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Nebivolol reduces asymmetric dimethylarginine in endothelial cells by increasing dimethylarginine dimethylaminohydrolase 2 (DDAH2) expression and activity

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Abstract

Asymmetric dimethylarginine (ADMA) has been reported to affect the synthesis of nitric oxide (NO) in endothelial cells by inhibiting endothelial NO synthase (eNOS) activity and to cause endothelial dysfunction in humans. This study was conducted in human umbilical vein endothelial cells (HUVECs) to evaluate the effect of nebivolol, a selective β1-adrenergic receptor antagonist, on ADMA concentration and on dimethylarginine dimethylaminohydrolase (DDAH2), the enzyme that regulates ADMA catabolism. Nebivolol dose-dependently decreased ADMA/symmetric dimethylarginine (SDMA) ratio (p from <0.01 to <0.001). This was parallelled by a dose-dependent increase in DDAH2 mRNA (p from <0.01 to <0.001) and protein expression (p from <0.01 to <0.001) and activity (p from <0.01 to <0.001). The small interference RNA (siRNA)-mediated knockdown of DDAH2 abolished the modification of DDAH2 expression ($p < 0.001$) and ADMA/SDMA ratio ($p < 0.001$) induced by nebivolol.

In conclusion, the results of this study demonstrate that nebivolol reduces ADMA concentration by increasing DDAH2 expression and activity. Our in vitro findings describe a novel vascular effect of nebivolol and clearly identify this compound as the first antihypertensive agent that modulates DDAH2 in endothelial cells.

Keywords: Nebivolol; β1-Adrenergic receptor antagonists; Endothelial nitric oxide synthase; Nitric oxide; Asymmetric dimethylarginine; Dimethylarginine dimethylaminohydrolase

1. Introduction

Essential hypertension is characterized by an impairment of endothelial function [1], a key step in the initiation/progression of atherosclerotic process. The hallmark of endothelial dysfunction is a reduction in the bioavailability of nitric oxide (NO) [2], principally determined by a reduction in its biosynthesis and/or oxidative inactivation.

Nebivolol is a third-generation β1-adrenergic receptor (AR) antagonist whose haemodinamic profile is different from that of classical β-blockers [3]. Experimental models have demonstrated that nebivolol has vasodilatory properties due to the release of NO [4,5] by a mechanism that is, so far, not completely known. There is evidence indicating that endothelial NO synthase (eNOS) is involved in the nebivolol effect since several groups [6–8] have described that the production of NO from the endothelium can be abolished by eNOS inhibitors [3,6–7]. At present, the mechanism by which nebivolol may modulate eNOS activity in endothelial cells remains controversial [9–11]. Nebivolol has also been shown to increase the production of NO by reducing its oxidative inactivation [12,13]. It is likely therefore that nebivolol may increase NO availability by at least two mechanisms: by reducing superoxide generation and by increasing eNOS activity.

Asymmetric dimethylarginine (ADMA) is a naturally occurring amino acid that circulates in plasma, is excreted in urine, and is found in tissues and cells [14]. It has aroused interest because it inhibits eNOS [15] and therefore has the potential to produce considerable biological effects, particularly endothelial dysfunction [16]. An emerging role for ADMA as a novel cardiovascular risk factor has also been recently reported [17]. ADMA together with symmetric dimethylarginine (SDMA) are synthe-

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sized in cells when arginine residues in proteins are methylated by the action of protein arginine methyltransferases [18]. Dimethylarginine dimethylaminohydrolase (DDAH) selectively degrades ADMA but not SDMA [19]. As a consequence the decrease of ADMA/SDMA ratio has already been reported to be characteristic of an increased DDAH2 activity [20]. Two isoforms of the enzyme exist: DDAH1 predominates in tissues containing neuronal NOS, whereas DDAH2 is more prevalent in tissues expressing eNOS [19].

Although nebivolol has been demonstrated to reverse endothelial dysfunction in essential hypertension [5] little is known about the underlying molecular mechanism of nebivolol action.

In this study we studied the effect of nebivolol on ADMA and DDAH2 induction and activity in endothelial cells in comparison with the beta-adrenoreceptor blocker atenolol.

