peroxidase labeled anti-rabbit IgG (H+L) (1:5000, PI-1000 Vector Laboratories, Burlingame, CA) for 1h at RT. The blot was developed using enhanced chemiluminescence (ECL) method (ECL, Amersham, Piscataway, NJ) and protein bands were visualized on an X-ray film.

The membrane was stripped using Re-blot Plus Western blot strong antibody stripping solution (Millipore, Canada). β -actin antibody (mouse monoclonal to beta Actin- loading control, Abcam- ab8226) was used as loading control (1: 5000, incubated for 2h at RT) followed by incubation with affinity purified secondary peroxidase labeled anti-mouse IgG (H+L) (1:5000, PI-2000 Vector Laboratories, Burlingame, CA) for 1h at RT. Protein was detected using ECL method and protein bands were visualized on X-ray film as described above. All incubations with primary and secondary antibodies were in TBST containing 0.2% BSA and membranes were washed between incubations with TBST. Average pixel intensity was obtained after image inversion from black to white pixels using the freeware ImageJ from the National Institutes of Health [18].

Figure 1A shows the relative stabilization of HIF-1 α in hypoxic rat brain prepared in each test buffer in lanes 4–6 in a representative blot. Non-hypoxic controls are shown in lanes 2 and 3. Stabilization of HIF-1 α is equivalent in samples prepared using HEPES buffer containing protease inhibitors (Fig. 1A, lane 4), CoCl₂ alone (lane 5) or protease inhibitors plus CoCl₂ (lane 6). Figure 1B is that of the membrane shown in Fig. 1A which was stripped and probed with β -actin antibody used as loading control. Figure 1C shows the individual average pixel intensity of each lane where the samples are prepared in different buffers as described above in Fig. 1A and are compared to lane 4 (mean $\pm$ SD, n = 3).

These data show that CoCl₂ when added to the buffer inhibits HIF-1 α degradation in mammalian brain as effectively as buffers containing protease inhibitors. This HIF-1 α stabilization is based on the principle that cobalt prevents both pVHL-independent and pVHL-dependent degradation of HIF-1 α [14,15,19]. This method also precludes the need for any special processing steps like freezing using liquid nitrogen.

In conclusion, this paper presents a simple modification to the extraction of HIF-1 α as depicted in Fig. 2. The proposed model in Fig. 2 depicts how HIF-1 α is degraded in the presence of oxygen (A), but stabilized when cobalt is present in buffer (B). Cobalt binds to either the iron center of HIF-hydroxylase or binds to HIF directly even if hydroxylation has been initiated, thus preventing degradation of HIF-1 α [15]. This buffer makes sample preparation for HIF-1 α less cumbersome, avoids usage of expensive protease inhibitors and eliminates the need for complex freezing protocols. There are potential applications of this buffer for sample preparation in a wide range of research fields including cancer, stroke and myocardial infarction and to study kinetics and time course of HIF-1 α stabilization and accumulation.

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Fig. 1.

Western blot depicting that cobalt chloride stabilizes HIF-1 α in the homogenization buffer during sample preparation of brain tissue.

(A) Representative Western blot of brain tissue from normoxic and 3 h normobaric 8 % oxygen exposed hypoxic rats. Lane 1 shows the molecular weight markers. Lanes 2 and 3 were loaded with samples from normoxic brain tissue. Lane 2 sample was prepared using HEPES buffer, containing the protease inhibitors sodium orthovanadate, phenylmethanesulfonyl fluoride and DL-dithiothreitol. Lane 3 sample was prepared using HEPES buffer containing cobalt chloride and no protease inhibitors. Lanes 4, 5 and 6 were loaded with hypoxic brain samples. Lane 4 sample was prepared using HEPES buffer

containing protease inhibitors as in lane 2. Lane 5 sample was prepared using, HEPES buffer containing cobalt chloride and no protease inhibitors. Lane 6 sample was prepared using HEPES buffer containing sodium orthovanadate and phenylmethanesulfonyl fluoride along with cobalt chloride. Lanes 7 and 8 are hypoxia induced and uninduced COS-7 nuclear extract used as positive and negative controls for HIF-1 α . Note the accumulation of HIF-1 α (~120 kDa) in tissue exposed to hypoxia (indicated by an arrow, lanes 4, 5 and 6). The stabilization of HIF-1 α is equivalent in buffers containing cobalt chloride with or without protease inhibitors (lanes 5 and 6 respectively). The numbers on the right of membrane indicate molecular weight markers in kDa and numbers on the top of membrane indicate the lanes.

(B) The membrane used to detect HIF-1 α (Fig. 1A) was stripped and probed with β -actin antibody used as loading control (~ 42 kDa) to show uniform protein concentration between lanes 2–6.

(C) Bar graph showing arbitrary units of pixel intensity after image inversion from black to white pixels (mean $\pm$ SD, n = 3). The individual pixel intensity of each lanes were compared to lane 4 where the animals are exposed to 8% O₂ and the samples prepared in HEPES buffer containing all protease inhibitors. The numbers (2–6) denotes the corresponding lanes in Fig. 1A. The graph indicates that stabilization of HIF-1 α with a buffer containing CoCl₂ (lane 5) is equivalent to buffer containing protease inhibitors without CoCl₂ (lane 4) or with CoCl₂ (lane 6). This also shows the reproducibility of the results and enzyme inhibitors are not required to stabilize HIF-1 α .

Fig. 2. Proposed model for CoCl_2 stabilization of HIF-1 α

(A) HIF-1 α degradation pathway by HIF-specific hydroxylase, an iron-binding protein in the presence of oxygen by hydroxylation of proline564 (P564) in HIF. The hydroxylated protein is ubiquitinated by pVHL, which is degraded by the proteosomal mechanism.

(B) In the presence of cobalt in the buffer, cobalt binds to the iron center of hydroxylase, thus inhibiting the enzyme activity. Cobalt can also directly bind with hydroxylated proteins and prevent the binding of HIF-1 α to pVHL, thereby stabilizing HIF-1 α during sample preparation. The "X" indicates where cobalt binds and blocks HIF-1 α degradation.