Effects of Antihypertensive Drugs on Cholesterol Metabolism of Human Mononuclear Leukocytes and Hepatoma Cells

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Objectives: Primary prevention trials of antihypertensive therapy have shown conflicting results on coronary events. Potential interference of antihypertensive agents with cellular lipid metabolism may alter the atherosclerotic risk of individuals.

Design and Methods: The effects of the calcium antagonist's verapamil, diltiazem, and nifedipine and of the beta-blockers propranolol and metoprolol on low density lipoprotein (LDL) receptor activity, cholesterol esterification rate, oleate incorporation in triglycerides and sterol synthesis were studied in freshly isolated human leukocytes and HEP G2 cells.

Results: Up to a concentration of 3–10 μmol/L, verapamil, propranolol, and metoprolol led to an increased cellular content of 125I-LDL by an inhibition of degradation. In mononuclear cells verapamil stimulated accumulation and degradation. No effect on binding was observed. Diltiazem was only stimulatory on 125I-LDL processing in leukocytes. Beta blockers and verapamil significantly reduced the LDL mediated 14C-oleate incorporation in cholesterol esters. In the presence of 25-hydroxycholesterol the esterification was not diminished, which suggests that cholesterolacyltransferase (ACAT) was not affected per se. Whereas all the agents induced the synthesis of lanosterol, metoprolol inhibited cholesterol synthesis. None of the agents had a significant influence on 14C-oleate incorporation in triglycerides, suggesting a specific influence on cholesterol metabolism.

Conclusions: Antihypertensive drugs affect the cholesterol metabolism on a cellular level. Mechanisms are an interference with degradation of LDL and consequent alterations of cholesterol esterification. Using leukocytes as peripheral cells and HEP G2 as a model of human liver, these results may have importance when clarifying more precisely if cholesterol metabolism in leukocytes or hepatocytes. As monocytes, as precursor of foam cells (24) and hepatocytes (25) play the key role in the control of lipid metabolism and in the development of atherosclerosis it is necessary to clarify more precisely if cholesterol metabolism in these cells is susceptible for antihypertensive drug modulation. Used as a model was freshly isolated human mononuclear leukocytes and the highly differentiated human hepatoma-derived cell line HEP G2. Mononuclear leukocytes as HEP G2 cells were shown to possess LDL receptors (26) and HDL receptors (27). The LDL receptor pathway delivers free cholesterol to the enzyme acyl-CoA:cholesterol-acyltransferase that catalyzes cholesterol esterification (28). Also they have a functioning sterol synthesis cascade with a regulated HMG-CoA reductase (29) as well as the ability to secrete several lipoproteins (30). Therefore, in many ways both cell lines are a reliable model of the lipid metabolism in vivo.

The calcium antagonist's verapamil, diltiazem, are disappointing. Despite that blood pressure was lowered significantly, cardiac events remained similar in therapy and control groups (1). Even deleterious effects of calcium channel blockers given for hypertension on cardiac events have been reported (2). Negative metabolic effects of these agents especially on lipid metabolism were thought to be the reason of this phenomenon (3). Three lines of evidence link the theory of influencing coronary risk through lipid metabolism by antihypertensive therapy: First antihypertensives alter lipoprotein levels in men (for review see 4). Second atherosclerosis in animal models for example the rate of lipid accumulation in arteries was affected by antihypertensives such as verapamil (5), diltiazem (6), propranolol (7), and metoprolol (8). Third these agents can alter lipid metabolism at the cellular level (9–23). Whereas most of these studies, often measuring only single metabolic pathways were undertaken with fibroblasts (9–11,14), vascular cells (13,15,17), or mouse macrophages (19–21) only some (22,23) used human leukocytes or hepatocytes. As monocytes, as precursor of foam cells (24) and hepatocytes (25) play the key role in the control of lipid metabolism and in the development of atherosclerosis it is necessary to clarify more precisely if cholesterol metabolism in these cells is susceptible for antihypertensive drug modulation. Used as a model was freshly isolated human mononuclear leukocytes and the highly differentiated human hepatoma-derived cell line HEP G2. Mononuclear leukocytes as HEP G2 cells were shown to possess LDL receptors (26) and HDL receptors (27). The LDL receptor pathway delivers free cholesterol to the enzyme acyl-CoA:cholesterol-acyltransferase that catalyzes cholesterol esterification (28). Also they have a functioning sterol synthesis cascade with a regulated HMG-CoA reductase (29) as well as the ability to secrete several lipoproteins (30). Therefore, in many ways both cell lines are a reliable model of the lipid metabolism in vivo.
and nifedipine and the beta blockers propranolol and metoprolol were chosen because they were widely used and the investigations of their influence on cellular lipid metabolism yielded conflicting results (9–23).

