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Amiodarone Decreases Gene Expression of Low-Density Lipoprotein Receptor at Both the mRNA and the Protein Level

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Amiodarone, a potent antiarrhythmic drug, decreases plasma and tissue triiodothyronine (T₃) and increases plasma cholesterol levels, resembling changes seen during hypothyroidism. The increase of serum cholesterol during amiodarone medication is associated with a decreased expression of the hepatic low-density lipoprotein (LDL) receptor mRNA. To further elucidate the mechanism of amiodarone-induced hypercholesterolemia, we investigated whether the decreased mRNA levels are the result of decreased transcription or increased degradation or both, and whether protein expression is decreased accordingly. Relative to pair-fed controls, amiodarone treatment increased plasma cholesterol by 69% and decreased expression of the mRNA encoding for the hepatic LDL receptor by 45%. To study this decrease in mRNA, we performed a run-on assay, from which it appears that amiodarone acts by decreasing LDL receptor mRNA expression 2.5-fold at the transcriptional level. The decay rate of liver LDL receptor mRNA, measured at different time points after injecting actinomycin D, was not different between amiodarone-treated and control animals (116 ± 32 minutes and 84 ± 10 minutes, P = .44). Hepatocytes in primary culture isolated from amiodarone-treated and control animals were used to determine specific binding of [¹²⁵I]-LDL to hepatic LDL receptors. Amiodarone decreased specific LDL binding and Scatchard analysis demonstrated that amiodarone treatment reduced the number of LDL receptors by 69%, without affecting the dissociation constant (Kd). In conclusion, amiodarone-induced hypercholesterolemia can be explained by decreased transcription of the LDL receptor gene, resulting in lower mRNA and protein levels.

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Materials and Methods

Amiodarone was a kind gift of Sanofi B.V. (Maassluis, The Netherlands). Formaldehyde, formamide, β-mercaptoethanol, and guanidine isothiocyanate were from Merck, Darmstadt, Germany. Collagenase (type H); actinomycin D, rat-tail collagen, and β-actin riboprobe (digoxigenin-labeled) were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany), and [¹²⁵I] (carrier-free) in NaOH from Amersham (Buckinghamshire, UK). Ham’s F10, Hanks balanced salt solution (HBSS) and fetal calf serum (FCS) were obtained from Biowithacker (Brussels, Belgium). Fatty acid-free bovine serum albumin (BSA) was from Sigma (St Louis, MO). The cDNA probe for human LDL receptor (pLDLR3) was a generous gift from Dr D.W. Russell (Dallas, TX). All other reagents were of the highest grade possible.

Animals

Male Wistar rats (weight, 270 to 325 g) were housed under normal conditions with free access to tap water. All animal experiments were approved by the local animal welfare committee. Rats were divided into two groups. Group A was fed ad libitum on standard laboratory chow, and treated daily with 10 mg/100 g body weight (BW) amiodarone by gastric tube for 2 weeks. Group B was treated daily with water by gastric tube, and served as pair-fed controls of group A.

Experiment 1 was designed to evaluate the stability of the mRNA encoding for the LDL receptor. Twenty-four hours after the last gastric

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tubing, rats were intraperitoneally injected with actinomycin D (0.2 mg/100 g BW; reconstituted in water 0.4 mg/mL at 4°C) at 30, 60, or 120 minutes before they were killed (three rats per time point). Blood (EDTA) was collected by cardiac puncture and plasma stored at −20°C for determination of plasma lipids and thyroid hormones. The liver was removed from each rat and immediately frozen and stored in liquid nitrogen for further analysis (described later).

Experiment II was designed to evaluate the effect of amiodarone on LDL receptor protein. Rats (four per group) were treated with amiodarone (group A) or water (pair-fed controls, group B) as described earlier. Twenty-four hours after the last gastric tubing, rats were anesthetized by intraperitoneal injection of Nembutal (Sanofi, France), and rat livers were isolated and perfused in a recirculating system at 37°C to isolate the parenchymal cells.

