European Journal of Pharmacology 636 (2010) 36–41

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Molecular and Cellular Pharmacology

Angiotensin II type 1 receptor blockers prevent tumor necrosis factor-α-mediated endothelial nitric oxide synthase reduction and superoxide production in human umbilical vein endothelial cells

Hiroki Kataoka [a], Ryuichiro Murakami [a,*], Yasushi Numaguchi [a], Kenji Okumura [a,b], Toyoaki Murohara [a]

[a] Department of Cardiology, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan
[b] Department of Cardiovascular Medicine, Nagoya University School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan

ARTICLE INFO

Article history:
Received 1 September 2009
Received in revised form 13 January 2010
Accepted 17 March 2010
Available online 29 March 2010

Keywords:
Angiotensin II type 1 receptor blocker
Tumor necrosis factor-α
Endothelial nitric oxide synthase
Superoxide
Human umbilical vein endothelial cell

ABSTRACT

Decrease in endothelial nitric oxide synthase (eNOS) expression is one of the adverse outcomes of endothelial dysfunction. Tumor necrosis factor-α (TNF-α) is known to decrease eNOS expression and is an important mediator of endothelial dysfunction. We hypothesized that an angiotensin II type 1 (AT1) receptor blocker would improve endothelial function via not only inhibition of the angiotensin II signaling but also inhibition of the TNF-α-mediated signaling. Therefore we investigated whether an AT1 receptor blocker would restore the TNF-α-induced decrease in eNOS expression in cultured human umbilical vein endothelial cells (HUVEC). Pretreatment of HUVEC with an antioxidant (superoxide dismutase, α-tocopherol) or AT1 receptor blockers (olmesartan or candesartan) restored the TNF-α-dependent reduction of eNOS. The AT1 receptor blocker decreased the TNF-α-dependent increase of 8-isoprostane. The superoxide dismutase activities in HUVEC were stable during AT1 receptor blocker treatment, and the AT1 receptor blocker did not scavenge superoxide directly. The AT1 receptor blocker also decreased TNF-α-induced phosphorylation of IκBα and cell death. These results suggest that AT1 receptor blockers are able to ameliorate TNF-α-dependent eNOS reduction or cell injury by inhibiting superoxide production or nuclear factor-κB activation.

1. Introduction

Impairment of the endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) pathway in dysfunctional endothelia is considered to be a major contributor to the pathogenesis of various cardiovascular diseases. The expression level of eNOS in the vessel wall is one of the important determinants of the production of NO (Wilcox et al., 1997), but NO production is also dependent on other factors: the availability of substrate L-arginine and cofactors for eNOS tetrahydrobiopterin (Stuehr, 1999), the phosphorylation status of eNOS (Dimmeler et al., 1999), and the presence of reactive oxygen species, that can inactivate NO (Rubanyi and Vanhoutte, 1986).

Tumor necrosis factor-α (TNF-α) is a major pro-inflammatory cytokine, and is predominantly synthesized by macrophages infiltrating adipose tissue (Weisberg et al., 2003) in obese individuals. Obesity is well known as an independent risk factor for myocardial infarction, stroke or type 2 diabetes mellitus. In these cases, insulin resistance and endothelial dysfunction are strongly related to obesity (Lteif et al., 2005), and TNF-α seems to be an important cause of both insulin resistance (Sethi and Hotamisligil, 1999) and endothelial dysfunction (Picchi et al., 2006). In aged spontaneously hypertensive rats, protein expression of eNOS in the aorta was significantly lower and serum TNF-α level was significantly higher compared to in the control rats (Chou et al., 1998). Cell culture experiments also have shown that TNF-α decreases eNOS expression by reducing the half-life of mRNA encoding for endothelial NO synthase (Yan et al., 2008; Yoshizumi et al., 1993). Thus TNF-α is regarded as an important mediator linking metabolic disorder, aging, and inflammation to endothelial dysfunction.

