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Carvedilol prevents low-density lipoprotein (LDL)-enhanced monocyte adhesion to endothelial cells by inhibition of LDL oxidation

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Abstract

Cultured human umbilical vein endothelial cells oxidize low-density lipoproteins (LDL), assessed as increase in thiobarbituric acid reactive substance formation and oxidized LDL-induced cytotoxicity (lactate dehydrogenase (LDH) release). Endothelial cell-generated oxidized LDL also enhances the adhesiveness of endothelial cells to monocytes. Carvedilol, a new vasodilating β-adrenoceptor antagonist, inhibits the oxidation of LDL by endothelial cells and reduces oxidized LDL-induced LDH release from endothelial cells in a concentration-dependent manner with IC_{50} values of 2.56 and 1.38 μM, respectively. Moreover, carvedilol inhibits oxidized LDL-induced adhesion of monocytes to the endothelial cells in a similar concentration-dependent manner. Under the same conditions, propranolol, atenolol, pindolol and labetalol had only weak or no consistent effects on both LDL oxidation by endothelial cells and adhesion of monocytes to the endothelial cells. Monoclonal antibodies against human intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) or E-selectin (ELAM-1) partially blocked oxidized LDL-stimulated adhesion of endothelial cells to monocytes. The inhibitory effects of carvedilol on LDL oxidation and monocyte adhesion to endothelial cells may protect blood vessels from atherosclerotic processes associated with oxidized LDL-induced injuries.

Keywords: Carvedilol; Low-density lipoprotein (LDL); Antioxidant; Monocyte; Adhesion

1. Introduction

Endothelium dysfunction has been demonstrated in experimental models of hypertension and atherosclerosis which suggests that modified endothelium exists early in vascular response to injury (Luscher and Noll, 1994; Novosel et al., 1994). Furthermore, patients with coronary artery disease and congestive heart failure demonstrated dysfunctional endothelium. Therefore, vascular protection has recently been considered as an area for future concentrated investigation in hypertension and heart failure (Buhler et al., 1994). The endothelium located on the luminal surface of the vascular wall is vulnerable to attack by a variety of deleterious factors, among which oxidized low-density lipoprotein (LDL) is considered of major importance (Steinberg, 1993). Thus, it has been repeatedly demonstrated that oxidized LDL causes endothelial injury which results in pro-inflammatory transformation of the endothelium such as to enhance leukocyte adherence to the blood vessel wall, and further exacerbation of the damage to the endothelial layer (Frostegard et al., 1990). It is also important to note that endothelial cells also actively contribute to the production of oxidized LDL and thereby augment and amplify the damage (Henriksen et al., 1981; Morgan et al., 1993). Therefore, inhibition of oxidative modification of LDL could lead to significant endothelial and vascular protection. Carvedilol is a new vasodilating β-adrenoceptor antagonist for treatment of hypertension. Carvedilol is also being studied in clinical trials for use in chronic stable angina and congestive heart failure (Ruffolo et al., 1991). Recently, carvedilol has been shown to reduce infarct size in several different models of acute myocardial infarction in five different species (Feuerstein et al., 1994). The mechanisms for this extraordi-

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nary cardiac protection by carvedilol has not fully been explored. Clearly, β-blocking activity could well serve for reduction in cardiac work and hence an antiischemic effect. However, recent studies also demonstrated that carvedilol is a potent antioxidant in swine cardiac ventricular membranes and brain tissue homogenates (Yue et al., 1992a, b). The present study was designed to investigate whether carvedilol prevents oxidation of LDL by cultured human umbilical vein endothelial cells and therefore reduces oxidized LDL-induced cellular damage and adhesiveness of the endothelial cells to monocytes, a crucial step for migration of monocytes across the vascular intima (Muller, 1995). As a comparison, the antioxidant activity of some commonly used β-adrenoceptor antagonists was also evaluated.

