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THE EFFECT OF GAMMALINOLENIC ACID ON THE SUBFRACTIONS OF PLASMA HIGH DENSITY LIPOPROTEIN OF THE RABBIT

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Abstract—The effect of dietary supplementation with evening primrose oil (containing 70% gammalinolenic acid) on the concentration of plasma lipids and lipoproteins of the New Zealand White rabbit was investigated. No significant changes were observed in the concentrations of plasma cholesterol or triglycerides during the treatment, although an increase in high density lipoprotein (HDL) cholesterol ($P < 0.01$) was observed at 4 weeks of evening primrose oil intake and 2 weeks after withdrawal. However, when HDL subpopulations were resolved by gradient gel electrophoresis, major alterations were observed in the distribution of HDL subfractions. These included an increase in HDL_{2b} ($P < 0.001$) and HDL_{3c} ($P < 0.001$) and the appearance of very large particles of HDL. These findings suggest that supplementation of diets with n-6 fatty acids may be effective in the long-term prevention of atherosclerosis.

During the last decades, many studies have established a direct relationship between the concentrations of plasma cholesterol and low density lipoprotein (LDL†) and the development of atherosclerosis and coronary heart disease (CHD) [1, 2]. There is consistent evidence that low levels of dietary linoleic acid are related to a high risk of CHD [3]. These findings have been leading many healthy individuals towards the intake of essential fatty acids (EFAs) in order to decrease their risk of developing heart disease. EFAs of both the n-3 and n-6 series have consistently been shown to lower the concentrations of both total plasma cholesterol and LDL cholesterol [4]. It has been suggested that this effect is due mainly to a physiological regulation of cholesterol metabolism [5]. However, the long-term prevention of atherosclerosis does not depend exclusively upon lowering the levels of plasma cholesterol and more attention is now focussed on the protective role of high density lipoprotein (HDL) against CHD [2, 6–8]. An inverse relationship has been observed between the concentration of HDL and the incidence of CHD [9], possibly due to the role of this lipoprotein in the process of reverse cholesterol transport [8, 10]. Specific subfractions of HDL, rather than total HDL, appear to be involved in this process, whereby surplus cholesterol is removed from peripheral tissues and transported to the liver for excretion as bile salts [8]. Since gammalinolenic acid (GLA) has been suggested to have antiatherogenic properties [11], it is pertinent to study the effects of dietary supplementation with evening promrose oil (EPO) containing a high concentration of GLA on the subfractions of HDL. For this purpose, the rabbit was chosen as an animal model since the overall distribution of plasma lipoprotein and the composition of HDL in this species are similar to those of humans [12]. Furthermore, a similar profile of HDL subfractions has been observed in both species in recent studies in our laboratory.

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† Abbreviations: apo, apolipoprotein; CHD, coronary heart disease; EFAs, essential fatty acids; EPO, evening primrose oil; GLA, gammalinolenic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Six female New Zealand White rabbits were used in this study at the age of approximately 6 months. The animals weighed, on average, 3.7 kg. Rabbits were caged individually in a room with controlled temperature (20°) and cycled with 12 hr darkness and light. The animals were fed a standard laboratory chow diet (Rabbit maintenance R14, Biosure Ltd, Cambridgeshire, U.K.), which along with water was available *ad lib.*

The manipulation of rabbits in this study was approved by the Home Office.

A preparation of EPO containing 70% GLA (Scotia Pharmaceuticals Ltd) was dissolved in diethyl ether (AnalaR) and sprinkled over the standard chow in order to provide the animals with a supplement of 40 mg GLA/kg/day, which accounted for less than 1% of the caloric intake. The animals were frequently weighed during the study and their food intake was closely monitored.

Blood (20 mL) was drawn from a marginal ear vein of unfastened, calm, unanaesthetized and non-fasting animals. The blood samples were collected in vials containing EDTA (final concentration 1.3 mg/mL).

