Carvedilol, a new antihypertensive, prevents oxidation of human low density lipoprotein by macrophages and copper

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Summary

Growing evidence indicates that oxidized low-density lipoprotein (LDL) may promote atherogenesis. Therefore, inhibition of LDL oxidation may impede this process. Carvedilol is a vasodilating, β-adrenoceptor blocking agent. As a new antihypertensive drug, carvedilol is unique by virtue of its potent antioxidant activity. Therefore, we tested the ability of carvedilol to inhibit the oxidation of LDL by either macrophages or Cu²⁺. Carvedilol inhibited LDL oxidation by macrophages in a dose-dependent manner, with an IC₅₀ value of 3.8 μM, as assessed by a thiobarbituric acid reactive substance (TBARS) assay. Under the same conditions, propranolol showed only a mild inhibitory effect (IC₅₀ > 100 μM), while pindolol, atenolol and labetalol had almost no effect. Carvedilol, at 10 μM, almost completely inhibited the macrophage-induced increase in electrophoretic mobility of LDL, while other β-blockers at 50–300 μM had no significant effect. Carvedilol inhibited superoxide release from mouse macrophages, which correlated well with its inhibition of LDL oxidation. Carvedilol also inhibited Cu²⁺-induced LDL oxidation with an IC₅₀ value of 17 μM, while all other β-blockers were inactive up to 300 μM. These observations suggest that carvedilol might not only be an effective antihypertensive drug, but might also be effective in prevention of atherosclerosis.

Key words: Antioxidant; Atherogenesis; LDL; β-blocker

Introduction

Oxidation of low density lipoprotein (LDL) by endothelial cells, smooth muscle cells or monocytes/macrophages has been identified as a key step in the progress of atherogenesis [1]. Oxidized LDL could promote atherogenesis by direct
cytotoxicity, a chemotactic effect on monocytes, an inhibitory effect on macrophage motility, or initiation of foam cell formation by macrophages which take up oxidized LDL via their scavenger receptor [1,2]. The recent demonstration of the existence in vivo of oxidatively modified LDL in human and animal atherosclerotic plaques further supports this hypothesis [3,4]. Moreover, a protective effect of probucol against LDL oxidation has been implicated in the antiatherogenic effect of this drug [5,6]. Therefore, inhibition of LDL oxidation has been suggested as a novel approach to impede atherogenesis [7].

Carvedilol is a new vasodilator/β-adrenoceptor antagonist used for the treatment of mild to moderate hypertension [8]. More recently, carvedilol has been shown to reduce the extent of myocardial injury following coronary artery occlusion in several experimental models [9,10]. Interestingly, the capacity of carvedilol for cardioprotection exceeded that of propranolol given at equivalent β-blocking doses, suggesting that carvedilol might have additional cardioprotective action unrelated to β-adrenergic blockade. Recent studies from this laboratory have shown that carvedilol inhibits oxygen radical-mediated lipid peroxidation in swine ventricular membranes with potency similar to the 21-aminosteroid antioxidant, U 74500A [11], and scavenges superoxide released from human neutrophils [12]. Therefore, the present study was designed to assess the effect of carvedilol on the oxidation of human LDL by mouse macrophages and copper ions, as estimated by measuring the formation of TBARS [7,13] and electrophoretic mobility [14].

Materials and Methods

Materials

Drugs and other reagents were purchased from the following sources: Carvedilol (SKF 105517, racemic form) from Boehringer Mannheim (Germany) or SmithKline Beecham (King of Prussia, PA); propranolol, labetalol, atenolol, pindolol, 2-thiobarbituric acid, cupric sulfate, ferricytochrome c (Cyto c) (type III, horse heart) and phorbol myristate acetate (PMA) from Sigma (St Louis, MO); celiprolol from Rhone-Poulenc-Rorer (King of Prussia, PA); malonaldehyde bis(dimethyl acetal) (MDA) from Aldrich (Milwaukee, WI). Carvedilol was solubilized in dimethylsulfoxide (DMSO) and further diluted with 0.9% NaCl. The final concentration of DMSO was less than 1%. Other β-blockers were dissolved in ethanol and then diluted with 0.9% NaCl. In the final assay, the highest concentration of ethanol employed was less than 1%. Human low density lipoprotein (LDL, 1.019–1.063 g/ml, more than 10 preparations) was provided by Biotechnology Research Institute (Rockville, MD) or Sigma, and was prepared from fresh human plasma and kept in 0.15 M NaCl–0.01% EDTA (pH 7.4). Prior to use, the LDL was dialyzed at 4°C for 24 h against 3 changes of at least 150 vols. of 0.15 M NaCl (pH 7.4) to remove the EDTA. The LDL was stored at 4°C under N2 and used within 1 week.

