Biochemical and Biophysical Research Communications 457 (2015) 324–327

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

7-Ketocholesterol induces the reduction of KCNMB1 in atherosclerotic blood vessels

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ARTICLE INFO

Article history:
Received 16 December 2014
Available online 7 January 2015

Keywords:
Atherosclerosis
7-Ketocholesterol
Hypertension
KCNMB1
HA-VSMC

ABSTRACT

Hypertension is a high-risk symptom in atherosclerotic patients, and vascular rigidity is one of the main factors leading to hypertension. β1-Subunit of BKCa channel (KCNMB1; MaxiKβ1) has been reported as a modulator of vascular flexibility. To determine the relationship between atherosclerosis and KCNMB1, we studied some atherogenic factors affecting vascular tone. Blood of atherosclerotic patients shows increased concentration of 7-ketocholesterol (7K), which has been studied as a harmful lipid to blood vessels. Our data showed that KCNMB1 was significantly down-regulated in the presence of 7K, in a dose-/time-dependent manner in vascular smooth muscle cells (VSMCs). And, the reduction of KCNMB1 was confirmed in cell images of 7K-stimulated VSMCs and in vessel tissue images of *ApoE* knock-out mice. To determine whether aryl hydrocarbon receptor (AhR) was involved in the reduction of KCNMB1 by 7K-stimulation, protein level of AhR was analyzed by Western blot. Our data showed that the reduction of KCNMB1 was modulated through the AhR pathway. In conclusion, results of our study suggest that 7K induces the reduction of KCNMB1 through the AhR pathway.

1. Introduction

Atherosclerosis is a chronic, inflammatory disease characterized by accumulation of lipids (such as cholesterol crystals, oxysterols, modified fatty acids, aldehydes, and lysophospholipids) and fibrous elements in the coronary artery [1,2]. In atherosclerotic patients, flexibility of the inflammatory blood vessel is decreased, and blood pressure is elevated. The relationship of hyperpiesia and atherosclerosis has been studied, however, the mechanisms are not well known.

Oxysterols are derived non-enzymatically, either from the diet or from *in vivo* oxidation, or are formed enzymatically during cholesterol catabolism. The most abundant oxysterol in human blood is 27-hydroxycholesterol (27OH-chol). Other abundant oxysterols are 7α-hydroxycholesterol (7αOH-chol), 7-ketocholesterol (7K), and 7β-hydroxycholesterol (7βOH-chol). However, the major types of oxysterols in atherosclerotic plaques are 7K and lower levels of 7αOH-chol, 7βOH-chol, and 27OH-chol [3,4]. Compared with normal tissues or blood, a much higher 7K: cholesterol ratio has been reported for atherosclerotic plaques [5]. 7K predisposes human aorta smooth muscle cells (SMCs) to Fas-mediated cell death [6], and induces interleukin-6 (IL-6), an inflammatory and atherogenic cytokine, via the p38 MAPK pathway in human vascular smooth muscle cells (hVSMCs) [7]. Many studies have reported the toxicity of 7K, however, a relationship of 7K and hypertension is unclear.

Chronic hypertension is known to be associated with increased morbidity and mortality from stroke, coronary artery disease, congestive heart failure, renal disease, and so on [8,9]. Arterial rigidity is persistently increased as a result of malfunction of vessel relaxation in chronic hypertension [9]. Arterial rigidity is regulated by functional balance of the ion channels responsible for cellular

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http://dx.doi.org/10.1016/j.bbrc.2014.12.109
0006-291X/

depolarization and hyperpolarization. The increased arterial rigidity is mainly related to depolarization of smooth muscle, which may have resulted from dysfunction of ion channels responsible for cell membrane hyperpolarization [10]. The membrane depolarization activates voltage-dependent l-type Ca^{2+} channels, induces an increase in Ca^{2+} influx and global intracellular Ca^{2+} level, and causes vessel constriction [11,12]. Large-conductance Ca^{2+}-activated K^+ (BKCa or MaxiK) channels are believed to play an important role in hyperpolarization of vascular smooth muscle cells (VSMCs) [13]. BKCa channels are comprised of an alpha gene (*kcnma1*) encoding a pore-forming subunit and one of four beta genes (*e.g.*, *kcnmb1*) encoding cell-restricted modulatory subunits [14–16]. KCNMB1 confers heightened channel sensitivity to calcium and voltage in VSMCs, which provides for efficient fine-tuning of vascular rigidity [17,18]. The functionality of KCNMB1 has been studied extensively, however, much less is known regarding its transcriptional regulation. In a recent study, expression of the calcium channel protein is influenced by hypoxia-induced factor-1 (HIF-1) and histone deacetylase [19].