Blood samples were taken immediately prior to treatment, after 2 and 4 weeks of EPO intake and after 2 and 12 weeks of withdrawal. For the final

Table 1. Mean concentrations ± SD of total plasma cholesterol and lipoprotein cholesterol (mg/dL) in the New Zealand White rabbit during the course of treatment with EPO (N = 5)

		EPO supplementation		After EPO withdrawal	
	Before EPO	2 weeks	4 weeks	2 weeks off	12 weeks off
Plasma	79.5 ± 12.1	91.8 ± 12.8	96.0 ± 12.2	88.8 ± 7.8	85.0 ± 19.2
VLDL	3.6 ± 1.5	3.6 ± 1.1	7.7 ± 3.6	6.3 ± 0.9*	7.3 ± 2.8
	(5.4%)	(5.3%)	(6.9%)	(7.1%)	(11.2%)
LDL	25.7 ± 10.8	23.3 ± 6.0	44.5 ± 13.7	27.2 ± 13.2	20.8 ± 1.0
	(38.3%)	(34.8%)	(40.2%)	(30.6%)	(32.0%)
HDL	37.9 ± 8.2	40.0 ± 8.6	58.4 ± 8.8*	55.4 ± 8.5*	36.7 ± 9.5
	(56.4%)	(59.9%)	(52.9%)	(62.3%)	(56.8%)

* $P < 0.01$ in relation to basal values.
Percentage of cholesterol recovered in lipoproteins is shown in brackets.

Table 2. Mean concentrations ± SD of total plasma triglycerides and lipoprotein triglycerides (mg/dL) in the New Zealand White rabbit during the course of treatment with EPO (N = 5)

			After EPO withdrawal	
	Before EPO	4 weeks on EPO treatment	2 weeks off	12 weeks off
Plasma	83.6 ± 12.5	69.0 ± 11.6	73.2 ± 13.4	87.9 ± 9.4
VLDL	28.0 ± 2.4	14.5 ± 3.7*	22.2 ± 5.2	32.7 ± 8.4
	(49.5%)	(32.9%)	(38.3%)	(58.0%)
LDL	11.7 ± 3.4	12.7 ± 5.5	15.2 ± 3.9	17.8 ± 5.7
	(20.7%)	(28.9%)	(26.2%)	(31.5%)
HDL	16.9 ± 4.8	16.9 ± 8.6	20.6 ± 14.0	5.9 ± 2.1*
	(29.8%)	(38.2%)	(35.5%)	(10.5%)

* $P < 0.01$ in relation to basal values.
Percentage of triglycerides recovered in lipoproteins is shown in brackets.

bleeding, the animals were given an overdose of Sagatal (Pentobarbitone Sodium B.P. Vet) injected into a marginal ear vein, and the blood was collected by cardiac puncture.

Plasma was prepared by low speed centrifugation at 4° and lipoproteins were isolated by flotation using the Beckman preparative ultracentrifuge model L2-65B, with the 50Ti rotor; densities were adjusted with solid NaBr. Very low density lipoprotein (VLDL), LDL and HDL were separated by ultracentrifugation at densities 1.020, 1.055 and 1.250 g/mL, for 18, 18 and 40 hr, respectively, at 105,400 *g* at 16°. Previous trials had shown that isolation of very large and/or very small rabbit HDL was optimized at these density intervals. No significant amounts of LDL or apolipoprotein B (apo B) were present in the HDL fraction separated at these densities, as determined by gradient gel electrophoresis and by SDS electrophoresis of delipidized lipoproteins.

Cholesterol and triglyceride concentrations were measured in total plasma and isolated lipoprotein fractions using enzymatic assay kits (cholesterol CHOD-PAP diagnostic kit and triglyceride, fully enzymatic kit, Boehringer).

Subfractions of HDL were resolved on 4–30% linear polyacrylamide gradient gels (Pharmacia PAA 4/30) by electrophoresis as previously described [13]. HDL (20 μL), isolated by ultracentrifugation, was applied to the gels which had been pre-equilibrated in Tris–borate buffer (90 mM Tris–HCl, 80 mM boric acid, 2.5 mM EDTA; pH 8.5) at 70 V for 20 min. A reference protein mixture (HMW Calibration Kit, Pharmacia) was included on each gel. The gels were thereafter submitted to 20 V for 20 min, 70 V for 30 min and 120 V for 24 hr. Following electrophoresis, the gels were fixed in 10‰ (w/v) sulphosalicylic acid for 1 hr prior to staining in 0.1% (w/v) Coomassie brilliant blue R in methanol:acetic acid:water (5:1:4, by vol.) for 16 hr. Gels were destained for several days in methanol:acetic acid:water (5:7.5:87.5, by vol.) and scanned using a BioRad model 620 video densitometer. The scan data were processed in an IBM/XT personal computer. The R_f value of the individual bands on each gel was calculated using the ratio of migration distance of the band relative