**Design and Methods**

As methods, three major pathways of cellular lipid metabolism were measured: 125I-LDL processing as an indicator of LDL receptor activity, 14C-Oleate incorporation in cholesterol esters to address ACAT activity, and 14C-acetate incorporation in various sterols as a marker for cholesterol biosynthesis. 14C-acetate incorporation in thin layer chromatographic separated sterols was chosen because experiments with 3H2O were not possible due to the relative high radioactive load of this substance. This limitation was tolerable since the aim was to measure relative changes rather than absolute rates of enzyme activities. For this purpose it was shown that 14C-acetate incorporation in sterols correlates sufficiently with the relative rate of cholesterol synthesis (31).

**Materials**

Bovine serum albumin (BSA, essentially fatty acid free), (+)-propranolol, diltiazem, verapamil, cholesterol (5-cholesten-3β-ol), 25-hydroxycholesterol (5-cholestene-3β, 25-diol 3-acetate), and lanosterol (3β-hydroxy-8,24-lanostadiene) were obtained from Sigma (Munich, Germany). 125I-Sodium hydroxide solution, (2-14C) acetic acid sodium salt, (1-14C)-oleic acid, (1,2-3H)-cholesteryl oleate, and (1,2,3H) cholesterol were purchased from Amersham (Amersham, UK). Minimum essential medium, penicillin/streptomycin-solution, and fetal calf serum were from Gibco (Glasgow, UK). Cell culture flasks were from Falcon (Oxnard, CA, USA). All other chemicals were reagent grade from Merck (Darmstadt, Germany). Metoprolol was a gift from Astra (Wedel, Germany).
Isolation of LDL and Preparation of Lipoproteins

Human LDL (density 1.019–1.063 g/mL) was isolated from plasma of normolipaemic healthy subjects by sequential ultracentrifugation as described by Havel (32). 

\[ ^{125} \text{I-LDL was prepared by the iodine monochloride method of McFarlane (33) as modified by Shepard et al. (34). After iodination, free iodine was removed by passage through a QAE-Sephadex A-50 anion exchange column and sterilized by 0.45 \mu m Millipore filtration. Analysis of iodinated lipoprotein substrates showed, that <1% of the radioactivity was due to free iodine and <0.02% of the radioactivity represents trichloroacetic acid-soluble noniodine material. Specific activity of the preparations was expressed as cpm per nanogram protein content and was usually in the range among 80 and 150 cpm/ng.}\]

Isolation and Incubation of Mononuclear Leukocytes

Peripheral blood mononuclear leukocytes were isolated by the method of Böyum (35) from heparinized blood obtained from healthy subjects after an overnight fast. Ten milliliters of blood was diluted with an equal volume of 0.15 M NaCl Ficoll-Hypaque (Lymphoprep®, Nyegaard & Co., Oslo, Norway) was layered under the mixture of blood and saline in 50 mL conical tubes (Falcon, Oxnard, CA, USA) and centrifuged at 400 \text{x} \ g for 40 min at room temperature. The resultant band of mononuclear leukocytes was collected, cells were washed twice in RPMI-1640 (Gibco) and then resuspended in RPMI-1640 containing 100 U/mL penicillin and 100 \mu g/mL streptomycin (Gibco). Cells were then plated at a density of 2 \times 10^6 cells/500 \mu L in 22 mm 12-well tissue culture clusters (Costar, Cambridge, USA) and incubated in a humidified atmosphere with 5% \text{CO}_2 at 37°C. In some assay's insulin (bovine, Sigmachemie, Munich, Germany) was added at zero time (concentrations see figure legends). Purity of cell preparations was routinely assessed by staining smears with Wright-Giemsa stain. Differential counts before and after incubations showed that 85–95% of the cells were lymphocytes, the remainder were monocytes.
CELL CULTURE

HEP G2 cells (American Tissue Type Culture Collection, Waldorf, MD, USA) were grown in Dulbecco's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin-solution and 1% glutamin. Fresh medium was added every third day. Cell viability as assessed by trypan blue exclusion was 95% before and after incubation up to 20 h with and without antihypertensive agents in lipid-free medium.