Cell culture

Mouse resident peritoneal macrophages were obtained from male CD-1 mice (20–30 g) (Charles River, Raleigh, NC). Ca2+, Mg2+-free phosphate-buffered saline (pH 7.4) was injected into the peritoneal cavity, then macrophages were collected by peritoneal lavage and pelleted by centrifugation (1000 rev./min for 5 min). The cells were resuspended in Ham’s F10 cell medium containing 100 µg/ml of gentamicin, plated into 35-mm dishes at 4 × 10^6 cells/ml (1 ml per dish) and allowed to adhere for 2 h in a 37°C humidified 5% CO2 incubator. Non-adherent cells were removed with 2 washes of Ham’s F10 medium. To test the ability of carvedilol or other agents to inhibit LDL peroxidation, unless otherwise stated, the cells were preincubated with the test agent at 37°C for 20 min, LDL was added (100 µg protein/dish) and incubation continued for 24 h.

TBARS assay

The extent of LDL peroxidation was estimated as the formation of TBARS [13]. At the end of the incubation, the contents of the dishes were removed and centrifuged for 10 min at 1000 rev./min and the supernatant was used for the TBARS assay. An equal volume of 20% trichloroacetic acid was added to each sample followed by an equal volume of 1% thiobarbituric acid and the mixture heated for 30 min in a boiling water bath. The superna-
tant was removed after centrifugation at 3000 rev./min for 10 min and the TBARS was measured on a Perkin-Elmer fluorescence spectrophotometer (Model 650-10S) with excitation at 520 nm and emission at 552 nm. The amounts of TBARS are expressed in nmol equivalent MDA from a standard MDA curve prepared with malonaldehyde bis(dimethyl acetal).

Copper ion-induced autooxidation of LDL

Copper ion-induced autooxidation of LDL was produced by incubation of LDL (100 µg protein/ml) in 0.15 M saline (pH 7.4) with copper sulfate (20 µM) for 4 h at 37°C and the TBARS were measured [7,14].

LDL gel electrophoresis

Increases in electrophoretic mobility of LDL were determined by one-dimensional agarose electrophoresis. This was performed by running 5 µl of sample at 90 V for 35 min using pre-made agarose gels (Universal™ Gel, Ciba-Corning, Medfield, PA) and staining with 0.025% Fat Red 7B in 60% methanol following the manufacturer’s instructions. Results are expressed as relative electrophoretic mobilities compared with the migration of native (unmodified) LDL [15].

Measurement of superoxide (O$_2^-$) released from mouse macrophages

Superoxide (O$_2^-$) was measured as described previously [12]. Briefly, macrophages (1 × 10$^6$) were seeded in microtiter wells (96-well plate, Nunc, high affinity) and solutions containing PMA (10$^{-7}$ M) and Cyto c (0.32 mM) were applied to each of three wells. Three wells received Cyto c and HEPES buffer to measure basal, unstimulated release of O$_2^-$. One well served as an assay blank which contained Cyto c, buffer and 700 units SOD to confirm that Cyto c reduction was inhibitable by SOD. Changes in the optical density were measured intermittently on a MAX microplate reader at 550 nm. O$_2^-$ release was calculated using the following conversion: nmol O$_2^-$ = ([mean O.D. of three test wells) – (mean O.D. of three regent blank)] × 15.9 [12].

Fig. 1. Concentration-dependent inhibition of macrophage-induced LDL oxidation by carvedilol. LDL (100 µg protein/dish) was incubated for 24 h with macrophages in Ham’s F-10 medium in the presence or absence (control) of the test agent. The LDL oxidation was estimated by the formation of TBARS and expressed in nmol equivalent MDA as described in Methods. TBARS produced in cell-free conditions (medium + LDL) was subtracted from those of the dishes containing macrophages. Each point for carvedilol is the mean ± S.E.M. of 3–5 experiments done in duplicate, while the data for other β-blockers are the average of two experiments done in duplicate. The inset in Fig. 1 is the time course of LDL oxidation by mouse macrophages. LDL (100 µg protein/ dish) was incubated in Ham’s F-10 medium at 37°C in the presence (4 × 10$^6$ cells) or absence (LDL alone) of macrophages for various time intervals. The data shown are means of duplicate determinations from a representative experiment.
Statistics
Data in text and figures are mean ± S.E.M. values. Statistical analysis was performed as indicated in the figure legends; significant difference was accepted at $P < 0.05$.

