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Radical-scavenging and iron-chelating properties of carvedilol, an antihypertensive drug with antioxidative activity

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Abstract

Carvedilol, an antihypertensive agent, has been in clinical use for several years. In addition to its function as a β-blocker, carvedilol has been shown to act as an antioxidant. However, there is some controversy as to how carvedilol achieves its antioxidative ability: by radical scavenging or ion chelation? We therefore used a method of radical generation independent of metal ions to investigate the antioxidative properties of carvedilol. We showed that carvedilol decreased low-density lipoprotein (LDL) oxidation induced by a peroxyl radical-generating system [2,2′-azobis(2-amidinopropane)hydrochloride]. Formation of thiobarbituric acid-reactive substances, lipid hydroperoxides, and newly generated epitopes on oxidised LDL was used to monitor LDL oxidation. We further showed that carvedilol was consumed during reaction with peroxyl radicals. However, carvedilol showed no reaction with nitrogen-centered radicals (1,1-diphenyl-2-picrylhydrazyl and 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]), which are often used in assays for determining antioxidative properties. On the other hand, we found that carvedilol acted as a chelator of ferric ions. Using mass spectrometry and NMR spectroscopy, we observed complex formation with free and acetylacetonate-complexed ferric ions. The binding constant with Fe^{3+} was in the range of 10^5 L/mol. From our data, we concluded that carvedilol acts as both a metal chelator and a radical scavenger *in vitro*. However, it is selective in reacting with different radicals and is not an electron-donating radical scavenger as is α-tocopherol. Therefore, taking into account the low physiological concentration, the antioxidative properties reported earlier may not solely be explained by its radical-scavenging activity.

Keywords: Carvedilol; Antioxidant; Free radicals; Radical scavenger; Chelator; LDL

1. Introduction

Free radicals are thought to be involved in the development of a number of pathological conditions and diseases, especially of the cardiovascular system [1,2]. The radicals involved in those processes are mainly oxygen-centered radicals such as superoxide, hydroxyl, or peroxyl radicals. The administration of antioxidative drugs or natural products to treat or prevent such conditions is of great interest [3]. Thus, the characterisation of the antioxidant activities of these compounds is necessary and may be performed by a variety of methods [4–10]. There are differences in the mechanisms of antioxidative actions. Therefore, different methods to investigate antioxidative properties could give rise to quite different results [10].

Carvedilol is used as an antihypertensive drug. Besides being a β-adrenoceptor blocker, carvedilol has been reported to act as an antioxidant *in vitro* and *ex vivo* [11–19]. This antioxidative function would serve as an additional benefit given that carvedilol is used in the treatment of patients with hypertension, which is often accompanied by cardiovascular disorders. However, the mechanism of its antioxidative action is still unclear. In numerous works reporting the antioxidative behaviour of carvedilol, different systems for radical generation were used, including autoxidation of dihydroxyfumaric acid in the presence or absence of iron ions [14,15], ferrous iron with ascorbic acid [17],

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Abbreviations: ABTS, 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]; AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; DPPH, 1,1-diphenyl-2-picrylhydrazyl; IgG, immunoglobulin G; LDL, low-density lipoprotein; LPO, lipid peroxide; TBARS, thiobarbituric acid-reactive substance; and apo B, apolipoprotein B.

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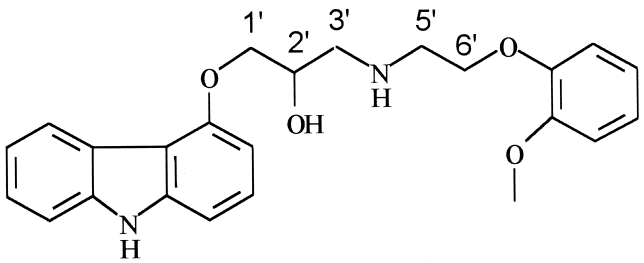

Fig. 1. Chemical structure of carvedilol.

stimulated macrophages [12,13], and enzymatic systems [17]. From these works, it is not possible to unequivocally decide whether carvedilol scavenges free radicals already generated or whether it prevents radical formation. While one group of researchers excluded formation of complexes with ferric ions [12], other groups reported ferric iron complexation by carvedilol [19,20].

In order to shed new light on these problems, we investigated the influence of carvedilol on LDL oxidation induced independently from metal ions as well as the iron-chelating properties of carvedilol.

2. Materials and methods

2.1. Materials

Carvedilol, (±)-1-[carbazoloyl-(4)-oxyl]-3-[2-methoxyphenoxy)] ethylamino-2-propanol, was kindly provided by Roche Diagnostics. Goat anti-rabbit IgG, goat anti-mouse IgG, BSA, probucol, Tween 20, DPPH, the Lowry protein test kit, and the non-ionic detergent Igepal CA-630 were purchased from Sigma Chemical Co. Aprotinin was from Bayer. Rabbit antiserum against human apolipoprotein B was from Behring AG, the Eu^{3+} labelling kit (DELFIA® No. 1244-302) was obtained from Wallac Oy and AAPH from Polysciences Europe. All other reagents were from Merck. Ferric acetylacetonate, $Fe(acac)_3$, was synthesised as described elsewhere [21].

