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Radical scavenging and NO-releasing properties of selected β-adrenoreceptor antagonists

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Abstract
It is claimed that novel β-adrenolytic drugs possess superior antioxidant properties as compared to classical selective or non-selective β-adrenoceptor antagonists. Here we tested this notion by analyzing radical scavenging properties of selected β-adrenolytic drugs and their ability to release nitric oxide in biological preparations. Selective β_1-adrenolytics such as nebivolol, atenolol, metoprolol and non-selective β-adrenolytics with α1-receptor blocking properties such as carvedilol and labetalol were chosen for analysis. NO-releasing properties of nebivolol and carvedilol distinguished third generation β-adrenolytics from their older counterparts while the reactivity towards hydroxyl and peroxyl radicals discerns only carvedilol but not nebivolol. Thus, superior clinical efficacy of third generation β-adrenolytics may be related to their ability to release NO rather then to their direct antioxidant properties.

Keywords: *Nebivolol, carvedilol, radical scavenging, NO release*

Introduction

β-Adrenoreceptor antagonists (β-adrenolytics, β-blockers) are commonly used in the treatment of cardiac dysrythmias, hypertension, coronary artery disease and heart failure. It is widely accepted that these drugs are beneficial because they protect cardiovascular system against excessive adrenergic stimulation exerted via β-adrenoreceptor. However, recently published experimental and clinical data seem to suggest that some among the novel β-adrenolytic drugs such as nebivolol or carvedilol afford additional cardioprotective effects unrelated to β-adrenoreceptor blockade. Indeed, nebivolol is an unique cardioselective β-adrenoceptor antagonist because apart from its β_1-adrenolytic activity it possesses also vasodilating properties attributed to endothelial nitric oxide (NO) [1–3]. NO-dependent vasodilating effects of nebivolol was shown in different vascular beds and species including humans [2,4–6]. Moreover, independently on hypotensive action, chronic treatment with nebivolol, but not with atenolol, reversed endothelial dysfunction in patients with arterial hypertension [7,8] as well as in experimental animal model of hypertension [9]. In contrast to nebivolol, carvedilol is an adrenergic

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antagonist with non-selective β- and α1-receptor blocking properties. Surprisingly, it possesses ancillary properties, similar to nebivolol. In patients with coronary disease carvedilol improved NO-dependent endothelial function [10], while *in vitro* it released NO from vascular endothelium [11]. Interestingly, pleiotropic endothelial activity of nebivolol or carvedilol is not shared by other β-adrenolytic drugs, for instance, atenolol or metoprolol.

On the other hand, it was claimed that direct antioxidant activity of nebivolol and carvedilol was, at least in part, responsible for the beneficial endothelial and cardiovascular action of these drugs. Furthermore, in numerous studies distinct antioxidant properties of nebivolol and carvedilol were demonstrated [12–16] and it was suggested that superior antioxidant activity of these drugs as compared to their older counterparts could, at least in part, be explained by their better direct scavenging abilities towards the reactive oxygen species (ROS) [17–27].

Direct scavenging of superoxide radical anion, $O_2^{\cdot-}$, by β-adrenolytics is unlikely and effect of these drugs on $O_2^{\cdot-}$ seems to be related to the inhibition of NAD(P)H oxidase in inflammatory cells [24,28,29]. Indeed, nebivolol and carvedilol diminished superoxide ion production in inflammatory cells, through the inhibition of NAD(P)H oxidase activity [24,28,29] and this activity of nebivolol and carvedilol was not accompanied by the direct reactivity of these compounds with superoxide radical anion. Even at high concentrations these β-adrenolytic drugs did not inhibit the reaction of $O_2^{\cdot-}$ with cytochrome *c*, whereas it was inhibited by superoxide dismutase SOD [25–28].

On the other hand, direct radical scavenging properties of nebivolol and carvedilol are not consistently understood. For example, it has been reported that carvedilol inhibits lipid peroxidation by scavenging free radicals [23], while other experiments show that carvedilol rather sequesters ferric ion [27,30]. From those results it might be concluded that carvedilol does not act as a radical-scavenging antioxidant but that it does act as an antioxidant against iron-induced lipid peroxidation by sequestering ferric ion. In numerous works reporting the antioxidative properties of carvedilol and other β-adrenolytics, different systems for radical generation were used, including methods requiring the presence of iron ions and enzymatic systems [23–25,31–34]. From these results, it is not possible to unequivocally decide whether carvedilol scavenges free radicals already generated or whether it prevents radical formation [34].

Taking the above into consideration, the main objective of this study was to determine radical scavenging properties of selected β-adrenolytic drugs in reference to their ability to release NO in biological preparations. For our analysis we have chosen selective β_1-adrenolytics such as nebivolol, atenolol, metoprolol and non-selective β-adrenolytics with α1-receptor blocking properties such as carvedilol and labetalol. To elucidate mechanisms of the radical scavenging processes, we investigated a reactivity of this group of compounds with hydroxyl and peroxyl radicals. In addition we also investigated a reactivity of indole analog of carvedilol (carbazole group replaced by indole moiety) and methyl-2-hydroxypropylamine (HPMA), representing a central fragment of all investigated compounds that is considered to be responsible for their physiological interaction with the β-adrenoreceptors. On the other hand, NO-dependent coronary vasodilator activity of the selected β-adrenolytic drugs was assayed in the isolated guinea pig heart. We have shown previously that this model is suitable for the detection of the NO-dependent endothelial action of cardiovascular drugs [4,35,36].

Experimental

Compounds

The β-adrenolytics were obtained from Berlin-Chemie (nebivolol), Roche (carvedilol), or from Sigma-Aldrich (atenolol, metoprolol, labetalol). Indole analog of carvedilol was synthesized by Dr Grażyna Groszek from the Department of Chemistry, Rzeszow University of Technology and obtained as a gift. Other chemicals were commercially available from Sigma-Aldrich: KBr, NaN_3, KSCN, KH_2PO_4, Na_2HPO_4, ABTS (2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diamonium salt), PNBA (4-Nitrobenzoic acid). The aqueous solutions were prepared using water purified by a Millipore-Milli-Q system.

