Biochemical and Biophysical Research Communications 445 (2014) 43–47

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

A cellular stress response (CSR) that interacts with NADPH-P450 reductase (NPR) is a new regulator of hypoxic response

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ARTICLE INFO

Article history:
Received 18 January 2014
Available online 31 January 2014

Keywords:
NADPH-P450 reductase
Cellular stress response
Hypoxic response

ABSTRACT

NADPH-P450 reductase (NPR) was previously found to contribute to the hypoxic response of cells, but the mechanism was not clarified. In this study, we identified a cellular stress response (CSR) as a new factor interacting with NPR by a yeast two-hybrid system. Overexpression of CSR enhanced the induction of erythropoietin and hypoxia response element (HRE) activity under hypoxia in human hepatocarcinoma cell lines (Hep3B), while knockdown of CSR suppressed them. This new finding regarding the interaction of NPR with CSR provides insight into the function of NPR in hypoxic response.

1. Introduction

Hypoxia inducible factor (HIF)-1α is a critical regulatory factor of cellular response to hypoxia during normal development or pathological processes such as cardiovascular disease and cancer [1,2]. The heterodimer of HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT) binds to the hypoxic-response element (HRE) and promotes the expression of genes involved in glycolysis, angiogenesis, cell survival, etc. [3]. In a previous study, we found that NADPH-P450 reductase (NPR) is an important factor in the activation of HIF-1 [4]. NPR is a membrane-bound protein containing an N-terminal positioned flavin mononucleotide (FMN) binding domain linked to a NADPH binding domain via a flavin adenine dinucleotide (FAD) domain [5]. The primary functions of NPR are to donate electrons to microsomal cytochrome P450s (P450s) [6] which metabolize xenobiotic and endogenous compounds. NPR is also required for the catalysis of several enzymes including squalene monooxygenase [7], cytochrome b5 [8], fatty acid desaturase [9], 7-dehydrocholesterol reductase [10] and heme oxygenase [11]. These various electron-accepting partners of NPR suggest an important role of NPR in a multitude of physiological processes. We found that the inhibition of NADPH-dependent enzymes or the knockdown of NPR significantly suppressed HIF-1α activation and the induction of erythropoietin (EPO) expression in Hep3B cells, suggesting that NPR is necessary for hypoxic response [4]. Otto et al. [12] have demonstrated that the embryos of NPR-deficient mice exhibit a low expression level of HIF-1α following vascular endothelial growth factor (VEGF) and EPO, as well as inhibition of vasculogenesis and hematopoiesis. However, the mechanism behind this contribution of NPR to hypoxic response remains unknown.

In the present study, we used a yeast two-hybrid system with a human heart cDNA library to search a new protein interacting with NPR, and identified a cellular stress response (CSR). We found that NPR interacts with CSR, and that CSR promotes the hypoxic response of Hep3B cells.

2. Materials and methods

2.1. Materials

The Matchmaker GAL4 two-hybrid system 3 and a human heart cDNA library were obtained from Clontech Laboratories (Mountain View, CA). PNGase F and the Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). Fetal bovine serum (FBS) and penicillin–streptomycin solution were purchased from Sigma Chemical (St. Louis, MO), and pBAsi-hU6 Neo vector and SYBR premix Ex Taq II were purchased from Takara Bio (Shiga, Japan). Dulbecco's modified Eagle's medium (DMEM) and anti-DYKDDDDK (Flag) antibody were purchased from Wako Pure

Abbreviations: NPR, NADPH-P450 reductase; CSR, cellular stress response; HIF-1, hypoxia-inducible factor-1; EPO, erythropoietin; P450, cytochrome P450; EET, epoxyeicosatrienoic acid; HRE, hypoxia response element; PBS, phosphate-buffered saline.