Table 3. Mean percentage ± SD of basic profile of five subfractions of HDL (mean particle size: 7.7 – 10.9 nm) resolved by gradient gel electrophoresis during the course of dietary supplementation with GLA for the New Zealand White rabbit (N = 5)

		EPO supplementation		After EPO withdrawal	
Subfraction	Before EPO	2 weeks	4 weeks	2 weeks off	12 weeks off
2b	6.8 ± 3.7	9.9 ± 2.6*	13.8 ± 5.1†	16.7 ± 6.9†	7.8 ± 5.0
2a	30.0 ± 13.2	18.3 ± 3.5*	22.8 ± 5.3	20.3 ± 4.0	21.6 ± 8.0
3a	42.4 ± 9.7	26.4 ± 13.3*	22.8 ± 8.2†	26.5 ± 6.5†	44.5 ± 7.3
3b	19.5 ± 2.6	22.7 ± 6.7	20.2 ± 5.3	22.2 ± 6.3	25.0 ± 8.6
3c	1.3 ± 0.8	22.7 ± 6.9†	20.4 ± 6.8†	14.3 ± 4.4†	2.1 ± 1.0

The results presented in this table exclude the relative percentage of the two larger lipoprotein particles which were present only during GLA treatment. This table therefore represents a comparison of the variation of the five basic subfractions of HDL of the New Zealand White rabbit.

Subfractions of HDL are defined as described in Materials and Methods.

* $P < 0.01$, † $P < 0.001$ in relation to basal values, 1 week before administration of EPO.

to the migration distance of bovine serum albumin in the standard lane of the gel [14]. Using this procedure, HDL from the New Zealand White rabbit was resolved into five subfractions with a profile that resembled that of human HDL [13, 14] though the R_f values of the rabbit subfractions were lower than those of the human counterparts, showing that the rabbit has HDL subfractions of larger particle size. Estimates of the mean particle diameter of each subfraction were obtained from the plots of the peak R_f values against the diameters of the corresponding human HDL fractions given by Blanche *et al.* [14] and Chang *et al.* [15]. The rabbit subpopulations of HDL were defined as HDL_{2b} (mean R_f 0.550; mean particle diameter 10.9 nm), HDL_{2a} (0.602; 10.1 nm), HDL_{3a} (0.665; 9.4 nm), HDL_{3b} (0.764; 8.3 nm) and HDL_{3c} (0.805; 7.7 nm). In addition, two further subpopulations were observed in rabbits treated with EPO. These were designated HDL_{1b} (0.401; 12.8 nm) and HDL_{1a} (0.327; 14.4 nm), respectively, by analogy with HDL_1 [16]. The percentage distribution was calculated from the peak areas within the R_f limits of the above subfractions. The values obtained by this procedure are not affected by the non-linear relationship that has been shown to exist between pore diameter and migration distance [17].

Separation of apo E-poor and apo E-rich HDL subfractions was performed by heparin–Sepharose affinity chromatography [18] and quantitated according to Griffin *et al.* [19].

RESULTS

Effect of EPO on plasma cholesterol, triglycerides and lipoprotein levels

All of the animals tolerated well the intake of 70% GLA for 4 weeks and no behavioural abnormality was observed.

One rabbit showed very high basal levels of total plasma cholesterol and LDL cholesterol; the results from this animal were therefore not included in the main group. This animal was, however, submitted to the same procedures but the results are presented separately. All the results presented below are therefore for five rabbits.

The mean pre-treatment concentration of total plasma cholesterol was 79.5 ± 12.1 mg/dL and no significant difference in this value was observed during treatment. The distribution of cholesterol among the different lipoprotein fractions showed significant changes at the 4th week of EPO intake and immediately after EPO withdrawal. The most significant alteration was an increase in HDL cholesterol ($P < 0.01$) which then returned to basal values after 12 weeks of EPO withdrawal (Table 1).