Results

Human LDL was oxidized by mouse macrophages in a time-dependent manner and the production of TBARS reached a plateau after 24 h of incubation (inset in Fig. 1). Therefore, all data relating to macrophages presented here were obtained following 24 h incubation. The production of TBARS in the presence (LDL + cells) or absence (LDL alone) of the macrophages was 18.4 ± 1.0 nmol and 2.6 ± 0.4 nmol MDA equivalent/mg protein ($n = 15$), respectively. Increasing the number of macrophages per dish increased the production of TBARS (data not shown).

The ability of carvedilol and four commonly used β-blockers to inhibit LDL oxidation by macrophages was compared. As shown in Fig. 1, carvedilol dose-dependently inhibited LDL oxidation by the macrophages with an IC$_{50}$ value of 3.8 µM. Carvedilol at the maximal concentration tested did not have an apparent effect on the cells as determined by the trypan blue exclusion test. Propranolol inhibited LDL oxidation by the macrophages, but was much weaker than carvedilol. Increasing concentrations of propranolol (over 100 µM) resulted in an apparent effect on macrophage viability. Pindolol, labetalol and atenolol up to 300 µM were unable to prevent LDL oxidation by the macrophages. Under the same conditions, the IC$_{50}$ value of probucol was 0.86 ± 0.41 µM ($n = 3$).

The effect of a 72-h preincubation with carvedilol on the ability of macrophages to oxidize LDL was also investigated. As shown in Fig. 2, pretreatment of macrophages with carvedilol significantly reduced the ability of the cells to oxidize LDL in a dose-dependent fashion with an IC$_{50}$ value of 1.8 µM.

The effect of carvedilol on the electrophoretic mobility of LDL submitted to oxidative modification by the macrophages is shown in Table 1. A marked increase from 7.5 ± 0.3 to 13.6 ± 1.2 mm in the electrophoretic mobility of LDL incubated with macrophages for 24 h indicated lipid perox-

<table>
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<tr>
<th>Incubation conditions</th>
<th>Relative electrophoretic mobility</th>
<th>$P$</th>
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<tbody>
<tr>
<td>Native LDL</td>
<td>1.0</td>
<td></td>
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<tr>
<td>LDL + cell + vehicle (control)</td>
<td>1.78 ± 0.11</td>
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<tr>
<td>LDL + cell + carvedilol 1 µM</td>
<td>1.55 ± 0.04</td>
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<tr>
<td>LDL + cell + carvedilol 3 µM</td>
<td>1.43 ± 0.02</td>
<td>&lt;0.05</td>
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<tr>
<td>LDL + cell + carvedilol 10 µM</td>
<td>1.14 ± 0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL + cell + carvedilol 30 µM</td>
<td>1.06 ± 0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL + cell + propranolol 50 µM</td>
<td>1.50</td>
<td></td>
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<tr>
<td>LDL + cell + pindolol 300 µM</td>
<td>1.54</td>
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<tr>
<td>LDL + cell + atenolol 300 µM</td>
<td>1.50</td>
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LDL (200 µg/ml) was incubated for 24 h in Ham's F-10 medium in 35 mm dishes containing macrophages in the presence or absence of the drugs. The electrophoretic mobility of LDL was determined in agarose gels as described in Methods. Results are means ± S.E.M. of 4–6 independent experiments. The data for propranolol, pindolol and atenolol are means of 2 experiments. The electrophoretic mobility of the native LDL was 7.5 ± 0.3 mm ($n = 7$).
Fig. 3. Effect of carvedilol on PMA-induced O$_2^-$ release from mouse macrophages. Cells were treated with drug for 20 min at 37°C and then activated by addition of PMA (0.1 μM). O$_2^-$ formation was monitored at 550 nm by measurement of ferricytochrome c reduction and the incubation time was 60 min as described in Methods. Each point represents the mean ± S.E.M. (n = 3–5). The inset is the time course of PMA (0.1 μM)-stimulated production of O$_2^-$ from mouse macrophages. Each point is the average of duplicate samples.

oxidation of LDL and an increased negative charge in the LDL molecule. Carvedilol reduced the relative electrophoretic mobility dose-dependently, while other β-blockers at the concentrations indicated had no significant effect.

PMA (0.1 μM) induced a time-dependent increase in O$_2^-$ production by mouse macrophages and the production of O$_2^-$ reached a plateau at 60 min (inset in Fig. 3). As such, all data were obtained at 60 min incubation following stimulation. The production of O$_2^-$ increased from a basal value of 0 to 1.84 ± 0.14 nmol (n = 5). Carvedilol inhibited PMA-induced O$_2^-$ production in a dose-dependent manner with an IC$_{50}$ value of 1.39 μM (Fig. 3).

Figure 4 shows the correlation between inhibition of LDL oxidation and inhibition of superoxide release by carvedilol in mouse macrophages.

