The lipid-lowering effects of 3-hydroxy-3-methylglutaric acid and bile acid drainage in WHHL rabbits

J. L. M. Van Niekerk*, Th. Hendriks*, J. A. Gevers Leuven†, L. Havekes† and H. H. M. de Boer*

*Department of General Surgery, St Radboud University Hospital, Nijmegen, The Netherlands and †Gaubius Institute, Health Research Division TNO, Leiden, The Netherlands

(Received 27 October 1983/27 February 1984; accepted 29 March 1984)

Summary

1. The effect of 3-hydroxy-3-methylglutaric acid (HMG) on serum cholesterol levels was investigated in Watanabe heritable hyperlipidaemic (WHHL) rabbits.
2. Oral administration of HMG resulted in a reduction of serum cholesterol by 39%.
3. Bile acid drainage, by means of either cholestyramine medication or partial ileal bypass (PIB) surgery, also led to significant reductions in circulating cholesterol, by 35 and 59% respectively.
4. Intraperitoneal injection of HMG after PIB surgery further reduced serum cholesterol by 35%.
5. Fibroblasts from the WHHL rabbits did not show high-affinity binding, uptake or degradation of 125I-labelled low density lipoprotein (LDL).
6. The working mechanism of these lipid-lowering therapies in WHHL rabbits is discussed in relation to recent literature.
7. The significant reductions in circulating cholesterol induced by HMG warrant further investigation into the use of this compound in the management of familial hypercholesterolaemia.

Key words: cholestyramine, 3-hydroxy-3-methylglutaric acid, hypercholesterolaemia, hypolipidaemic drugs, WHHL rabbit.

Abbreviations: HMG, 3-hydroxy-3-methylglutaric acid; LDL, low density lipoprotein; PIB, partial ileal bypass; VLDL, very low density lipoprotein; WHHL, Watanabe heritable hyperlipidaemic.

Introduction

Among the risk factors for atherosclerotic cardiovascular disease, hypercholesterolaemia is thought to be one of the most important. Consequently, therapy in familial hypercholesterolaemia aims to induce a maximal lowering of serum cholesterol.

While diet and drug treatment may be sufficient in groups of patients with the heterozygous form of the disease, homozygous familial hypercholesterolaemia remains a very difficult condition to treat and has failed to respond adequately to a variety of drug regimens [1, 2].

The search for more effective lipid-lowering treatments in homozygous familial hypercholesterolaemia has been hampered by the fact that no suitable animal model for this human disease has been available. Recently, a unique experimental model has been developed in the form of the Watanabe heritable hyperlipidaemic (WHHL) rabbit [3]. Hypercholesterolaemia in these animals shows great similarity to the human form of the disease [4]. Experiments in our laboratory have revealed that surgical intervention in the form of a partial ileal bypass (PIB) greatly reduces serum cholesterol levels in WHHL rabbits [5].

Drugs which may possibly affect serum cholesterol levels are bile acid resins, e.g. cholestyramine, and competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol synthesis. The present paper reports on the lipid-lowering
potential of one such inhibitor, 3-hydroxy-3-methylglutaric acid (HMG), in WHHL rabbits and compares its effects with those obtained by bile acid drainage.

Methods
Homozygous female WHHL rabbits were bred by crossing and back-crossing between New Zealand White rabbits and WHHL rabbits (a generous gift from Dr Y. Watanabe).

The animals were divided into two groups. Group I \((n = 5)\) first received HMG orally during 6 weeks; subsequently, a PIB was constructed and the animals were given HMG orally for 4 weeks, followed by HMG intraperitoneally for 6 weeks. Group II \((n = 7)\) received cholestyramine for 6 weeks and thereafter a combination of cholestyramine and HMG (fed orally) also during 6 weeks.

The rabbits were caged individually and fed \textit{ad libitum} on a chow diet (LK04; Hope Farms, Woerden, The Netherlands). During the cholestyramine studies the animals were fed a daily dose of 3 g of cholestyramine (Questran; a gift from Bristol-Myers BV, The Netherlands) which had been added to the normal food mixture before pelleting. HMG (Sigma Chemical Company, St Louis, MO, U.S.A.) was dissolved in drinking water and given in a dose of 100 mg per day. During one experimental period a similar dose, dissolved in 5 ml of physiological saline, was injected intraperitoneally each day. The PIB operation was performed as described elsewhere [5].

Blood samples were collected after the animals had been deprived of food overnight. Cholesterol in serum was measured by the CHOD-PAP method [6, 7] and triglycerides by an enzymatic method [8].