Chronic administration of an angiotensin II type 1 (AT1) receptor blocker has been shown to ameliorate endothelial function (Schiffrin et al., 2000) and increase NO bioavailability (Prasad and Quyyumi, 2004). There are numerous reports showing that angiotensin II harms the endothelium and that its blockade helps to prevent endothelial dysfunction. However, it is unknown whether AT1 receptor blocker treatment restores the TNF-α-induced decrease of eNOS expression through a direct effect on the endothelium. Accordingly, we investigated whether TNF-α and AT1 receptor blockers would affect cultured human umbilical vein endothelial cells (HUVEC) with regard to (1) eNOS expression, (2) amount of oxidative stress, (3) ability to scavenge superoxide, (4) phosphorylation of IκBα and (5) cell death.

* Corresponding author. Tel.: +81 52 744 2168; fax: +81 52 744 2210.
E-mail address: ryuichi@med.nagoya-u.ac.jp (R. Murakami).

0014-2999/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.03.025

2. Materials and methods

2.1. Materials

RNH-6270 (an active metabolite of olmesartan medoxomil) and CV-11974 (an active metabolite of candesartan) were generously provided by Daiichi Sankyo Company, Ltd. (Tokyo, Japan) and Takeda Pharmaceutical Company, Ltd. (Osaka, Japan), respectively. Superoxide dismutase (SOD), xanthine oxidase and hypoxanthine were purchased from Sigma (St. Louis, MO). Human angiotensin II was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Recombinant human TNF-α, and anti-TNF-α antibody, enzyme-linked immunosorbent assay (ELISA) kit for TNF-α and interleukin (IL)-1β (Quantikine) were from R&D systems (Minneapolis, MN). The anti-eNOS antibody and anti-phospho-IκBα (Ser 32/36) antibody were from BD Biosciences (San Jose, CA) and BioSource International (Camarillo, CA), respectively. The 8-Isoprostane EIA kit was from Cayman Chemical (Ann Arbor, MI), and the Cell Death Detection ELISA^{PLUS} kit was from Roche Diagnostics (Basel, Switzerland).

2.2. Preparation of the conditioned media and cell culture

U937 cells, the human leukemic monocyte lymphoma cell line, were purchased from ATCC. U937 cells were grown to confluence at 37 °C in a humidified incubator containing 5% CO_2 in RPMI1640 medium with 10% fetal bovine serum (FBS; Gibco/Invitrogen, Carlsbad, CA). To attach the floating cells to culture dishes, U937 cells were stimulated with 200 nM phorbol 12-myristate 13-acetate (PMA). After cell adhesion, cells were washed twice with phosphate buffered saline, followed by 48 h of starvation with new RPMI1640 containing 0.2% FBS. Then the media were changed to fresh RPMI1640 without serum. After 24 h, these culture media were collected and centrifuged for 5 min at 1500×*g* and the supernatants were stored at −80 °C. For preparation of the conditioned medium, we mixed these supernatants with fresh Dulbecco's Modified Eagle's Medium (DMEM) in a volume ratio of 1:40. HUVEC and endothelial cell growth medium (EGM-2) were obtained from Sanko-Jyunyaku Co. (Tokyo, Japan). EGM-2 contains 5% FBS, hydrocortisone, basic fibroblast growth factor, vascular endothelial growth factor, R3 insulin-like growth factor-1, ascorbic acid, epidermal growth factor. HUVEC were grown to confluence in EGM-2 medium. Cell cultures between passages 4 and 6 were used for each experiment. Before each pharmacological intervention, HUVEC were starved with DMEM containing 4.5 g/l D-glucose, 2% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco/Invitrogen) for 12 h. All HUVEC experiments were performed using DMEM unless otherwise specified.

2.3. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting

For assessment of eNOS expression, HUVEC grown on 6-well plates were treated properly and then lysed in heated Laemmli buffer (Sigma). The eNOS expression and IκBα phosphorylation were assessed by SDS-polyacrylamide gel electrophoresis and Western blotting as described previously (Murakami et al., 2006). Immunoblot detection was performed using ECL reagents (Amersham Biosciences, Tokyo, Japan) for chemiluminescence. The chemiluminescence intensity on eNOS or phospho-IκBα was quantified and corrected with β-actin.