Hypertension and atherosclerosis have been studied by many researchers, and KCNMB1 have also been well studied. However, the involvements among the factors are unclear. In this study, we show that hypertension caused by atherosclerosis is related to a down-regulation of KCNMB1 by oxysterol.

2. Materials and methods

2.1. Cell culture

Human aortic vascular smooth muscle cells (HA-VSMCs) were purchased from American Type Culture Collection (ATCC; Manassas, VA), and were grown in Kaighn's modification of Ham's F-12 (F-12K) medium (ATCC) containing 15% fetal bovine serum (FBS). The cells were used at passage 7–9. The cells were maintained in a humidified incubator at 37 °C under 5% CO_2 atmosphere.

2.2. Reagents

Cholesterol and 7-ketocholesterol (7K) were purchased from Sigma–Aldrich, and 7α-hydroxy-cholesterol (7αOH-chol) and 7β-hydroxy-cholesterol (7βOH-chol) were purchased from Steraloid (Newport, RI). 27-hydroxy-cholesterol (27OH-chol) was purchased from Santa-Cruz (Delaware Avenue, CA). The lipids were dissolved in absolute ethanol. Primary antibody against human MaxiKβ (KCNMB1) was purchased from Santa-Cruz (Delaware Avenue, CA). Antibody against AhR was purchased from Abcam (Cambridge, CB4 0FL, UK). Antibody against β-actin was purchased from Calbiochem (La Jolla, CA). Horseradish peroxydase-conjugated secondary antibodies against goat, rabbit, and mouse IgG were purchased from Santa-Cruz, and AlexaFluo 488-conjugated secondary antibody was purchased from Invitrogen (Carlsbad, CA).

2.3. Western blot analysis

For whole cell lysates, cells were collected after treatment with or without oxysterols. The cells were lysed with lysis buffer (1% SDS, 1 mM $NaVO_3$, 10 mM Tris–HCl, pH 7.4) containing protease inhibitor cocktail (Sigma–Aldrich). The protein content of each sample was determined using the bicinchoninic acid method (BCA, Pierce, Rockford, Il). The samples were separated by SDS-PAGE, followed by transfer to PVDF membranes (Millipore Corp., Bedford, MA). After incubation for one hour with 5% skim milk in 0.1% Tween 20/TBS to block non-specific binding sites of the primary antibody, the membrane was probed with indicated primary antibodies at 4 °C overnight. After washing three times with 0.1% Tween 20/TBS for 15 min each, the membrane was incubated with HRP-conjugated secondary antibodies for one hour at room temperature. After washing three times with the washing buffer for 15 min each, bands were detected using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

2.4. Immunocytofluorescence

Cells cultured on a coverslip placed in a 6-well plate were washed with PBS, and fixed for 20 min with 1% paraformaldehyde at room temperature. After incubation with blocking solution (5% skim milk in PBS) for one hour to reduce non-specific binding, the cells were incubated at 4 °C overnight with antibody against KCNMB1 diluted in blocking solution (1:100). After washing three times with PBS for 15 min each, the cells were incubated for one hour with indicated secondary antibody diluted in PBS (1:250) at room temperature in darkness. After washing three times with PBS for 15 min each, the slides were mounted in Vectashield™ (Vector Laboratories, Burlingame, CA) and visualized using a confocal microscope.

2.5. Immunohistofluorescence

Mouse slide samples were kindly gifted from Professor Koanhoi Kim. The tissues were washed with PBS, and fixed for 20 min with 4% paraformaldehyde at room temperature; after incubation with blocking solution (3% BSA in PBS) for one hour to reduce non-specific binding, they were incubated at 4 °C overnight with antibody against KCNMB1 diluted in blocking solution (1:50). After washing three times with PBS for 15 min each, the slides were incubated for one hour with indicated secondary antibody diluted in PBS (1:100) at room temperature in darkness. After washing three times with PBS for 15 min each, the slides were mounted in Vectashield™ (Vector Laboratories, Burlingame, CA) and visualized using a confocal microscope.