For measuring LDL receptor activity in WHHL rabbits from group II and in normal rabbits, fibroblasts from the respective rabbits were isolated from explants of a skin biopsy and cultured as described elsewhere [9]. LDL was isolated from normal human serum by density gradient ultracentrifugation [10]. The isolated LDL was labelled with \(^{125}\)I [11] and stabilized by the addition of lipoprotein-depleted serum (LPDS, 20% by vol.). Eighteen hours before the binding experiment, the culture medium was replaced with medium containing 20% LPDS instead of 20% serum. Thereafter the cells were incubated with different amounts of \(^{125}\)I-LDL in the same medium in the presence or absence of an excess of unlabelled LDL. After 2.5 h of incubation at 37°C the binding, internalization and degradation of \(^{125}\)I-LDL were measured essentially as described by Goldstein \textit{et al.} [12]. The values obtained after incubation in the presence of an excess of unlabelled LDL were subtracted from the respective values obtained in the absence of unlabelled LDL in order to obtain high-affinity binding, internalization and degradation.

The effects of various treatments on serum cholesterol levels were evaluated statistically by comparing values obtained during treatment with the values obtained in the previous control period using a non-parametric test based on the average of Wilcoxon two-sample statistics computed for each rabbit separately [13]. For this test weeks of treatment and control periods were selected where no significant common trend in the values could be established by means of Kendall's test for concordance (Friedman test, [14]). The effect of cholestyramine plus HMG was compared with that of cholestyramine alone by computing for each rabbit the difference, \(\nu_0\), of the mean of the (last) two serum cholesterol levels during cholestyramine administration and for the preceding control period and the corresponding difference, \(\nu_1\), for the combined treatment and applying a one-sample \(t\)-test on \(\nu_2 - \nu_1\).

Results
Body weight in the two groups of animals remained unaffected by any of the treatments. An exception was group I during the first HMG regimen. Here, animals which were rather young and still growing at the start of the experiment had gained some weight after the 6 weeks period \((2400 \pm 80\text{ g at 25 weeks vs 2220} \pm 130\text{ g at 19 weeks of age})\). The level of serum triglycerides also remained essentially unchanged over the experimental period in both groups.

HMG, given orally over 6 weeks, lowered serum cholesterol considerably (Fig. 1). Levels at 23 and 25 weeks were significantly \((P < 0.01)\) reduced as compared with those obtained at 17 and 19 weeks. Average serum cholesterol during HMG treatment was lowered from 18.7 \pm 0.9 (SD) to 11.5 \pm 2.8 mmol/l. The subsequent PIB procedure resulted in a sustained decrease of serum cholesterol. Comparison of any of the values measured before PIB, at 27, 29 or 31 weeks of age, with those obtained after PIB, at 33, 35, 37, 49 and 51 weeks, shows a very significant \((P < 0.01)\) drop in serum cholesterol. Oral administration of HMG after surgery had no additional effect on circulating cholesterol, at least not within 4 weeks. However, if HMG was given intraperitoneally, serum cholesterol levels decreased further. Concentrations measured at 59, 60 and 61 weeks were
3-Hydroxy-3-methylglutaric acid in WHHL rabbits

**FIG. 1.** Effect of HMG medication on serum cholesterol levels in WHHL rabbits, before and after partial ileal bypass surgery. Results are depicted as means ± SD (n = 5).

**FIG. 2.** Effects of cholestyramine and of cholestyramine plus HMG (oral) medication on serum cholesterol levels in WHHL rabbits. Results are depicted as means ± SD (n = 7).

significantly lower than those observed in weeks 49 and 51 (P < 0.01). Also, values obtained in weeks 56, 57 and 58 were significantly (P = 0.05) lower than those measured in weeks 49 and 51. Average serum cholesterol during intraperitoneal HMG administration was thus reduced from 6.9 ± 0.7 (49 and 51 weeks) to 4.5 ± 0.4 (59, 60 and 61 weeks) mmol/l.

Cholestyramine also reduced serum cholesterol in WHHL rabbits (Fig. 2). Concentrations at 38 and 40 weeks were very significantly lower as compared with those measured at 32 and 34 weeks (P < 0.01). Average serum cholesterol at these measuring points was lowered from 15.2 ± 2.2 to 9.9 ± 1.1 mmol/l. A combination of cholestyramine and HMG (oral) did result in a reduction of similar size and level of significance. Average serum cholesterol decreased from 14.8 ± 2.6 (42 and 44 weeks) to 8.7 ± 2.8 (48 and 50 weeks) mmol/l.

Statistical comparison of the effects induced by cholestyramine alone and by the simultaneous use of cholestyramine and HMG gives no support for any additive effect induced by HMG in this experiment.

Fibroblasts were isolated and cultivated from three WHHL rabbits and one New Zealand White rabbit. The results presented in Fig. 3 show that the fibroblasts from the WHHL rabbits did not show significant high-affinity binding, uptake or degradation of 125I-LDL as compared with the high-affinity binding, uptake and degradation of 125I-LDL measured in fibroblasts from the normal rabbit.

