Expression of human cholesterol 7α-hydroxylase in atherosclerosis-susceptible mice via adenovirus infection

Gina L. MOORE*, Christian A. DREVON†, Dietrich MACHLEDER‡, John D. TRAWICK*, Alan McCLELLAND§, Soumitra ROY§, Russette LYONS†, Robert JAMBOU§ and Roger A. DAVIS‖

Adult mice from the atherogenic C57BL/6J strain were infected with an adenovirus vector containing the human 7α-hydroxylase cDNA (AV17h1) and fed a chow diet, human 7α-hydroxylase mRNA and enzyme activity doubled compared with that in mice infected with an adenovirus vector (AV1Null) alone. In AV17h1-infected mice fed on a high fat cholic acid (HFCA) diet, mRNA expression and activity of both the endogenous and adenovirus (human) 7α-hydroxylase were repressed. AV17h1-infected mice fed on a HFCA diet and killed at mid-light had increased 7α-hydroxylase activity and mRNA compared with mice killed at mid-dark. Since expression of AV17h1 is driven by a constitutive Rous sarcoma virus promoter, the repression of human 7α-hydroxylase by the HFCA diet was unexpected. In spite of this post-transcriptional repression by the HFCA diet, AV17h1-infected mice expressed the human 7α-hydroxylase mRNA, causing its enzyme activity to be 3-fold greater than in AV1Null-infected mice. In AV17h1-infected mice, the 7α-hydroxylase enzyme activity varied as a linear function of human mRNA abundance. In conclusion, the accumulation of apolipoprotein B-containing lipoproteins in plasma of C57BL/6 mice fed on the HFCA diet was not reduced by longer-term (2 weeks) 7α-hydroxylase expression, probably because of its diminished expression caused by the diet and hepatic inflammation from the adenovirus infection. These results may suggest that adenovirus is effective in promoting longer-term (2 weeks) expression of 7α-hydroxylase.

INTRODUCTION

Cholesterol 7α-hydroxylase (7α-hydroxylase) is a liver-specific gene product that is rate-limiting in the bile acid-synthetic pathway [1–10]. Since the excretion of bile acids is the major quantitative pathway responsible for removing cholesterol from the body, hepatic expression of 7α-hydroxylase plays an essential role in maintaining cholesterol homeostasis [1–10]. Expression of 7α-hydroxylase is affected by hormones, diet and other physiological conditions (reviewed in [5,6]). Two major dietary effectors of 7α-hydroxylase expression are cholic acid and cholesteryl, having opposite effects. In several species, dietary cholic acid decreases whereas cholesteryl increases the expression and activity of 7α-hydroxylase. We have reported that in C57BL/6 mice, cholic acid is required as a component of the cholesterol-rich diet to: (1) decrease the expression of hepatic low-density lipoprotein (LDL) receptors; (2) repress the expression of 7α-hydroxylase; and (3) cause changes in plasma lipoproteins (i.e. increased very-low-density lipoprotein [VLDL]/LDL [10]). Decreased high-density lipoprotein (HDL) has been linked to increased susceptibility of these mice to develop atherosclerosis [11–13].

Short-term (2 days) adenovirus-mediated gene transfer of cholesterol 7α-hydroxylase in hamsters results in significantly decreased plasma LDL levels caused by increased hepatic LDL-receptor-mediated uptake [14]. The present study aimed to answer the following two questions. (1) Is adenovirus an effective gene-delivery system for longer-term studies (2 weeks) of cholesterol 7α-hydroxylase? (2) Does adenovirus-mediated expression of 7α-hydroxylase ameliorate diet-induced hypercholesterolaemia in C57BL/6 mice which are susceptible to atherosclerosis?