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http://dx.doi.org/10.1016/j.bbrc.2014.01.110
0006-291X/

Chemicals (Osaka, Japan), Isogen from Nippon Gene (Toyama, Japan), Revert AidTM M-MuLV Reverse Transcriptase from MBI Fermentas (Vilnius, Lithuania), and GenePORTERTM2 was from Gene Therapy Systems (San Diego, CA).

2.2. Yeast two-hybrid assay

The Matchmaker GAL4 two-hybrid system was used to perform yeast two-hybrid screening as described previously [13]. The full length of the NPR cDNA described previously [4] was inserted into pGBK T7 vector, and used as bait. The Human Heart Matchmaker cDNA Library was used as a pGAD library.

2.3. Isolation of CSR cDNA and preparation of constructs

The entire coding regions of human *CSR1* and *CSR2* were isolated from Hep3B cells by PCR. The primers for *CSR1* and *CSR2* were 5′-CGGATATCGAGACCATGAAAGTGAGGTC-3′ (forward primer for *CSR1/2*; underline, *Eco*RV site; double underline, start codon), 5′-GCTCTAGATCAGTAGAAGCTCTGGCTTC-3′ (reverse primer for *CSR1*; underline, *Xba*I site; double underline, stop codon), and 5′-CCGCTCGAGTCATATCCGATTATGTGAAA-3′ (reverse primer for *CSR2*; underline, *Xho*I site; double underline, stop codon). The amplified *CSR1* or *CSR2* cDNA was inserted into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) or pcDNA-3 × Flag vector with *Eco*RV and *Xba*I, or *Eco*RV and *Xho*I, respectively. Full-length *NPR* cDNA was inserted into pCMV-Myc vector (Clontech Laboratories) with *Sfi*I and *Sal*I sites. For the knockdown of CSR1/2 with shRNA, a specific sequence for *CSR1/2* was inserted into the pBAsi-hU6 Neo vector following the manufacturer's instructions. The two targeting sequences of *CSR1/2* were as follows: sequence 1, 5′-AAGTGGACTCTCTCTCCGAAG-3′; sequence 2, 5′-AAGTCTGTCTCCATCATGCTG-3′.

2.4. Cell culture and treatment

The human hepatocarcinoma cell line, Hep3B, was obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging, and Cancer of Tohoku University (Sendai, Japan). HEK293T cells were a gift from Professor Shintaro Suzuki of Kwansei Gakuin University. Hep3B cells were cultured in DMEM containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) and maintained at 37 °C in 5% CO_2 and 95% air (normoxia). For hypoxic stimulation, the cells were cultured in a multi-gas incubator, APM-30D (Astec, Shizuoka, Japan), with 1% O_2 and 5% CO_2 (hypoxia) for 6 h. For the overexpression or knockdown of CSR, cells were transfected with the plasmids with calcium phosphate or GenePORTERTM2.

2.5. Glycosidase treatment of cell lysates

Cells were transfected with *CSR1* or *CSR2*/pCDNA-3 × Flag, and cell lysates containing 100 μg protein in PBS with 0.25% sodium dodecyl sulfate (SDS) and 0.05 M dithiothreitol (DTT) were incubated at 95 °C for 5 min. NP40 (final 1%) and PNGase F (10U) were then added to the sample, and the mixture was incubated at 37 °C overnight.

2.6. Preparation of antibodies

NPR antibody was prepared as previously described [4]. The antibody against human CSR was prepared in rabbits using the method described previously [4]. Full-length human *CSR2* was inserted into pET-50b vector (Merck, Darmstadt, Germany), and CSR protein was expressed in BL21 (DE3). The expressed CSR was purified by preparative electrophoresis with Mini Prep Cell (Bio-Rad, Hercules, CA) following the manufacturer's instructions, and used for the preparation of antibody in rabbits. All experiments were conducted in accordance with guidelines on the welfare of experimental animals and with the approval of the Ethics Committee on the use of animals of Kwansei Gakuin University.