MEASUREMENT OF LDL RECEPTOR ACTIVITY

$^{125}$I-LDL processing was determined as described by Goldstein et al. (36). Cells were cultured in 6 well 1 mL culture dishes for 3–4 days (HEP G2) at 37°C in a 5% CO$_2$ atmosphere or additionally processed without preincubation (leukocytes). Then the fetal calf medium was removed and DMEM without protein or lipid was added with and without agents. After 20 h the dishes received 500 μL of 20% BSA in RPMI, $^{125}$I-LDL (10 μg/mL) and in control dishes native LDL (250 μg/mL) for the determination of unspecific binding. After an incubation period of 6 h (37°C, 5% CO$_2$) cells were chilled on ice. The medium was discharged and the degradative products were determined as the trichloroacetic-acid soluble noniodine material. The adherent cells were washed three times with 0.5% BSA-Trisbuffer (pH 7.4) and three times with buffer without BSA. Dextran sulfate buffer (1 mL of a 4 mg/mL solution) with 10 mM Hepes was then added. After 1 h incubation (4°C) the supernatant was counted for the binding value. The adherent cells were dissolved in 0.1% NaOH and counted for the internalization value. An aliquot was also taken for the determination of the protein content. Specific values for binding, internalization and degradation were expressed by subtracting the values with excess unlabelled LDL (250 μg/mL) from the total activity.

Figure 3 — Effects of various concentrations of verapamil, propranolol, diltiazem, and metoprolol on the incorporation of $^{14}$C-oleate in triglycerides by HEP G2 cells. Cells were incubated for 20 h in lipid free medium with and without agents. Oleate incorporation in triglycerides was measured in the presence of 250 μg/mL LDL (○) or 15 μg/mL 25-hydroxycholesterol (■). Results were expressed as the mean of three different experiments ± SEM.
MEASUREMENT OF CHOLESTEROL ESTERIFICATION AND 14C-OLEAT INCORPORATION IN TRIGLYCERIDES

Cholesterol esterification was measured as described by Goldstein et al. (36). Preincubation with agents was similar to the LDL receptor assay. At 20 h dishes received either unlabelled LDL (250 μg/mL) or 25-hydroxycholesterol (15 μg/mL). After 5 h 10 μL of a (1–14C)-oleate-albumin complex solution (10000 dpm/nmol) was added for additional 2 h incubation. Reaction was stopped on ice, and the cell monolayers were washed for six times with phosphate-buffer. Lipids were then extracted in situ by the addition of 1 mL 3:2 (vol/vol) hexane/isopropanol. A 3H-Cholesterol-Oleate standard (30000 dpm) was then added. The solvent was then evaporated and lipids developed on silica gel plates with heptane-ethyl-ether-acetic acid (45:15:0.5-vol/vol). The spots corresponding to cholesterol ester (as marked by a cholesterol-oleate standard) and to triglycerides (as marked by a triolein standard) were identified with iodine vapor and cut from the chromatogram for counting. Cellular protein was determined from the adherent cells after lipid extraction. Procedural losses were corrected with the help of the 3H-Standard.

MEASUREMENT OF 14C-ACETIC ACID INCORPORATION IN STEROLS

Cells were pretreated as described in the other assays. After 20 h, dishes received 25 μL of (2-14C) acetate (7.4 μBq; 203 μBq/mmol) and the incubation continued for 2 h. Total lipids were extracted as described in the esterification assay. Saponification was then performed with methanolic potassium hydroxide after 30000 dpm of (1,2-3H)-cholesterol was added as an internal standard. The nonsaponifiable fraction was then extracted three times with hexane and after evaporation developed on Silica linear K plates in dichlormethane + 0.5% methanol. The spots corresponding for cholesterol or lanosterol were cut for counting after identification with iodine vapor. Total incorporation in sterols was determined after addition of all chromatographic spots. Results were expressed in relation to the cellular protein,

Figure 4 — Effects of various concentrations of verapamil, propranolol, diltiazem, and metoprolol on the incorporation of 14C-acetate in sterols. HEP G2 cells were incubated in lipid free medium with and without agents. 14C-acetate incorporation in total sterols (●), lanosterol (□), and cholesterol (○) was determined by thin layer chromatography. Results were expressed as the mean of three different experiments ± SEM.
determined after dissolving the adherent cells in 0.1% NaOH. A correction for procedural losses was made with the 3H-Cholesterol standard (recovery over 80%).