2. Materials and methods

2.1. Materials

Human LDL (1.019–1.063 g/ml), prepared from fresh human plasma and kept in 0.15 M NaCl-0.01% EDTA (pH 7.4), was provided by Biotechnology Research Institute (Rockville, MD, USA). Prior to use, the LDL was dialyzed at 4°C for 24 h against 3 changes of at least 150 volumes of 0.15 M NaCl (pH 7.4) to remove the EDTA. The LDL was stored at 4°C under N_2 and used within 1 week. Cu^{2+}-oxidized LDL was prepared as described previously (Yue et al., 1992c). Carvedilol was provided by SmithKline Beecham (King of Prussia, PA, USA). Propranolol, atenolol, pindolol, labetolol and PKH_2-GL green fluorescent cell linker kit were purchased from Sigma (St. Louis, MO, USA). Monoclonal anti-human ICAM-1, VCAM-1 and ELAM-1 antibodies were purchased from Genzyme (Cambridge, MA, USA). $Na^{51}CrO_4$ (1 mCi/ml) was obtained from Amersham Corp. (Arlington Heights, IL, USA).

2.2. Cell culture

Human umbilical vein endothelial cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in Medium 199 supplemented with 20% heat-inactivated fetal calf serum, heparin (100 μg/ml), acidic recombinant fibroblast growth factor (25 ng/ml) and gentamicin in a humidified environment of 5% CO_2/95% air at 37°C. Cells were initially cultured in T150 flasks and then subcultured into 48-well tissue culture plates for the adhesion study or 6-well plates for the thiobarbituric acid reactive substance formation study. Cells from passages 13–18 were used in this study.

2.3. Measurement of LDL oxidation by human umbilical vein endothelial cells

The extent of LDL oxidation was estimated as the formation of thiobarbituric acid reactive substances (Hoff et al., 1992). The confluent endothelial cells, cultured in 6-well plates and changed to Ham's F10 medium for 24 h, were incubated with LDL (100 μg/ml) for 4 h unless otherwise indicated. At the end of the incubation period, the contents of the dishes were removed and centrifuged for 10 min at 1000 rpm and the supernatant was used for thiobarbituric acid reactive substance formation assays. To test the ability of carvedilol or other agents to inhibit LDL peroxidation the cells were preincubated with the test agent at 37°C for 20 min prior to the addition of LDL.

2.4. Cytotoxicity assay

Cellular injury was assessed by measuring the amount of lactate dehydrogenase (LDH) released from the endothelial cells into the medium (Kuzuya et al., 1991). At the end of incubation, 50 μl of medium from each well was removed and LDH activity was determined by use of an LDH reagent following the manufacturer's instructions (Sigma). Each LDH activity was compared with that released from cells after the addition of 0.1% Triton X-100 (as total LDH release), and expressed as percentage of total release.

2.5. Adhesion assay

Human monocytes were isolated from fresh blood as described previously (Wang et al., 1995). The assays for monocyte adherence to endothelial cells were carried out according to the method described by Suzuki et al. (1989). The ^{51}Cr-labeled monocytes were added (4×10^5/well) to endothelial cell monolayers cultured in 48-well plates, which had been incubated with LDL (100 μg/ml) or vehicle for 4 h, unless otherwise described, and then washed 3 times with Ham's F-10 medium. After a 2 h co-incubation, non-adherent cells were removed by gentle washing twice with buffer solution. Adherent monocytes were lysed by adding 0.2 ml of 0.2% sodium dodecyl sulfate (SDS) and assayed for ^{51}Cr activity. Monocyte adherence to the endothelial cells was expressed as percentage of total cells added, such that % adherence = [(cpm harvested)/(cpm added)] × 100.

For photographic analysis, monocytes were labeled with a fluorescent dye using a PKH2-GL staining kit as described previously (Yuan and Fleming, 1990). Briefly, 1 ml of the diluent containing 5 ml of fluorescent dye was added to 1 ml of monocyte suspension and incubated for 10 min at room temperature, and then 2 ml

of fetal bovine serum was added to stop the uptake of the dye into the cells. The cells were washed once with Ham's F-10 medium and resuspended in the same buffer at 4×10^5 cells/ml.