**LDL Receptor mRNA Determination**

RNA was isolated from liver samples using a modified Chomczynski single-step RNA isolation method.8,9 mRNA was isolated from total RNA using the polyAT tract mRNA isolation system from Promega (Madison, WI). A 5-µg aliquot of polyA+ RNA was denatured and electrophoresed in 1% agarose/formaldehyde vertical gels. The separated RNAs were transferred to nylon membranes (Boehringer Mannheim) by pressure blotting and membranes were fixed using UV crosslinking (1 J/m²). The filters were hybridized at 55°C with a digoxigenin-labeled LDL receptor RNA probe. Hybridization and detection were performed as described previously.10 The blots were rehybridized at 68°C with a β-actin probe, labeled with digoxigenin, to standardize the amount of mRNA in each lane. The hybridization pattern of three identical experiments were analyzed. Several exposures of each blot were done on x-ray film (Fuji RX, Japan) and the relative amount of the mRNA was calculated using Eagle Eye II video-imaging system and OneD-scan software (Stratagene, La Jolla, CA). The exposure time was plotted against the intensity of each band. The optimal exposure time chosen was the one with the highest band intensity within the linear range, which was determined for each blot separately. The ratio between LDL receptor and β-actin was calculated.

The nuclear run-on assay was performed as described using digoxigenin-labeled uridine triphosphate (UTP) on nuclei isolated from livers of amiodarone-treated and control rats,10,11 and signals were quantified using a Lumimager (Boehringer Mannheim, Germany).

**LDL-Binding Assay**

*Isolation of rat hepatocytes.* Rat parenchymal cells were isolated as described previously.11 Briefly, the liver was perfused with a carbogen saturated buffer containing 142 mmol/L NaCl, 6.7 mmol/L KCl, and 6.7 mmol/L Hepes, pH 7.4. The liver than was removed and transferred to an ice-cold serum-free Ham’s F10 medium containing 2% BSA. Fresh albumin-containing medium was then added containing 5 to 100 µg/mL [125I]-LDL in the presence or absence of 500 µg/mL unlabeled LDL. Each incubation was performed in triplicate. The cells were incubated for 2 hours at 4°C. After incubation, the cells were washed five times with 0.15 mmol/L NaCl buffered with 0.05 mmol/L Tris/HC1 pH 7.4 containing 0.2% BSA, and twice with buffer without BSA. Finally, the cells were lysed with 1 mL 0.1-mmol/L NaOH and the radioactivity counted. The protein content of the samples was determined using a Biorad (München, Germany) protein assay.

**Assays**

Plasma cholesterol concentrations were determined by a fully enzymatic, kinetic UV method (Cobas analyzer; Boehringer Mannheim). Thyroid hormones were measured with in-house radioimmunoassays (RIAs),14 and thyroid-stimulating hormone (TSH) by a specific rat TSH RIA. Reagents used in the TSH-RIA were r-TSH-RP-2 (AFP-5153B) as reference preparation, anti r-TSH-S-5 (C 21381) as antibody, and r-TSH-I-8 (AFP-8334B) as antigen for iodination (courtesy of National Institute of Arthritis, Diabetes, Digestive & Kidney Diseases, Bethesda, MD).

**Calculations and Statistical Analysis**

Student’s t test was used to evaluate difference in serum cholesterol and thyroid hormones between amiodarone-treated and control rats. Data from experiment I and II were analyzed by ANOVA. The Excel 5 statistical package (Microsoft, Redmond, WA) was used.

Calculation of the binding of LDL to LDL receptor protein in experiment II was performed as follows. Specific binding was calculated by subtracting nonspecific binding from the total binding. The radioligand binding analysis program LIGAND (Biosoft, Cambridge, UK) was used to estimate the affinity constant and maximal-binding capacity (MBC). We obtained nonlinear Scatchard plots, most probably caused by an inequality of the affinity constant of the labeled and unlabeled ligand.17 The ratio (a) between the affinity constant of the unlabeled ligand and the labeled ligand, and the ratio (b) between the concentration of unlabeled and labeled ligand were calculated from the binding data according to Hollemans and Bertsom18 and Hollemans and Bertins17 and subsequently applied to calculate the MBC and the affinity constant for the unlabeled ligand.