Synthesis of methyl-2-hydroxypropylamine

To a stirred aqueous solution (40%) of methylamine (50 g; 0.61 mole of $MeNH_2$) kept at −5°C propylene oxide (8.7 g; 0.15 mole) was added dropwise over three hours. The stirring was continued overnight at room temperature. The excess of methylamine and water were distilled off on the rotary evaporator and the residue was shaken with solid potassium carbonate, filtered, and extracted with ethyl ether. The extract was thoroughly dried over anhydrous $MgSO_4$ and fractionally distilled under reduced pressure to give 10.4 g (78%) of methyl-2-hydroxypropylamine as colorless liquid, b.p. 62–63°C/18 mm Hg. ^{1}H NMR (Brucker 250 MHz, D_2O) δ ppm: 1.15 (d, 3H, $J = 6.2$ Hz, CH_3-N), 2.31 (s, 3H, CH_3), 2.52 (d, 2H, $J = 6.0$ Hz, CH_2), 3.91 (m, 1H, CH–O).

Pulse radiolysis

The pulse radiolysis experiments were carried out with high energy (6 MeV) electron pulses (2–7 ns) generated from ELU-6 linear electron accelerator. The dose absorbed per pulse was determined with N_2O

saturated aqueous solution of KSCN (0.01 M), assuming $G((SCN)_2^{\cdot-}) = 6.0$ and $\varepsilon((SCN)_2^{\cdot-}) = 7600\,M^{-1}\,cm^{-1}$ (G represents yield of radicals per 100 eV of energy absorbed and ϵ is molar extinction coefficient at 475 nm) [37]. The dose delivered per pulse was within the range 2–9 Gy. The spectra of the transient products were obtained with the higher dose pulses, whereas the rate constants were determined with lower doses to minimize interference of radical–radical reactions. Details of the pulse radiolysis system are given elsewhere [38,39].

Pulse radiolysis of neutral water produces three highly reactive species: e_{aq} (2.6), $^{\cdot}OH$ (2.7), $H^{\cdot}$ (0.6) in addition to the formation of less reactive products: H_2O_2 (0.7), H_2 (0.45), H_3O^+(2.6) (numbers in parentheses are the G values determined 100 ns after an electron pulse) [40].

In order to study the reaction of β-adrenolytics (β-blockers) with the hydroxyl radicals the aqueous solutions were saturated with the N_2O in order to convert e_{aq} into hydroxyl radicals (reaction 1, $k = 8.7 \times 10^9 M^{-1}\,s^{-1}$) [41,42] and to remove oxygen

$$e_{aq} + N_2O \rightarrow {}^{\cdot}OH + OH^- + N_2 \quad (1)$$

$$\beta - \text{blocker} + {}^{\cdot}OH \rightarrow \text{products}. \quad (2)$$

Kinetic analysis was done with Levenberg–Marquardt algorithm. The first-order rate constant values (k_{obs}) were evaluated from the plot of ΔA vs. time. The bimolecular rate constants were determined from the slope of the linear plot of k_{obs} vs. solute concentration. Alternatively to the direct observation of the reaction of β-adrenolytics with hydroxyl radical based on the products build-up (reaction 2), the rate constant of this reaction (k_2) was determined by the competition kinetics methodology. The competition between reactions of hydroxyl radical with β-adrenolytics (reaction 2) and one of the standard hydroxyl radical scavengers like SCN^- (thiocyanate anion) $ABTS^{2-}$ 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate ion) or $PNBA^-$ (*p*-nitrobenzoate ion) (reactions 3–6) was monitored as a function of relative concentration of β-adrenolytic drug to competitor. The concentration of the competitor was kept constant throughout the experiment at the appropriate level to secure the scavenging of all hydroxyl radicals, even in the presence of minimal amounts of β-adrenolytics

$$SCN^- + {}^{\cdot}OH \rightarrow SCN^{\cdot} + OH^- \quad (3)$$

$$SCN^- + SCN^{\cdot} \rightarrow (SCN)_2^{\cdot-} \quad (4)$$

$$ABTS^{2-} + {}^{\cdot}OH^- \rightarrow ABTS^{\cdot-} \quad (5)$$

$$PNBA^- + {}^{\cdot}OH \rightarrow PNBA^- - OH^{\cdot}(\text{adduct}). \quad (6)$$

The products formed in the reactions 4–6 strongly absorb at:

$(SCN)_2^{\cdot-}$, $\lambda_{max} = 475$ nm (the rate constant of the formation of $(SCN)_2^{\cdot-}$, $k_3 = 1.1 \times 10^{10}\,M^{-1}\,s^{-1}$), $ABTS^{\cdot-}$, $\lambda_{max} = 415$ nm ($k_5 = 1.2 \times 10^{10}\,M^{-1}\,s^{-1}$), ($PNBA^- - OH^{\cdot}$), $\lambda_{max} = 420$ nm ($k_6 = 2.6 \times 10^9\,M^{-1}\,s^{-1}$),

and they do not overlap with absorption of the reaction products of primary interest [40].

In the competition kinetics approach the absorbance (A) of the product generated in the reaction with competitor (reactions 3–6) depends on the rate constants of reactions with both competitor and the studied β-adrenolytic drug assuming that both these species are in excess compare to the hydroxyl radical concentration:

$$\frac{A_0 - A}{A} = \frac{k_{\beta-\text{blocker}}}{k_{\text{competitor}}} \frac{[\beta - \text{blocker}]}{[\text{competitor}]} \quad (7)$$

where A_0 is the absorbance of product of the competitor reaction in the absence of β-adrenoreceptor antagonist. Therefore the reciprocal of the absorption A, at any moment of the reaction, should be a linear function of β-adrenolytic drug concentration:

$$\frac{1}{A} = \frac{1}{A_0} + \frac{1}{A_0} \frac{k_{\beta-\text{blocker}}}{k_{\text{competitor}}} \frac{[\beta - \text{blocker}]}{[\text{competitor}]}. \quad (8)$$

The concentrations of β-adrenolytic drug and competitor were chosen so that the reaction was completed during the pulse and the [β-blocker]/[competitor] relation varied over a suitable range.