EXPERIMENTAL

Animals and diets

Female C57BL/6J mice, 10–12 weeks old, were obtained from Jackson Laboratory, Bar Harbor, ME, U.S.A. The mice were housed in a room with a reverse-light cycle (lights from 18:00 h to 06:00 h). They were fed Chow and water ad libitum. After 1 week, the mice were divided randomly without consideration of body weight into two groups: 30 were fed on Purina rodent breeder chow (chow diet), and 60 were fed on a Foodtech ‘high fat’ chow diet supplemented with 15% fat, 1.25% cholesterol and 0.5% sodium cholate [high-fat cholic acid (HFCA) diet], as described [1]. After 3 weeks, blood (100 µl) was obtained from the retro-orbital plexus and mice were injected with the adenovirus or saline control, as described below. Mice were maintained on the same diets for 2 more weeks and then killed via exsanguination under isoflurane anaesthesia. Among the different

Abbreviations used: AV17h1, adenovirus vector containing the human cholesterol 7α-hydroxylase cDNA; AV1Null, adenovirus vector alone; HFCA diet, chow diet supplemented with 15% fat, 1.25% cholesterol and 0.5% sodium cholate; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein; poly(A)+, polyadenylated; RSV, Rous sarcoma virus.  † To whom correspondence should be addressed.
dietary groups, there was no difference in the amount of food consumed or body weight gain throughout the study. Four mice were deleted from the study because of tail vein aneurysm or abnormal appearance before tail vein injection.

Recombinant adenovirus
A cDNA clone containing the human 7α-hydroxylase coding region and no 3'-untranslated region was provided by Dr. Daniel Soltis, Sandoz Pharmaceuticals. This sequence was found to be identical with the previously described allele [13]. The cDNA was ligated into the adenovirus shuttle vector pAVS6 and was used to construct a replication-deficient adenovirus vector [14]. The resulting recombinant adenovirus was designated AV17h1.

Mouse tail vein injection of recombinant adenovirus
Mice were injected with one of the following: the human 7α-hydroxylase adenovirus (AV17h1), the adenovirus vector without insert (AVINull) or the saline vehicle (Hanks buffered-saline solution) via the tail vein. Each mouse received a vector dose of 109 plaque-forming units in a volume of 200 µl of Hanks balanced-salt solution.

7α-Hydroxylase activity in isolated mouse liver microsomes
Mouse liver microsomes were isolated immediately after the mice had been killed, placed in liquid nitrogen and then stored at −70°C. The 7α-hydroxylase assay included microsomes (0.5–1.0 mg of protein) incubated with [4-14C]cholesterol in the form of liposomes, for 60 min at 37°C in the presence of an NADPH-generating system [15]. The incubation was terminated by the addition of 2 ml of 100% ethanol containing 1 µg/ml butylated hydroxytoluene. The reaction mixture was extracted three times with 2 ml of hexane, then the organic layer was washed with 1 ml of PBS, pH 7.4. The hexane extracts were treated with steroid 3α-oxidase and then separated using reverse-phase HPLC [16]. The results are expressed as specific activities and were calculated using UV quantification of 7α-hydroxycholesterol mass produced over 30 min incubation (pmol/min per mg of protein). Since there was no significant difference in the weight of the livers or the yield of microsomal protein/g of liver among the different groups, specific activities should be a valid indicator of the relative activity of 7α-hydroxylase/mouse.

Isolation of RNA
The livers were cut out and immediately placed in liquid N2 and stored at −70°C. Frozen livers were immediately homogenized in 5 ml of solution D (4 M guanidinium thiocyanate/25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) using a Tekmar tissuemizer. Total RNA was isolated as described previously [17]. Polyadenylated [poly(A)+] mRNA was isolated using Collaborative Biomedical Product Type 3 oligo(dt)-cellulose (Bedford, MA, U.S.A.) [18]. The cellulose mixture was divided up into ten RNase-free 1.7 ml microcentrifuge tubes, and total RNA in 100 µl of loading buffer (0.5 M LiCl/10 mM Tris/HCl, pH 7.5, 1 mM EDTA, pH 8.0, 0.1% SDS) was added to the cellulose mixture, which was mixed by inversion, wrapped in parafilm and gently rocked at room temperature overnight. RNA/oligo(dt)-cellulose mixture was washed with 0.5 ml of loading buffer twice. Then poly(A)+ mRNA was eluted from the oligo(dt)-cellulose by adding 1.0 ml of elution buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.05% SDS) to the tube, which was vortex-mixed briefly, rocked at 37°C for 5 min and centrifuged at 10000 g for 30 s. The supernatant was pipetted into a 2.0 ml microcentrifuge tube, and cold 100% ethanol was added to precipitate mRNA.