**DATA ANALYSIS**

Values were expressed as means of \( n \) experiments (indicated in the legends of figures) ± SEM. Zero percent was defined as results of control dishes without active agent. The significance of differences between means was established by Student’s \( t \)-test. As software WINSTAT\(^\circ\) (Kalmia Inc.) was used.

**Other Assays**

Cellular protein was determined with the Lowry method (37) using a bovine albumin solution as a standard.

**Results**

Figure 1 shows the effects of verapamil, propranolol, diltiazem, and metoprolol on \( {^{125}}I \)-LDL metabolism in HEP G2 cells. After 20 h incubation in lipid free medium the rate of specific degradation was suppressed by increasing concentrations of verapamil, propranolol, and metoprolol up to 55% in the highest concentration (30 \( \mu \)mol/L). Diltiazem had...
only a slight effect. Nearly corresponding to the inhibition of degradation an increase of intracellular accumulation of \(^ {125}\text{I}-\text{LDL}\) was observed. Verapamil showed significant effects beginning at 1 \(\mu\text{mol/L}\) (15% increase of degradation). Comparable to degradation diltiazem had little effect on the internalization. Binding was not significantly affected by any drug. With measurement of cholesterol esterification another step of cholesterol metabolism was studied (Figure 2). An impressive suppression was seen in the LDL (250 \(\mu\text{g/mL}\)) mediated \(^{14}\text{C}-\text{oleate}\) incorporation in cholesterol by verapamil, propranolol, and metoprolol. No significant effect was observed when 25-hydroxycholesterol (15 \(\mu\text{g/mL}\)) was used as a direct substrate for the acyltransferase. Comparable to the LDL receptor diltiazem had little if any effect on cholesterol esterification. Figure 3 shows the agents effect on \(^{14}\text{C}-\text{oleate}\) incorporation in triglycerides. An effect that was small (maximum 10%) was only seen in the highest concentrations used. There was no significant difference between the LDL (250 \(\mu\text{g/mL}\)) mediated or the 25-hydroxycholesterol (15 \(\mu\text{g/mL}\)) mediated activity. The agents effect on \(^{14}\text{C}-\text{acetate}\) incorporation in lanosterol, cholesterol, and total sterols were shown in Figure 4. All drugs increased the incorporation rate in lanosterol from +270% (verapamil 30 \(\mu\text{mol/L}\)) to +60% (diltiazem 30 \(\mu\text{mol/L}\)). Only metoprolol significantly affected the incorporation rate in cholesterol (−54%) and total sterols (−37%) at a concentration of 30 \(\mu\text{mol/L}\), whereas verapamil, propranolol, and diltiazem were neutral in this regard. Figure 5 showed the effects of propranolol and the alpha-receptor stimulating agent alpha-methyl-norepinephrine on \(^ {125}\text{I}-\text{LDL}\) processing in mononuclear leukocytes. In contrast to the \(\beta\)-blocker alpha-methylnorepinephrine inhibited LDL uptake up to 50% (16). Figure 6 showed the effects of the three calcium antagonists verapamil, diltiazem, and nifedipine on LDL processing in mononuclear leukocytes. Whereas nifedipine was neutral in this regard, diltiazem and more effectively verapamil increased the cellular association of the lipoprotein.

### Discussion

The widely used antihypertensives verapamil, diltiazem, nifedipine, propranolol, and metoprolol had drug and cell specific effects on cholesterol metabolism in human mononuclear and HEP G2 cells (Figures 1,2,4–6). Their effects seem to be limited to cholesterol metabolism since oleate incorporation in triglycerides was not influenced by these agents (Figure 3).