The trichloromethylperoxyl radicals ($CCl_3O_2^{\cdot}$; $E^{\circ}(CCl_3O_2^{\cdot}/CCl_3O_2^-) \geq 1.0$ V) [42] were generated in the system containing 48% 2-propanol, 48% water and 4% carbon tetrachloride (v/v). In this solvent the trichloromethyl radicals ($CCl_3^{\cdot}$) are formed upon ionization as a major radiolysis product in reactions of CCl_4 with aqueous electron, hydrogen atoms and 2-propanol ketyl radicals (reactions 9–12).

$$CCl_4 + e_{aq} \rightarrow CCl_3^{\cdot} + Cl^- \quad (9)$$

$$CCl_4 + H^{\cdot} \rightarrow CCl_3^{\cdot} + H^+ + Cl^- \quad (10)$$

$$OH^{\cdot} + (CH_3)_2CHOH \rightarrow (CH_3)_2C^{\cdot}OH + H_2O \quad (11)$$

$$CCl_4 + (CH_3)_2C^{\cdot}OH \rightarrow CCl_3^{\cdot} + H^+ + Cl^- + (CH_3)_2CO \quad (12)$$

$$CCl_3^{\cdot} + O_2 \rightarrow CCl_3O_2^{\cdot}. \quad (13)$$

In dioxygen- or air-saturated solutions the trichloromethylperoxyl radicals (reaction 13) are formed rapidly and quantitatively ($G = 6.2 \pm 0.5$) [43,44]. The rates of their reaction with β-adrenolytics were determined by following the buildup of absorption of the products since the peroxyl radicals exihibit only weak absorption in UV region. Second-order rate constants were determined from the slopes of the first-order rates vs. solute concentrations.

In order to identify the products of one-electron oxidation of β-adrenolytics their reaction with strong oxidants like $N_3^{\cdot}$ radicals or dibromide radical anions $Br_2^{\cdot -}$ was studied. We have found that $Br_2^{\cdot -}$ ($E^{\circ}(Br_2^{\cdot -}/2Br^-) = 1.63$ V) [41,42] which is a stronger oxidant than $N_3^{\cdot}$($E^{\circ}(N_3^{\cdot}/N_3^-) = 1.33$ V) [41,42] is more suitable for that purpose as it allows to monitor the oxidation reaction both by the $Br_2^{\cdot -}$ decay and product formation (radical cation of β-adrenoceptor antagonist). Dibromide radical anions, $Br_2^{\cdot -}$, were generated by the pulse radiolysis carried out in the solution of KBr saturated with N_2O, so that the $^{\cdot}OH$ radicals react with bromide anions to form oxidizing species. The rate constants of the reactions of dibromide radical anions with β-adrenolytics were determined on the basis of $Br_2^{\cdot -}$ decay at 360 nm [41,42]

$$Br^- + {}^{\cdot}OH \rightarrow {}^{\cdot}Br + OH^- \qquad (14)$$

$$^{\cdot}Br + Br^- \rightarrow Br_2^{\cdot -}. \qquad (15)$$

In some cases, when dibromide radical anion absorption interfered with the weak absorption of the products, $N_3^{\cdot}$ radicals were used as oxidizing species. Pulse radiolysis of aqueous solution of NaN_3 leads to the formation of $N_3^{\cdot}$ radicals in reaction of $^{\cdot}OH$ radicals with N_3^-

$$N_3^- + {}^{\cdot}OH \rightarrow N_3^{\cdot} + OH^-. \qquad (16)$$

Quantum chemical calculations

The geometries of all species were optimized by the B3LYP density functional method [45,46] as implemented in the Gaussian 03 suite of programs [47]. The above calculations were done at the B3LYP/6-31G* level. Relative energies were calculated at the same level including ZPE correction.

Bioassay of NO-dependent coronary vasodilation in the isolated guinea pig heart

The details of the method were described elsewhere [48]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and the experimental procedures used in the present study were approved by the local Animal Research Committee.

Briefly, guinea pigs of both sexes and body weight of 300–400 g G were anaesthetised with pentobarbital (30–40 mg kg^{-1} body weight). Their hearts were isolated, washed in ice-cold saline, and mounted in Langendorff apparatus of Hugo Sachs Electronics (HSE). Guinea pig hearts were perfused retrogradely through aorta under a constant perfusion pressure of 60 mm Hg with Krebs-Henseleit buffer of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.64, $NaHCO_3$ 24.88, K_2PO_4 1.18, glucose 5.55, sodium pyruvate 2.0, equilibrated with 95% O_2 + 5% CO_2 at 37°C in the oxygenator with rotating disc (HSE). The hearts were paced with 273 impulses per min through two platinum electrodes placed in the right atrium. Left ventricular pressure (LVP) was measured using the fluid-filled balloon inserted into the left ventricle and connected to a pressure transducer (Isotec HSE). The end diastolic pressure was adjusted to be less than 10 mm Hg. The dP/dt_{max} and dP/dt_{min} values were calculated from LVP signal by an analogue differentiation amplifier (DIF module HSE). Coronary flow was monitored by ultrasonic flowmeter (HSE). LVP, dP/dt_{max}, dP/dt_{min} and coronary flow were calibrated once a day before the experiment and then continuously displayed throughout the experiment and finally analysed using the specially-designed software (PSCF. EXE-IGEL, Poland). Before experiments hearts were equilibrated for 30 min to reach the steady-state conditions.