**Discussion**

The possibility of using HMG as a hypolipidaemic drug in familial hypercholesterolaemia has been investigated only incidentally. Lupien and co-
workers have reported its protective action in diet-induced hypercholesterolaemia in rabbits [15] and subsequently its use in 36 heterozygous [16] and one homozygous [17] patient, where the drug appeared to be effective in lowering serum cholesterol, at the same time being well-tolerated and non-toxic. The present results, obtained with a new animal model for familial hypercholesterolaemia, supply further evidence for the lipid-lowering potential of this compound. In all five rabbits of group I treated with HMG over a period of 6 weeks, serum cholesterol was lowered considerably. The average reduction was 39%, ranging from 24 to 58%. In this respect, HMG appears to be equally effective in WHHL rabbits as compactin [18] which is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Compactin was recently shown to be effective in heterozygous patients [19, 20].

Cholestyramine also reduced serum cholesterol levels in WHHL rabbits (Fig. 2). Cholestyramine and HMG showed no additive effects in our experiment, while a combination of cholestyramine and compactin has been reported to work synergistically in dogs [21] and heterozygous patients [20]. However, at present we cannot exclude the possibility that part of the HMG, a carboxylic acid of low acidic strength, was bound by the anion exchanger cholestyramine. In this case, optimal absorption of HMG into the circulation would have been prevented.

Earlier findings in our laboratory show that PIB strongly reduces serum cholesterol and inhibits atherogenesis in WHHL rabbits [5]. Present results indicate that, if HMG was given orally after operation it remained uneffective, presumably because of impaired absorption from the gut. However, intraperitoneal administration of HMG resulted in a further reduction of serum cholesterol. This underlies the potential powerful combination of bile acid drainage by means of PIB and cholesterol synthesis inhibition as a therapy in familial hypercholesterolaemia. This way, serum cholesterol levels in WHHL rabbits could be lowered from 18.7 ± 0.9 mmol/l to 4.5 ± 0.4 mmol/l, a reduction of 76%.

Although Shimada et al. [22] have reported some LDL receptor activity on fibroblasts derived from WHHL rabbits, the bulk of current evidence seems to indicate these animals to be receptor-negative, i.e. entirely without LDL receptors [4]. Present results (Fig. 3) show that our strain of WHHL rabbits is also receptor-negative. This leaves the problem of how to explain the lipid-lowering effects of the various therapies. Bile acid drainage, through PIB or cholestyramine treatment, supposedly causes serum levels of LDL-cholesterol to fall by means of ultimately increasing the number of LDL receptors. Inhibitors of cholesterol synthesis, such as compactin and HMG, are also thought to stimulate production of LDL receptors in the liver [23]. Therefore, these therapies are not expected to be effective in receptor-negative familial hypercholesterolaemia homozygotes. The fact that HMG, cholestyramine and PIB significantly lower serum cholesterol

FIG. 3. High-affinity binding (a), internalization (b) and degradation (c) of 125I-labelled LDL by fibroblasts isolated from three WHHL rabbits (●, ○, △) and from one normal rabbit (■). The values represent ng of 125I-LDL bound (a), internalized (b) or degraded (c) per mg of cell protein and are depicted as means of triplicate determinations.
levels in homozygous WHHL rabbits then must mean that these therapies lower serum cholesterol through some other mechanism.

It has been shown (cf. [4]) that the rabbit liver takes up predominantly β-VLDL (IDL). Only a small amount of β-VLDL is converted to LDL and sequestered as such. Rabbit hepatocytes contain, next to the LDL receptor, a receptor for chylomicrons. Binding of chylomicrons to this receptor is competitively reduced by β-VLDL [24]. Therefore, it seems possible that β-VLDL is taken up by the rabbit liver by means of the chylomicron receptor, which is normally developed in the WHHL rabbit [24]. Bile acid drainage and inhibition of cholesterol synthesis could then stimulate production of this type of hepatic receptor and thus exert their serum cholesterol lowering effect in WHHL rabbits.

It remains to be seen if HMG works equally well in patients with homozygous familial hypercholesterolemia. In particular, its application in patients with the receptor-negative subtype seems questionable, since the human liver takes up β-VLDL almost exclusively after conversion to LDL (cf. [4]). However, it certainly appears worthwhile to investigate further the effectiveness of HMG in patients with the receptor-deficient form of the disease.

Acknowledgments

The authors are grateful to the staff of the Central Animal Laboratory of the Medical Faculty (head: Dr W. J. van der Gijde) for their excellent efforts in breeding the WHHL rabbits, and to Dr Ph. van Elteren (Department of Statistical Support, University of Nijmegen) for his statistical analysis of the experimental data.

References

role for hepatic low density lipoprotein receptors in vivo in the dog. Proceedings of the National Academy of Sciences U.S.A., 78, 1194-1198.