2.2. Lipoprotein isolation

Lipoproteins were isolated from plasma of young healthy fasting donors, with serum lipoprotein (a) levels lower than 1 mg/dL as described [22]. Kallikrein inactivator (aprotinin; 100,000 U/L), Pefabloc (11.2 mg/L), and EDTA (1 g/L) were present during all steps of lipoprotein preparation to prevent lipid peroxidation and apo B cleavage by contaminating bacteria or proteinases. LDL (1.019–1.063 g/mL) was obtained by sequential ultracentrifugation by adjusting the density with KBr. LDL protein was measured by the method of Lowry *et al*. [23] with BSA as standard, pooled, and stored sterile-filtered at 4°.

2.3. Oxidation of LDL with AAPH

Pooled LDL from 3 donors (1:1:1; 1.65 mg/mL protein) was extensively dialysed in 10 mM phosphate buffer pH 7.4, which was carefully degassed and then saturated with nitrogen. Oxidation of native LDL was performed by incubating 0.3 mg/mL of LDL protein with 2 mM AAPH and 10 μM EDTA in the presence or absence of carvedilol at 37°. At intervals up to 24 hr, the reaction was stopped at 4°. Immediately thereafter, the amount of generated LPO [24] and TBARS [25] was estimated.

2.4. Solid-phase fluorescence immunoassay

To investigate the influence of carvedilol on the formation of oxidation-specific epitopes on AAPH-oxidised LDL, a binding assay was performed as described earlier [26]. In brief, microtitration plates were coated with 200 μL LDL (10 μg/mL) in 10 mM phosphate buffer (pH 7.4) containing 1 g/L of EDTA at 4° overnight. After washing three times with washing buffer (10 mM phosphate buffer, pH 7.4, containing 9 g/L of NaCl and 0.2 g/L of Tween 20) using a microtitration plate washer (Wallac Oy), 300 μL of blocking buffer (10 mM phosphate buffer, 1 g/L of EDTA, 10 g/L of BSA pH 7.4) was added to the wells to block the remaining binding sites for 90 min at 25°. The wells were then washed three times and 200 μL of OB/04 [27] (1:200 dilution) or rabbit anti-human apo B (1:10,000 dilution) in assay buffer pH 7.4 (10 mM phosphate buffer, 1 g/L of EDTA) was incubated for 1 hr at 25°. After three washes, the amount of bound antibodies was detected by adding 200 ng/mL of anti-mouse IgG or anti-rabbit IgG labelled with europium (DELFIA, Eu^{3+} labelling kit 1244-302, Wallac Oy) according to the manufacturer's description. After a 1-hr incubation at 25°, plates were washed six times and 200 μL of enhancement solution (Wallac Oy) per well was added to measure the fluorescence counts with a Victor fluorescence reader (Wallac Oy). The final result for oxidation-specific epitopes on AAPH-oxidised LDL was expressed as the ratio of OB/04 (counts)/apo B (counts).

2.5. Reaction of carvedilol with peroxyl radicals

Carvedilol (5 μM) was incubated in the presence of 20 mM AAPH in 50% methanol at 37° for up to 3 hr. At the times indicated, aliquots were taken and the carvedilol concentration was measured by HPLC. The HPLC system consisted of an ERC vacuum degasser, a Flux Rheos 4000 binary gradient pump, a pulse dampener, and a Jasco 821-FP fluorescence detector. Chromatography was achieved using a Bischoff 150 × 3-mm column filled with Prontosil 120-3-OH, 3-μm particles. Acetic acid (0.5%) in a mixture of water, acetonitrile, and methanol (4:1:5, v/v/v) with a flow rate of 400 μL/min was used as mobile phase. Carvedilol was detected by fluorescence with an excitation

wavelength of 285 nm and an emission wavelength of 340 nm [28].

2.6. Reactions of carvedilol with nitrogen-centered radicals

The radical-scavenging activity of carvedilol was tested using two different radical sources. The commercial testkit, Randox Total Antioxidant Status, is based on the formation of the radical cation of $ABTS^+$ and its scavenging by antioxidants [29,30]. The solutions of the testkit were prepared according to the instructions and contained chromogen, consisting of 6.1 μM metmyoglobin and 610 μM ABTS, substrate (250 μM hydrogen peroxide), and a standard of 1.6 mM Trolox. The assay was performed as a kinetic measurement of the absorbance at 620 nm up to 7 min [9,31]. In a cavity of a 96-well plate, 5 μL of sample and 250 μL chromogen solution were mixed. The reaction was started by adding 50 μL substrate. When a detergent was used, 2% Igepal was added to the chromogen solution. Carvedilol was added dissolved in ethanol. Ethanol had no effect on radical formation.

In a second system, the scavenging of DPPH was measured mixing 500 μL of a solution of carvedilol in ethanol with 100 μL of an ethanolic solution of DPPH (300 μM) [32,33]. After 20 min, the absorption at 515 nm was measured. Trolox was used as standard.

