Video Article

Co-immunoprecipitation Assay Using Endogenous Nuclear Proteins from Cells Cultured Under Hypoxic Conditions

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Abstract

Low oxygen levels (hypoxia) trigger a variety of adaptive responses with the Hypoxia-inducible factor 1 (HIF-1) complex acting as a master regulator. HIF-1 consists of a heterodimeric oxygen-regulated α subunit (HIF-1α) and constitutively expressed β subunit (HIF-1β) also known as aryl hydrocarbon receptor nuclear translocator (ARNT), regulating genes involved in diverse processes including angiogenesis, erythropoiesis and glycolysis. The identification of HIF-1 interacting proteins is key to the understanding of the hypoxia signaling pathway. Besides the regulation of HIF-1α stability, hypoxia also triggers the nuclear translocation of many transcription factors including HIF-1α and ARNT. Notably, most of the current methods used to study such protein-protein interactions (PPIs) are based on systems where protein levels are artificially increased through protein overexpression. Protein overexpression often leads to non-physiological results arising from temporal and spatial artifacts. Here we describe a modified co-immunoprecipitation protocol following hypoxia treatment using endogenous nuclear proteins, and as a proof of concept, to show the interaction between HIF-1α and ARNT. In this protocol, the hypoxic cells were harvested under hypoxic conditions and the Dulbecco's Phosphate-Buffered Saline (DPBS) wash buffer was also pre-equilibrated to hypoxic conditions before usage to mitigate protein degradation or protein complex dissociation during reoxygenation. In addition, the nuclear fractions were subsequently extracted to concentrate and stabilize endogenous nuclear proteins and avoid possible spurious results often seen during protein overexpression. This protocol can be used to demonstrate endogenous and native interactions between transcription factors and transcriptional co-regulators under hypoxic conditions.

Video Link

The video component of this article can be found at https://www.jove.com/video/57836/

Introduction

Hypoxia occurs when inadequate oxygen is supplied to the cells and tissues of the body. It plays a critical role in various physiological and pathological processes such as stem cell differentiation, inflammation and cancer[1,2]. Hypoxia-inducible factors (HIFs) function as heterodimers composed of an oxygen-regulated α subunit and a constitutively expressed β subunit also known as ARNT[3]. Three isoforms of the HIF-α subunits (HIF-1α, HIF-2α and HIF-3α) and three HIF-β subunits (ARNT/HIF-1β, ARNT2 and ARNT3) have been identified to date. HIF-1α and ARNT are ubiquitously expressed, whereas HIF-2α, HIF-3α, ARNT2 and ARNT3 have more restricted expression patterns[4]. The HIF-1 protein complex is the key regulator of the hypoxia response. Under hypoxic conditions, HIF-1α becomes stabilized, then translocates to the nucleus and dimerizes with ARNT[5]. Subsequently, this complex binds to specific nucleotides known as hypoxia responsive elements (HREs) and regulates the expression of target genes involved in diverse processes including angiogenesis, erythropoiesis and glycolysis[6]. In addition to this "canonical" response, the hypoxia signaling pathway is also known to crosstalk with multiple cellular response signaling pathways such as Notch and Nuclear Factor-kappa B (NF-κB)[7,8,9].

The identification of novel HIF-1 interacting proteins is important for a better understanding of the hypoxia signaling pathway. In contrast to ARNT, which is insensitive to oxygen levels and constitutively expressed, HIF-1α protein levels are tightly regulated by cellular oxygen levels. At normoxia (21% oxygen), HIF-1α proteins are rapidly degraded[10,11]. The short half-life of HIF-1α at normoxia presents specific technical challenges for the detection of the protein from cell extracts, as well as for the identification of HIF-1α-interacting proteins. Furthermore, several transcription factors including those of the HIF-1 complex translocate into the nucleus under hypoxic conditions[12,13,14]. Most of the current methods used for PPI studies are performed using non-physiological overexpression of proteins. Such protein overexpression has been reported to cause different cellular defects through multiple mechanisms including resource overload, stoichiometric imbalance, promiscuous interactions,