Throughout the treatment with EPO containing 70% GLA, the mean total plasma concentration of triglycerides did not alter significantly, although a decrease of 17.5% was observed at 4 weeks of EPO intake (Table 2). However, at 4 weeks of treatment, the concentration of triglycerides had significantly decreased in VLDL ($P < 0.01$), while a relative increase in HDL triglycerides was observed (from 29.8% to 38.2% of triglycerides recovered in lipoproteins). After 12 weeks of EPO withdrawal, the concentration of VLDL triglycerides had returned to basal values.

Effect of EPO on the subfractions of HDL

Major changes were observed in the distribution of subpopulations of HDL as determined by gradient gel electrophoresis (Fig. 1 and Table 3). The most outstanding alteration was the appearance of large HDL particles, with diameters greater than those of HDL_{2b}, after 2 weeks of EPO treatment. These particles were not detectable in any of the rabbits after 12 weeks of EPO withdrawal. This alteration was accompanied by an increase in the relative proportion of subfractions HDL_{2b} ($P < 0.001$) and HDL_{3c} ($P < 0.001$), both subfractions returning to basal values after 12 weeks of EPO withdrawal.

The pre-treatment concentration of apo E-rich HDL isolated by heparin–Sepharose affinity chromatography was 17.2 ± 4.7 mg/dL (44.9 ± 3.6% of the total plasma HDL). No significant change in these values was observed during or after EPO treatment (20.2 ± 8.7 and 22.5 ± 4.9 mg/dL at 2

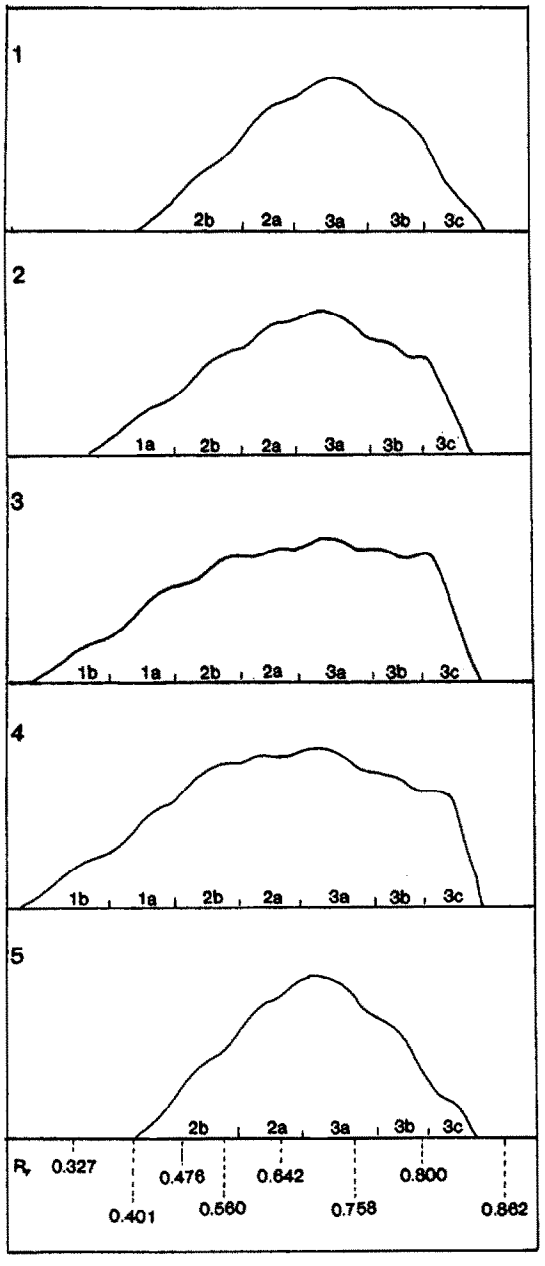

Fig. 1. Polyacrylamide gel scans showing the effect of EPO on the distribution of HDL subfractions in the rabbit. Prior to EPO treatment (1), 2 weeks (2) and 4 weeks of EPO intake (3), 2 weeks after (4) and 12 weeks after EPO withdrawal (5). HDL subfractions were defined as described in Materials and Methods. The patterns shown here are from a single rabbit and are typical of those of all six animals studied.

and 4 weeks of EPO intake, respectively, and 23.0 ± 4.0 mg/dL after 2 weeks of EPO withdrawal).