2.6. Statistical analysis

Statistical analysis (one-way ANOVA) was performed using PRISM version 5.0 (GraphPad software, San Diego, CA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Relationship between KCNMB1 and oxysterols

To determine which oxysterols influenced KCNMB1 in blood of atherosclerotic patients, HA-VSMCs were stimulated for 48 h with 5 μg/ml cholesterol, 7K, 7αOH-chol, 7βOH-chol, and 27OH-chol, and proteins extracted from the cells were analyzed by Western blotting.

KCNMB1 was significantly decreased in the presence of 7K, but not by cholesterol or other oxysterols (Fig. 1A). To confirm this result, we investigated the reduction of KCNMB1 with 7K. The cells were treated for indicated times with indicated concentration of 7K. As shown in Fig. 1B, KCNMB1 of HA-VSMCs was significantly reduced by treatment with 5 and 10 μg/ml of 7K. However, an increase in dying cells was observed in cells treated with 10 μg/ml of 7K. KCNMB1 was also affected by 7K-treatment time. In Fig. 1C, the channel protein was decreased at 48 h after treatment with 5 μg/ml of 7K. These results demonstrated that 7K affected the protein level of KCNMB1.

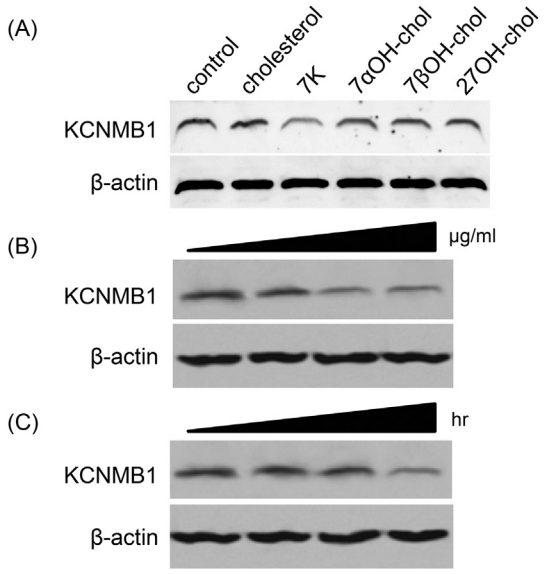

Fig. 1. Reduction of KCNMB1 by 7K. (A) HA-VSMCs (1×10^6 cells/100 mm culture dish) were culture for 48 h with 5 μg/ml of indicated lipids. Proteins harvested from the cells were analyzed with Western blot. (B, C) The cells were stimulated for 48 h with various concentrations of 7K, and were maintained for various times with 5 μg/ml of 7K. The harvested samples were analyzed with Western blot. Results are representative of 3 independent experiments.

3.2. Visualization of KCNMB1 damaged by 7K

Next, we visualized the change of KCNMB1 on HA-VSMCs by stimulation with 7K. The protein was detected in accordance with cytoplasmic membrane in normal condition (Fig. 2, upper panel).

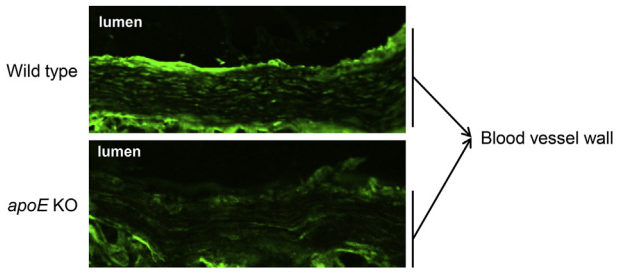

Fig. 3. Visualization of the KCNMB1 reduction in aortic tissue of *apoE* knock-out mouse. The tissue slides embedding murine aorta tissues were immuno-blotted as described in Materials & Methods. Results are representative of 3 independent experiments. Upper panel: tissue of wild type mouse; lower panel: tissue of *apoE* knock-out mouse.

However, the protein was poorly detected on cells in the presence of 7K (Fig. 2, lower panel). These results demonstrated that KCNMB1 was affected in the presence of 7K.