and pathway modulation[15,16]. In terms of PPI studies, protein overexpression can lead to false positive, or even false negative, results depending on the protein properties and functions of the overexpressed proteins. Therefore, the current methods for PPI studies have to be modified in order to reveal the physiologically relevant PPIs under hypoxic conditions. We have previously demonstrated the interaction between HIF-1 and the Ets family transcription factor GA-binding protein (GABP) in hypoxic P19 cells, which contributes to the response of the *Hes1* promoter to hypoxia[17]. Here, we describe a co-immunoprecipitation protocol to study PPIs between endogenous nuclear proteins under hypoxic conditions. The interaction between HIF-1α and ARNT is shown as a proof of concept. This protocol is suitable for demonstrating the interactions between transcription factors and transcriptional co-regulators under hypoxic conditions, including but not limited to the identification of HIF-1 interacting proteins.

Protocol

This protocol section, which uses human embryonic kidney 293A (HEK293A) cell,s follows the guidelines of human research ethics committee in Nanyang Technological University, Singapore.

1. Induction of Hypoxia in HEK293A Cells

1. Prepare four 10 cm dishes and seed 3–5 x 10^6 HEK293A cells per dish in 10 mL Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/mL penicillin and 100 mg/mL streptomycin. Culture the cells in a 37 °C, 5% CO_2 incubator.
   NOTE: Cell seeding should be carried out in a biological safety cabinet (BSC). The surfaces of all materials to be placed in the BSC should be wiped with 70% ethanol.
2. 24 h after seeding, when the cells have reached 80–90% confluency, put two dishes into the hypoxia subchamber in the incubator glovebox (see **Table of Materials**) with 1% O_2 and 5% CO_2 at 37 °C, and keep the other two dishes at normoxia (21% O_2, 5% CO_2 at 37 °C) for 4 h. Use one set of normoxic and hypoxic cells to evaluate the hypoxia treatment by western blot and utilize the other set of cells for the co-immunoprecipitation experiments.
   NOTE: The oxygen levels can be set at 0–5%, and the duration of the hypoxia treatment can be varied depending on the cell type and study objective.

2. Whole Cell and Nuclear Extraction

NOTE: See **Table 1** for information on buffers used in this protocol.

1. **Harvest the normoxia control cells.**
   1. Remove the culture media by aspiration and rinse the cells with 10 mL DPBS (PH 7.0–7.2) using a 10 mL pipette.
      NOTE: Avoid touching the cell monolayer with the pipette. During the washing, gently pipette the DPBS down the wall of the cell culture plate to avoid cell loss.
   2. Pipette 5 mL ice-cold DPBS into the plate and scrape the cells off the surface of the plate in ice-cold PBS with a cell scraper.
   3. **Transfer the cell suspension into 15 mL conical tubes and keep on ice.**

2. **Harvest the cells cultured under hypoxic conditions.**
   1. Pre-equilibrate the DPBS to hypoxic conditions by placing an uncovered **1**00 mL experiment glass reagent bottle filled with DPBS in the hypoxia subchamber (1% O_2 and 5% CO_2 at 37 °C) for 24 h in advance.
   2. Approximately 1 h prior to harvesting the hypoxia treated cells, place an ice box containing ice into the processing chamber of the glovebox, which has been equilibrated to 1% O_2 and 5% CO_2. Transfer the bottle containing the pre-equilibrated hypoxic DPBS from the hypoxia subchamber to the processing chamber and place it on ice.
   3. 4 h following hypoxia treatment, transfer the cells from the hypoxia subchamber to the processing chamber that has been pre-equilibrated to 1% O_2 and 5% CO_2.
   4. Remove the culture media by aspiration and rinse the cells once with 10 mL ice-cold pre-equilibrated hypoxic DPBS with a 10 mL pipette.
   5. Add 5 mL ice-cold pre-equilibrated DPBS with a 5 mL pipette and dislodge the cells by scraping with a cell scraper.
   6. Tilt the cell culture plate and collect the detached cells using a 10 mL pipette. Transfer the cell suspension in DPBS into 15 mL conical tubes and keep on ice.
   7. Open the door between the processing and buffer chambers of the glovebox, both of which have been pre-equilibrated to 1% O2 and 5% CO_2. Transfer the 15 mL conical tubes on ice containing hypoxia treated cells from the processing chamber to the buffer chamber. Open the door of the buffer chamber and remove the cells completely from the glovebox.

