Placenta 35 (2014) 109–116

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Increased NFAT5 expression stimulates transcription of Hsp70 in preeclamptic placentas

J.K. Park [a,b], T.G. Kang [a], M.Y. Kang [b], J.E. Park [a], I.A. Cho [b], J.K. Shin [a,b], W.J. Choi [a,b], S.A. Lee [a,b], W.S. Choi [b,c,1], H.M. Kwon [d], J.H. Lee [a,b], W.Y. Paik [a,b,*,1]

[a] Department of Obstetrics and Gynecology, School of Medicine, Gyeongsang National University, JinJu, South Korea
[b] Institute of Health Sciences, School of Medicine, Gyeongsang National University, JinJu, South Korea
[c] Department of Anatomy, School of Medicine, Gyeongsang National University, JinJu, South Korea
[d] School of Nano-Bioscience and Chemical Engineering, Ulsan National Institute and Science and Technology, Ulsan, South Korea

ARTICLE INFO

Article history:
Accepted 13 December 2013

Keywords:
Preeclamptic placenta
HIF-1α
NFAT5
Hsp70

ABSTRACT

Objective: We investigated the expression of heat shock protein 70 (Hsp70), nuclear factor of activated T cells 5 (NFAT5), and hypoxia-induced factor-1α (HIF-1α) in the placentas of normal and preeclamptic pregnancies and in human placental hypoxia models *in vitro* to examine the regulatory mechanisms of placental Hsp70 expression.

Methods: The expression levels of HIF-1α, NFAT5, and Hsp70 were examined in placental samples from 10 females with preeclampsia and 10 normotensive control patients and in human choriocarcinoma trophoblast cells treated with 1 mM $CoCl_2$ by western blotting. Using models of placental hypoxia, pharmacological inhibition of HIF-1α with chetomin and shRNA knockdown and overexpression of NFAT5 were performed to investigate the roles of HIF-1α and NFAT5 in induction of Hsp70 by placental hypoxia.

Results: The levels of HIF-1α, NFAT5, and Hsp70 expression were significantly higher in the preeclamptic compared to normal placentas. In the placental hypoxia models, the expression of HIF-1α, NFAT5, and Hsp70 were significantly higher after 3, 6, and 12 h of 1 mM $CoCl_2$ treatment, respectively. Pharmacological inhibition of HIF-1α suppressed the induction of NFAT5 and Hsp70 at the protein level. shRNA knockdown of NFAT5 suppressed the induction of Hsp70 protein and overexpression of NFAT5 stimulated the induction of Hsp70 mRNA and protein in models of human placental hypoxia *in vitro*.

Conclusion: HIF-1α positively regulates the induction of NFAT5 and Hsp70 by placental hypoxia and NFAT5 stimulates transcription of Hsp70 in response to placental hypoxia in models of human placental hypoxia *in vitro*.

1. Introduction

Preeclampsia, a hypertensive, pregnancy-specific disorder, has long been analyzed in terms of its association with cellular stress. However, preeclampsia, a multi-system disorder that affects maternal vascular function and fetal growth, remains one of the most serious complications of pregnancy. The pathophysiology of preeclampsia is unclear, but accumulating evidence suggests that an excessive maternal systemic inflammatory response to pregnancy with systemic oxidative stress and resultant endothelial damage plays a crucial role in the pathogenesis of the preeclampsia [1,2].

Heat shock proteins (Hsps) are known primarily as intracellular proteins with molecular chaperone and cytoprotective functions [3]. However, a growing body of evidence suggests that extracellular Hsp70 may serve as a danger signal to the innate immune system and that it may contribute to the establishment of autoimmune diseases [4–6]. In preeclampsia, serum Hsp70 levels are increased, and reflect systemic inflammation and oxidative stress [7,8]. Nevertheless, the regulation of Hsp70 in healthy individuals, as well as in patients with preeclampsia, has not been fully identified. The increase in accumulation of Hsp70 under conditions of stress is achieved by transcriptional activation of tonicity-enhancer binding protein (TonEBP or nuclear factor of activated T cells, NFAT5) in the urinary concentrating mechanism [9]. TonEBP/NFAT5 is a member of the Rel/NFκB/NFAT family of signal transcription

* Corresponding author. Department of Obstetrics and Gynecology, School of Medicine, Gyeongsang National University, 90 Chilam-dong, Jinju 660-702, South Korea. Tel.: +82 55 750 8152; fax: +82 55 759 1118.
E-mail address: wypaik@gnu.ac.kr (W.Y. Paik).
[1] Both authors contributed equally to this work (Co-correspondence).

0143-4004/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.placenta.2013.12.005

factors [10]. A recent study suggested that activation of NFAT5 in the inner medulla of the kidney is induced by hypoxia in addition to hypertonicity [11]. The function of NFAT5 is well-known in the kidney, but other functions are under investigation; *e.g.*, an immunomodulatory function in T cells [12,13].