3.3. Reduction of KCNMB1 in atherosclerotic aortic root tissue

To study KCNMB1 expression *in vivo*, we investigated with C57BL/6 $apoE^{-/-}$ mice. The knockout and wild-type mice were maintained for two months with a high-fat diet, sacrificed, and aortic roots were harvested. The tissues were processed to frozen sample with OCT compound, and were prepared on tissue-slides. The signal of KCNMB1 was strongly expressed on the blood vessel wall of the aortic root from wild-type. In particular, the signals were expressed on the endothelial layer and smooth muscle cells in the intima. However, KCNMB1 was reduced in the inflammatory blood vessel wall of *apoE* knock-out mice. This result demonstrated the relationship between inflammation of blood vessel and KCNMB1 expression.

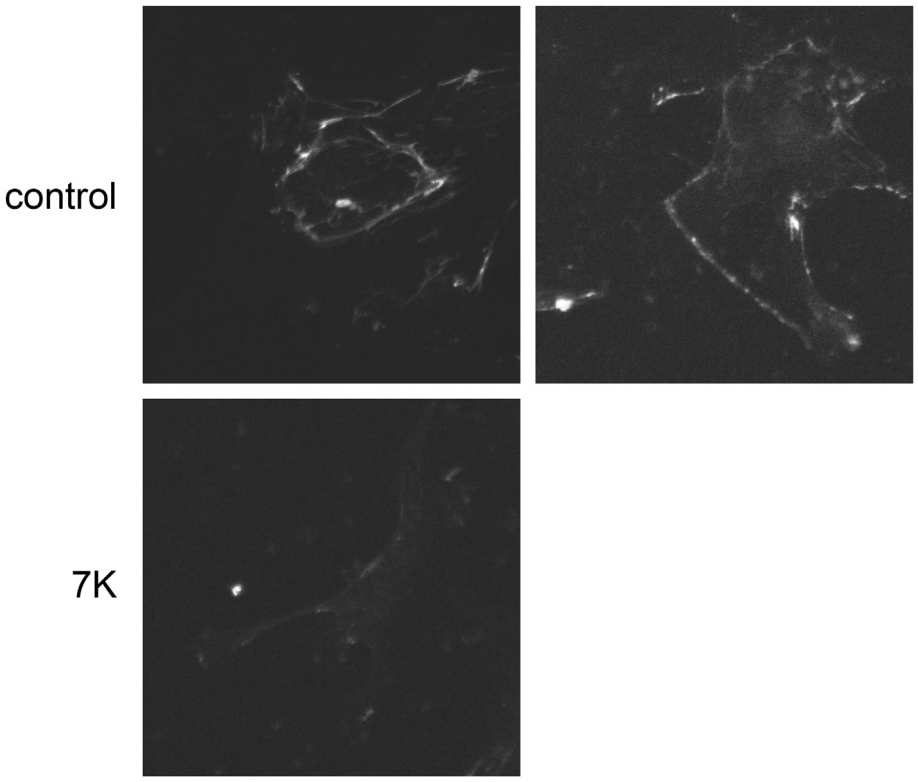

Fig. 2. Visualization of the KCNMB1 reduction by 7K on VSMCs. HA-VSMCs (1×10^6 cells/100 mm culture dish) were culture for 48 h with 5 μg/ml of 7K on coverslip. The cells were fixed, and were immuno-blotted with fluorescence. Results are representative of 3 independent experiments. Upper panels: control; lower panel: 7K-stimulated cell.

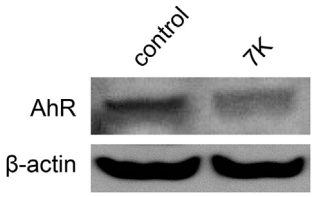

Fig. 4. Decrease of AhR protein level by 7K. HA-VSMCs (1×10^6 cells/100 mm culture dish) were culture for 48 h with 5 μg/ml of 7K, and were harvested. The proteins were analyzed with Western blot. Results are representative of 3 independent experiments.