3. Pellet both the normoxic cells from 2.1 and the hypoxic cells from 2.2 by centrifugation at 1,000 x *g* for 5 min at 4 °C.
4. **Prepare the whole cell extracts.**
   1. Resuspend the cell pellets in 500 µL of ice-cold radio immunoprecipitation assay (RIPA) lysis buffer containing 50 mM Trisaminomethane Hydrochloride (Tris-HCl) pH 8.0, 150 mM sodium chloride (NaCl), 1% tergitol-type NP-40 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), supplemented with 1x protease inhibitor cocktail (see **Table of Materials**) by pipetting the cell pellets up and down several times.
   2. Transfer the cell lysates into new 1.5 mL microcentrifuge tubes and keep on ice for 30 min with occasional vortexing.
   3. Centrifuge the cell lysates at 13,000 x *g* for 10 min at 4 °C.
   4. Collect the supernatant, aliquot 50 µL of the supernatant into 1.5 mL microcentrifuge tubes, and store at -80 °C.

5. **Prepare the nuclear extracts using a nuclear extraction kit (see Table of Materials).**

1. Gently resuspend the cell pellets in 500 µL of lysis buffer NL supplemented with 1x protease inhibitor cocktail and 0.1 M dithiothreitol (DTT) by pipetting the cell pellets up and down several times.
2. Add 25 µL of detergent solution NP to the cell suspension and vortex for 10 s at maximum speed.
3. Centrifuge at 10,000 x *g* for 5 min at 4 °C.
4. Collect the supernatant (cytoplasmic extracts), aliquot 50 µL of the supernatant into 1.5 mL microcentrifuge tubes, and store at -80 °C.
5. Resuspend the pellet containing cell nuclei in 500 µL of lysis buffer NL supplemented with 1x protease inhibitor cocktail and 0.1 M DTT by vortexing for 5 s at maximum speed.
6. Centrifuge at 10,000 x *g* for 5 min at 4 °C and save the nuclear pellet.
7. Resuspend the nuclear pellet in 50 µL of Extraction Buffer NX1 supplemented with 1x protease inhibitor cocktail by pipetting the pellet up and down several times.
8. Incubate for 30 min on ice, vortexing for 10 s every 5 min at maximum speed.
9. Centrifuge at 12,000 x *g* for 10 min at 4 °C.

6. **Desalt the nuclear extracts.**
   1. Collect the supernatant from step 2.5.9 and transfer into the mini dialysis devices with a maximum volume of 100 µL per unit.
   2. Cap the mini dialysis devices and place them in a flotation device.
   3. Put the flotation device in a beaker containing 500 mL of pre-chilled dialysis buffer (20mM Tris-HCl pH 7.4, 20% glycerol, 100mM potassium chloride (KCl), 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT) and incubate for 30 min at 4 °C with gentle stirring.
      Caution: PMSF is hazardous. Avoid direct contact with skin or inhalation.
   4. Collect the samples from the corner of the mini dialysis devices and transfer into new 1.5 mL microcentrifuge tubes.
   5. Centrifuge at 12,000 x *g* for 10 min at 4 °C, aliquot 25 µL of each supernatant into a 1.5 mL microcentrifuge tube, and store at -80 °C.

3. Evaluation of the Hypoxia Treatment by Detection of the Protein Expression and Subcellular Localization of HIF-1α

1. Determine the protein concentration of the whole cell or nuclear/cytoplasmic extracts using the microplate assay of a bicinchoninic acid (BCA) protein assay kit according to manufacturer's instructions[18].
2. Dilute the cell lysates in 1x Laemmli sample buffer containing 5% 2-Mercaptoethanol and boil at 95 °C for 5 min.
   Caution: Do not touch the surface of the heating block, since it may cause burns.
3. **Separate the proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).**
   1. Load equal amounts of protein (25 µg) into each well of a SDS-PAGE precast gradient gels (4–20%), along with 3 µL of the molecular weight marker.
   2. Run the gel in running buffer containing 2.5 mM Tris, 19.2 mM glycine, 0.01% SDS, PH8.3 for 30 min at 200 V.