Northern blotting and quantification of mRNA
Relative abundance of specific mRNAs was determined by Northern-blot analysis [18]. Poly(A)+ mRNA (20 µg) was denatured in a solution containing 100% deionized formamide, 37°C (1/100) formaldehyde and 20 × borate buffer (65 mM sodium tetraborate, 0.62 M boric acid, 0.5 M EDTA, pH 8.0), then loaded on to a 0.8% agarose/3% formaldehyde/1 × borate gel and electrophoresed for 3 h at constant current (70 mA). The gel was briefly washed with water, then with 10 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) and transferred to a nylon membrane (Zetaprobe GT; Bio-Rad). The membrane containing the transferred mRNA was UV-cross-linked in Stratagene Stratalinker and subsequently prehybridized in 20 ml of prehybridization-hybridization solution (7% SDS, 12 mM NaH2PO4 and 12 mM NaCl) at 44°C for 30 min. The blots were sequentially hybridized with 32P-labelled (nick-translated) probes corresponding to: (1) the 1200 bp EcoRI fragment of a cDNA clone for human 7α-hydroxylase (obtained by PCR), a 1640 bp EcoRI fragment of a cDNA clone for rat 7α-hydroxylase (a gift from Dr. David Russell) [2] and a 2000 bp fragment of a cDNA encoding β-actin [19]. Blots were first probed with the human 7α-hydroxylase cDNA, stripped in a low-salt buffer solution (0.1 × SSC, 0.05% SDS) at 70°C for 24 h, then reprobed with the rat 7α-hydroxylase cDNA. The size of the human 7α-hydroxylase (no 3'-untranslated region; approx. 2.2 kb) meant that it could be easily separated from the multiple forms of the endogenous mouse mRNA. Therefore, even though there was cross-hybridization of the human and mouse mRNA with the human and rat cDNA probes, quantification of each mRNA was not affected (Figure 1). The blots were stripped and reprobed for β-actin before being analysed by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The amount of 7α-hydroxylase RNA was quantified relative to that of β-actin [18] using the ImageQuant program from Molecular Dynamics.

Serum lipid determinations
Serum (100 µl) was fractionated into VLDL/LDL and HDL using a buffer consisting of 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl and 0.01% NaN03 on a Superoxie 6 FPLC column (Pharmacia). Aliquots of each lipoprotein fraction were subjected to robotic lipid analysis using commercially available enzymic kits and calibration standards (Sigma) [1].

Statistical analysis
All data are reported as means ± S.D. Statistical significance was determined by Student’s t test. Values of P ≤ 0.05 were considered to be significant.

RESULTS
Effect of atherogenic diet on expression of endogenous 7α-hydroxylase mRNA and activity
In rats, expression of 7α-hydroxylase (activity and mRNA) is sensitive to feeding schedule (diurnal variation), being highest at mid-dark and lowest at mid-light [5,6]. To determine the effects of the atherogenic diet and the adenovirus itself (i.e. AVINull vector) on the maximum level of endogenous 7α-hydroxylase, mice were killed during the mid-dark period. Without adenovirus...
Table 1  Dietary effects on the enzyme activity and expression of mouse 7α-hydroxylase mRNA

C57BL/6 mice were fed on either HFCA or chow diet. After 3 weeks, they were injected with either saline or AV1Null adenovirus and maintained on the same diet for 2 weeks. They were then killed at mid-dark. The amount of 7α-hydroxylase mRNA was quantified relative to β-actin mRNA by Northern-blot analysis. 7α-Hydroxylase activity was determined in hepatic microsomes. Values are presented as means ± S.D. for the number of mice indicated. N.D. Not detectable. *P < 0.01 compared with values for chow-fed mice.

<table>
<thead>
<tr>
<th>Injection</th>
<th>Diet</th>
<th>Mouse 7α-hydroxylase mRNA</th>
<th>7α-Hydroxylase activity (pmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>HFCA</td>
<td>N.D.</td>
<td>2.1 ± 0.6 (n = 6)</td>
</tr>
<tr>
<td>Saline</td>
<td>Chow</td>
<td>1.6 ± 1.4 (n = 7)</td>
<td>25.9 ± 7.5 (n = 7)*</td>
</tr>
<tr>
<td>AV1Null</td>
<td>HFCA</td>
<td>N.D.</td>
<td>2.6 ± 1.4 (n = 9)</td>
</tr>
<tr>
<td>AV1Null</td>
<td>Chow</td>
<td>0.21 ± 0.11 (n = 6)</td>
<td>8.3 ± 2.8 (n = 7)*</td>
</tr>
</tbody>
</table>

Adenovirus-mediated expression of human 7α-hydroxylase mRNA and activity

Mice eating the chow diet (mid-dark period) exhibited 20-fold higher expression of the mRNA encoding human 7α-hydroxylase than those fed on the HFCA diet (Table 2). These data indicate that the HFCA diet reduced the expression of the adenovirus-derived human 7α-hydroxylase.