2.4. Measurement of endothelial cell death and oxidative stress markers in culture

For quantitative determination of cell death, we measured histone-associated DNA fragmentation using a quantitative sandwich-enzyme-immunoassay (Cell Death Detection ELISA^{PLUS}) according to the manufacturer's instructions. For sample preparation, HUVEC were grown on 24-well plates and treated with 150 nM RNH-6270 or 120 nM CV-11974 for 1 h, followed by 12 h of co-incubation with 50 pg/ml TNF-α. After centrifugation at 200×*g* for 10 min and removal of supernatant, cells were lysed and applied to streptavidin-coated assay plates. Formation of 8-isoprostane (8-iso prostaglandin F2α) was measured using a 8-isoprostane-acetylcholinesterase conjugate competitive enzyme-immunoassay kit (8-Isoprostane EIA Kit) according to the manufacturer's instructions. For sample preparation, HUVEC were grown on 24-well plates with 2 ml of medium and treated with 150 nM RNH-6270 for 1 h, followed by 24 h of incubation with 50 pg/ml TNF-α. After centrifugation at 200×*g* for 10 min, the medium was collected and applied to assay plates. The remaining cells were lysed and the amounts of protein were determined. 8-isoprostane levels were expressed as ng/mg protein.

2.5. Measurement of total superoxide dismutase activity in cell lysate

The ability of cells to dismutate superoxide anion was measured using a commercially available kit (Superoxide Dismutase Assay Kit; Cayman Chemical) according to the manufacturer's instructions. For sample preparation, HUVEC were grown on 6-well plates and treated with RNH-6270 for 1 h followed by 24 h of incubation with 50 pg/ml TNF-α. After the removal of medium, cells were harvested with 500 μl of cold HEPES buffer [20 mM HEPES, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose], sonicated and centrifuged at 1000×*g* for 5 min at 4 °C. The supernatant was collected and applied to assay plates. The protein concentration of the supernatant was determined and SOD activity was expressed as U/mg protein.

2.6. Measurement of TNF-α, IL-1β, and angiotensin I and II in culture media

The culture media of HUVEC treated with TNF-α and RNH-6270 were collected, and the amounts of TNF-α and IL-1β in the media were measured by an ELISA kit (Quantikine) according to the manufacturer's instructions. Angiotensin I or II levels were estimated by radioimmunoassay.

2.7. Statistical analysis

Results of the experimental studies are presented as the means ± S.D. Statistical differences between continuous variables and treatment groups were determined by one-way analysis of variance followed by Scheffe's post hoc test. A probability value of <0.05 was regarded as significant.

3. Results

3.1. TNF-α decreased eNOS expression and AT1 receptor blockers restored it

First, we examined whether TNF-α secreted from activated macrophages would actually decrease eNOS expression in HUVEC. For this purpose, we used U937 cells, which is a human monocyte-derived cell line. Stimulation with PMA leads U937 cells to attach to the plastic well and differentiate into macrophages. We prepared a conditioned medium, which was a mixture of the culture medium of PMA-stimulated U937 cells and fresh DMEM. An ELISA assay revealed that the final conditioned medium contained 39.2 ± 9.5 pg/ml TNF-α. When HUVEC were treated for 24 h with the conditioned medium, the protein expression of eNOS was significantly reduced by 60%. This eNOS reduction was fully restored by pretreatment of HUVEC with neutralizing anti-TNF-α antibody. Next, we tested the effects of AT1 receptor blockers on eNOS reduction in the conditioned medium. Pretreatment of HUVEC with RNH-6270, an active metabolite of olmesartan medoxomil, partially restored the reduction of eNOS (Fig. 1A). We also tested the effects of the pro-inflammatory cytokines IL-1β and IL-6. The concentration of IL-1β in the conditioned medium was 0.92 ± 0.07 pg/ml, but