4. **Transfer the proteins to from the gel to the nitrocellulose membrane.**
   1. Assemble the transfer sandwich (filter paper-gel-membrane-filter paper) with the gel on the anode side and the membrane on the cathode side of the cassette.
   2. Place the cassette in the transfer tank filled with transfer buffer containing 2.5 mM trisaminomethane (Tris), 19.2 mM glycine and 20% methanol.
      CAUTION: Methanol is flammable and should be stored inside the flammable liquid storage cabinet.
   3. Carry out the transfer for 1 h at 100 V in the cold room.

5. Block the blot in 10 mL blocking buffer containing 50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.1 Tween 20 and 5% non-fat milk for 1 h at room temperature on a shaker.
6. Incubate the blot with anti-HIF-1α antibody (1/500 dilution) in the same blocking buffer overnight at 4 °C.
7. Wash the blot 3 times for 5 min each time with 50 mL TBS-T.
8. Incubate the blot with horseradish peroxidase (HRP)-conjugated secondary antibody (1/1,000 dilution) in 10 mL TBS-T containing 5% non-fat dry milk for 1 h at room temperature.
9. Wash the blot 3 times for 5 min each time with 50 mL TBS-T.
10. Mix 500 µL each of enhanced chemilumescent (ECL) reagent A and B in a 1.5 mL microcentrifuge tube and vortex briefly.
11. Apply the ECL substrate to the blot and incubate for 1 min at room temperature.
12. **Capture the chemiluminescent signals using a charge-coupled device (CCD) camera-based imaging system.**
    1. Drain excess ECL substrate by touching the edge of the membrane with a tissue paper and place the membrane in a sheet protector.
    2. Place the membrane on the sample tray of the CCD camera-based imaging system.
    3. Launch image processing software (see **Table of Materials**) and capture the images with the following settings: File → New protocol → Single channel → Protocol Setup → Gel imaging (Application: Chemi; Imaging Area: Bio-Rad Ready gel; Imaging Exposure: The software will automatically optimize the exposure time for intense bands) → Run protocol
       NOTE: The exposure time can be set manually to achieve the optimal images.

4. Immunoprecipitation and Detection of the Immunoprecipitated Proteins

1. Wash 50 µL of protein A/G sepharose beads in 500 µL of TBS buffer in 1.5 mL microcentrifuge tubes and pellet the beads by centrifugation at 3,000 x *g* for 2 min at 4 °C.
2. Discard the supernatant and resuspend the beads in 100 µL of TBS buffer.
3. Add 2 µL of mouse monoclonal anti-ARNT antibody (1.4 mg/mL) or mouse Immunoglobulin G (IgG) (1.4 mg/mL) that was prepared by reconstituting 0.7 mg mouse IgG in 500 µL TBS buffer.

4. Place the 1.5 mL microcentrifuge tubes containing the beads in a tube rotator and incubate for 2 h at 10 rpm in the cold room.
   NOTE: Handle the tubes gently and keep the suspension containing the beads at the bottom of the tube.
5. Pellet the beads by centrifugation at 3,000 x *g* for 2 min at 4 °C and discard the supernatants.
6. Dilute 200 µg of the nuclear protein lysate obtained in step 2.6.5 in 800 µL of the IP buffer consisting of 50 mM Tris-HCl (pH 7.4), 180 mM NaCl, 20% glycerol, 0.2% NP-40 and 1x protease inhibitor cocktail. Incubate the lysate with the antibody-coupled beads obtained in step 4.5 overnight at 4 °C.
7. Pellet the beads by centrifugation at 3,000 x *g* for 2 min at 4 °C, discard the supernatants and wash the beads 3 times with 1 mL ice-cold TBS containing 0.2% NP-40.
8. Boil the beads in 50 µL Laemmli sample buffer at 95 °C for 5 min.
9. Centrifuge the beads at 10,000 x *g* for 5 min at 4 °C, collect the supernatant, and discard the beads.
   NOTE: The supernatant can be stored at 4 °C for the short term or -20 °C for the long term.
10. Detect the presence of HIF-1α from the immunoprecipitated protein complexes by western blot as previously described in step 3.3 onwards.
    NOTE: Protein quantification is not required for this step. Load full volume (50 µL) of the supernatant of each sample into each well of a SDS-PAGE precast gradient gels (4–20%)