2.7. Immunoprecipitation

Hep3B cells were washed with PBS and lysed in buffer (50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl and 0.5% NP40) with a protease inhibitor cocktail. After centrifugation at 14,000*g* for 15 min, the supernatant was incubated with anti-NPR antibody overnight at 4 °C. Protein A-Sepharose (GE Healthcare, Little Chalfont, UK) was then added, and the mixture was incubated for 1 h at 4 °C. The immunocomplexes were precipitated and washed with buffer (50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl and 0.05% NP40), then analyzed by immunoblotting with anti-NPR or -CSR antibody. In HEK293T cells, *NPR*/pCMV-Myc and *CSR1* or *CSR2*/pCDNA-3 × Flag were transfected. Cells were washed with PBS and lysed in lysis buffer (50 mM Tris–HCl buffer (pH 7.5) containing 0.2% Triton X-100) with a protease inhibitor cocktail. After centrifugation, the supernatant was incubated with anti-DYKDDDDK (Flag) antibody for 1 h at 4 °C. Protein A-Sepharose was then added, and the mixture was incubated for 1 h at 4 °C. The immunocomplexes were precipitated and washed with buffer (50 mM Tris–HCl buffer (pH 7.5) containing 0.05% Triton X-100). The precipitate was analyzed by immunoblotting with anti-Flag or -Myc antibody.

2.8. Isolation of RNA and reverse-transcription PCR

Total RNA was extracted from cells with Isogen following the manufacturer's instructions, and converted to cDNA by reverse transcription. Real-time PCR was performed with SYBR premix Ex Taq II following the manufacturer's instructions. PCR was performed in a Thermal Cycler Dice Real Time System Single TP850 (Takara). The primers for human *EPO* (Acc No. NM_000799) were 5′-ATGTGGATAAAGCCGTCAGTGG-3′ (forward) and 5′-CTGGAGTGTCCATGGGACAG-3′ (reverse). The primers for human *histone* (Acc No. NM_003548) were 5′-TATCGGGCTCC AGCGGTCATGTC-3′ (forward) and 5′-GGATCGAAACGTGCAAAGCTG GAG-3′ (reverse). The primers for human *β-actin* (Acc No. NM_001101) were 5′-CAAGAGATGGCCACGGCTGCT-3′ (forward) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (reverse).

2.9. Luciferase reporter assay

pGL3-SV40HRE containing two copies of the HRE of the *EPO* gene was prepared as described previously [4]. pGL3-SV40HRE (0.5 μg) and pRL-TK (25 ng) were co-transfected with *CSR1* or *CSR2*/pcDNA-3 × Flag into Hep3B cells in a 24-well plate with GenePORTERTM2. After incubation for 24 h, the culture medium was replaced with fresh medium. After incubation for 6 h under hypoxia, luciferase activity was assayed with a luminometer (Lumat LB9507; Berthold, Bad Wildbad, Germany) using the Dual-Luciferase Reporter Assay System.

2.10. Statistical analysis

Statistical analysis was carried out with the Student's *t*-test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Exploration of NPR-binding protein

To examine new interacting proteins with NPR, the yeast two-hybrid system was used to screen the human heart cDNA library, using NPR as bait. Because NPR interacts with various P450s, we used the heart as a library in which the expression levels of P450s are low compared with other organs [14]. We obtained clones that encoded the CSR1 and CSR2 common coding region after sequencing analysis and database searches.

3.2. Interaction of NPR with CSR

In Hep3B cells, endogenous CSR1 and CSR2 were detected. To verify the interaction of NPR with CSR1 or CSR2 in mammalian cells, immunoprecipitation was performed using anti-NPR antibody. Both endogenous CSR1 and CSR2 were successfully co-precipitated with NPR (Fig. 1A), indicating the interaction of these proteins *in vivo*. A subsequent reciprocal immunoprecipitation using anti-CSR antibody was also performed, but we were unable to detect co-immunoprecipitation, possibly due to low expression levels of endogenous CSR1 or CSR2. Next, CSR1 or CSR2 fused by 3 × Flag and NPR fused by Myc were co-overexpressed in HEK293T cells, and immunoprecipitation was performed using anti-Flag antibody (Fig. 1B and C). Myc-fused NPR was detected by anti-Myc antibody, indicating an interaction between CSR1/CSR2 and NPR.