In a cell free system, Cu$^{2+}$ (20 μM) induced LDL autooxidation in a time-dependent manner. The formation of TBARS reached a plateau after 4 h incubation (inset in Fig. 5), which is in accordance with a previous study [14]. Increasing the LDL concentration increased the amount of TBARS (data not shown). Copper-induced LDL autooxidation was also inhibited by carvedilol with an IC$_{50}$ value of 17 μM as shown in Fig. 5. Under the same conditions, other β-blockers up to 300 μM had no significant effect.
Fig. 5. Effect of carvedilol on Cu^{2+}-induced LDL oxidation. LDL (100 μg protein/ml) was incubated in 0.9% NaCl (pH 7.4) at 37°C with or without drugs for 5 min, then CuSO_{4} (20 μM) was added. The incubation continued for 4 h and TBARS was assayed. In the absence of Cu^{2+}, TBARS content was below the detectable level. Each point for carvedilol represents the mean ± S.E.M. of 4–5 experiments done in duplicate. The data for other β-blockers are the average of two experiments done in duplicate. The inset is the time course of Cu^{2+}-induced LDL oxidation. LDL (100 μg/ml) was incubated in the presence or absence of 20 μM CuSO_{4}. Samples were taken at the indicated time points for the determination of TBARS. Results are the means of two experiments performed in duplicate.

Discussion

The results of the present study clearly demonstrate that carvedilol can markedly prevent macrophage- and Cu^{2+}-induced LDL oxidation. Although some β-blockers have been previously reported to inhibit iron-catalyzed lipid peroxidation [16], the present study is, to our knowledge, the first demonstration of a protective effect of a β-blocker against LDL peroxidation, by both a cell-and metal ion-mediated mechanism.

It has been reported that O_{2}^{•−} is an important oxygen radical in mediating LDL oxidation by smooth muscle cells [17] and human monocytes [18,19] and that O_{2}^{•−} dismutase (SOD) inhibits LDL oxidation by smooth muscle cells [17]. Our previous study demonstrated that carvedilol scavenges O_{2}^{•−} in human neutrophils [12] and more recently, the free radical-scavenging activity of carvedilol has been further confirmed by electron paramagnetic ressonance techniques [20]. In order to examine whether carvedilol inhibits LDL oxidation by inhibiting O_{2}^{•−}, we studied the effect of carvedilol on O_{2}^{•−} released from mouse macrophages. The results show that carvedilol inhibits O_{2}^{•−} with an IC_{50} value (1.4 μM) similar to the IC_{50} value for inhibition of LDL oxidation in macrophages (3.8 μM) and the correlation between inhibition of LDL oxidation and inhibition of O_{2}^{•−} by carvedilol is good (r = 0.93). We also observed that macrophage-induced LDL oxidation was reduced by 50–60% in the presence of SOD (100 μg/ml) (data not shown). Taken together, the results suggest that protection by carvedilol against cell-induced LDL oxidation may be, in part, through its capacity to scavenge O_{2}^{•−} radical. However, the inhibition of Cu^{2+}(20 μM)-induced LDL oxidation by carvedilol was less marked than the oxidation elicited by macrophages. The difference in carvedilol potency in the two systems might be due to the fact that oxidative modification of LDL is far more complex than a simple free radical mechanism [21]. Moreover, the mechanism of LDL oxidation by
transition metals still remains to be clarified [14].

The effective concentration of carvedilol for inhibition of LDL oxidation by macrophages is above 1 μM (Fig. 1). The plasma peak concentrations of carvedilol in human volunteers have been reported to be approximately 0.3 μM following an oral dose of 50 mg [22]. Therefore, the clinical relevance of the anti-LDL oxidation property of carvedilol must be considered. Carvedilol is a highly lipophilic compound with a partition coefficient value of 3 (log[octanol/H₂O], unpublished data) which is similar to that of propranolol [23]. It has been reported that accumulation of propranolol in Purkinje fibers and platelets reaches concentrations up to 30–40-fold over plasma concentrations [24,25]. It was also reported that hydrophobic calcium channel blockers reside in membranes at concentrations two orders of magnitude more than in the aqueous phase [26]. The present results that macrophages pretreated with carvedilol at 300–600 nM for 72 h showed a lower capability to oxidize LDL (Fig. 2), suggest that carvedilol might enter and accumulate in the cells. Although we do not know the exact levels of carvedilol in either the cells or the LDL particles, it is conceivable that an effective level of carvedilol for inhibition of LDL oxidation may be attainable in vivo. The observation that carvedilol markedly inhibits human LDL oxidation by macrophages or copper suggests that carvedilol may have antiatherogenic effects. This novel action of carvedilol would increase its therapeutic value as an antihypertensive, since hypertensive patients are at risk to develop atherosclerosis.

References