Results on the hypercholesterolaemic rabbit

As discussed above, one rabbit showed a very high basal level of total plasma cholesterol and LDL cholesterol (162.0 and 91.7 mg/dL, respectively). During the course of treatment with EPO, this hypercholesterolaemic rabbit showed similar changes in plasma lipids and lipoprotein concentrations to those of the other animals. Total plasma cholesterol remained between 150.7 and 200.3 mg/dL, while HDL cholesterol increased by 15.5% at the 4th week of treatment, when compared to basal levels. Total plasma triglyceride concentration decreased by 46% at 4 weeks of EPO intake, with HDL triglycerides increasing by 13%. The increases in concentration of HDL_{2b} and HDL_{3c} and the appearance of subpopulations of HDL_{1b} and HDL_{1a} was similar to the changes in the normolipidaemic rabbits.

DISCUSSION

The present study has demonstrated that dietary administration of EPO to New Zealand White rabbits, while producing no significant alteration in the levels of total plasma cholesterol or triglyceride, resulted in large changes in the concentration and distribution of HDL subpopulations. The small increases in the concentration of total plasma HDL is consistent with that reported recently for normolipidaemic human subjects treated with n-3 and n-6 EFAs [4]. The effect of n-6 fatty acids on the distribution of HDL subfractions has not been reported previously.

Studies on HDL subfractions may be of considerable importance in understanding the protective effect of HDL against the development of CHD. The increase in the concentrations of HDL_{2b} and HDL_{3c}, as well as the appearance of larger HDL_{1a} and HDL_{1b} subpopulations found in the present study is consistent with an increase in the rate of reverse cholesterol transport. It has been suggested that pre-β HDL, which probably corresponds to HDL_{3c}, is the only lipoprotein particle of size sufficiently small to permit penetration through the interstitium of the artery wall [20]. This small lipoprotein particle may therefore be effective in promoting the efflux of surplus cholesterol from the arterial wall [9]. At the same time, cholesteryl ester- and apo E-rich HDL of the size of HDL_{2b} are implicated in the removal of cholesterol by the liver [8, 16]. Recent reports of reduced levels of HDL_{2b} in coronary survivors and high coronary risk subjects [21–23] provide clinical evidence for the importance of this subfraction in relation to CHD.

The appearance of larger particles of HDL during GLA administration may have resulted from an increase in the activity of lipoprotein lipase which hydrolyses triglycerides in chylomicrons and VLDL with the formation of larger particles of HDL [24]. Alternatively, the larger particles of HDL may have been formed by an increased activity of lipid transfer protein, which is required for the redistribution of cholesteryl ester between HDL and both LDL and VLDL [25] and involves an equimolar transfer of triglycerides to HDL from LDL and VLDL [26]. Since the rabbit has been reported to be deficient in hepatic lipase [27], the formation of large HDL particles by these reactions could not be compensated for by hepatic lipase hydrolysis of HDL triglycerides, thus resulting in much larger HDL particles. Unsaturated fatty acids have been shown to modulate the activities of lecithin:cholesterol acyltransferase

[28], lipoprotein lipase [29] and lipid transfer protein [30]. It has been postulated that these effects are related to an increased fluidity of the lipoprotein surface conferred by the unsaturated fatty acids [30]. The significant increase in the concentration of small HDL_{3c} particles during treatment with GLA may also arise through an enhancement in the redistribution of constituents between HDL particles produced by the activating effect of GLA on cholesteryl ester transfer protein as suggested by Lagrost and Barter [31] on the basis of *in vitro* studies.

The absence of any significant diferences in apo E-containing HDL may be due to the simultaneous appearance of very small (apo E-deficient) and very large (apo E-rich) HDL, suggesting that supplementation with GLA has no effect on the rate of apo E synthesis.

The incidental presence of one hypercholesterolaemic rabbit which showed similar alterations in the HDL profile to those of the normocholesterolaemic rabbits suggests that dietary supplementation of n-6 EFAs might have similar effects in both normal and hypercholesterolaemic subjects.

In conclusion, this study demonstrates that administration of EPO to rabbits significantly alters the distribution of HDL subspecies which might reflect an increase in the rate of reverse cholesterol transport. However, a proper understanding of the effect of GLA on the lipoprotein system and the development of atherosclerosis must await a clearer knowledge of the role of subfractions of HDL in the development and regression of the atherosclerotic lesion.

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