2.7. Mass spectrometry

All mass spectrometric analyses were performed on a Finnigan LCQ mass spectrometer with an electrospray ion source. Nitrogen was used as spray gas. The settings of the ion source were: capillary temperature, 200°; capillary voltage, 3 V; tube lens offset, 30 V; spray voltage, 3.5 kV. Samples dissolved in acetonitrile/water (9:1, v/v) were introduced via a syringe pump with a flow rate of 5 μL/min. In MS/MS experiments, the isolation width was set at 2 and collision energy at 18%.

2.8. NMR spectroscopy

All NMR experiments were carried out on a Varian Unity INOVA NMR spectrometer operating at a ^1H frequency of 600 MHz. A solution of 2 mM carvedilol in acetonitrile-d_3/H_2O (9:1, v/v) was titrated with a 60-mM solution of $FeCl_3$ in the same solvent. Suppression of the water signal was achieved by the WATERGATE sequence [34]. 1D and 2D TOCSY, 2D COSY, and 2D ROESY spectra were recorded on the sample before metal addition and at the end of the titration for resonance assignments. One hundred twenty-eight scans were accumulated for each 1D spectrum of 8K data points. All 2D experiments were acquired as matrices of 1024*512 complex points and zero filled to 2K*1K data points. A 60° phase-shifted squared sine bell window function was applied prior to Fourier transformation. Phase sensitivity was achieved by the time-proportional phase incrementation scheme in t_1 (TPPI) [35].

To obtain binding constants and lifetimes of free and complexing carvedilol, the concentrated $FeCl_3$ solution was added to final concentrations of 0.2, 0.4, 0.6, 0.8, 1, 1.5, and 2 mM Fe^{3+}. Due to the rapid chemical exchange between free and complexing carvedilol, the observed chemical shift (σ_{obs}) is an average of both. It is given by the equation

$$\sigma_{obs} = \frac{[C_{tot}] - [C_{Fe}]}{[C_{tot}]}\,\sigma_C + \frac{[C_{Fe}]}{[C_{tot}]}\,\sigma_{C_{Fe}} \quad (1)$$

where δ_C and $\delta_{C_{Fe}}$ are the chemical shifts of a certain signal in carvedilol in free and complexed form, $[C_{Fe}]$ is the concentration of complex formed, and $[C_{tot}]$ and $[Fe_{tot}]$ are the total concentrations of carvedilol and Fe^{3+}, respectively. The concentration of the complex depends on the binding constant (K_b) according to

$$[C_{Fe}] = 0.5\left([Fe_{tot}] + [C_{tot}] + \frac{1}{K_b}\right) - \sqrt{0.25\left([Fe_{tot}] + [C_{tot}] + \frac{1}{K_b}\right)^2 - [Fe_{tot}][C_{tot}]} \quad (2)$$

Since all parameters except the binding constant are known, the latter can be obtained by a non-linear one-parameter fit to Eqn 1, by substituting $[C_{Fe}]$ from Eqn 2.

2.9. Statistical analysis

A two-sided Student's *t*-test was performed for testing the significance of differences with $P < 0.001$ as the criterion for very high significance.

3. Results

LPOs and TBARS were estimated in LDL oxidised by 2 mM AAPH in the presence of 0, 100, and 200 μM carvedilol. Fig. 2a shows a time-dependent increase in LPO content in all incubations. In the presence of carvedilol, formation of LPO was significantly decreased from 6 hr on. The amount of LPO was reduced to 70% and 57% in the presence of 100 and 200 μM carvedilol, respectively, compared to AAPH-oxidised LDL without carvedilol. The effect was more pronounced in the 24-hr incubation: 54% and 33% of LPOs were formed.

The results of the TBARS assay (Fig. 2b) showed a similar pattern compared to the LPO assay: a time-dependent formation of TBARS in all incubations, although the onset was delayed compared to the formation of LPOs. From the 6-hr incubation on, the formation of TBARS was reduced in the presence of carvedilol: after 6 hr 100 and 200 μM carvedilol reduced TBARS release to 75% and 55%, respectively, compared to AAPH-oxidised LDL without

