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Effect of DL-nebivolol, its enantiomers and metabolites on the intracellular production of superoxide and nitric oxide in human endothelial cells

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Abstract

Nebivolol, a third generation selective β_1-adrenoceptor (β_1-AR) antagonist, has been reported to reduce intracellular oxidative stress and to induce the release of nitric oxide (NO) from the endothelium. Nebivolol is also subjected to a complex metabolic process where glucuronidation, aromatic and alicyclic hydroxylation are the major pathways leading to several metabolites. We have studied the effect of nebivolol, its enantiomers and metabolites on intracellular oxidative stress and NO availability in human umbilical vein endothelial cells (HUVECs). Furthermore, since the receptors involved in this endothelial effect of nebivolol remain controversial, we have studied this matter by the use of antagonists of β-AR. DL-Nebivolol, D-nebivolol and L-nebivolol significantly reduced the formation of reactive oxygen species (ROS) and superoxide induced by oxidized-low density lipoprotein (ox-LDL), although the racemic and L-form were significantly more active than D-nebivolol in this activity. A marked decrease in the availability of intracellular NO was found in HUVECs exposed to ox-LDL and this parameter was normalized by the prior incubation with DL-nebivolol, D-nebivolol and L-nebivolol; the effect of racemate was mainly mimicked by its L-enantiomer. eNOS activity significantly increased by a 5-min contact of HUVECs with DL-nebivolol and L-nebivolol, but not with D-nebivolol, and a similar pattern was observed when the intracellular calcium increase was measured. The metabolites A2, A3′, A12 and A14 but not A1, A3 and R 81,928, derived from different metabolic pathways, retained the antioxidant activity of the parent racemic compound DL-nebivolol, reducing the intracellular formation of ROS and superoxide. The effects of DL-nebivolol on intracellular formation of NO, eNOS activity and intracellular Ca^{2+} were partially antagonized by the antagonists of $\beta_{1\text{-}2}$-AR nadolol or by the β_3-AR antagonist SR59230A and further antagonized by their combination or by ($\beta_{1\text{-}2\text{-}3}$-AR antagonist bupranolol. In conclusion, this study shows that the NO releasing effect of nebivolol is mainly due to its L-enantiomer; the racemate and its enantiomers possess a remarkable antioxidant activity that contributes to its effect on the cellular NO metabolism and the activation of β_3-AR through a calcium dependent pathway is involved in the mechanisms leading to the NO release.

Keywords: β-Adrenergic receptor; Nebivolol; Nitric oxide (NO); Oxidative stress; Reactive oxygen species (ROS)

1. Introduction

Nebivolol is a third generation beta-blocker, highly selective for the β_1-adrenoceptors (AR) and endowed with the ability to release nitric oxide from the cardiovascular endothelium [1]. It is a racemic mixture of two enantiomers, D- and L-nebivolol, and its antihypertensive activity is mainly ascribed to D-nebivolol, which shows an over 100-fold greater affinity for β_1-ARs than the L-enantiomer [2]. Both enantiomers [3], but particularly the L-form [4,5], possess an endothelium-dependent vasorelaxant effect that can be antagonized by NO synthase inhibitors. Owing to these characteristics nebivolol possesses a peculiar pharmacodynamic profile producing vasodilation as observed in several conditions and species (see Ref. [1] for review). Some receptors have been described as responsible for the activation of the endothelial effect of nebivolol, among them the β_2- [6,7] and β_3-[8–11] ARs, the estrogen receptors of the plasma membrane [12], $5HT_{1A}$ receptors [13] as commonly found for other β blockers, and the P2Y purinoceptor [14]. A further mechanism of action is due to the antioxidant properties of nebivolol that can increase the release of NO by reducing its oxidative inactivation [15]. Reactive oxygen species (ROS) are reactive derivatives of O_2 metabolism, including superoxide anion, hydrogen peroxide, hydroxyl radical and nitric oxide. It is well known that

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superoxide, can inactivate NO in a chemical reaction producing peroxynitrite, and nebivolol has been found to interfere with this inactivation by enhancing the bioavailability of NO [15,16].

Indeed the increase in endothelial NO release induced by nebivolol is highly dependent on the tissue/cell system and species examined, being possibly related to the different receptor type and/or coupled transduction pathway activated in each experimental setting.

Nebivolol is also subjected to a complex metabolic process where glucuronidation, aromatic and alicyclic hydroxylation are the major pathways leading to several metabolites [17,18]. Among the latter, it has been recently reported that the 4-keto derivative metabolite produced from the alicyclic hydroxylation, has a similar pattern of activity on NO availability as compared with nebivolol itself [15].

Therefore, the aim of the present study is to evaluate the effect of nebivolol, its enantiomers and metabolites on intracellular oxidative stress and NO availability and the interrelationships between them in human umbilical vein endothelial cells (HUVECs), chosen as primary human culture and as known and widespread model to study NO [19]. Furthermore, since the receptors involved in this endothelial effect of nebivolol remain controversial, we have studied this matter by using antagonists of β-AR.