2.6. Statistics

Data presented in text and figures are means ± S.E.M. unless otherwise stated. Statistical significance among groups was examined through one-way analysis of variance and Dunnett's multiple range test (Tallarida and Murray, 1987).

3. Results

3.1. LDL oxidation by human umbilical vein endothelial cells

Exposure of the endothelial cells to LDL resulted in a time-dependent increase in lipid peroxides as assessed by thiobarbituric acid reactive substance formation (Table 1). In the presence of endothelial cells, the production of thiobarbituric acid reactive substance from LDL after 4 h incubation was 5.5-fold higher than that of the control (without cell). Meanwhile, endothelial cells incubated with LDL released LDH in a similar time-dependent manner as observed for thiobarbituric acid reactive substance formation (Table 1). A 30% of the total LDH was released from the endothelial cells after incubation of LDL (100 μg/ml) with the cells for 4 h. Moreover, 22.3 ± 1.8% ($n = 4$) cell death was observed.

3.2. Inhibition of endothelial cell-mediated LDL oxidation by carvedilol

Pretreatment of the endothelial cells with carvedilol for 20 min before the addition of LDL produced a dose-dependent inhibition of LDL oxidation by the cells with an IC_{50} value of 2.56 μM, and prevented

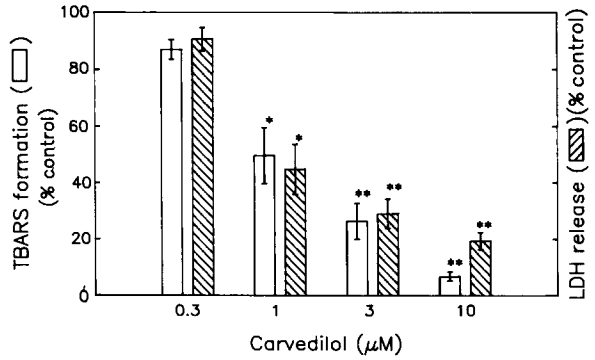

Fig. 1. LDL oxidation by the human umbilical vein endothelial cells and inhibition by carvedilol. The endothelial cells were preincubated with carvedilol or vehicle (control) for 20 min before the LDL (100 μg/ml) was added to the endothelial cells, and the incubation continued for 4 h. The LDL oxidation was estimated by the formation of thiobarbituric acid reactive substances, and the LDH release from the endothelial cells was determined as described in Materials and methods. Each point was the mean ± S.E.M. of 4 experiments performed in duplicate. $^*P < 0.05$; $^{**}P < 0.01$ vs. control (without carvedilol).

LDH release from the cells with an IC_{50} value of 1.38 μM (Fig. 1; Table 2). Under the same conditions, propranolol had much less effects on both LDL oxidation by endothelial cells and LDH release from the cells (Table 2). Other β-adrenoceptor antagonists, at concentration up to 100 μM, inhibited LDL oxidation by endothelial cells and LDH release from the cells by less than 50%.

3.3. Oxidized LDL induced adhesiveness of endothelial cells for monocytes

When the endothelial cells were incubated with LDL, an increase in the adhesiveness of the endothelial cells for monocytes was demonstrated (Fig. 2). The increase in the adhesion of monocytes to the endothelial cells was dependent on LDL concentration and the time of incubation. Similar results were also observed

Table 1
Time dependency of LDL oxidation by human umbilical vein endothelial cells and LDH release from the endothelial cells which were incubated with LDL

Parameter		Time (h)		
		2	4	6
TBARS[a] formation (O.D.)	LDL alone	0.01	0.13	0.30
	LDL with cells	0.36	0.74	1.20
LDH release (% total release)	Without LDL	1.31	2.02	2.38
	With LDL	10.80	30.10	49.88
Cell death	Without LDL		0	0.5
	With LDL		22.30	70.30

[a]TBARS: thiobarbituric acid reactive substances. The data are means of 4 determinations. LDL concentration was 100 μg/ml.