Representative Results

To assess the cellular response to hypoxia, the expression levels and subcellular localization of the components of the HIF-1 complex following hypoxia treatment were examined. HEK293A cells were cultured under hypoxic conditions for 4 h or kept at normoxia as controls. HIF-1α and ARNT protein levels were examined in whole cell or nuclear/cytoplasmic extracts by western blot. As expected, total HIF-1α levels were upregulated by hypoxia, whereas ARNT levels in total cellular lysates were not significantly altered (**Figure 1A**). In addition, hypoxia induced nuclear accumulation of both HIF-1α and ARNT in HEK293A cells (**Figure 1B**), which is consistent with previous reports, although cytoplasmic expression of ARNT was not detected in some of the tested cell lines[19].

Next, we demonstrated the interaction between HIF-1α and ARNT following hypoxia treatment. Nuclear extracts were prepared from HEK293A cells exposed to normoxic or hypoxic conditions for 4 h. Co-immunoprecipitation experiments were performed using nuclear extracts from HEK293A cells. As shown in **Figure 2**, HIF-1α was co-immunoprecipitated together with ARNT from the nuclear extracts of hypoxic HEK293A cells.

Taken together, the protocol described can be successfully used to induce a hypoxic response in cells and to further determine physiological protein binding of endogenously expressed HIF-1α/ARNT complexes within the nucleus.

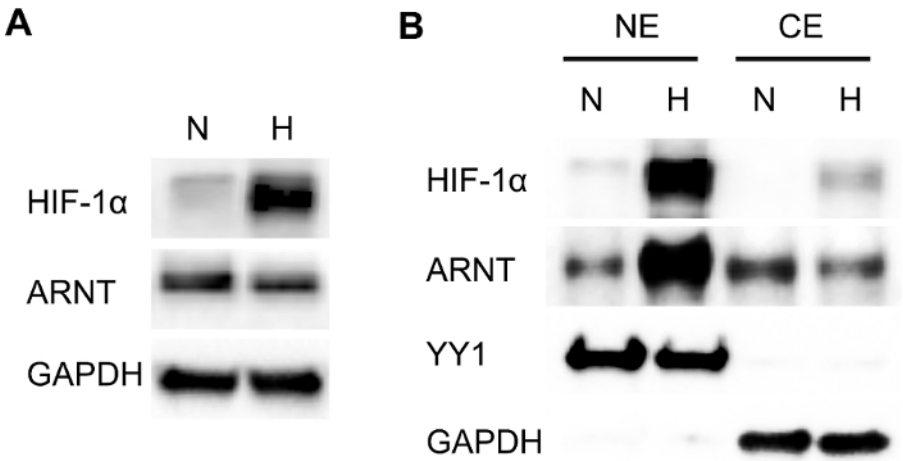

Figure 1: Regulation of protein expression and subcellular localization of HIF-1 components by hypoxia. (**A**) Immunoblot analysis of total HIF-1α or ARNT expression in HEK293A cells. The cells were exposed to hypoxia for 4 h (H) or kept at normoxia (N). Proteins from whole-cell extracts were separated by SDS-PAGE and analyzed by immunoblotting with anti-HIF-1α, anti-ARNT and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. GAPDH was used as a loading control. (**B**) Immunoblot analysis of HIF-1α and ARNT expression in subcellular fractions of HEK293A cells. HEK293A cells were cultured under hypoxic conditions for 4 h (H) or kept at normoxia (N). 25 µg of protein from nuclear or cytoplasmic extracts were analyzed by immunoblotting using anti-HIF-1α, anti-ARNT, anti-yin and yang 1 (YY1) and anti-GAPDH antibodies. YY1 and GAPDH were used as loading controls for nuclear and cytoplasmic fractions, respectively. Please click here to view a larger version of this figure.