3.4. Pathway involved in the decrease of KCNMB1 by 7K

To determine whether or not aryl hydrocarbon receptor (AhR), which is well known as one of the signaling receptors by 7K, is involved in the decrease of KCNMB1, we analyzed the AhR protein level. HA-VSMCs were cultured for 48 h with 5 μg/ml of 7K, and proteins from the cells were analyzed by Western blotting. As shown in Fig. 4, the protein level of AhR was significantly decreased by treatment with 7K. The result indicates that 7K affected AhR and influenced KCNMB1 through the signal.

4. Discussion

Vascular rigidity causes high risk diseases such as hypertension, thrombosis, cardiac infarction, and ischemia, and KCNMB1 is involved in blood vessel flexibility. Atherosclerotic patients commonly have symptoms of hypertension. A report showed that high-level cholesterol in blood modulates vascular reactivity to endothelin-1 by stimulating a pro-inflammatory pathway [20]. We conducted this study based on the presumption that oxysterols oxidized from high-level cholesterol affected vascular rigidity.

According to our results, among the oxysterols increased in blood of atherosclerotic patients, KCNMB1 was significantly reduced in the presence of 7K, in a dose- and time-dependent manner (Fig. 1), and the result can be visualized in Fig. 2. 7K induces apoptosis of VSMC, destruction of cholesterol homeostasis, blood vessel inflammation, and so on. A recent study demonstrated involvement of oxysterols in hypertension of preeclampsia [21]. The data demonstrated that 7K reduced the expression of KCNMB1 channel protein, and the result caused vascular rigidity. *In vivo* results also showed a reduction of KCNMB1 (Fig. 3). High-fat diet for *apoE* knock-out mice induces concentration of oxysterols and inflammatory factors in blood. We think that the increase in oxysterols induced the reduction of KCNMB1, and the main lipid leading to the reduction was 7K.

One study reported that 7K is a modulator for AhR [22]. To determine whether the receptor pathway is involved in the presence of 7K, we investigated the relationship of 7K and AhR. AhR is a receptor for xenobiotic ligands such as polychlorinated dibenzo-dioxins, dibenzofurans, 3-methylcholanthrene, benzo(*a*)pyrene, and benzoflavones. Ligand-bound AhR translocalizes into the nucleus, and the signal is transferred to the aryl hydrocarbon receptor nuclear translocator (ARNT). And, the AhR/ARNT signal pathway induces expression of cytochrome P450 and other genes [23]. Our results showed that protein level of AhR was decreased by the 7K-stimulation. Savouret et al. also showed that 7K competitively inhibits AhR signal transduction [22]. In accordance with these data, we assumed that the reduction of KCNMB1 induced by 7K was modulated through the AhR-ARNT signaling pathway.

In conclusion, our study showed that 7K induced the reduction of KCNMB1, a subunit of the Ca^{2+} channel, *in vivo* and *in vitro*, and was modulated by the AhR signaling pathway.

Acknowledgment

This research was supported by the 2013 Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (grant number: 2014027171). JK was supported by the PKNU Research Abroad Fund CD-2013-0914.