Table 2
IC_{50} values of carvedilol and propranolol for inhibition of LDL oxidation (TBARS formation), lactate dehydrogenase (LDH) release from human umbilical vein endothelial cells and oxidized LDL-induced adhesiveness of the endothelial cells for monocytes

Agent	IC_{50} (μM)		
	TBARS[a] formation	LDH release	Monocyte adhesion
Carvedilol	2.48 ± 0.34	1.43 ± 0.21	2.08 ± 0.40
Propranolol	121.40 ± 12.3	94.97 ± 10.33	95.72 ± 6.23

[a]TBARS: thiobarbituric acid reactive substances. The percent inhibition versus log concentration corresponding to 50% inhibition was expressed as the IC_{50} values. The values in the absence of the inhibitor were assumed as 100%. The data are the means ± S.E.M. ($n = 3$).

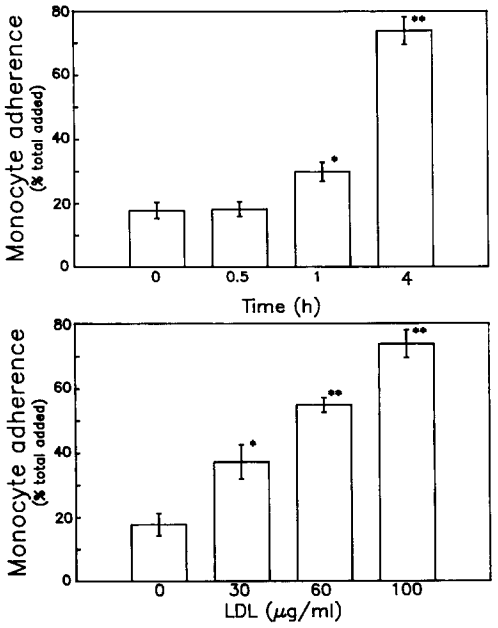

Fig. 2. Oxidized LDL stimulates adhesiveness of human umbilical vein endothelial cells for monocytes. The human umbilical vein endothelial cells in Ham's F-10 medium was incubated with LDL at the concentrations indicated for 4 h (low panel) or with 100 μg/ml of LDL for the time indicated (upper panel), and washed extensively before the addition of ^{51}Cr-labeled monocytes and co-incubation continued for 2 h. Nonadherent cells were removed by two gentle washings. Adherent monocytes were lysed and assayed for ^{51}Cr activity. Monocyte adherence to endothelial cells was expressed as percentage of total cells added. Each point was the average ± S.E.M. of 3 experiments performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control.

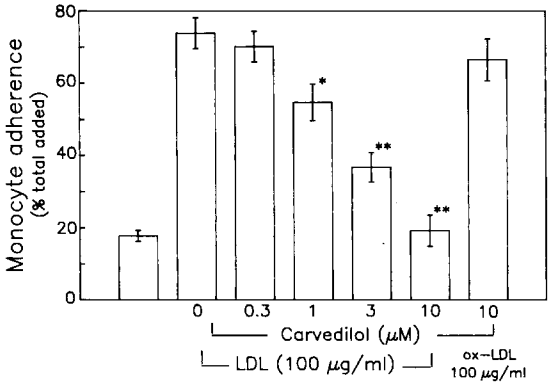

Fig. 3. Inhibition by carvedilol of oxidized LDL-induced adhesiveness of human umbilical vein endothelial cells to monocytes. Human umbilical vein endothelial cells were preincubated with carvedilol at the concentrations indicated for 20 min before LDL (100 μg/ml) was added and incubation continued for 4 h, and washed extensively before the ^{51}Cr-labeled monocytes were added, and co-incubation continued for 2 h. The adhesion assay was done as described in the legend to Fig. 2. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. endothelial cells incubated with LDL but without carvedilol.

when the endothelial cells were incubated with Cu^{2+}-oxidized LDL. At 100 μg/ml of oxidized LDL, the adhesion of monocytes to the endothelial cells reached a plateau at 2 h after incubation and 82 ± 4.5% of monocytes were adhered to the endothelial cells.