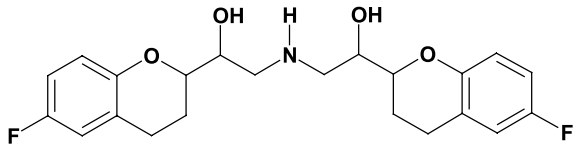
For studying coronary vasodilator responses β-adrenolytics were given as 1 min intracoronary infusions at the final concentration of 1–30 μM. In control experiments in absence of inhibitors the coronary vasodilator responses of selected β-adrenolytics were reproducible (data not shown). The involvement of endothelium-derived NO in coronary vasodilator responses to β-adrenolytics were assessed by pre-treatment with NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME) (100 μM). β-adrenolytics with vasodilator properties were infused twice: in the absence and in the presence of L-NAME, which was administered at least 20 min prior to infusion of a vasoactive substance.

Nebivolol, carvedilol and indole analogue of carvedilol were dissolved in the mixture of DMSO and water (v/v 1: 1), metoprolol, atenolol and labetalol were dissolved in water. All these compounds were infused into the coronary circulation at a rate of 0.03–0.09 ml min^{-1}. The rate of infusion was adjusted to the value of basal coronary flow. Infusion of vehicle slightly increased coronary flow by 1.07 ± 0.16 ml/min.

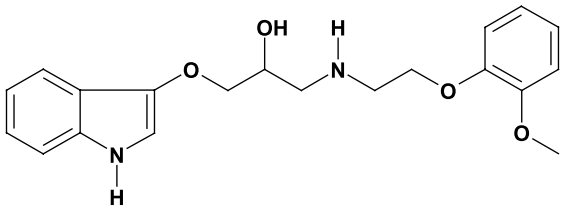
Duration of an experiment never exceeded three hours, up to this period of time quality of preparation of isolated guinea pig heart stayed unchanged.

Results and discussion

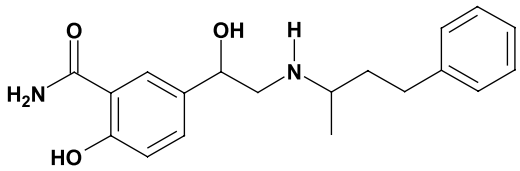
The following β-adrenoceptor antagonists were investigated:



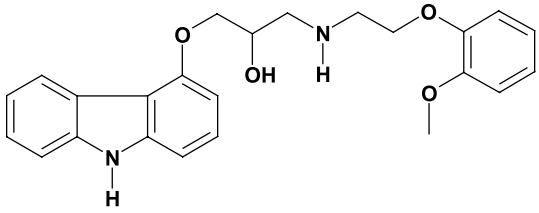
Nebivolol



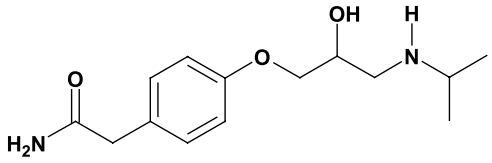
Indole analog of carvedilol



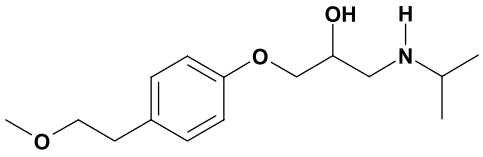
Labetalol



Carvedilol



Atenolol



Metoprolol

Radical scavenging

As the pulse radiolysis technique allows for the direct determination of the rate constants with ROS [49,50] this method was used in order to obtain the rate constants for the reactions of hydroxyl and peroxyl radicals with the β-adrenolytics.

Figures 1 and 2 present the transient absorption spectra obtained upon electron pulse irradiation of N_2O saturated aqueous solutions of six different β-adrenolytics at neutral pH (7.4–7.6). The reactivity of all investigated compounds with the hydroxyl radical which is the major reactive species under those experimental conditions is very high. However, because of the poor solubility of some β-adrenolytics in aqueous solutions direct monitoring of the kinetics of these reactions, based on the product build-up, could lead to erroneous evaluation of their rate constants. Therefore the competition kinetics was used to determine rate constants for the reactions of investigated β-adrenolytics with hydroxyl radical. Three different standard hydroxyl radical scavengers like SCN^- (thiocyanate anion), $ABTS^{2-}$ (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate ion) and $PNBA^-$ (p-nitrobenzoate ion) (see reactions 3–6 in Experimental Section) were used as β-adrenolytics competitors for scavenging of hydroxyl radicals. By monitoring the strong absorptions (A) characteristic for the reaction products of the competitors applied with hydroxyl radicals and their disappearance with the increasing concentration of β-adrenolytics the second order rate constant for the scavenging of hydroxyl radicals by β-adrenolytics can be found. Insets to Figures 1 and 2 present the dependence of $1/A$ vs. the ratio of the concentrations of the β-adrenolytic drug and competitor used in the experiment. Table I summarizes the estimated rate constants.

Because of the low solubility of carvedilol and large uncertainty of the obtained results (see inset to the Figure 1B) its indole analog was also investigated.

The satisfactory agreement can be noticed among the results obtained with different competitors, which eliminates errors arising from possible secondary reactions with some competitors. Such a case was evidently observed in the system: labetalol, $ABTS^{2-}$ and hydroxyl radical where the $ABTS^{\cdot-}$ (the product of the reaction of $ABTS^{2-}$ with the $^{\cdot}OH$) was also formed in the reaction of $ABTS^{2-}$ with the product of the reaction of labetalol with the $^{\cdot}OH$.