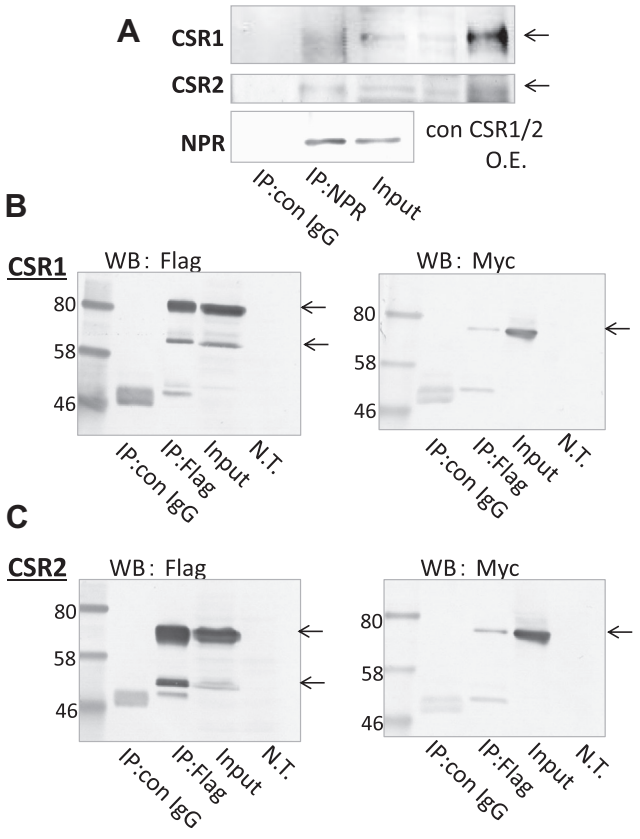

Fig. 1. Interaction of NPR with CSR. (A) Cell lysates of Hep3B were immunoprecipitated with control IgG or anti-NPR antibody, and analyzed by Western blotting with anti-NPR or anti-CSR antibody. (B and C) HEK293T cells were transfected with *NPR*/pCMV-Myc and *CSR1* (B) or *CSR2* (C)/pCDNA-3 × Flag. Cell lysates were immunoprecipitated with control IgG or anti-Flag antibody, and analyzed with indicated the antibodies. con, control; N.T., no transfection; O.E., overexpression.

3.3. N-glycosylation of CSR proteins

CSR proteins are the member of the class A scavenger receptor family, which are known to be glycoprotein. Several sites for predicted N-linked glycosylation have been found in CSR proteins [15], but to the best of our knowledge, the glycosylation of CSR proteins is not known. The CSR1/2 proteins overexpressed in Hep3B cells or HEK293T cells were detected at greater size than their predicted molecular weight by approximately 20 kDa. To analyze the glycosylation status of CSR proteins, cell lysates were incubated with N-glycosidase PNGase F, which cleaves N-glycans including high mannose, hybrid, and complex structures (Fig. 2). After digestion, the bands of CSR1 and CSR2 sifted down at around the predicted size, suggesting that CSR proteins were N-glycosylated.