3.4. Inhibition by carvedilol of LDL oxidation and prevention of monocyte adhesion to endothelial cells

The increase in monocyte adhesion to endothelial cells was inhibited when carvedilol was added to the endothelial cells with the LDL (Figs. 3 and 4), and the inhibitory effect of carvedilol was concentration-dependent (Fig. 3). Under the same conditions, propranolol showed a mild effect (Table 2). Other β-adrenoceptor antagonists, at concentrations up to 100 μM, inhibited

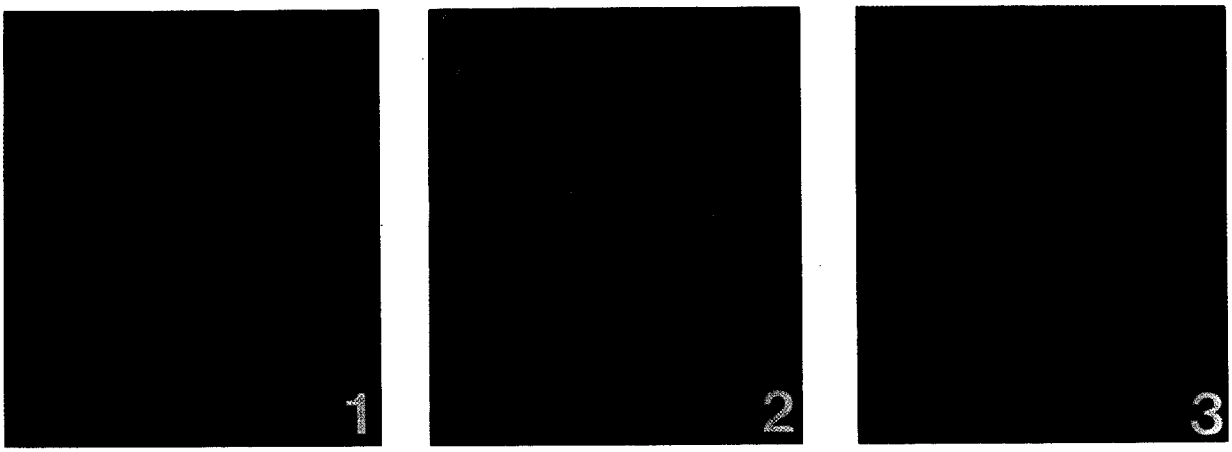

Fig. 4. Representative fluorescent photomicrographs of monocyte adhesion to human umbilical vein endothelial cells. The endothelial cells were incubated with vehicle (1 and 2) or carvedilol (3 μM) for 20 min, and then 100 μg/ml of LDL was added to (2) and (3). Incubation continued for 4 h at 37°C, and washed extensively before ^{51}Cr-labelled monocytes were added. After 2 h co-incubation, nonadherent cells were removed by two gentle washings. The adherent cells were examined immediately by fluorescence photomicrograph of a typical field of the endothelial cells. Magnification × 150.

Table 3
Effects of monoclonal anti-human ICAM-1, VCAM-1 and ELAM-1 antibodies on oxidized LDL-induced human umbilical vein endothelial cell adhesiveness to monocytes

Antibody (μg protein/ml)	Monocyte adhesion (% total added)		
	Anti-ICAM-1	Anti-VCAM-1	Anti-ELAM-1
0	51.02±4.54	51.02±4.54	51.02±4.54
5	53.53±2.74	50.47±2.70	49.28±2.36
20	39.75±1.30[a]	36.87±2.81[a]	51.62±3.81
50	24.03±3.37[b]	25.92±1.23[b]	32.01±2.40[b]

Human umbilical vein endothelial cells, cultured in 48-well plates, were incubated with LDL (100 μg/ml) or vehicle (basal) for 4 h and washed extensively with F10 medium. The antibody was added and incubated for 20 min before ^{51}Cr-labeled monocytes were added. Adhesion assay was performed as described in Materials and methods. The data are the means±S.E.M. of four measurements. The basal adhesion of monocytes to the endothelial cells was 10.14± 2.47.%. [a]$P < 0.05$; [b]$P < 0.01$ vs. control (without addition of corresponding antibody).