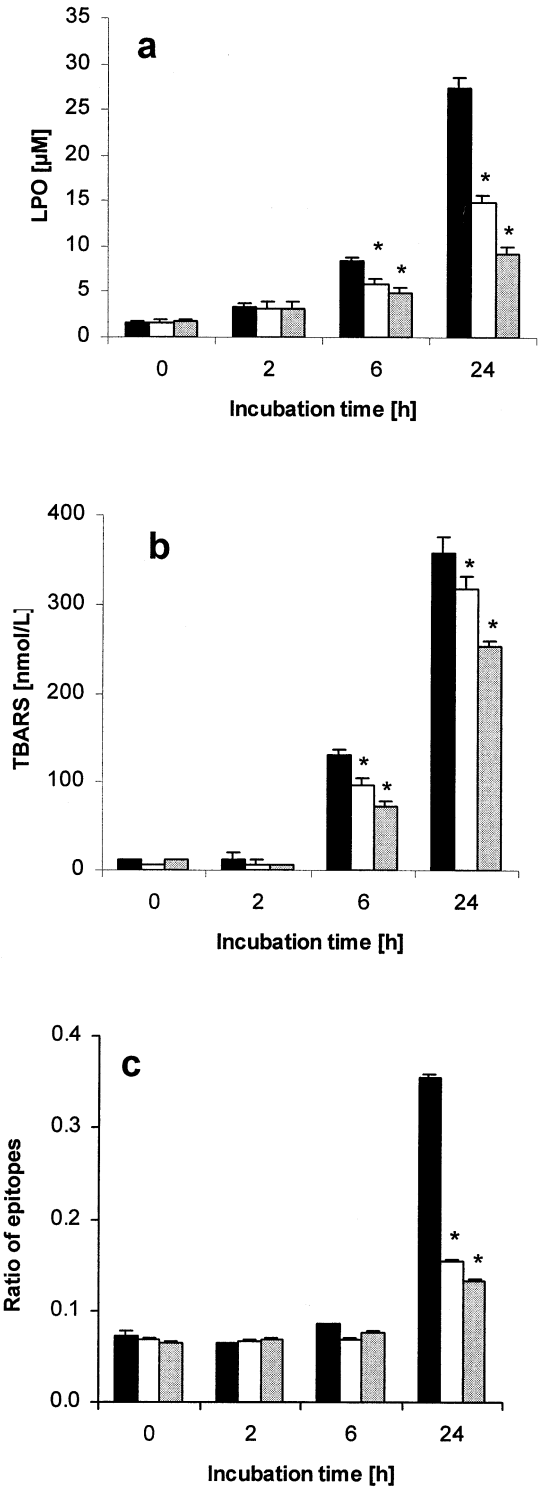

Fig. 2. Effect of carvedilol upon oxidation of LDL induced by AAPH during different time intervals. Pooled LDL (3 different donors; 1 + 1 + 1) was oxidised at 37° with 2 mM AAPH in the presence of 0 (closed bars), 100 μM (open bars), and 200 μM (grey bars) carvedilol. The degree of oxidation was recorded by measuring the formation of LPOs (a), the formation of TBARS (b), and the ratio of immunoreactivity against monoclonal antibodies OB/04 to polyclonal anti-apolipoprotein B (c). Values ± the range of two experiments performed in duplicate (a and b) or three experiments performed in triplicate (c) are given. Values marked with an asterisk differ very significantly from the corresponding control value without carvedilol ($P < 0.01$).

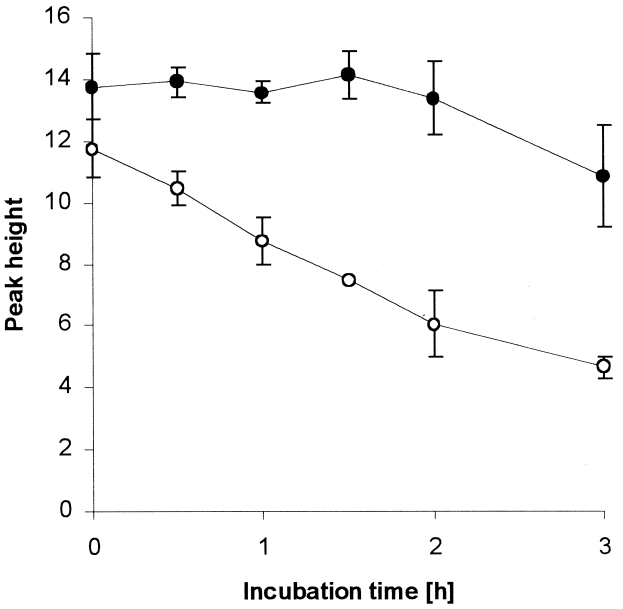

Fig. 3. Reaction of carvedilol with peroxyl radicals. Carvedilol (5 μM in 50% methanol) was incubated in the presence of 20 mM AAPH at 37°. At the times indicated, aliquots of the mixture were analysed by HPLC and the height of the carvedilol peak was determined. One assay was performed without (open circles) and another with (filled circles) purging the solutions with He. Values ± the range of two experiments are shown.

carvedilol. In the 24-hr incubation, carvedilol reduced the TBARS content to 89% and 71%, respectively.

A monoclonal antibody against oxidised LDL (OB/04) and a polyclonal antibody against apo B were used to characterise the protein part of LDL oxidised by 2 mM AAPH. As shown in Fig. 2c, the ratio of OB/04/apo B did not increase after a 2-hr incubation, and only slightly after 6 hr. After an incubation time of 24 hr, however, the ratio showed a pronounced increase in the absence of carvedilol. In the presence of 100 and 200 μM carvedilol, the increase in the ratio of OB/04/apo B was reduced to 43% and 37%, respectively, compared to the ratio obtained in the absence of carvedilol.