Mice killed during the mid-light period displayed 6-fold more human mRNA than those killed during the mid-dark period (Table 3). The increase in 7α-hydroxylase mRNA observed in mice killed during the mid-light period compared with those killed during the mid-dark period was derived from the adenovirus vector (human), whereas the endogenous (mouse) 7α-hydroxylase mRNA remained repressed (Figure 1). Moreover, the increased hepatic content of human 7α-hydroxylase mRNA observed in AV17h1-injected mice fed on the HFCA diet, killed during the mid-light period, was functionally expressed by a 70% increase in enzyme activity (Table 3). To examine further how expression of human 7α-hydroxylase influenced total enzyme activity, the relative abundance of human 7α-hydroxylase mRNA was correlated with the enzyme activity of hepatic microsomes (Figure 2). In mice from both dietary groups, enzyme activity varied as a linear function of human 7α-hydroxylase mRNA (Figure 2: r = 0.608, P < 0.01).

Effect of expression of human 7α-hydroxylase mRNA on enzyme activity and plasma cholesterol levels

In the chow-fed mice, both adenovirus vectors AV17h1 and AV1Null significantly increased VLDL/LDL cholesterol 3-fold, compared with saline only. This increase in VLDL/LDL chol-
Moreover, in spite of the 2–4-fold increase in 7α-hydroxylase activity on the HFCA diet showed the expected decrease in HDL cholesterol. Similar plasma levels of lipoproteins in mice receiving either the Saline Injection and diet Plasma VLDL/LDL HDL indicated.* was determined enzymically. Each value represents the means ± S.D. for the number of animals indicated. *P < 0.05 compared with values for chow-fed mice.

**Table 4 Effects of adenovirus infection on serum lipoprotein cholesterol levels**

Serum obtained from mice fed on chow or HFCA diet and killed during the mid-dark was separated by FPLC. The total cholesterol content of individual plasma and lipoprotein fractions was determined enzymically. Each value represents the means ± S.D. for the number of animals indicated. *P < 0.05 compared with values for chow-fed mice.

<table>
<thead>
<tr>
<th>Injection and diet</th>
<th>Plasma (µg/ml)</th>
<th>VLDL/LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chow (n = 7)</td>
<td>64 ± 11</td>
<td>9 ± 4</td>
<td>58 ± 8</td>
</tr>
<tr>
<td>HFCA (n = 5)</td>
<td>227 ± 71*</td>
<td>177 ± 48*</td>
<td>50 ± 24*</td>
</tr>
<tr>
<td>AV1Null</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chow (n = 7)</td>
<td>80 ± 11</td>
<td>24 ± 5</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>HFCA (n = 9)</td>
<td>254 ± 97*</td>
<td>201 ± 97*</td>
<td>53 ± 9</td>
</tr>
<tr>
<td>AV17h1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chow (n = 7)</td>
<td>79 ± 6</td>
<td>21 ± 3</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>HFCA (n = 9)</td>
<td>297 ± 73*</td>
<td>233 ± 79*</td>
<td>64 ± 16</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Our previous findings suggested that cholic acid is required for the repression of 7α-hydroxylase and the diet-induced hypercholesterolaemia [1]. To examine this hypothesis, we expressed 7α-hydroxylase in atherosclerosis-susceptible mice in a manner that presumably would not be affected by the HFCA diet. We expected that the adenovirus Rous sarcoma virus (RSV)-driven expression of 7α-hydroxylase would be insensitive to cholic acid repression, allowing us to determine the role of this bile-acid-repressible enzyme in the diet-induced changes in plasma lipoproteins [10,11]. Short-term (~3 days) studies of adenovirus gene therapy have shown that expression of the desired gene product was sufficiently great to observe a significant physiological response in the form of changes in lipid metabolism, e.g. 7α-hydroxylase [14], LDL receptors [21,22], LDL-receptor-related protein [23] and the apolipoprotein B-encoding enzyme [24]. Since a minimum of 2 weeks is required to reach steady state for plasma lipoproteins and the diurnal variation of 7α-hydroxylase, we examined whether adenovirus-driven expression of 7α-hydroxylase would be effective for this extended period of time. Although at the conception of these studies it was realized that hepatic inflammation was a time-related consequence of adenovirus gene delivery [20], it was not known whether this might affect gene therapy or lipoprotein metabolism.