2. Materials and methods

2.1. Cell cultures

Primary HUVECs were isolated by collagenase treatment of human umbilical veins from male or female newborns as described by Jaffe et al. [21]. Briefly, HUVECs were grown in 25-cm^2 flasks (Orange Scientific, Waterloo, Belgium) in human endothelial cell-specific medium EBM-2 (Cambrex Bio Science Walkersville, Walkersville, MD), supplemented with EGM-2 (Cambrex Bio Science Walkersville), in an incubator at 37 °C with 5% CO_2. Cells were identified as endothelial by their characteristic cobblestone morphology and the presence of von Willebrand factor by immunocytochemistry using a specific antibody (F-3520; Sigma–Aldrich, St. Louis, MO). When cells were at 75% confluence, culture medium was exchanged for M-199 (Invitrogen, Carlsbad, CA) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Invitrogen) for 24 h. Cells from passages 2 to 4 were seeded onto 75 cm^2 flasks and used for experiments.

Nebivolol (courtesy of Menarini Ricerche, Firenze, Italy) and atenolol (Sigma, St. Louis, MO) were dissolved in ethanol and in M-199.

2.2. ADMA and SDMA measurement

HUVECs were grown to confluence onto 25 cm^2 flasks, incubated by increasing amounts of nebivolol and atenolol (from 5 to 20 μmol/L) for 24 h and ADMA and SDMA measured in the medium by high-performance liquid chromatography (HPLC) as previously described [22].

Briefly, sample cleanup was performed by solid-phase extraction on polymeric cation-exchange columns using monomethylarginine as internal standard. After derivatization with *ortho*-phthaldialdehyde reagent containing 3-mercaptopropionic acid, ADMA and SDMA were separated by isocratic reversed-phase HPLC with fluorescence detection. The stable derivatives were separated with near baseline resolution. Using a sample volume of 0.2 mL, linear calibration curves were obtained with limits of quantification 0.01 μM for both ADMA and SDMA. Analytical recovery was 98–102%, and interassay CV was better than 3%.

2.3. Real-time RT-PCR quantification of DDAH2 mRNA

HUVECs were grown to confluence in 25 cm^2 flasks and incubated by increasing amounts of nebivolol and atenolol (from 5 to 20 μmol/L) for 12 h. Total RNA was extracted with a RNeasy Mini Kit (Qiagen) and was reverse transcribed using IScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time RT-PCR was conducted by iCycler thermocycler (Bio-Rad) using IQSYBR Green PCR SuperMix (Bio-Rad) and 300 pmol/mL each primer pair. Amplification condition was as follows: 95 °C for 5 min, 50 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Primers were designed by Beacon Design 4.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by MWG Biotech AG (Ebersberg, Germany): DDAH2, sense 5-GCTGCTAGAACTGCCACCTGAG-3 and antisense 5-GGGCTTTGCGGACTCCATCG-3, PCR fragment of 143 bp; β-actin, sense 5-TTGGCA ATGAGCGGTTCC-3 and antisense 5-AGCACTGTGTTGGCGTAC-3, PCR fragment of 148 bp. Transcript abundance, normalized to β-actin expression, was expressed as fold increase over a control sample (no addition of nebivolol or atenolol).

2.4. Western blotting analysis

HUVECs were grown to confluence in 75 cm^2 flasks, incubated by increasing amounts of nebivolol and atenolol (from 5 to 20 μmol/L) for 24 h and lysed with 0.1% SDS. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The DDAH2 goat polyclonal antibody (ab1383) was from Abcam (Cambridge, UK). Immunoreactive band was visualized with a horseradish peroxidase conjugated secondary antibody using an ECL detection kit (Amersham) at about 33 kDa and quantified by densitometric analysis after normalization to β-actin.

2.5. eNOS enzyme activity

HUVECs grown to confluence in 25 cm^2 flasks were incubated by increasing amounts of nebivolol and atenolol (from 5 to 20 μmol/L) for 24 h. The effect of nebivolol and atenolol on eNOS metabolism of ^{3}H arginine to ^{3}H citrulline was determined as described previously [23]. Briefly, HUVECs lysates were suspended in cold lysis buffer (0.3 M sucrose, 10 mM HEPES, 1% Nonidet P-40, 0.1 mM EDTA, 1 mM dithiothreitol, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, and 50 μM phenylmethylsulfonyl fluoride, pH 7.4) and vortexed. Cell lysates (150–250 μg of protein) were combined with NADPH (2 mM), $CaCl_2$ (230 μM), TB4 (3 μM), and ^{3}H-arginine (0.2 μCi, 10 μM) for 20 min at 37 °C. The assay volume was kept constant at 100 μL. To determine whether nebivolol altered inducible NOS activity, the assay was repeated with EDTA (1.7 mM) replacing calcium in the assay buffers.