Although an excessive maternal systemic inflammatory response to pregnancy with systemic oxidative stress is thought to be involved in the pathogenesis of preeclampsia, it is unclear how hypoxia regulates placental expression of Hsps. We hypothesized that increased Hsp70 expression in preeclamptic placentas reflects an excessive placental inflammatory response to placental oxidative stress and that NFAT5 stimulates transcription of Hsp70 in response to placental hypoxia. To address our hypothesis, we investigated the expression of Hsp70, NFAT5, and hypoxia-induced factor-1α (HIF-1α) in the placentas of normal and preeclamptic pregnancies and in human placental hypoxia models *in vitro* and investigated the roles of HIF-1α and NFAT5 in induction of Hsp70 by placental hypoxia.

2. Materials and methods

2.1. Human placental tissue samples

The experimental protocol used was peer-reviewed and approved by the Human Subject Research Committee of Gyeongsang National University Hospital, Jinju, Korea. Written informed consent, with permission to collect placental tissue, was obtained from 20 pregnant females who were scheduled to undergo cesarean section without labor at 35–40 weeks of pregnancy. Of these pregnancies, 50% ($n = 10$) were normal and 50% ($n = 10$) were preeclamptic.

Preeclampsia was diagnosed based on the presence of hypertension (blood pressure ≥140 mmHg systolic or ≥90 mmHg diastolic after 20 weeks of gestation in previously normotensive females) and proteinuria (urinary excretion ≥0.3 g of protein in a 24-h specimen, or ≥30 mg/dL or a reading ≥1+ on dipstick test in a random urine specimen). Severe preeclampsia was defined as HELLP syndrome, eclampsia, or preeclampsia with either severe hypertension (blood pressure ≥160 mmHg systolic or ≥110 mmHg diastolic) or severe proteinuria (proteinuria ≥2.0 g in a 24-h specimen, or a reading ≥2+ on dipstick test). All preeclamptic females in this study had "severe" preeclampsia on the basis of the ACOG criteria [14]. The control subjects had no evidence of chronic hypertension, preeclampsia, or gestational hypertension. No control or preeclamptic subjects had been diagnosed with any chronic disease, such as hypertension, diabetes mellitus, or renal disease, before becoming pregnant.

Tissues from five different portions of the placenta were sectioned into full-thickness samples of ~3 × 3 cm. The samples were then re-sectioned into pieces of ~1 × 1 cm, frozen in liquid nitrogen, and stored at −70 °C. One 1 × 1-cm piece from each of the five different portions of each placenta was used for protein extraction.

2.2. Double immunofluorescence staining

For histological studies, placental tissues were immunostained as described previously, with some modifications. Sectioned placental tissues were incubated for 1 h in blocking solution (5% normal goat serum, 1% bovine serum albumin, 0.5% Triton X-100, 0.05% sodium azide in 0.05 M PBS, pH 7.4), followed by 4 °C overnight incubation with mixed primary antibodies: monoclonal mouse anti-Hsp70 and polyclonal rabbit anti–HIF–1α or monoclonal mouse anti-Hsp70 and polyclonal rabbit anti-NFAT5 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). After several washes in 0.1 M PBS, the sections were incubated for 1 h with donkey anti-IgG conjugated to Alexa Fluor 488 (green; 1:1000 dilution; Invitrogen, Carlsbad, CA) or Alexa Fluor 594 (red; 1:1000 dilution; Invitrogen) as the secondary antibody. Sections were rinsed in PBS and wet-mounted using wet anti-fade reagent with DAPI (Invitrogen). Digital images using a BX51-DSU microscope (Olympus, Tokyo, Japan) were captured and documented. Omission of the primary antibodies served as a negative control to estimate non-specific interactions (Supplementary Fig. 1).

2.3. Cell lines and cell culture

The choriocarcinoma cell line JEG-3 cells were cultured according to the protocol recommended by the American Type Culture Collection (ATCC, Manassas, VA); *i.e.*, in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37 °C in a humidified 5% CO_2 incubator. When the cells reached 80% confluence, as determined by eye, the cells were detached by trypsinization.

2.4. Hypoxic treatment

In this study, $CoCl_2$ was used as a hypoxia-mimicking agent as reported previously [15]. To determine the appropriate amount of $CoCl_2$ (Wako, Osaka, Japan), 2×10^5 JEG-3 cells/mL were seeded in dishes 10 cm in diameter 24 h before treatment with 0, 100, 200, 400, 600, 800, or 1000 μM $CoCl_2$. For the experiments, the JEG-3 cells were cultured in the absence or presence of 600, 800, or 1000 μM $CoCl_2$ for 3, 6, 12, or 24 h.