**RESULTS**

Amiodarone treatment, as compared with pair-fed controls, resulted in a significant increase of plasma cholesterol and T₃ and a decrease of plasma T₄ (Table 1). Plasma TSH did not increase significantly.
The rate of LDL receptor mRNA disappearance after administration of the transcription inhibitor actinomycin D was determined by Northern blot analysis, using β-actin as internal standard (Fig 1A). The hybridization patterns of three identical experiments were analyzed. The relative mRNA expression at time zero of the pair-fed controls was taken as one. The amount of LDL receptor mRNA (log values) was plotted against time (Fig 1B). Treatment with amiodarone reduced the level of LDL receptor mRNA by approximately 45% relative to pair-fed controls, as evident from time-zero levels in Fig 2. The mRNA half-lives (t1/2) were calculated assuming first-order kinetics. The t1/2 of LDL-R mRNA in controls was not different from that in amiodarone-treated rats (84 ± 10 and 116 ± 32 minutes, respectively; P = .44, n = 3). We also performed a run-on experiment on nuclei isolated from livers of control rats and those treated with amiodarone. As can be seen in Fig 2, the transcription from the LDL receptor gene is reduced 2.5-fold in the amiodarone-treated animals compared with controls.

Figure 3 shows the specific binding of LDL to isolated hepatocytes. The binding of LDL to LDL receptors increased with increasing amounts of added LDL. The binding of LDL to hepatocytes isolated from amiodarone-treated rats was approximately two times lower than the binding to control hepatocytes. Data obtained from this experiment were also evaluated by Scatchard analysis. A representative plot is depicted in the insert of Fig 3. The slope and the intercept of the fitted line indicate the dissociation constant (Kd) of the binding of LDL to LDL receptors and the MBC or total number of LDL receptors, respectively. The Kd was not different between amiodarone-treated animals and pair-fed controls (Table 2), but the number of LDL receptors was decreased by 69% (Table 2).

**DISCUSSION**

In agreement with our previous study, amiodarone treatment resulted in an increase of plasma T4 and a decrease of plasma T3, explained by inhibition of T4 5'-deiodination in the liver. The
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Fig 2. Effect of amiodarone on LDL receptor gene transcription. Dots represent the result of a run-on experiment performed on nuclei purified from livers of control or amiodarone-treated rats. β-actin was used as a control to normalize the LDL receptor expression data.

Reduction in plasma T₃ in this study is comparable to that previously published.⁵,¹⁹ The plasma and tissue concentrations of rats treated with 10 mg/100 g BW closely resemble those in patients on long-term amiodarone treatment.²⁰ Plasma cholesterol increased by 69%; this cannot be explained by the mild increase of TSH, because levels up to 7.6 ng/dL in experimentally induced hypothyroidism do not increase plasma cholesterol as observed previously.³ In a previous study, we demonstrated that the increase in plasma cholesterol during amiodarone treatment is due to an increase in both LDL and HDL cholesterol, whereas triglyceride levels remained unchanged.⁶ In line with our previous study, the liver mRNA encoding the LDL receptor was decreased by approximately 45% after 2 weeks of amiodarone treatment. We evaluated the degradation of the LDL receptor mRNA by injecting actinomycin D at various time intervals before the animals were killed. The dose used was twice the dose shown to decrease total RNA transcription by more than 80% in rat liver.²¹ The rate of LDL receptor mRNA degradation was not different between the amiodarone-treated animals and the pair-fed controls, as evident by the similar slope of the regression lines (Fig 2). We calculated the t₁/₂ of the LDL receptor mRNA as 84 minutes in control rats. This figure is comparable to that obtained by other investigators using human cells.²²⁻²⁴ The only other report in the literature on the t₁/₂ of the LDL receptor mRNA in relation to thyroid hormone status is that by Ness et al.²⁵ They observed a t₁/₂ of approximately 30 minutes in young hypophysectomized rats, which did not change after treatment with T₃. The discrepancy with our results might be due to difference in age (adult v

Table 2. Effects of Amiodarone on Liver LDL Receptors

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MBC (ng/mg protein)</th>
<th>Kd (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>64 ± 7.5</td>
<td>77 ± 15</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>4</td>
<td>20 ± 8.8*</td>
<td>41 ± 21</td>
</tr>
</tbody>
</table>

NOTE. Values are means ± SE.
*P< .01 v controls.