3.4. Effects of overexpression or knockdown of CSR on EPO induction

To investigate the effects of CSR on hypoxic response, CSR1 or CSR2 alone, or both together were overexpressed in Hep3B cells, and the induction of the hypoxia-inducible gene *EPO* was analyzed (Fig. 3A and B). Overexpression of CSR2 enhanced the expression of *EPO* mRNA under hypoxia compared with mock-treated cells. On the other hand, the effect of CSR1 overexpression was low (Fig. 3B). CSR1 and CSR2 have an alpha-helical coiled-coils domain in which protein oligomerization is predicted to occur. Scavenger receptor proteins other than CSR are known to form trimers through the interactions of each coiled-coils domain [16]. Co-overexpression of CSR1 and CSR2 has been suggested to be more effective than overexpression of CSR1 or CSR2 alone in protecting cells from stress [15]. In the case of *EPO* induction under hypoxia by CSR, however, no additive effects of CSR1 and CSR2 were observed. CSR-knockdown cells were generated using two different targeting sequences for *CSR1/2*, and decreased CSR levels were confirmed by Western blotting compared with mock-treated cells or the cells transfected with *GFP*-targeting shRNA (Fig. 3C). The shRNA with *CSR*-targeting sequence 2 significantly decreased CSR2 levels, which significantly suppressed *EPO* mRNA levels under hypoxia compared with controls (Fig. 3D).

3.5. Effect of CSR on HRE reporter activity

The effect of CSR on the HRE activity of the *EPO* gene was analyzed by luciferase reporter assay. Overexpression of CSR1 or CSR2

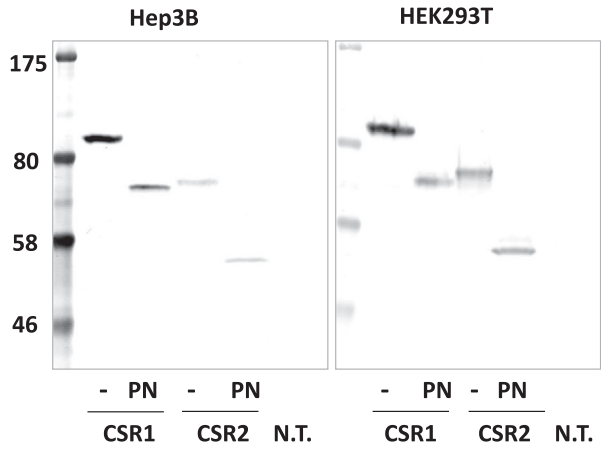

Fig. 2. Cleavage of CSR protein by PNGase F in Hep3B and HEK293T cells. Cells were transfected with *CSR1* or *CSR2*/pCDNA-3 × Flag. Cell lysates were incubated with PNGase F overnight at 37 °C, and analyzed by Western blotting with anti-Flag antibody. PN, PNGase F; N.T., no transfection.