Cell viability after exposure to hypoxic conditions was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were seeded at 2×10^5/mL on a 96-well plate, and treated with or without $CoCl_2$ for 24 h. Then, 30 μL of 1 mg/mL MTT (Sigma Chemical, St. Louis, MO) were added to each well, and the plate was incubated at 37 °C until a blue color appeared. The medium was aspirated and replaced with dimethyl sulfoxide. The absorbance at 560 nm was read using a microtiter plate reader.

2.5. Inhibition of HIF-1α by chetomin

Chetomin (Santa Cruz Biotechnology) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 1 mM stock solution. Cells were seeded in 100-mm dishes 24 h before treatment with 75 nM chetomin. Cells were pretreated with 75 nM chetomin or DMSO for 4 h at 37 °C under normoxic conditions. Then, the cells were exposed to hypoxic conditions (1 mM $CoCl_2$) or maintained in a normoxic environment and incubated for 24 h.

2.6. shRNA knockdown and overexpression of NFAT5

Recombinant lentiviruses expressing shRNA against NFAT5 or eGFP-control were produced as follows. Lentiviral pLKO.1-puro vectors encoding shRNA specific for NFAT5 or eGFP-control were purchased from Sigma TRC shRNA library. The NFAT5 shRNA sequence (TRCN0000020019) used was 5′-CCGGGCCCAGATTCAGTCAGAGTTACTCGAGTAACTCTGACTGAATCTGGGCTTTTT-3′. The eGFP-control shRNA sequence (SHC005, MISSION pLKO.1-puro eGFP shRNA Control) used was 5′-CCGGTACAACAGCCACAACGTCTATCTCGAGATAGACGTTGTGGCTGTTGTATTTTT-3′. For lentiviral production, 293T cells were transfected with pLKO.1 vector together with packaging plasmids encoding Gag/Pol, Rev, and VSV-G using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Culture media containing lentiviral particles were collected 48 and 72 h after transfection and filtered. Viral supernatants were pooled and stored at −80 °C. JEG-3 cells were infected with viruses in media. Forty-eight hours after infection, cells were lysed and lysates were processed for immunoblotting.

Cells were transfected with expression plasmids for pCMV-Tag (flag control), pCMV-Tag2C-Yc1 (Rel homology domain of NFAT5-c, NFAT5 dominant negative mutant) or pcDNA3.1 hNFAT5-c (accession no. NM_006599), using Lipofectamine 2000 (Invitrogen). Cells were grown to ~90% confluency in 60-mm^2 plates, transfected with 1 μg of each plasmid and assayed after 24 h.

2.7. Western blotting

Western blotting for HIF-1α, NFAT5, and Hsp70 was performed using total placental lysates or JEG-3 cell lysates. For cultured JEG-3 cells, the medium was aspirated, and the cells were scraped off into 1 mL of 1 × PBS. After centrifugation at 130× *g* for 3 min, the supernatant was discarded, and the collected cells were lysed. Placental tissues and JEG-3 cells were suspended in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin), and phosphatase inhibitors (1 mM sodium orthovanadate and 1 mM sodium fluoride), and lysed by repeated sonication with 2- to 5-s pulses. The lysates were clarified by centrifugation (10,000× *g*, 20 min, 4 °C), and the protein concentration in the supernatants was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. The lysates were diluted in sample buffer (0.15 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and boiled for 5 min. Equal amounts of protein (30 μg) were loaded into the lanes of 6–8% SDS-polyacrylamide gels, and the proteins were separated by electrophoresis. The separated proteins were transferred onto nitrocellulose membranes. The membranes were washed in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with one of the following primary antibodies: anti-HIF-1α, anti-NFAT5, or anti-Hsp70 (0.2 μg/mL; Santa Cruz Biotechnology). The membranes were washed and incubated with secondary antibody (1:10,000; Pierce). Immunoreactive proteins were detected by enhanced chemiluminescence using a western blotting system (Pierce).

2.8. Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using the Trizol reagent (Invitrogen) and converted into cDNA with the Reversed Transcription System (Promega) according to the manufacturer's protocol. Quantitative PCR was performed on a LightCycler 480 Real-time PCR System with Power SYBR Green PCR Master Mix (Roche Applied Science). Relative values of mRNA were normalized to the level of β-actin mRNA. The primers used in these experiments are as follows. Hsp70 (298 bp) forward, 5′-GTGCAGTGGCCTACAGGATT-3'and Hsp70 reverse, 5′-AGCGAGGAAGATCCTGCTTAT-3′; β-actin (248 bp) forward, 5′-CTGGCACCACACCTTCTACAATG-3'and β-actin reverse 5′-CCTCGTAGATGGGCACAGTGTG-3′.