References

[1] J.A. Berliner, J.W. Heinecke, The role of oxidized lipoproteins in atherogenesis, Free Radic. Biol. Med. 20 (1996) 707–727.
[2] J.A. Berliner, M. Navab, A.M. Fogelman, J.S. Frank, L.L. Demer, P.A. Edwards, A.D. Watson, A.J. Lusis, Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics, Circulation 91 (1995) 2488–2496.
[3] A.J. Brown, W. Jessup, Oxysterols and atherosclerosis, Atherosclerosis 142 (1999) 1–28.
[4] K.L. Carpenter, S.E. Taylor, C. van der Veen, B.K. Williamson, J.A. Ballantine, M.J. Mitchinson, Lipids and oxidised lipids in human atherosclerotic lesions at different stages of development, Biochim. Biophys. Acta 1256 (1995) 141–150.
[5] K.L. Carpenter, S.E. Taylor, J.A. Ballantine, B. Fussell, B. Halliwell, M.J. Mitchinson, Lipids and oxidised lipids in human atheroma and normal aorta, Biochim. Biophys. Acta 1167 (1993) 121–130.
[6] M.C. Rho, Y.K. Kim, J.S. Chang, H.S. Lee, J.A. Baek, M.Y. Chung, H.C. Lee, H.W. Lee, B.Y. Rhim, M.A. Reidy, K. Kim, 7-Ketocholesterol predisposes human aorta smooth muscle cells to Fas-mediated death, J. Mol. Cell. Cardiol. 39 (2005) 823–832.
[7] S.C. Sung, K. Kim, K.A. Lee, K.H. Choi, S.M. Kim, Y.H. Son, Y.S. Moon, S.K. Eo, B.Y. Rhim, 7-Ketocholesterol upregulates interleukin-6 via mechanisms that are distinct from those of tumor necrosis factor-alpha, in vascular smooth muscle cells, J. Vasc. Res. 46 (2009) 36–44.
[8] J. He, P.K. Whelton, Elevated systolic blood pressure and risk of cardiovascular and renal disease: overview of evidence from observational epidemiologic studies and randomized controlled trials, Am. Heart J. 138 (1999) 211–219.
[9] B. Folkow, Physiological aspects of primary hypertension, Physiol. Rev. 62 (1982) 347–504.
[10] N.J. Rusch, BK channels in cardiovascular disease: a complex story of channel dysregulation, Am. J. Physiol. Heart Circ. Physiol. 297 (2009) H1580–H1582.
[11] M. Ishiguro, G.C. Wellman, Cellular basis of vasospasm: role of small diameter arteries and voltage-dependent Ca2+ channels, Acta Neurochir. Suppl. 104 (2008) 95–98.
[12] H.J. Knot, M.T. Nelson, Regulation of arterial diameter and wall [Ca2+] in cerebral arteries of rat by membrane potential and intravascular pressure, J. Physiol. 508 (Pt 1) (1998) 199–209.
[13] J.E. Brayden, M.T. Nelson, Regulation of arterial tone by activation of calcium-dependent potassium channels, Science 256 (1992) 532–535.
[14] L. Pallanck, B. Ganetzky, Cloning and characterization of human and mouse homologs of the Drosophila calcium-activated potassium channel gene, slowpoke, Hum. Mol. Genet. 3 (1994) 1239–1243.
[15] Z. Jiang, M. Wallner, P. Meera, L. Toro, Human and rodent MaxiK channel beta-subunit genes: cloning and characterization, Genomics 55 (1999) 57–67.
[16] R. Behrens, A. Nolting, F. Reimann, M. Schwarz, R. Waldschutz, O. Pongs, hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family, FEBS Lett. 474 (2000) 99–106.
[17] Y. Tanaka, K. Koike, A. Alioua, K. Shigenobu, E. Stefani, L. Toro, Beta1-subunit of MaxiK channel in smooth muscle: a key molecule which tunes muscle mechanical activity, J. Pharmacol. Sci. 94 (2004) 339–347.
[18] S. Ghatta, D. Nimmagadda, X. Xu, S.T. O'Rourke, Large-conductance, calcium-activated potassium channels: structural and functional implications, Pharmacol. Ther. 110 (2006) 103–116.
[19] Y.T. Ahn, Y.M. Kim, E. Adams, S.C. Lyu, C.M. Alvira, D.N. Cornfield, Hypoxia-inducible factor-1alpha regulates KCNMB1 expression in human pulmonary artery smooth muscle cells, Am. J. Physiol. Lung Cell. Mol. Physiol. 302 (2012) L352–L359.
[20] D. Paris, T. Town, J. Humphrey, K. Yokota, M. Mullan, Cholesterol modulates vascular reactivity to endothelin-1 by stimulating a pro-inflammatory pathway, Biochem. Biophys. Res. Commun. 274 (2000) 553–558.
[21] A.C. Valbuena-Diez, F.J. Blanco, B. Oujo, C. Langa, M. Gonzalez-Nunez, E. Llano, A.M. Pendas, M. Diaz, A. Castrillo, J.M. Lopez-Novoa, C. Bernabeu, Oxysterol-induced soluble endoglin release and its involvement in hypertension, Circulation 126 (2012) 2612–2624.
[22] J.F. Savouret, M. Antenos, M. Quesne, J. Xu, E. Milgrom, R.F. Casper, 7-Keto-cholesterol is an endogenous modulator for the arylhydrocarbon receptor, J. Biol. Chem. 276 (2001) 3054–3059.
[23] M.S. Denison, S.R. Nagy, Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals, Annu Rev. Pharmacol. Toxicol. 43 (2003) 309–334.