Journal of Molecular and Cellular Cardiology 77 (2014) 20–28

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

Orphan nuclear receptor Nur77 is a novel negative regulator of endothelin-1 expression in vascular endothelial cells

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ARTICLE INFO

Article history:
Received 2 July 2014
Received in revised form 15 September 2014
Accepted 25 September 2014
Available online 2 October 2014

Keywords:
Nur77
Endothelin-1
Transcription
AP-1
c-Jun

ABSTRACT

Endothelin-1 (ET-1) produced by vascular endothelial cells plays essential roles in the regulation of vascular tone and development of cardiovascular diseases. The objective of this study is to identify novel regulators implicated in the regulation of ET-1 expression in vascular endothelial cells (ECs). By using quantitative real-time PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), we show that either ectopic expression of orphan nuclear receptor Nur77 or pharmacological activation of Nur77 by 6-mercaptopurine (6-MP) substantially inhibits ET-1 expression in human umbilical vein endothelial cells (HUVECs), under both basal and thrombin-stimulated conditions. Furthermore, thrombin-stimulated ET expression is significantly augmented in both Nur77 knockdown ECs and aort from Nur77 knockout mice, suggesting that Nur77 is a negative regulator of ET-1 expression. Inhibition of ET-1 expression by Nur77 occurs at gene transcriptional levels, since Nur77 potently inhibits ET-1 promoter activity, without affecting ET-1 mRNA stability. As shown in electrophoretic mobility shift assay (EMSA), Nur77 overexpression markedly inhibits both basal and thrombin-stimulated transcriptional activity of AP-1. Mechanistically, we demonstrate that Nur77 specially interacts with c-Jun and inhibits AP-1 dependent c-Jun promoter activity, which leads to a decreased expression of c-Jun, a critical component involved in both AP-1 transcriptional activity and ET-1 expression in ECs. These findings demonstrate that Nur77 is a novel negative regulator of ET-1 expression in vascular ECs through an inhibitory interaction with the c-Jun/AP-1 pathway. Activation of Nur77 may represent a useful therapeutic strategy for preventing certain cardiovascular diseases, such as atherosclerosis and pulmonary artery hypertension.

1. Introduction

ET-1, a 21-amino acid peptide, is released continuously and predominantly from vascular ECs and is of significant importance in the regulation of cardiovascular function [1]. Through binding to 2 types of receptors, namely ET_A and ET_B, ET-1 exerts multiple biological functions in the cardiovascular system, including vasoconstriction, and proinflammatory and mitogenic properties in vascular ECs and smooth muscle cells (SMCs) [2]. ET_A receptors are mainly expressed in vascular SMC and cardiac myocytes, whereas ET_B receptors are predominantly localized on ECs and certain vascular SMCs [3]. The binding of ET-1 to ET_A and ET_B receptors in vascular SMCs results in a potent vasoconstriction, whereas the activation of endothelial ET_B receptors by ET-1 stimulates the release of NO and prostacyclin and causes an endothelium-dependent vasodilation [4,5]. Recently, multiple lines of evidence suggest that increased expression of ET-1 has been implicated in the development of endothelial dysfunction, which is associated with many cardiovascular events, such as atherosclerosis and vascular complications in diabetes [6,7]. In fact, the increased expression of ET-1 has been documented in atherosclerotic lesions and human coronary artery diseases [8,9]. Furthermore, endothelial specific overexpression of ET-1 markedly increased the formation of atherosclerotic lesions in Apo-E knockout mice fed with a high fat diet [10], further implicating its essential role in the development of vascular diseases. In the lungs, increased ET-1 has been shown to cause potent vasoconstriction and vascular remodeling, hence, contributing significantly to the development of both primary and secondary pulmonary hypertension [11]. Accordingly, the ET-1 receptor antagonists have been shown to dramatically improve the clinical outcome of the patients with severe pulmonary artery hypertension [12].