To determine whether carvedilol reacts with AAPH-derived radicals, 5 μM carvedilol was incubated in the presence of 20 mM AAPH at 37°. Carvedilol was attacked by peroxyl radicals and its concentration was reduced to 50% within a few hours (Fig. 3). However, α-tocopherol under the same conditions was consumed completely within the first 30 min (data not shown). Purging of the assay mixture with He before the addition of AAPH stabilised carvedilol by preventing peroxyl radical formation. On the other hand, carvedilol showed no effect at all in an assay using DPPH radicals in ethanolic solution or in a commercial test kit for measuring the antioxidant capacity. In the latter system, cation radicals of ABTS are formed from hydrogen peroxide and metmyoglobin. Carvedilol had no effect in the presence or absence of the non-ionic detergent Igepal, while α-tocopherol and Trolox showed radical scavenging in both cases. In the assay based on the decolorisa-

tion of DPPH, Trolox and α-tocopherol showed a concentration-dependent radical-scavenging activity. Again, carvedilol had no effect at all, up to a fourfold excess compared to DPPH (data not shown).

Using electrospray mass spectrometry, possible complex formation of carvedilol with iron ions was investigated. The acetylacetonate complex of ferric iron, $Fe(acac)_3$, gave ions of m/z 376 and 254 according to $[Fe(acac)_2]^+$ and $[Fe(acac)_3 + Na]^+$, respectively. Carvedilol showed two peaks of m/z 407 and 813 according to $[M + H]^+$ and $[2M + H]^+$, respectively. After mixing carvedilol with $Fe(acac)_3$, the peaks resulting from the latter disappeared and new peaks with m/z values of 560, 660, and 682 emerged (Fig. 4a). These peaks can be interpreted as $[Fe(acac)carvedilol^-]^+$ (I), $[Fe(acac)_2carvedilol]^+$ (II), and $[Fe(acac)_2carvedilol^- + Na]^+$ (III), respectively. In an MS/MS experiment, it was shown that II (m/z 660) upon fragmentation gave mainly I by dissociation of one-molecule acetylacetone, and a further fragment with m/z 407 (Fig. 4b). The latter daughter ion was confirmed to be carvedilol by MS^4 compared with the authentic substance. MS/MS of I (m/z 560) gave daughter ions with m/z 335 and 364. This fragmentation is in accordance with a complex formation within the side chain of carvedilol, including the hydroxyl group on C2′ and the nitrogen atom.

Using NMR spectroscopy, we found that the addition of Fe^{3+} to a solution of carvedilol led to significant changes in the chemical shifts of resonances in the side chain of carvedilol, and almost unchanged signals from the aromatic protons. In the side chain, the changes in chemical shifts were most pronounced around the NH group (Fig. 5). In addition to changes in the chemical shifts of side chain protons, two additional signals appeared around 8 ppm after the addition of about 0.5 equivalents of metal ions. These signals exchange with water protons, as found by recording 1D spectra after varying periods of saturation of the water signal. A sensitivity-enhanced 1D ^{15}N-^1H HSQC showed no peaks around 8 ppm, indicating that these two additional signals are caused by OH groups. As the relative intensity of these signals points towards two OH groups, we hypothesize that in the complex between carvedilol and Fe^{3+} at least two relatively slowly exchanging OH groups are coordinated to the iron.

Monitoring the chemical shifts in side chain protons as a function of the Fe^{3+} concentration led to a titration curve with a steep linear increase and sudden plateauing caused by a tight binding between iron and carvedilol (data not shown). In such a case, almost all iron that is brought into solution is bound to carvedilol until no more binding sites are available. The binding stoichiometry can then be easily extracted from this turning point, which is at about 0.5 equivalents of iron. A 1:2 (iron:carvedilol) complex is therefore most likely. The binding constant was obtained by least-squares fitting of Eqn 1 to the experimental titration curves of each side chain proton by the Mathematica® program (Wolfram Research, Inc.). The obtained binding constants averaged to give a value of 76,000 ± 13,000 L/mol.