LDL-induced adhesion of monocytes to endothelial cells less than 50%. When the endothelial cells were incubated with Cu^{2+}-oxidized LDL, the inhibitory effect of carvedilol on monocyte adhesion to endothelial cells was not observed (Fig. 3), indicating that carvedilol exerted to inhibit oxidized LDL formation rather than the direct effect on oxidized LDL.

3.5. Effects of monoclonal antibodies against human ICAM-1, VCAM-1 and ELAM-1 on oxidized LDL-activated adhesiveness of endothelial cells to monocytes

Human umbilical vein endothelial cells were cultured with LDL (100 μg/ml) or vehicle for 4 h and then washed with F10 medium. Anti-ICAM-1, -VCAM-1 or -ELAM-1 antibody was added and incubated for 20 min before monocytes were added. As shown in Table 3, oxidized LDL-induced adhesion of monocytes to the endothelial cells was partially blocked by anti-human ICAM-1 or anti-human VCAM-1 antibody in a concentration-dependent manner. At 1:20 dilution, anti-ICAM-1 and anti-VCAM-1 antibody reduced oxidized LDL-induced adhesion of endothelial cell for monocytes by 49.9% and 45.9% ($P < 0.01$), respectively. Anti-human ELAM-1 antibody only at 1:20 dilution inhibited adhesion of monocytes to the endothelial cells by 33.3% ($P < 0.01$). In addition, the nonimmune immunoglobulin (IgG) had no effect.

4. Discussion

Exposure of human umbilical vein endothelial cells to LDL resulted in a time-dependent increase in thiobarbituric acid reactive substance formation, indicating an oxidation of LDL by endothelial cells. Meanwhile, an increase in cytotoxicity of LDL to endothelial cells, as measured by LDH release from the endothelial cells, was also observed with a corresponding time-dependent manner, suggesting that the cytotoxicity was due to the formation of oxidized LDL. After 4 h incubation with 100 μg/ml of LDL, more than 20% of endothelial cells died. It was previously observed in this laboratory that vascular smooth muscle cells oxidized LDL slower than that of endothelial cells. Under similar conditions, comparable levels of LDL oxidation by vascular smooth muscle cells was achieved by 24 h of incubation. These results suggested that endothelial cells are highly active in oxidation of LDL, and also highly sensitive to the cytoxic effects of oxidized LDL. Due to the significant effects of oxidized LDL on endothelial cells found after 4 h of co-incubation with LDL, this time point, 4 h, was chosen in the present study for observation of interaction between endothelial cells and monocytes.

Carvedilol produced a concentration-dependent inhibition of LDL oxidation by human umbilical vein endothelial cells, and was considerably more potent than the other β-adrenoceptor antagonists tested; in this respect, the present data concur with our previous observations for inhibition of Fe^{2+}-mediated lipid peroxidation in ventricular membranes and brain homogenates (Yue et al., 1992a, b). Moreover, carvedilol protected against LDL-induced cytotoxicity to endothelial cells with an IC_{50} value that was similar to the IC_{50} value for inhibition of LDL oxidation, which far exceeded that of all other β-adrenoceptor blockers. These results clearly suggest that the protective effect of carvedilol was due to its inhibition of LDL oxidation.

Two general mechanisms for cell-catalyzed oxidation of LDL have been proposed: first, release of oxygen free radicals from cells, such as superoxide and hydrogen peroxide which can further become hydroxyl radicals to attack LDL; second, generation of lipoperoxides within the cell followed by transfer of these into the LDL and initiation of chain reaction in the LDL (Steinberg, 1993). Carvedilol has been demonstrated to be a free radical scavenger for oxygen radicals both in aqueous phase and lipid phases (Yue et al., 1992a, 1993); therefore inhibition of oxidation of LDL by carvedilol could be via both mechanisms described above. This could also explain why other β-adrenoceptor antagonists such as propranolol virtually lack a protective effect on LDL oxidation by the endothelial cells, since no direct oxygen radical scavenging capacity was noted for these agents (Yue et al., 1992a).