The results presented above suggest that all the investigated compounds possess very good scavenging properties towards hydroxyl radical. It seems therefore interesting to determine whether it can be assigned to the functional hydroxypropylamine group common for all β-adrenoreceptors or it is a result of the presence of aromatic fragments in these molecules. It is known that hydroxyl radical can react with organic molecules through one-electron transfer, hydrogen abstraction or addition to unsaturated bonds. Therefore, the side aromatic fragments of the compounds could be responsible for the formation of adducts with hydroxyl radicals while the central 2-hydroxypropylamine

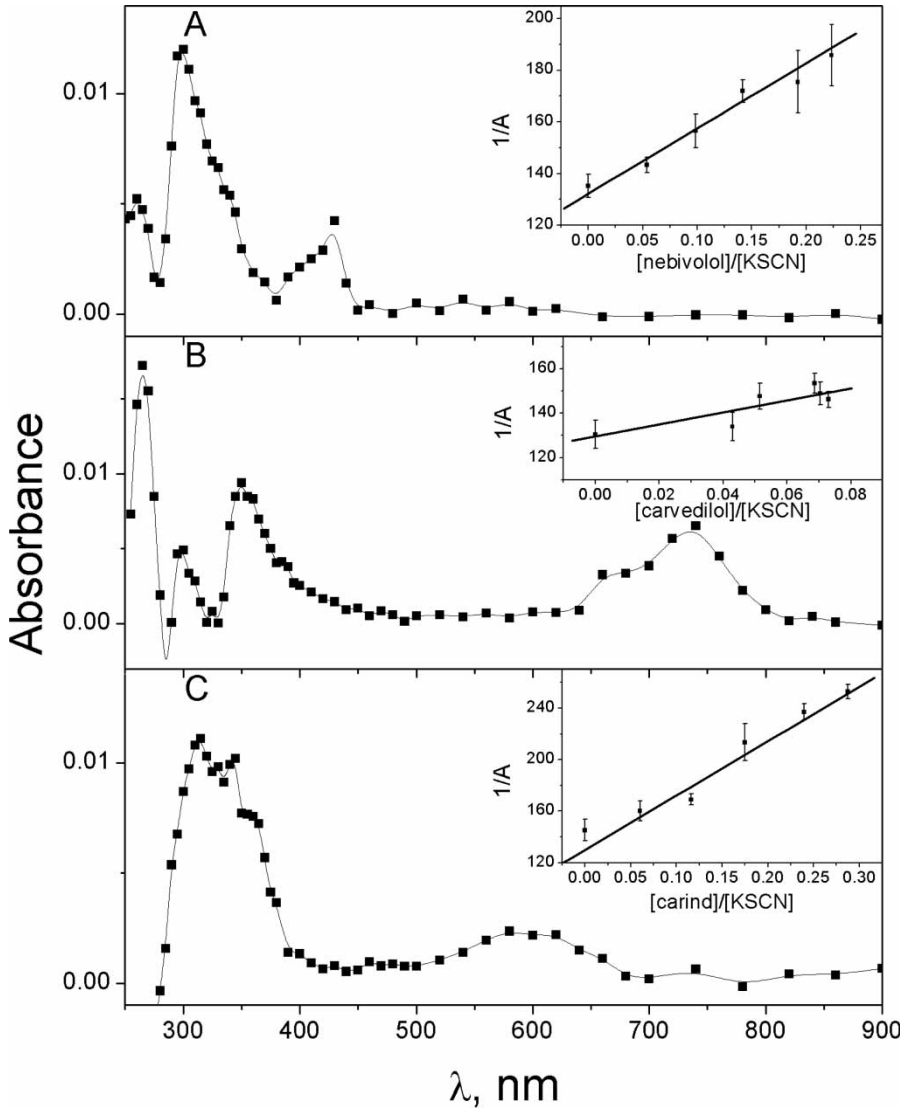
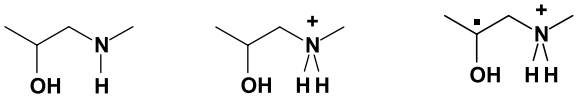


Figure 1. Transient absorption spectra of products of the reaction of β-adrenolytics with hydroxyl radicals. Spectra obtained 20 μs after the pulse radiolysis of β-adrenolytics aqueous solution containing 5 mM phosphate buffer (pH 7.4–7.6) and saturated with N_2O. Inset: Dependence of the reciprocal of competitor absorbance vs. β-adrenolytic drug to competitor concentration ratio. A—nebivolol [saturated solution]; B—carvedilol [saturated solution]; C—indole analog of carvedilol [0.05 mM]. Radiation dose: A—5 Gy, B, C—9 Gy. Optical path—10 mm.

fragment of the molecule could be responsible for the hydrogen atom abstraction. One-electron oxidation could take place from the aromatic side rings or central amine group. As most of the investigated β-adrenolytics, except carvedilol ($pK_a = 7.96$), had similar pK_a values above 9, they all occur in the protonated form (protonation of the amine fragment) under the experimental conditions. The lower pK_a value of carvedilol was attributed to the inductive effect of the β-O-atom which lowers the basicity of the amino group [51]. Therefore, one-electron oxidation of this fragment is less likely although the hydrogen atom abstraction could be still possible [52]. According to B3LYP/6-31G* calculations the abstraction could take place at the β carbon atom of hydroxypropyl group. The bond dissociation energy, calculated as a difference in the relative energies of formed ketyl radical and hydrogen atom minus that for the species before hydrogen atom abstraction (i.e. protonated HPMA) is on the order of 88.5 kcal/mol. The reaction leading to the ketyl radicals is characteristic for the reaction of hydroxyl radicals with alcohols. Quite stable ketyl radicals are also formed upon the protonation of radical anions of some aromatic ketons [53,54].