Because of critical roles of ET-1 in the cardiovascular system, its expression is tightly controlled and primarily regulated at the gene transcriptional levels [13]. Several transcriptional factors, including AP-1,

Abbreviations: ET-1, endothelin-1; qRT-PCR, quantitative real-time PCR; HUVEC, human umbilical vein endothelial cell; EMSA, electrophoretic mobility shift assay; siRNA, small interference RNA; NBRE, Nur77-binding response element; 6-MP, 6 mercaptopurine; ELISA, enzyme-linked immunosorbent assay.

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http://dx.doi.org/10.1016/j.yjmcc.2014.09.027
0022-2828/

GATA-2, vascular endothelial zinc finger 1 (Vezf1), forkhead box O (FOXO), and NF-κB, have been identified to bind to the ET-1 promoter region and transcriptionally regulate the ET-1 expression [14–17]. The AP-1 binding site is located at −108 bp of human ET-1 promoter and recruits both c-Fos and c-Jun, which is of importance for the high basal levels of ET-1 promoter activity in ECs [14]. In addition, PKC dependent activation of AP-1 has also been shown to mediate the increased ET-1 expression stimulated by thrombin, angiotensin II, and high-density lipoprotein [18–20]. Recently, the nuclear receptors have emerged as critical regulators for the ET-1 expression in the cardiovascular system. For instance, through directly binding to the promoter of ET-1, both the mineralocorticoid receptor and the glucocorticoid receptors have been shown to mediate the aldosterone dependent induction of ET-1 expression [13]. Importantly, activation of several nuclear receptors, including estrogen receptor (ER), retinoic acid receptor (RAR), farnesoid X receptor (FXR), and peroxisome proliferator-activated receptors (PPAR) α and γ, has been shown to attenuate the ET-1 expression in vascular cells [19,21–23], further implicating the functional importance of the nuclear receptor superfamily in the regulation of cardiovascular function.

NR4A receptors are immediate-early genes that are regulated by various physiological stimuli including growth factors, hormones, and inflammatory signals and involved in a wide array of important biological processes, including cell apoptosis, brain development, glucose and lipid metabolism, and vascular remodeling [24]. The NR4A subfamily consists of 3 well-conserved members, Nur77 (NR4A1), Nurr1 (NR4A2), and NOR-1 (NR4A3), respectively. Like other nuclear receptors, NR4A receptors consist of an N-terminal transactivation domain, a central 2-zinc-finger DNA-binding domain, and a C-terminal ligand-binding domain. So far, no ligands have been identified for these receptors and therefore they are classified as orphan receptors. Recently, there has been much attention paid to the function of these receptors in the cardiovascular system [25]. In vascular smooth muscle cells, the expression of Nur77 and NOR-1 was significantly induced by atherogenic stimuli, such as platelet-derived growth factor-BB, epidermal growth factor, and α-thrombin, and overexpression of Nur77 has been shown to inhibit cell proliferation and attenuate vascular injury-induced neointimal formation in vivo [26,27]. NR4A nuclear receptors are also induced in vascular ECs by several stimuli, such as hypoxia, TNF-α, and vascular endothelial growth factor, and modulate EC growth, survival, and angiogenesis [28,29]. Most importantly, our recent study has implicated Nur77 as a potent negative regulator for the pro-inflammatory responses in ECs via a selective inhibition of NF-κB pathway [29]. Recently, Nur77 has been shown to inhibit the development of atherosclerosis through its anti-inflammatory responses in microphages [30,31]. Whether Nur77 elicits vascular protective effects through regulating ET-1 production, however, is undetermined.

In the present study, we investigated the effect of Nur77 on the expression of ET-1 in HUVECs. Our results demonstrated that Nur77 potently inhibited the ET-1 production under both basal and thrombin stimulated conditions. Mechanistically, we found that Nur77 inhibits ET-1 production at the gene transcriptional levels, through attenuating the expression of c-Jun, which is a critical component for the activation of AP-1 transcriptional pathway.