In hepatoma cells the main effect of the beta blockers and verapamil was an inhibition of intracellular, \(^ {125}\text{I}-\text{LDL}\) degradation. This leads to an increased intracellular accumulation of the lipoprotein. Cholesterol derived from this source does not stimulate its esterification (Figure 2) and, therefore, may be trapped in different cellular regulatory pools of cholesterol (38). Toxic effects or a direct inhibition of cholesterol acyltransferase was unlikely, since no significant inhibition by the drugs was seen in the presence of 25-hydroxycholesterol that was taken up without surface receptors and acts as a direct substrate of the enzyme. The drug effect may lead to a reduced availability of extracellular cholesterol and should, therefore, lead to an increased cholesterol de novo synthesis. In these experiments this was only true for the lanosterol fraction that represents for only 15% of \(^{14}\text{C}-\text{acetate}\) incorporation in sterols. However, we measured \(^{14}\text{C}-\text{acetate}\) incorporation in sterols and not HMG-CoA-reductase activity directly, it could be deduced, that the drug enhanced cellular accumulation of LDL delivers cholesterol for cellular demands by a hitherto uncharacterized mechanism (39) or reduces overall cholesterol turnover. Our results were in contradiction to Bell (22) who observed stimulated cholesterol esterification by propranolol in rat liver, suggesting a species specific regulation.

Compared to verapamil, diltiazem only affected lipid metabolism of mononuclear leukocytes. In these cells \(^ {125}\text{I}-\text{LDL}\) accumulation and degradation was stimulated. According to the neutral effects in liver cells diltiazem and nifedipine did not alter blood lipid levels (40,41) whereas two studies showed a significant decrease of serum cholesterol.
levels under verapamil therapy (42,43) fitting to the observed enhancement of liver uptake. A study of Daugherty et al. (21) yields a similar dissociation between verapamil and diltiazem effects regarding cholesterol ester accumulation of rabbit macrophages. Whereas verapamil leads to a significant reduction of $^{3}$H-oleate uptake, diltiazem was without any effect. According to Filipovic and Buddecke (14) data for fibroblasts a stimulation of LDL receptor number can be assumed in the leukocytes but not in HEP G2 cells, since surface binding was not influenced by the drug (Figure 1).

Metoprolol, as the only substance, leads to a suppression of LDL degradation as well as to an inhibited $^{14}$C-acetate incorporation in sterols. An suppression of LDL degradation as well as to an inhibited number can be assumed in the leukocytes but not in HEP G2 cells, since surface binding was not influenced by the drug (Figure 1). A study (44) showed that calcium channel blocker mediated inhibition of cholesterol esterification in macrophages was accompanied by enhanced excretion of cholesterol from the cells. If cholesterol synthesis would be blocked by the same drug (as could be extrapolated by the inhibited incorporation of $^{14}$C-acetate in cholesterol by metoprolol) peripheral cholesterol levels may be lowered.

Four of five agents affected at least one parameter of lipid metabolism in liver and peripheral cells in opposite directions (Table 1). The major differences are a stimulation by verapamil and diltiazem of LDL degradation in mononuclear, but an inhibition or no effect in HEP G2 cells. If the measured effects only partly exist in vivo, they may be of importance besides known other effects of antihypertensive agents (45). In this context it may be of interest that the concentrations of the agents we used are within the therapeutic range (1 $\mu$mol/L).

The mechanisms how the tested agents influence cellular lipid mechanism may be in accordance to their lipophilicity rather than involvement of specific processes (46). A chloroquinelike effect that explains reduced lysosomal LDL degradation was also described (11,12). Another fascinating mechanism was proposed as a theory how antihypertensive agents affect lipid metabolism—their antioxidative properties (46). As previously described, calcium antagonists and beta blockers differ widely in their potential of inhibiting LDL oxidation also following their degree of lipophilicity (47,48).

In summary, calcium channel and beta receptor blockers affect hepatic and peripheral cellular lipid metabolism in opposite directions. These “side-effects” have to be taken into account when a long-term therapy with these agents was considered. If the more neutral drugs nifedipine and metoprolol are superior to metabolic active drugs as propranolol or verapamil has to be shown in clinical trials. Additional laboratory studies are needed to measure drug effects on HMG-CoA-reductase ACAT and LDL receptor directly by DNA techniques (49) to clarify the role of cellular lipid metabolism in the atherogenic process and its modulation by drugs (50).

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References