HPMA protonated HPMA ketyl radical generated from protonated HPMA

As a model representing the central fragment of the β-adrenolytics we investigated the reactivity of

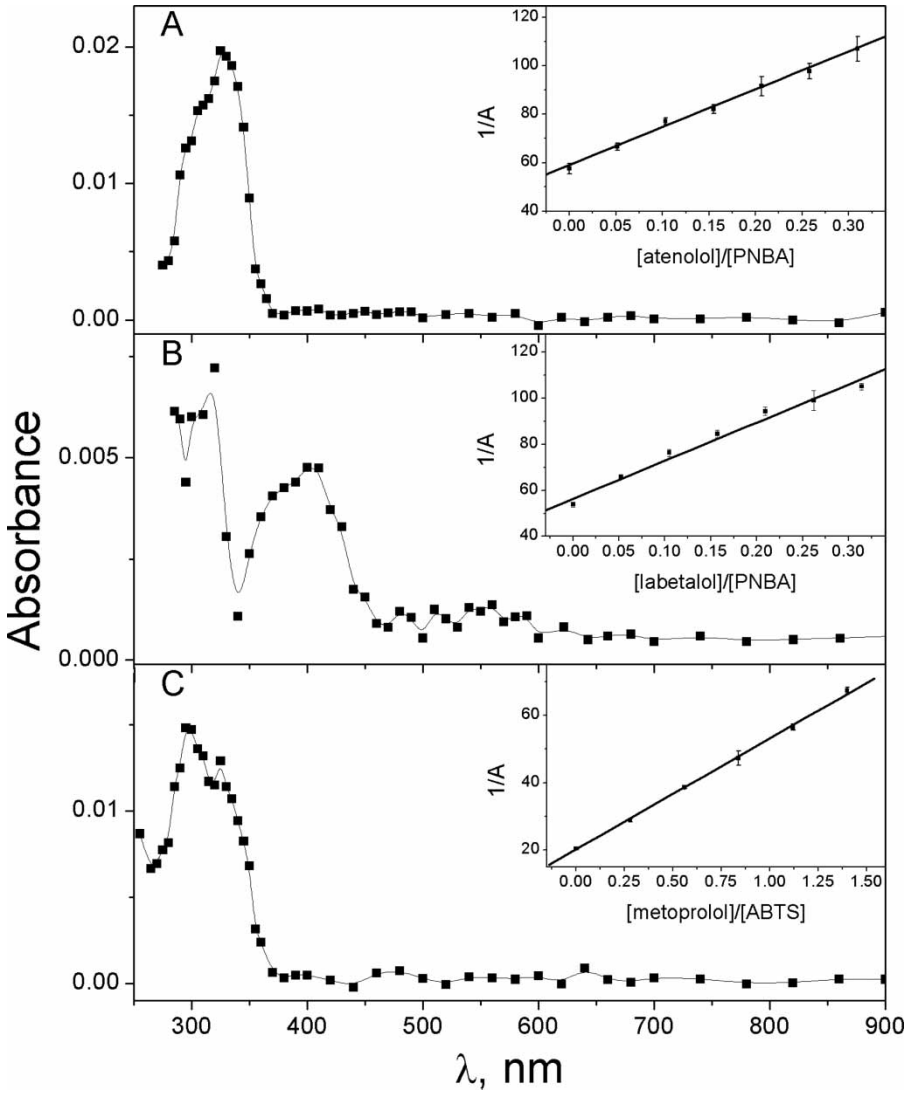


Figure 2. Transient absorption spectra of products of the reaction of β-adrenolytics with hydroxyl radicals. Spectra obtained 20 μs after the pulse radiolysis of β-adrenolytics aqueous solution containing 5 mM phosphate buffer (pH 7.4–7.6) and saturated with N_2O. Inset: dependence of the reciprocal of competitor absorbance vs. β-adrenolytic drug to competitor concentration ratio. A—atenolol [0.2 mM]; B—labetalol [0.2 mM]; C—metoprolol [0.2 mM]. Radiation dose: A, B—10 Gy, C—6 Gy. Optical path—10 mm.

the methyl-2-hydroxypropylamine, HPMA. This compound also occurs in protonated form under the experimental conditions ($pK_a = 10.2$). The rate constant for the reaction of this amine with hydroxyl radical was determined by the competition kinetics methods and was found to be one-order lower ($k = 9 \times 10^8\,M^{-1}\,s^{-1}$), than that found for β-adrenolytics themselves. Therefore, the scavenging properties of the central fragment of the molecules cannot explain high reactivity of these pharmaceuticals towards hydroxyl radicals. It may be concluded that capabilities of β-adrenolytics to act as hydrogen atom donors are rather small. Moreover, this implies that the central fragment of the investigated species, responsible for their β-receptor antagonism, is not modulated by the reaction with hydroxyl radicals.

It is suggested that most of the antioxidant activity of carvedilol is provided by the carbazole moiety, which by itself, can actively inhibit peroxidation [23,55]. It is very likely that carbazole moiety plays also a key role in the hydroxyl radical scavenging process. It has been shown previously for indole, tryptophan and their derivatives that the primary reaction between the hydroxyl radical and those aromatic systems is an addition [56,57]. In some cases, however (low ionization potential of the aromatic fragment, acidic environment) an adduct formation may be followed by the formation of the radical cation of the species (direct one-electron oxidation or acid-catalyzed dehydration). It is evident from the spectra presented in Figure 1B,C that reaction of hydroxyl radicals with carvedilol and its indole analog leads to the products, which possess absorption bands in the visible region of the spectrum characteristic for the radical cations of carbazole and indole [53,58–60]. Similar spectra were obtained upon direct one-electron oxidation of these two

Table I. Rate constants for the reactions of β-adrenolytics with hydroxyl radicals and dibromide radical anions.

Compound	k (β-adrenolytic drug+$^{\cdot}OH$) ($M^{-1}s^{-1}$)	k (β-adrenolytic drug+$Br_2^{\cdot-}$) ($M^{-1}s^{-1}$)
Nebivolol	$(1.9 \pm 0.2)10^{10}$⋆	$(7 \pm 1)10^{7}$
Carvedilol	$(2.4 \pm 0.5)10^{10}$⋆	$(5 \pm 1)10^{8}$
Indole analog of carvedilol	$(3.2 \pm 0.2)10^{10}$⋆	
Atenolol	$(6.9 \pm 0.4)10^{9}$⋆; $(9.4 \pm 0.9)10^{9}$†; $(7.0 \pm 0.3)10^{9}$‡	$(1.3 \pm 0.2)10^{6}$
Labetalol	$(1.9 \pm 0.2)10^{10}$⋆; $(1.1 \pm 0.07)10^{10}$‡	$(2.7 \pm 0.1)10^{6}$
Metoprolol	$(1.8 \pm 0.06)10^{10}$†; $(1.9 \pm 0.1)10^{10}$‡	$(6.1 \pm 0.2)10^{6}$
HPMA	$(9 \pm 1)10^{8}$⋆	

Competition reaction with⋆ SCN^-.† $ABTS^{2-}$.‡ $PNBA^-$.