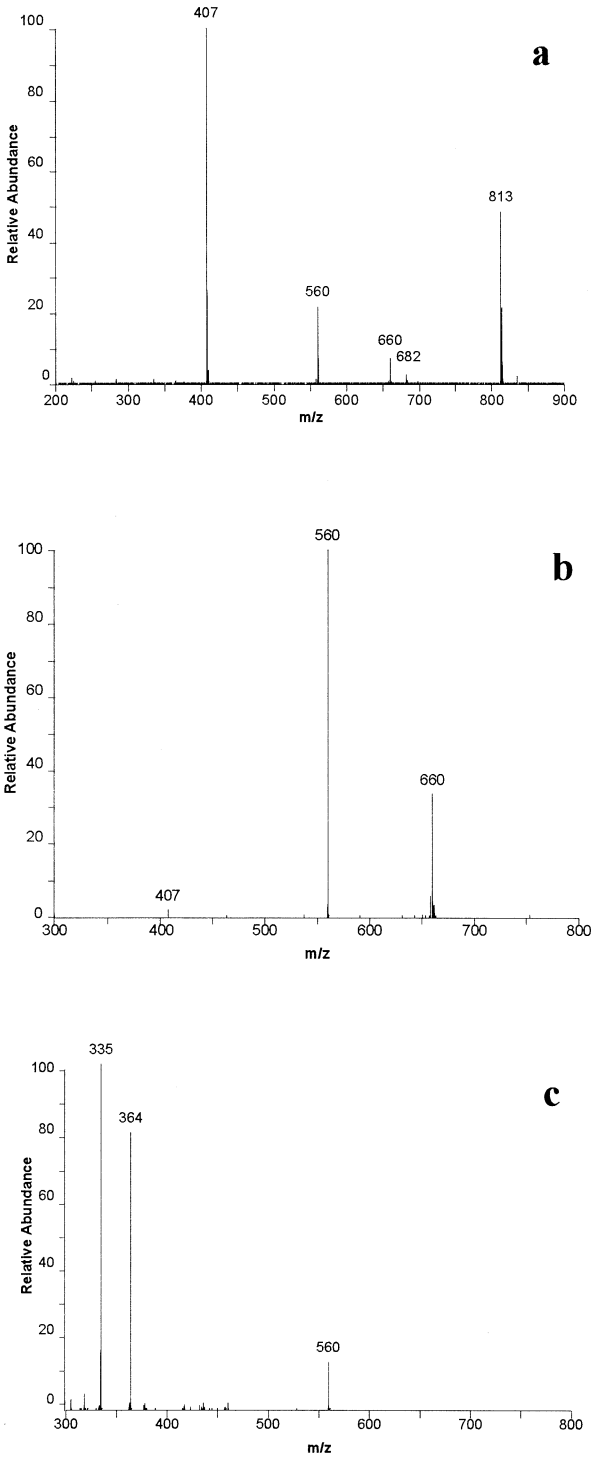

Fig. 4. Mass spectra of reaction products of carvedilol with $Fe(acac)_3$. Carvedilol (500 μM) was mixed with $Fe(acac)_3$ (100 μM) in acetonitrile/water 9:1 (v/v), and the mixture was introduced into an electrospray ion source by a syringe pump. The mass spectrum of the mixture (a) and the daughter spectra of the peaks with m/z 660 (b) and 560 (c) are shown.

Since just one signal was observed during the course of the titration, the lifetime of free and complexing carvedilol

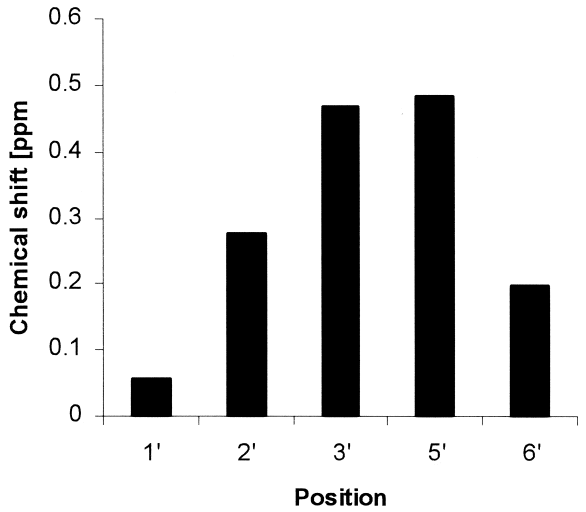

Fig. 5. Change in chemical shifts of side chain protons of carvedilol upon addition of Fe^{3+}. NMR spectra of carvedilol (1 mM in acetonitrile) were recorded before and after addition of 3 mM $FeCl_3$. The changes in the chemical shifts upon the addition of $FeCl_3$ are given.

must be shorter than the inverse frequency difference σ_C-$\sigma_{C_{Fe}}$ of the signal shifted most upon complex formation. As the protons at position H-4′ were shifted by about 150 Hz during the Fe^{3+} additions, the lifetime of free carvedilol must be less than about 0.007 sec.

4. Discussion

Carvedilol is a lipophilic agent whose most pronounced antioxidative effect has come to be expected in the lipid fraction of serum. *In vitro* oxidation of LDL can be stimulated by different methods. Two commonly used ways to initiate lipid peroxidation in LDL are the addition of transition metal ions such as copper or iron or incubation with macrophages in transition metal ions containing media. Both methods have been used to investigate the effect of carvedilol on LDL oxidation, with carvedilol showing an inhibitory effect in both cases [17]. However, in a recently published article, 5 and 10 μM carvedilol were not found sufficient to protect LDL against copper-mediated lipid peroxidation [20]. As these oxidation assays depend on the presence of metal ions, they are not able to distinguish between a radical-scavenging or an ion-chelating activity of the antioxidant under investigation. We therefore used an agent that acts independently from metal ions, generating peroxyl radicals for induction of LDL oxidation. Our results demonstrate for the first time an inhibitory effect of carvedilol on the generation of TBARS, lipid hydroperoxides, and epitopes against oxidised LDL. However, the concentration of carvedilol in our experiments was one order of magnitude higher than in other studies [20]. The effects of carvedilol on TBARS formation are similar whether copper ions or AAPH are used for initiation [11].

Formation of oxidation-specific epitopes on LDL appears delayed compared to the production of LPO and TBARS. In this formation, the effect of carvedilol is most pronounced. This might be due to a possible scavenging of the initial radicals that could directly damage the protein moiety of LDL and to a reduction in the formation of intermediates of lipid peroxidation, leading to conformational changes in or modifications of the protein moiety. Such a two-stage mechanism would likely be more effective compared to the scavenging of initial radicals alone. The reduction in epitopes is in agreement with a recent report where the autoantibody titer of antioxidatively modified LDL was found to be decreased in a group of hypertensive patients treated with carvedilol compared to a control group [36].