It is well established that adhesion of monocytes to the endothelium is an early event in atherogenesis (Beekhuizen and Furth, 1993; Elliot and Finn, 1993) and a crucial step for migration of monocytes across the vascular intima (Muller, 1995). In the present study, a significant increase in the adhesion of monocytes to

endothelial cells was demonstrated upon the endothelial cell preincubation with LDL. Similar results were also demonstrated when the endothelial cells were exposed to Cu^{2+}-oxidized LDL. These results are consistent with two recent reports in which a monocyte-like cell line, U937, was found to adhere to human umbilical vein endothelial cells treated with oxidized LDL (Frostegard et al., 1990) and oxidized LDL stimulated the adhesion of monocytes to rabbit aortic endothelial cells (Berliner et al., 1990).

Carvedilol, when added to endothelial cells together with the LDL, produced a concentration-dependent inhibition of monocyte adhesion to the endothelial cells. However, carvedilol had no effect on leukocyte adhesion to endothelial cells if Cu^{2+}-oxidized LDL was used to induce endothelial cell adhesiveness or after the endothelial cells were incubated with LDL for 4 h. The IC_{50} values of carvedilol for inhibition of monocyte adhesion to endothelial cells was 2.17 μM which is similar to the IC_{50} value for inhibition of thiobarbituric acid reactive substance formation. All other β-adrenoceptor antagonists showed weak or no consistent effect in either test. These results indicate a coupling between two processes, namely inhibition of endothelial cell-dependent LDL oxidation and prevention of enhanced adhesion of monocytes to the endothelial cells.

The specific adhesion molecules which mediate the adhesiveness of endothelium to leukocytes have not been established as yet. In the present study, we used monoclonal antibodies against human ICAM-1, VCAM-1 and ELAM-1 to examine the contribution of each of these adhesion molecules expressed by human endothelium (Beekhuizen and Furth, 1993; Jang et al., 1994) in the oxidized LDL-induced adhesiveness of endothelial cells to monocytes. Anti-ICAM-1 or VCAM-1 antibodies inhibited oxidized LDL-induced monocyte adhesion to human umbilical vein endothelial cells in a concentration-dependent manner. Anti-ELAM-1 antibody showed significant though mild inhibitory effect on monocyte adhesion to endothelial cells only at the highest concentration. The results indicate that these three adhesion molecules might be involved in oxidized LDL-induced adhesiveness of endothelial cells to monocytes. It has been reported that a lag time for maximal expression of these adhesion molecules on endothelial cells extends over 2–6 h (Sluiter et al., 1993). This time window is consistent with our observation that marked increase in monocyte adhesion to human umbilical vein endothelial cells induced by oxidized LDL was established at 4 h after stimulation. Further studies, however, to directly detect the expression of these adhesion molecules on oxidized LDL-stimulated endothelial cells are necessary. Moreover, other adhesion molecules and the interactions between different molecules should also be considered.

In conclusion, our study showed a high capacity of human umbilical vein endothelial cells to oxidize LDL and that such oxidized LDL enhance monocyte adhesion to the endothelial cells. The adhesion molecules, ICAM-1, VCAM-1 and ELAM-1, and especially the former two, might be involved in these processes. Carvedilol inhibits oxidation of LDL by endothelial cells, reduces oxidized LDL-induced cellular injury and prevents monocyte adhesion to the endothelial cells. The increasing evidence indicates that endothelial injury is implicated in the pathology of hypertension and coronary heart diseases, and lower levels of the antioxidants vitamin C and thiols in blood were found in hypertensive subjects than controls, reflecting their roles as the first-line defence against oxidative damage or greater oxidative consumption (Tse et al., 1994; Poppel et al., 1994); the antioxidant activity and the endothelial protective effect of carvedilol would increase its therapeutic value as an antihypertensive agent.

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