β-adrenolytics in reactions with azide radical or bromide radical anion and are presented in Figure 3.

In case of other β-adrenolytics the spectra obtained by direct one-electron oxidation of those compounds possess less characteristic absorption bands, mainly in the UV region of the spectrum, but we can conclude that one-electron oxidation is not the major pathway of their reactivity. However, similar high rate constants found for the reactions of hydroxyl radicals with all investigated β-adrenolytics may indicate quite similar mechanism of these reactions, probably through the initial adduct formation. Subsequent formation of the radical cations in some cases (carvedilol and its indole analog) can effectively mask process of initial adduct formation.

Similar results were obtained for the reaction of trichloromethylperoxyl radical ($CCl_3O_2^{\cdot}$) with investigated β-adrenolytics. Due to its strong oxidative properties this radical shows high reactivity towards organic and biological materials and therefore allows

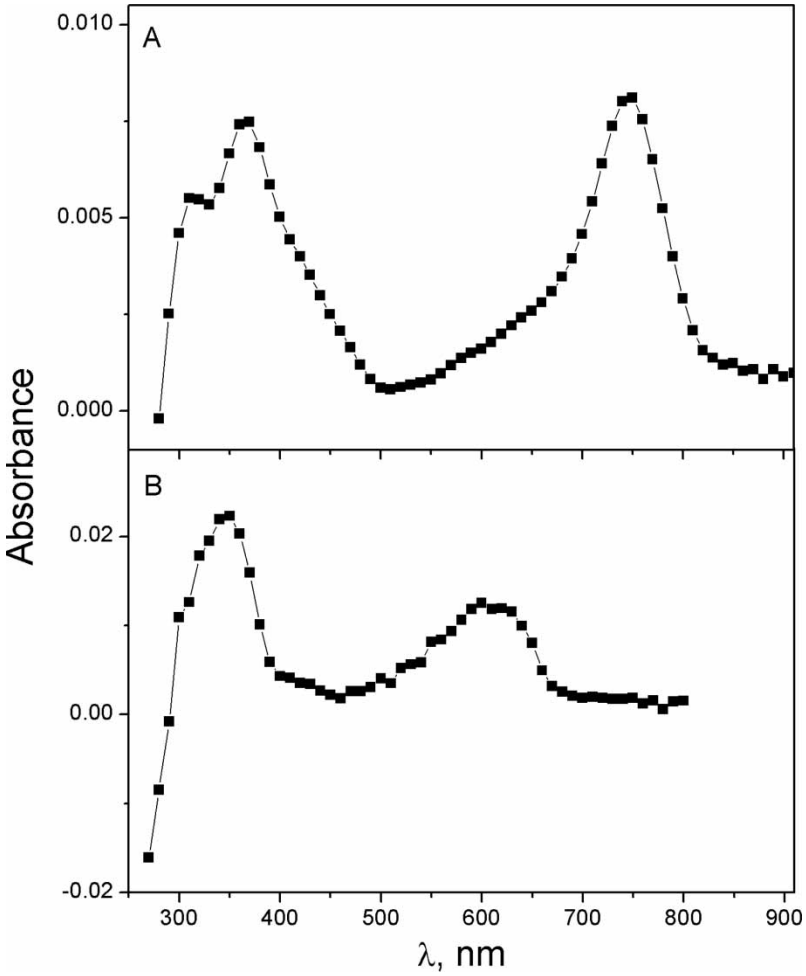


Figure 3. Spectra of the transient products of one-electron oxidation of β-adrenolytics by $N_3^{\cdot}$ radicals. Spectra obtained upon pulse radiolysis of N_2O saturated aqueous solutions of NaN_3 [50 mM] and A—carvedilol [< 0.1 mM] or B—indole analog of carvedilol [< 0.1 mM]. Spectra collected 20 μs after the electron pulse. Radiation dose-80 Gy. Optical path—10 mm.

to estimate the upper limits of rate constants of the reactions with peroxyl radicals. Among the investigated β-adrenolytics the fastest reaction was observed for carvedilol. Peroxyl radicals react with organic compounds by a mechanism involving one-electron oxidation or hydrogen abstraction. In the case of carvedilol the reaction unambiguously involved an oxidation mechanism since the same product was formed as that observed in the reaction with hydroxyl radical or strong one-electron oxidants (Figures 1B and 3A). The oxidation of carvedilol remained, however, not very rapid ($k = 1.3 \times 10^7\,M^{-1}\,s^{-1}$) as compared to the known examples of the reactivity of this peroxyl radical with organic, easy oxidized compounds, approaching $1 \times 10^9\,M^{-1}\,s^{-1}$ [43,44,61]. The mechanism of the reaction of this species with other β-adrenolytics may be different as the formation of one-electron oxidation products was not observed. In fact, in the group of investigated β-adrenolytics carvedilol remained the most easily ionized compound. The rate constant for the reaction of carvedilol with dibromide radical anion was one/two orders of magnitude higher than for the rest of the drugs studied (Table I).