2. Materials and methods

2.1. Cell culture

Human umbilical vascular ECs (HUVECs) were purchased from ATCC and cultured in EBM-2 medium (Lonza) supplemented with EGM-2 BulletKit (Lonza). Human pulmonary artery smooth muscle cells were purchased from ATCC and cultured in SmGM-2™ Growth Medium (Lonza). EA.hy926 cells and HEK293 cells were purchased from ATCC and cultured in DMEM supplemented with 10% FBS.

2.2. Mice

Nur77 knockout mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and animals were maintained on a C57BL/6 and 129SvJ hybrid background. $Nur77^{+/-}$ mice were crossed to obtain the wildtype and knockouts. Wildtype (WT) and Nur77 knockouts (KO) (10–12 weeks of age, 5–6 per group) were subjected to intraperitoneal injection (i.p.) of thrombin (70 units/g body weight). 3 h after injection, aorta from WT and Nur77 KO were then collected and total RNA was isolated by using TRIzol reagent (GIBCO/BRL) according to the manufacturer's instruction. The expression of ET-1 was then quantitated by quantitative real-time PCR (qRT-PCR). This study was reviewed and approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University.

2.3. Adenovirus construction

Adenoviruses harboring wild-type Flag-tagged Nur77 (Ad-Nur77) and Nor1 (Ad-NOR1) were made using AdMax (Microbix) as previously described [29]. The viruses were propagated in Ad-293 cells and purified using $CsCl_2$ banding followed by dialysis against 20 mmol/l Tris-buffered saline with 10% glycerol.

2.4. Electrophoretic mobility shift assay (EMSA)

HUVECs were transduced with Ad-Lac Z and Ad-Nur77 for 48 h and then treated with or without 10 U/ml thrombin (Haematologic Technologies) for 4 h; subcellular fractions were prepared as described previously [29]. Electrophoretic mobility shift assay (EMSA) was performed with Odyssey® IRDye® 700 infrared dye labeled double-stranded oligonucleotides coupled with the EMSA buffer kit (LI-COR Bioscience) according to the manufacturer's instructions. The specificity of the binding was examined using competition experiments, where 100-fold excess of the unlabelled oligonucleotides were added to the reaction mixture prior to add the infrared dye labeled oligonucleotide. The gel supershift assay was performed by adding c-Jun antibody (Cell Signaling) for AP-1 prior to the addition of the fluorescently labeled probe.

2.5. Western blot

Cell lysates were made using RIPA buffer (Thermo Scientific) containing 25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and proteinase inhibitor cocktail containing 2 mM PMSF, 20 μg/ml aprotinin, and 10 μg/ml leupeptin. Supernatants were resolved by SDS-PAGE, and transferred to nitrocellulose (BioRad). Blots were blocked with 5% nonfat milk in PBS with 0.1% Tween 20 (PBST) and then developed with diluted antibodies for Flag (1:1000 dilution; Genescript), Nur77 (1:250 dilution; BD Biosciences), GAPDH (1:2000 dilution; Santa Cruz Biotechnology) and c-Jun (1:1000 dilution; Cell Signaling) at 4 °C overnight, followed by incubation with goat anti-rabbit IgG (H + L) (DyLight 680 conjugated, Thermo Scientific) or goat anti-mouse IgG (H + L) (DyLight 800 conjugated, Thermo Scientific) for 1 h.

2.6. Co-immunoprecipitation

Immunoprecipitation was performed as described previously [29]. After preclearing for 1 h at 4 °C with protein A/G agarose (Sigma Aldrich), anti-flag M2 agarose (Sigma Aldrich) was added, and immune complexes were collected after overnight incubation at 4 °C. To identify the binding domains of Nur77 and c-Jun, HEK293 cells were transiently transfected with flagged tagged Nur77 mutant and Myc-tagged c-Jun mutant cDNAs using FuGENE 6 as described by the manufacturer (Roche Applied Science). Coimmunoprecipitation of Nur77 and c-Jun in HEK293 cells was performed essentially as described [29]. The