2. Materials and methods

2.1. Cell culture and treatment

HUVECs were isolated from human umbilical veins according to the method of Jaffe et al. [20] and used at passage 2–4. The cells were grown in 75 cm^2 culture flasks (Falcon, Becton Dickinson, Lincoln Park, USA), filled with 10 ml of M-199 (Sigma, St. Louis, USA) containing 10% fetal calf serum (Seromed, Berlin, Germany), 2 mM glutamine (Seromed, Berlin, Germany), 30 μg ml^{-1} endothelial cell growth supplement (Sigma, St. Louis, USA), 100 μg ml^{-1} heparin (Sigma, St. Louis, USA), 100 U ml^{-1} penicillin–streptomycin (Sigma, St. Louis, USA), 100 μg ml^{-1} streptomycin (Sigma, St. Louis, USA) and 2.5 μg ml^{-1} anphotericin (Sigma, St. Louis, USA). The flasks were incubated at 37 °C, 100% humidity and 5% of CO_2. The medium was refreshed every 2 days. At the beginning of each experiment the cells were harvested by trypsinisation, using 0.05% trypsin (Sigma Co, St. Louis, USA) and 0.537 mM ethylenediamine tetraacetic acid (EDTA) in phosphate buffered saline without calcium and magnesium (Seromed, Berlin, Germany). The trypsin was inactivated by dilution, and the cells were washed and counted. Cells were plated at a concentration of 40,000 cells cm^{-2} on a multiwell plate (9.6 cm^2 $well^{-1}$) (Falcon, Becton Dickinson, Lincoln Park, USA), grown for 2 days and then used for the incubations.

HUVECs were harvested and characterized as to acetylated low-density lipoprotein (LDL) binding and factor VIII expression, according to established and previously described techniques [21]. To assess cell survival, hexosaminidase, a stable cytosolic enzyme released by cells when they undergo lysis, was measured according to the method of Landegren [22].

DL-Nebivolol, D-nebivolol and L-nebivolol along with some metabolites (metabolites A1, A2, A3, A3′, A12 and A14, and R 81,928 from Berlin-Chemie, Berlin, Germany) were dissolved in ethanol and then diluted at the final concentration in culture medium M199. Identical dilutions of the solvent were prepared and used as control. Since racemic nebivolol was previously shown to reduce in a concentration-dependent manner the intracellular increase of ROS and superoxide and to reduce them by 50% at 10 μM [15], this concentration was chosen to compare the racemic nebivolol, its enantiomers and metabolites in this study.

2.2. LDL isolation and oxidation

Whole blood, obtained by venipuncture from healthy volunteers (with informed consent) after 12 h of fasting was collected into vacutainer tubes (Becton Dickinson, Meylan, France) containing EDTA (1 mg ml^{-1}), and processed for LDL separation in 1 day by sequential flotation in NaBr solution containing 1 mg ml^{-1} EDTA [23]. Cu^{2+} modified LDL (1.7 mg protein ml^{-1}) was prepared by exposure of LDL to 5 μM $CuSO_4$ for 18 h at 37 °C, and the extent of LDL oxidation was determined by thiobarbituric acid-reactive substances, as previously described [24]. Protein was measured by the Pierce BCA protein assay reagent [25]. Oxidative stress in endothelial cells was induced by the addition of oxidized (ox)-LDL at the concentration of 50 μg protein ml^{-1} as previously reported [15].

2.3. ROS and superoxide measurement

Intracellular ROS and superoxide levels were measured by following the oxidation of 2′,7′-dichlorofluorescein diacetate and hydroethidine by flow cytometry (Coulter Corporation, Hialeah, FL, USA) as described by Cominacini et al. [15]. HUVECs were first preincubated with DL-nebivolol, D-nebivolol, L-nebivolol (experiments performed in triplicate on five separate occasions), metabolites A1, A2, A3, A3′, A12, A14, and R 81,928 (experiments performed in quadruplicate on three separate occasions) at the concentration of 10 μM for 30 min at 37 °C. The cells were then exposed to ox-LDL (50 μg protein ml^{-1}) for 10 min at 37 °C in the presence of 5 mM arginine (Sigma, St. Louis, MO, USA) and 3 μM tetrahydrobiopterin (TB4; Sigma) to prevent superoxide formation by endothelial nitric oxide synthase (eNOS). Furthermore, to test the contribution of β-AR on ROS and superoxide levels after preincubation with DL-nebivolol, the ($\beta_{1\text{-}2}$-AR antagonist nadolol (10 μM; Sigma) and the β_3-AR antagonist SR59230A (2 μM; Tocris, Bristol, UK) were used.

2.4. NO measurement

Intracellular NO was measured by utilizing the fluorescent indicator 4,5 diaminofluorescein diacetate in flow cytometry [15,26]. HUVECs were incubated with 10 μM of DL-nebivolol, D-nebivolol and L-nebivolol or 100 nM bradykinin (Sigma), as a positive control, for 30 min at 37 °C. In other experiments (per-