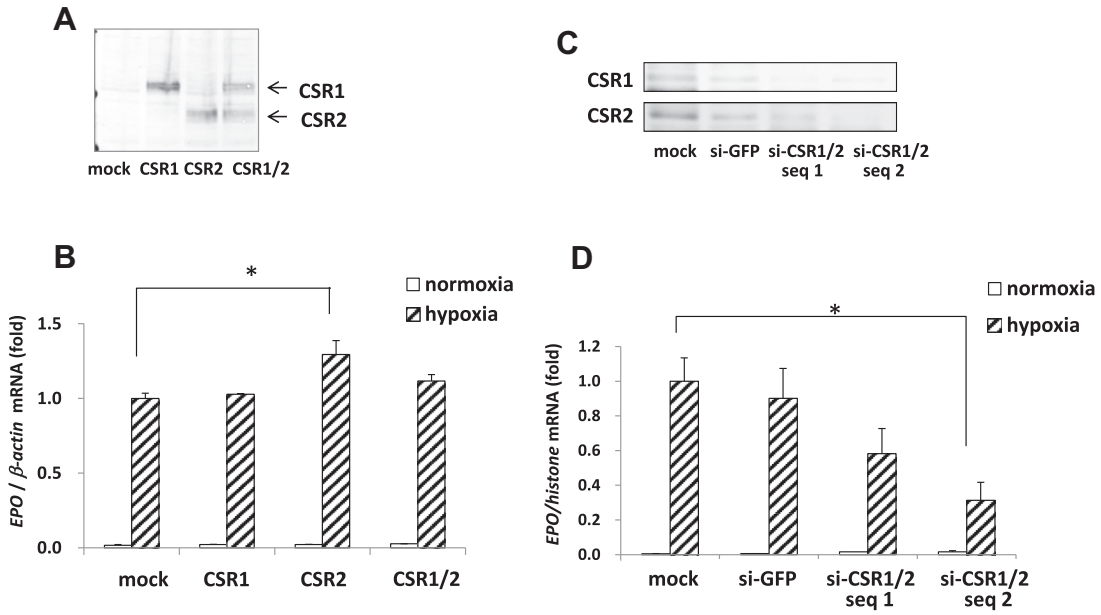

Fig. 3. *EPO* induction in CSR-overexpressing or CSR-knockdown cells. (A) *CSR1*, *CSR2* or *CSR1*/*CSR2* in pCDNA-3 × Flag was overexpressed in Hep3B cells, and detected by Western blotting with anti-Flag antibody. (C) CSR-knockdown cells were generated using shRNA targeting *CSR1*/*2* sequences, and knockdown of CSR was confirmed by Western blotting with anti-CSR antibody. (B and D) Cells were cultured under normoxic or hypoxic (1% O_2) conditions for 6 h, and *EPO* mRNA levels were analyzed by real-time PCR. The ratio of *EPO* mRNA to *β-actin* or *histone* mRNA under hypoxia in mock-treated cells was set at 1. EPO, erythropoietin. Values are the mean ± SD for three separate experiments. $^{*}p < 0.05$, significantly different from mock treatment.

enhanced HRE reporter activity under hypoxia, and the effect of CSR2 was higher than that of CSR1 (Fig. 4). Taken together, these results suggest that CSR promotes the transcriptional activity of HIF-1α. No additive effect of CSR1 and CSR2 was also observed on HRE activity as well as *EPO* mRNA levels.

4. Discussion

In the present study, CSR was identified as a new factor that interacts with NPR. CSR was isolated as a macrophage scavenger receptor-like protein, and has three putative functional domains: a membrane-spanning region, an alpha-helix coiled-coil domain, and a collagen-like domain [12]. CSR has two isoforms, CSR1 (full-length) and CSR2 which lacks a C-terminal collagen-like domain. NPR is a membrane-bound protein localized in the endoplasmic reticulum (ER), while CSR also has a predicted transmembrane domain and localizes in ER-Golgi apparatus [15]. We also revealed the localization of CSR in microsomal fraction as well as NPR by immunoblotting (data not shown). Therefore, the co-localization of these proteins in the membrane is predicted. One of the functions of CSR is to protect cells from oxidative stress, such as UV irradiation or exposure to hydrogen peroxide, by scavenging the reactive oxygen species (ROS) [15]. CSR1 has also been identified as a protein that is consistently down-regulated in prostate cancer tissue with high methylation of *CSR1* promoter, and a decrease in CSR has been found to be associated with cancer progression [13]. However, the primary function of CSR has not yet been clarified. Because of the function of NPR as an electron donor from NADPH to several enzymes including P450s and heme oxygenase 1, NPR may regulate CSR functions through electron transfer. However, to the best of our knowledge, the enzymatic activity of CSR, for instance in scavenging ROS, has not yet been confirmed. It has also been shown that NPR promotes the oligomerization and stability of heme oxygenase 1, and prevents its translocation to the nucleus [17]. Therefore, the interaction of NPR with CSR may be important to maintain the stability of CSR proteins in the membrane.