Fig 3. Effect of amiodarone treatment on expression of LDL receptor. Specific binding of [¹²⁵I]-LDL to LDL receptors in isolated hepatocytes from amiodarone-treated (I) and control rats (i) (values are mean ± SE of 4 independent experiments). Insert: Scatchard analysis of binding of LDL to LDL receptors in isolated hepatocytes from amiodarone-treated (I) and control animals (i). Representative plot shown.
young) or hormonal status (intact vs hypophysectomized rats). The latter seems more plausible, because rats easily become hypercholesterolemic after hypophysectomy, by which they lose their natural resistance to develop high cholesterol levels when the cholesterol content of the diet is increased. Because the stability of the LDL receptor mRNA is not affected, whereas its gene expression is, we conclude that the amiodarone-induced decrease in LDL receptor mRNA is caused by decreased transcription.

Our second set of experiments demonstrates that amiodarone also decreases the expression of the LDL receptor at the protein level: the number of LDL receptors in the liver of amiodarone-treated animals were on average 69% lower than that of pair-fed controls. This is evident from Scatchard analyses of the binding of LDL to isolated hepatocytes in primary culture. The experiments were performed at 4°C to prevent internalization of LDL receptors. Although the number of LDL receptors is in the same order of magnitude as reported by others, the observed Kd is higher than reported. This could (partly) be the result of the collagenase treatment during the preparation of the primary hepatocytes. It is interesting to note that amiodarone did not decrease the affinity of the LDL receptor for LDL binding, although the variation in the Kd between the experiments was rather large. Given the inherent variability of the assays, we believe that amiodarone decreases the LDL receptor mRNA and protein levels about equally. An additional mechanism by which the number of cell surface receptors is decreased could be via a amiodarone-induced decrease in growth hormone concentration. It has been shown that growth hormone can restore LDL receptor expression in livers of hypophysectomized rats. On the other hand, we have recently obtained evidence using transfection experiments that points to a thyroid hormoneresponsive element in the promoter of the LDL receptor gene (F. Hudig, unpublished observations, 1997) and we therefore suggest that at least a large part of the amiodarone effect is direct via the LDL receptor promoter. Another way in which amiodarone could influence the number of LDL receptors on the cell surface could be a non-genomic effect on LDL receptor recycling, as has been shown for the β-adrenergic receptor.

The present results strengthen the hypothesis that a local hypothyroid-like condition in the liver is connected to the amiodarone-induced hypercholesterolemia. Hypothyroidism in experimental animals is, in addition to the increase of plasma cholesterol, associated with a decrease in hepatic LDL receptor mRNA, which is not caused by changes in mRNA stability. The expression of the LDL receptor protein in hepatocytes in culture from hypothyroid rats is also decreased relative to euthyroid controls. Amiodarone mimics these effects of thyroid hormone deficiency. The mechanism by which amiodarone exerts these hypothyroid-like effects remains to be elucidated. Thyroid hormones increase the expression of LDL receptors in rat liver both at mRNA and at the protein level. Putative thyroid hormone-responsive elements are present in the promoter region of the gene encoding for the LDL receptor. It might thus be that the poten inhibition of extrathyroidal T3 production induced by amiodarone, which causes a very low T3 content of several tissues, including the liver, which results in a low T3 receptor occupancy and thereby decreases transcription of the LDL receptor gene. The recently observed inhibition of the binding of T3 to its nuclear aT and β receptors by desethylamiodarone, the major metabolite of amiodarone, could reduce the occupancy of T3 receptors even further. We have recently shown that T3 counteracts the amiodarone-induced increase in plasma cholesterol and decrease in liver LDL receptor gene expression, which suggests that the inhibitory effect of amiodarone on LDL receptor gene expression is mediated via T3-dependent pathways. However, other mechanisms are also possible. It has been shown that amiodarone, a lipophilic drug that accumulates to a great extent in liver and adipose tissue, stiffens membranes. This could interfere with the internalization of membrane-bound receptors and affect the number of membrane receptors, like the LDL receptor.

We conclude that the amiodarone-induced increase of plasma cholesterol levels can be explained by a reduced transcription of the LDL receptor gene, which results in a decrease of the LDL receptor protein on hepatocytes. It is likely that the reduced transcription is related to an amiodarone-induced hypothyroid-like condition in the liver.

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