From our data, we may conclude that the inhibitory effect of carvedilol in high concentrations *in vitro* is due to a scavenging of free radicals. However, we cannot distinguish between scavenging of the initial radicals generated by AAPH and the scavenging of product radicals formed during lipid peroxidation, since both may occur. Carvedilol is consumed slowly by incubation with AAPH, but only in the presence of oxygen. Therefore, we conclude that carvedilol does not react with AAPH itself or with carbon-centred radicals generated from it in the first stage, but with the peroxyl radicals or hydroperoxides generated in the presence of oxygen [37,38]. In our experiments, the proposed radical scavenging activity of carvedilol was confirmed. However, the consumption of carvedilol occurs very slowly compared to α-tocopherol. Moreover, carvedilol does not scavenge the nitrogen-centred radicals DPPH and $ABTS^+$, and thus does not act as an electron-donating antioxidant as α-tocopherol does. The radicals DPPH and $ABTS^+$ are frequently used for the determination of the antioxidative properties of compounds or biological samples, but when they are used, an antioxidative property of carvedilol cannot be detected. At low physiological concentrations of carvedilol compared to the experimental conditions, a radical-scavenging effect *in vivo* is not very likely.

Another mechanism of antioxidative action, the complex formation of carvedilol with iron ions, has been investigated using photometric methods with conflicting results [12,19]. Using mass spectrometry, we found the formation of products mixing carvedilol with an acetylacetonate complex of ferric iron, with carvedilol displacing acetylacetonate from the complex. One of those products released carvedilol upon fragmentation. The fragmentation pattern of another allows us to conclude that complex formation occurs in the side chain of the molecule. Complex formation was confirmed and elucidated in detail by NMR spectroscopy. The changes in chemical shifts clearly indicate an involvement of the nitrogen atom in the side chain in complex formation. While the antioxidative activity of carvedilol was attributed to the carbazole moiety of the molecule [16], we differentiate between radical scavenging and complex formation. Radical scavenging is increased in the hydroxylated metabolite

of carvedilol [17] and may indeed be localised to the carbazole ring system. However, complex formation is clearly attributable to the side chain of carvedilol. The antioxidative properties found with metal-dependent methods may arise from both radical scavenging and metal chelation. We cannot attribute the major role to one of these two effects. As under physiological conditions free iron ions are not available, radical scavenging may be the relevant mechanism. The question is, are physiological concentrations of carvedilol sufficient for this effect, taking into account that the lipophilic compound may accumulate in LDL? However, under pathological conditions, iron ions may be released from their protein-bound form [1,39], and complex formation may then contribute to the antioxidative action of carvedilol. As only traces of iron ions are to be expected and these are complexed readily by carvedilol, the serum concentrations within reach (0.3 μM after a dose of 50 mg [13]) may be enough for a protective effect by iron chelation.