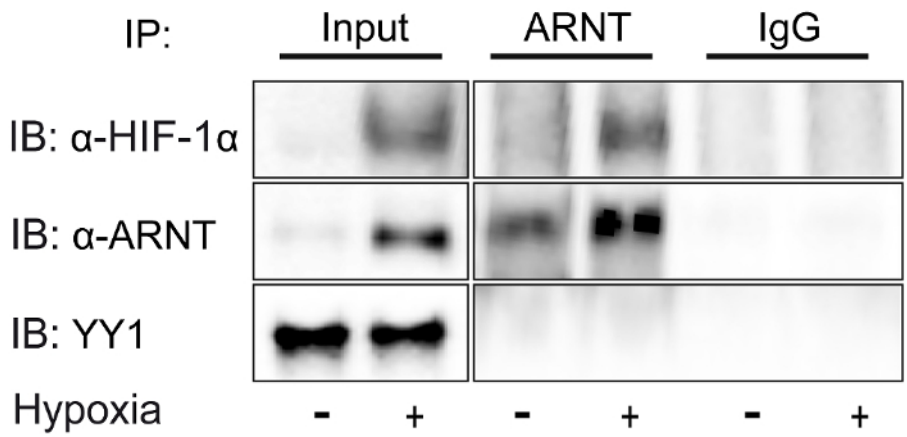

Figure 2: HIF-1α interacts with ARNT under hypoxic conditions. HEK293A cells were exposed to hypoxia for 4 h or kept at normoxia as controls. Nuclear extracts from HEK293A cells were prepared and immunoprecipitations were performed using an anti-ARNT antibody. Nuclear extracts (inputs) and immunoprecipitated proteins were analyzed by immunoblotting using anti-HIF-1α, anti-ARNT and anti-YY1 antibodies. YY1 was used as a loading control for the inputs while IgG was used as the negative control for the co-immunoprecipitation experiments. Please click here to view a larger version of this figure.

Solution	Components	Comments
Dialysis buffer	20 mM Tris-HCl (pH 7.4), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT	Add PMSF and DTT immediately before use.
Running buffer	2.5 mM Tris-HCl (PH 8.3), 19.2 mM glycine and 0.01% SDS	Mix 100 mL 10x Tris/Glycine/SDS buffer with 900 mL ddH_2O.
Transfer buffer	2.5 mM Tris-HCl (PH 8.3), 19.2 mM glycine and 20% methanol	Mix 100 mL 10x Tris/Glycine buffer with 200 mL methanol and 700 mL ddH_2O.
TBS buffer	50 mM Tris-Cl (pH 7.6) and 150 mM NaCl	Mix 100 mL 10x TBS buffer with 900 mL ddH_2O.
TBS-T buffer	50 mM Tris-Cl (pH 7.6), 150 mM NaCl and 0.1% Tween 20	Mix 100 mL 10x TBS buffer with 900 mL ddH_2O and 1 mL of Tween 20.
Blocking buffer	50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.1% Tween 20 and 5% non-fat milk	Dissolve 5 g of Blotting-Grade Blocker in 100 mL TBS-T buffer.
IP buffer	50 mM Tris-HCl (pH 7.4), 180 mM NaCl, 20% glycerol, 0.2% NP-40 and 1x protease inhibitor cocktail	Add protease inhibitor cocktail immediately before use.

Table 1: Solution preparation.

Discussion

The HIF-1 complex is a master regulator of cellular oxygen homeostasis and regulates a plethora of genes involved in different cellular adaptive responses to hypoxia. Identification of novel HIF-1 interacting proteins is important for the understanding of hypoxic signal transduction. Co-immunoprecipitation experiments are commonly used for PPIs studies to delineate cellular signal transduction pathways. However, protein overexpression is still widely used and this may lead to experimental artifacts. In addition, HIF-1α is a highly unstable protein and it becomes rapidly degraded during re-oxygenation[11]. In addition, hypoxia triggers nuclear translocation of HIF-1 components in most mammalian cell lines. Therefore, conventional co-immunoprecipitation protocols have to be optimized for the identification of physiologically relevant HIF-1 interacting proteins following hypoxia treatment. Here, we describe a modified co-immunoprecipitation protocol that is used to demonstrate the interaction between HIF-1α and ARNT at hypoxia.