NO release

In the isolated guinea pig heart basal coronary flow was $9.54 \pm 0.41\,ml\,min^{-1}(n = 33)$. Nebivolol and carvedilol increased coronary flow in a concentration-dependent manner while atenolol, labetalol, metoprolol, did not cause vasodilation but slightly reduced coronary flow (Figure 4). Moreover, indole analogue of carvedilol also did not cause coronary vasodilation. The comparison of vasoactive properties of selected β-adrenolytics given at a concentration of 10^{-5} M is shown in Figure 4C. In the presence of NOS inhibitor, L-NAME (10^{-4} M) coronary vasodilator responses to nebivolol and carvedilol were substantially inhibited (by 50–75%). Endothelium-dependent response induced by acetylcholine (10^{-7} M) or by bradykinin ($3x10^{-9}$ M) was inhibited by L-NAME to a similar degree (65–75%), while vasodilation induced by NO donor, S-nitroso-N-acetylpenicillamine (SNAP) (10^{-7} M) was not affected (data not shown). In contrast to L-NAME, indomethacin did not change coronary vasodilator response induced by nebivolol, carvedilol, acetylcholine or bradykinin. These results clearly indicate that among cardioselective β-adrenolytics that we tested, nebivolol but not atenolol or metoprolol is endowed with endothelial action. In turn, among non-selective β/α1-adrenolytics carvedilol but not its indole analogue or labetalol is able to release NO from coronary endothelium. Our results demonstrating the unique properties of nebivolol and carvedilol to stimulate production of NO from coronary endothelium seem to go in line with clinical studies showing

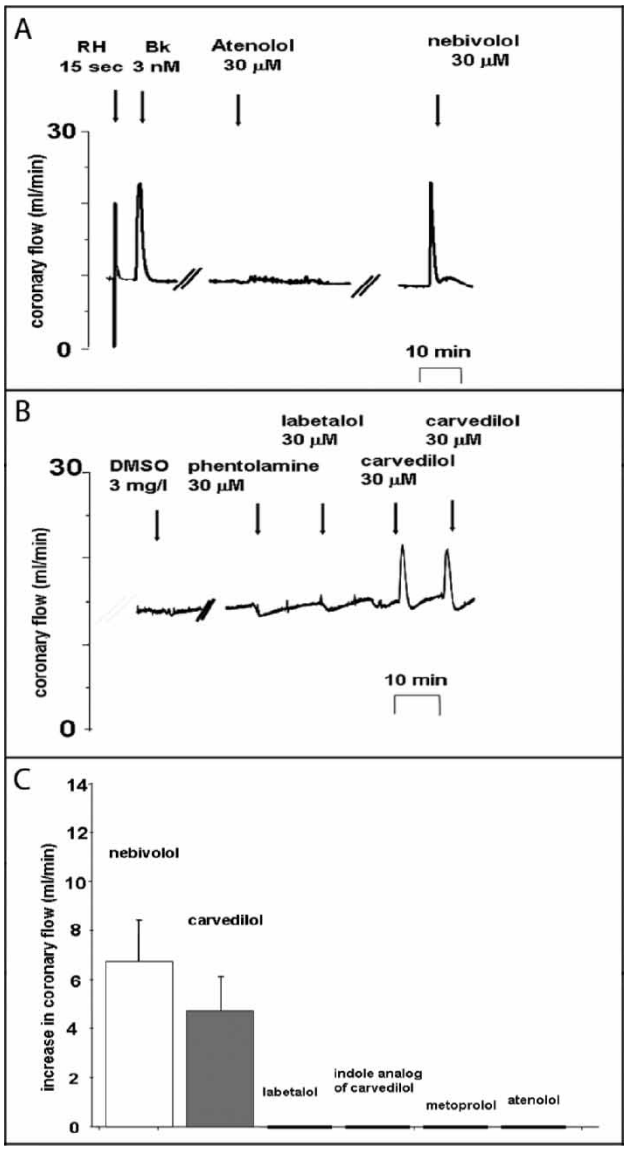


Figure 4. Comparison of vasoactive properties of selected β-adrenolytics in the isolated guinea pig heart perfused according to Langendorff technique. A—original tracings of the experiments showing coronary vasodilation induced by nebivolol in comparison with atenolol and classical endothelium-dependent responses. B—original tracings of the experiment showing coronary vasodilation induced by carvedilol in comparison with labetalol. Non-selective α-antagonist (phentolamine) or α_1-antagonist prozosin (not shown) induced a transient vasoconstriction similarly to labetalol. C—summarized data of vasoactive responses induced by various β-adrenoceptor antagonists given at 10 μM concentration.

the ability of nebivolol and carvedilol, but not atenolol [7,8,10] to improve NO-dependent endothelial function.

Conclusions

On the basis of our results we can exclude that direct ROS-scavenging activity of nebivolol or carvedilol contributes to their unique benefits reported in clinical trials [62–65] and cited above experimental

studies. Indeed, beneficial endothelial action including the release of NO from endothelium, characteristic for nebivolol or carvedilol is not shared by other β-adrenolytics such as atenol, metoprolol or labetalol. Furthermore, we revealed that carvedilol (and its indole analog) undergoes one-electron oxidation in contrast to other β-adrenolytics, including nebivolol. One could expect that formation of radical ions of carvedilol and its indole analog should differentiate the biological properties of these compounds from those which scavenge the radicals through a more stable adduct formation or hydrogen abstraction. Apparently, this is not the case as carvedilol and nebivolol possess similar ancillary properties.

From the perspectives of free radical chemistry, a positive charge distribution in radical cation of carvedilol would affect its physiological interactions with biological tissue to different extent than the interactions of neutral radicals of the remaining β-adrenolytics. Moreover, a different reactivity of the species formed could be expected. Most likely, the radical cations would be neutralized through back electron transfer or deprotonation, while radical species will still possess a free radical nature although less reactive than the hydroxyl or peroxyl radical themselves.

In contrast NO-releasing properties of nebivolol and carvedilol shown here and previously [2,4–6,11] distinguish third generation β-adrenolytics from their older counterparts. Still the mechanism of NO-release by these drugs and normalization of NO/cGMP/sGC signaling in vascular remains to be determined [29,66].

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