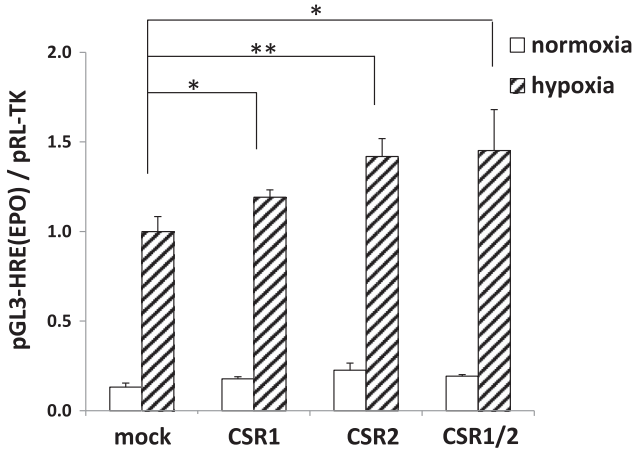

Fig. 4. HRE luciferase activity in CSR-overexpressing cells. pGL3-SV40HRE of *EPO* and pRL-TK vector was co-transfected with *CSR1*, *CSR2*, or *CSR1*/*2* in pcDNA-3 × Flag into Hep3B cells. Cells were incubated under hypoxia for 6 h, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System. EPO, erythropoietin; HRE, hypoxia response element. Values are the mean ± SD for three separate experiments. $^{*}p < 0.05$, significantly different from mock treatment. $^{**}p < 0.01$, significantly different from mock treatment.

Previously, we found that NPR promotes hypoxic response [4], but the mechanism was not clear. We also found that epoxyeicosatrienoic acids (EETs), which are produced from arachidonic acids by cytochrome P450 and NPR, promotes HIF-1α activation and the induction of EPO and VEGF under hypoxia [18], as does the overexpression of EET-producing P450s, CYP2C8 and CYP3A4 in Hep3B cells [18,19]. This raises the possibility that NPR regulates hypoxic response though the activation of EET-producing P450. However, inhibition of CYP2C and 3A4 did not bring about complete suppression of the hypoxic response although the inhibition or knockdown of NPR completely inhibited the hypoxic response, suggesting that another mechanism may also be involved in the

functions of NPR. In the present study, in addition of the action of EETs, we found that CSR that interacts with NPR has facilitative effects on hypoxic response, as does NPR, and that knockdown of CSR significantly suppressed hypoxic response. Therefore, the interaction of NPR with CSR may be involved in the regulation of hypoxic response by NPR. In this mechanism, it is possible that the function of CSR on scavenging free radicals may modulate HIF-1α activity. Various reports have indicated a relationship between the redox state of cells and HIF-1α activity; mitochondrial ROS stabilizes HIF-1α during hypoxia through the inhibition of prolyl hydroxylase (PHD) activity [20], meanwhile, it has been suggested that a reducing environment is required to stabilize HIF-1α in neurons under hypoxia [21]. Furthermore, the reduction of cysteine residue in HIF-1α by redox factor 1 (Ref-1) is important in its transactivation via enhancement of the interaction with CBP/p300 [22,23]. Therefore, it is possible that the change in cellular redox state by CSR affects HIF-1α activation.

In the present study, we found N-glycosylation of CSR1/2 protein. In the sequence of CSR protein, 11 putative glycosylation sites have been identified [15]. Protein glycosylation is a feature of scavenger receptors, and is critical for protein localization and ligand binding. Scavenger receptor class A (SR-A) is an original member of the class A scavenger receptor family, and its glycosylation is suggested to be necessary for its traffic to the plasma membrane, and for the internalization of the ligand, oligomeric amyloid-β peptide or acetylated low-density lipoprotein (LDL) [24]. To the best of our knowledge, the function of CSR1 or CSR2 as a receptor has not yet been demonstrated, but the glycosylation of CSR may affect its interaction with NPR or the regulation of the hypoxic response.

Finally, the present new finding of the interaction of NPR with CSR is expected to expand knowledge of the function of NPR and of new biological functions of CSR. Further study is necessary to elucidate the significance of this interaction and the mechanisms of the promotion of hypoxic response by CSR.

Acknowledgments

This study was supported in part by the Project to Assist Private Universities in Developing Bases for Research of the Ministry of Education, Culture, Sports, Science and Technology-Japan. It was also supported in part by a Grant-in-aid from Kwansei Gakuin University.

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