To avoid the degradation of HIF-1α during re-oxygenation, the hypoxic cells were harvested inside the processing chamber of the incubator glovebox which was pre-equilibrated to hypoxic conditions. The DPBS wash buffer was also equilibrated to hypoxic conditions before usage. If there is no hypoxia workstation available for processing, the hypoxic cells can be washed with pre-equilibrated hypoxic DPBS and thereafter harvested quickly on ice. Considering that hypoxia can trigger the nuclear translocation of HIF-1 components and that protein overexpression may induce artefactual results, endogenous nuclear proteins were used in this protocol. The use of nuclear extracts in the co-immunoprecipitation protocol has also been reported by others[20]. It represents a technical advantage for the study of interactions between nuclear proteins, because it minimizes the chance of nuclear proteins from being diluted out by whole-cell proteins. Furthermore, the use of nuclear extracts may be helpful for the reduction of the non-specific interactions, especially from highly abundant irrelevant cytoplasmic proteins, and for the improvement of nuclear protein stability by minimizing exposure to proteases present in the cytoplasm. The use of endogenous

nuclear extracts instead of whole cell extracts for the co-immunoprecipitation shows a significant technical improvement, as we can only detect the interaction between HIF-1α and GABPα using endogenous nuclear extracts but not using whole cell extracts[17].

In the described protocol, several conditions can be further optimized to improve results. We induced hypoxia in HEK293A cells by treating them with 1% oxygen for 4 h. However the oxygen levels and the duration of the hypoxia treatment can be varied for different cell types and adjusted according to the aims of the study. For instance, HIF-1α and HIF-2α can be differentially regulated by hypoxia in a cell type specific manner, indicating their distinct roles in different biological contexts. It has been shown that in neuroblastoma cells, HIF-1α is most active during short periods of intense hypoxia (1% O_2), whereas HIF-2α is also active under mild hypoxia (5% O_2) and becomes more active following chronic hypoxia treatment (48–72 h of hypoxic exposure)[21]. 0–5% O_2 levels are commonly used for *in vitro* hypoxia treatments, where 1–5% O_2, 0.1–1% O_2 and 0–0.1% O_2 are frequently defined as mild hypoxia, hypoxia and anoxia, respectively[22]. Salt concentration is another parameter that requires optimization, especially since it plays a critical role for ionic interactions in PPIs[23]. Nuclear extracts were used in this study with the nuclear proteins usually extracted using a buffer containing high concentrations of salt. Therefore, the nuclear extracts may have to be desalted prior to the co-immunoprecipitation experiments. Here, we desalted the nuclear extracts using a dialysis buffer containing 100 mM KCl, and mixed the dialyzed extracts with the IP buffer containing 180 mM NaCl proportionally to achieve a final salt concentration close to 150 mM, which is similar to physiological intracellular PPIs conditions. The final salt concentration can be modified depending on the properties of the PPIs of interest. In this protocol, GAPDH was used as a loading control for whole cell extracts and cytoplasmic fractions. We did not observe any significant hypoxia-induced regulatory effect on GAPDH expression in HEK293A cells. However, GAPDH has previously been shown to be upregulated by hypoxia in certain cell types[24]. With this in mind, one may need to use alternative proper loading controls when necessary[25]. Separately, we observed a significant level of background signal stemming from either the beads or antibodies used in the current protocol. To reduce the background, one can prolong the washing steps (10–15 min) or perform more than 3 washes (5–10 times). Alternatively, the ionic strength of the wash buffer can also be increased by titrating the salt concentration from 150 to 500 mM during washes. The lysates can also be pre-cleared by incubating with protein A/G sepharose beads for 1 h at 4 °C with rotation.

This protocol is limited to nuclear extracts and as such may not be suitable for the study of PPIs within other cellular compartments such as mitochondria and the endoplasmic reticulum. Similar to other conventional co-immunoprecipitation protocols, this method cannot be used to study PPIs in real-time or to determine if the PPIs are direct or indirect.

In summary, we provide a modified co-immunoprecipitation protocol for the identification of novel physiologically relevant HIF-1 interacting proteins. This protocol is also suitable for the study of the interactions between transcription factors and transcriptional co-regulators under hypoxic conditions. Although this protocol is designed specifically for the co-immunoprecipitation experiments under hypoxia conditions, a part of the described protocol for the normoxia control cells can also be used to study the nuclear PPIs under normoxia conditions.

Disclosures

The authors declare no conflicts of interest.

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