Acknowledgments

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References

[1] Halliwell B, Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 1984;219:1–14.
[2] Halliwell B, Gutteridge JM. Free radicals in biology and medicine. 2nd Edn. Oxford: Clarendon Press, 1989.
[3] McCall M, Frei B. Can vitamins materially reduce oxidative damage in humans? Free Radic Biol Med 1999;26:1034–53.
[4] Wayner DD, Burton GW, Ingold KU, Locke S. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins. FEBS Lett 1985;187:33–7.
[5] Whitehead TP, Thorpe GH, Maxwell SR. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. Anal Chim Acta 1992;266:265–77.
[6] Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med 1993;14:303–11.
[7] Ghiselli A, Serafini M, Maiani G, Azzini E, Ferro-Luzzi A. A fluorescence-based method for measuring total plasma antioxidant capability. Free Radic Biol Med 1995;18:29–36.
[8] Winston WW, Regoli F, Dugas AJ, Fong JH, Blanchard KA. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. Free Radic Biol Med 1998;24:480–93.
[9] Arnao MB, Cano A, Acosta M. Methods to measure the antioxidant activity in plant material. A comparative discussion. Free Rad Res 1999;31:S89–96.
[10] Regoli F, Winston GW. Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. Toxicol Appl Pharmacol 1999;156:96–105.
[11] Feuerstein GZ, Hamburger SA, Smith EF, Bril A, Ruffolo RR. Myocardial protection with carvedilol. J Cardiovasc Pharmacol 1992;19:138–41.
[12] Yue T-L, Cheng HY, Lysko PG, McKenna PJ, Feuerstein R, Gu JL, Lysko KA, Davis LL, Feuerstein G. Carvedilol, a new vasodilator and beta adrenoceptor antagonist, is an antioxidant and free radical scavenger. J Pharmacol Exp Ther 1992;263:92–8.
[13] Yue T-L, McKenna PJ, Lysko PG, Ruffolo RR, Feuerstein GZ. Carvedilol, a new antihypertensive, prevents oxidation of human low density lipoprotein by macrophages and copper. Atherosclerosis 1992;97:209–16.
[14] Yue T-L, McKenna PJ, Ruffolo RR, Feuerstein GZ. Carvedilol, a new β-adrenoceptor antagonist and vasodilator antihypertensive drug, inhibits superoxide release from human neutrophils. Eur J Pharmacol 1992;214:277–80.
[15] Yue T-L, McKenna PJ, Gu JL, Cheng HY, Ruffolo RR, Feuerstein GZ. Carvedilol, a new antihypertensive agent, prevents lipid peroxidation and oxidative injury to endothelial cells. Hypertension 1993;22:922–8.
[16] Feuerstein GZ, Yue T-L, Ruffolo RR. Carvedilol update: a multiple action antihypertensive agent with antioxidant activity and the potential for mycardial and vascular protection. Drugs Today 1993;29:401–19.
[17] Feuerstein R, Yue T-L. A potent antioxidant, SB209995, inhibits oxgen radical-mediated lipid peroxidation and cytotoxicity. Pharmacology 1994;48:385–91.
[18] Maggi E, Marchesi E, Covini D, Negro C, Perani G, Bellomo G. Protective effects of carvedilol, a vasodilating β-adrenoceptor blocker, against *in vivo* low density lipoprotein oxidation in essential hypertension. J Cardiovasc Pharmacol 1996;27:532–8.
[19] Tadolini B, Franconi F. Carvedilol inhibition of lipid peroxidation. A new antioxidative mechanism. Free Radic Res 1998;29:377–87.
[20] Noguchi N, Nishino K, Niki E. Antioxidant action of the antihypertensive drug, carvedilol, against lipid peroxidation. Biochem Pharmacol 2000;59:1069–76.
[21] Johnson A, Everett FW. A comparison of proton and deuteron nuclear magnetic resonance of some paramagnetic transition metal complexes. J Am Chem Soc 1972;94:1419–25.
[22] Jürgens G, Taddei-Peters WC, Költringer P, Petek W, Chen Q, Greilberger J, Macomber PF, Butman BT, Stead AG, Ransom JH. Lipoprotein(a) serum concentration and apolipoprotein(a) phenotype correlate with severity and presence of ischemic cerebrovascular disease. Stroke 1995;26:1841–8.
[23] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[24] el-Saadani M, Esterbauer H, el-Sayed M, Goher M, Nassar AY, Jürgens G. A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. J Lipid Res 1989;30:627–30.
[25] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1987;52:302–10.
[26] Greilberger J, Schmut O, Jürgens G. *In vitro* interactions of oxidatively modified low density lipoprotein with type I, II, III, IV, and V collagen, laminin, fibronectin, and poly-D-lysine. Arterioscler Thromb Vasc Biol 1997;17:2721–8.
[27] Hammer A, Kager G, Dohr G, Rabl H, Ghassempur I, Jürgens G. Generation, characterization, and histochemical application of monoclonal antibodies selectively recognizing oxidatively modified apoB-containing serum lipoproteins. Arterioscler Thromb Vasc Biol 1995;15:704–13.
[28] Reiff K. High-performance liquid chromatographic method for the determination of carvedilol and its desmethyl metabolite in body fluids. J Chromatogr 1987;413:355–62.
[29] Rice-Evans C, Miller NJ. Total antioxidant status in plasma and body fluids. Methods Enzymol 1994;234:279–93.
[30] Miller NJ, Rice-Evans C. Factors influencing the antioxidant activity determined by the $ABTS^{\cdot+}$ radical cation assay. Free Radic Res 1997;26:195–9.

[31] Strube M, Haenen GR, Van Den Berg H, Bast A. Pitfalls in a method for assessment of total antioxidant capacity. Free Radic Res 1997;26: 515–21.
[32] Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958;181:1199–200.
[33] Yamaguchi T, Takamura H, Matoba T, Terao J. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. Biosci Biotechnol Biochem 1998;62: 1201–4.
[34] Piotto M, Saudek V, Sklenar V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. J Biomol NMR 1992;2:661–5.
[35] Marion D, Wuthrich K. Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurements of 1H–1H spin–spin coupling constants in proteins. Biochem Biophys Res Commun 1983;113:967–74.
[36] Maggi E, Marchesi E, Ravetta V, Martignoni A, Finardi G, Bellomo G. Presence of autoantibodies against oxidatively modified lipoprotein in essential hypertension: a biochemical signature of an enhanced *in vivo* low-density lipoprotein oxidation. J Hypertens 1995;13:129–38.
[37] Barclay LR, Locke SJ, MacNeil JM, Van Kessel J, Burton GW, Ingold KU. Autoxidation of micelles and model membranes. Quantitative kinetic measurements can be made by using either water-soluble or lipid-soluble initiators with water-soluble or lipid-soluble chain-breaking antioxidants. J Am Chem Soc 1984;106: 2479–81.
[38] Niki E. Free radical initiators as source of water- or lipid-soluble peroxyl radicals. Methods Enzymol 1990;186:100–8.
[39] Bolann B, Ulvik RJ. Release of iron from ferritin by xanthine oxidase. Role of superoxide radical. Biochem J 1987;243:55–9.