Our results support the following three conditions: (1) adenovirus gene therapy allows longer-term (2 weeks) expression of human 7α-hydroxylase in mice; (2) unexpectedly, the HFCA diet diminishes the expression of 7α-hydroxylase in spite of it being driven by a constitutive RSV promoter; (3) longer-term (2 weeks) adenovirus transfection is associated with a 3-fold increase in VLDL/LDL cholesterol. This is probably caused by an inflammatory reaction [20] and the unexpected diminished expression of 7α-hydroxylase in mice fed on the HFCA diet. However, our results provide valuable new insights into the regulation of 7α-hydroxylase by diet and the obstacles that must be overcome to design effective gene therapies.

Adenovirus gene delivery of human 7α-hydroxylase provided longer-term (2 weeks) expression of human 7α-hydroxylase mRNA and activity. This conclusion is based on the data showing a 3-fold increase in 7α-hydroxylase activity in mice fed on the HFCA diet and injected with AV17h1 (Tables 2 and 3), as compared with those treated with the AV1Null vector or the saline vehicle alone (Table 1). We also observed that, in mice fed on the HFCA diet, the activity of 7α-hydroxylase correlated linearly with the relative abundance of the human 7α-hydroxylase mRNA (Figure 2). Other studies on hamsters fed on a cholesterol-rich diet (without cholic acid) showed that short-term (2 days) adenovirus-delivered expression of 7α-hydroxylase caused marked reduction in plasma LDL levels [14]. Differences in animals (hamsters compared with mice), diets (chow and without cholic acid) and time of duration may explain the different effects of 7α-hydroxylase on plasma lipoprotein levels. Differences in the promoters used (RSV in this study and cytomegalovirus in the hamster study) may also contribute to the ability to express 7α-hydroxylase at levels sufficient to affect diet-induced hypercholesterolaemia. Whereas adenovirus gene delivery provides a convenient method of examining short-term physiological responses, our results suggest that our adenovirus vector driven by RSV and expressed for 2 weeks in C57BL/6 mice is associated with an inflammatory response that greatly alters lipoprotein metabolism. Our results do not indicate that other adenovirus vectors administered to other animal models will promote similar inflammatory-induced changes in lipoprotein metabolism.
Two observations show that the HFCA diet diminished adenovirus expression of human \( \alpha \)-hydroxylase. First, independently of the time of death, mice fed on the HFCA diet expressed significantly less human \( \alpha \)-hydroxylase mRNA than those fed on the chow diet (Table 2). Second, when fed on the HFCA diet, mice expressed less \( \alpha \)-hydroxylase mRNA during the mid-dark period, when they were fasting (Figure 2 and Table 3). The diurnal variation in expression of human \( \alpha \)-hydroxylase in mice fed on the HFCA diet argues against the possibility that diet may have affected the number of \( \alpha \)-hydroxylase expression-competent virus particles in the liver. The mechanism responsible for the reduction in adenovirus expression of human \( \alpha \)-hydroxylase caused by the HFCA diet is not known. A ‘bile acid’ response element has been identified in the promoter of \( \alpha \)-hydroxylase [25–27]. This element is not present in our adenovirus response element or repression of RSV-promoter-driven ex- 

mRNA expression by a mechanism unrelated to the ‘bile acid’ work). Our present data suggest that the HFCA diet may change RSV-promoter-driven gene products (R. A. Davis, unpublished work). Our present data suggest that the HFCA diet may change mRNA expression by a mechanism unrelated to the ‘bile acid’ response element or repression of RSV-promoter-driven expression. Elucidation of this mechanism should provide a clearer understanding of how the HFCA diet represses \( \alpha \)-hydroxylase mRNA expression and enzyme activity.

This study was supported by the National Institutes of Health grants no. HL37195 and HL52005 and by the Research Council of Norway.

REFERENCES