EFFECT OF NICOTINIC ACID ON CHOLESTEROL METABOLISM IN MONKEYS

AMANY A. MAGIDE AND N. B. MYANT

Medical Research Council Lipid Metabolism Unit, Hammersmith Hospital, London

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SUMMARY

1. Single subcutaneous injections of nicotinic acid lowered the serum free fatty acid and triglyceride concentration in two non-fasting Rhesus monkeys.
2. Injections of nicotinic acid repeated daily for 2–4 weeks led to a substantial fall in serum cholesterol concentration, comparable with that obtained in humans given therapeutic doses of nicotinic acid by mouth.
3. The fall in serum cholesterol concentration was accompanied by a decrease in hepatic synthesis of cholesterol but there was no change in the faecal excretion of endogenous steroids.
4. The rate of synthesis of cholesterol, estimated from the faecal excretion of endogenous steroids and from the serum specific radioactivity curve, was such that a moderate degree of inhibition would have accounted for the observed decrease in the amount of circulating cholesterol during nicotinic acid treatment.

Key words: nicotinic acid, serum cholesterol concentration, serum lipids, faecal steroids in monkeys.

In 1955, Altschul, Hoffer & Stephen showed that nicotinic acid, given by mouth in large doses, lowers the serum cholesterol concentration in normal and hypercholesterolaemic human subjects. Since then, nicotinic acid has been used extensively in the treatment of primary hypercholesterolaemia though the mechanism of its effect on serum cholesterol has never been clearly established. Miettinen (1968) and Sodhi, Horlick & Kuchchodkar (1969) have reported an increase in faecal excretion of endogenous neutral steroids during prolonged treatment with nicotinic acid but others have failed to confirm this (Moutafis, Myant, Mancini & Oriente, 1971). Nicotinic acid has been reported to inhibit cholesterol synthesis in man (Parsons, 1961; Moutafis & Myant, 1971). Miettinen (1971), on the other hand, observed a stimulation of cholesterol synthesis in hypercholesterolaemic subjects given nicotinic acid in doses sufficient to lower the serum cholesterol concentration.

Observations on laboratory animals have not resolved these contradictions. In doses comparable, on the basis of body weight, with those used clinically, nicotinic acid lowers serum...
cholesterol concentration in rabbits (Altschul, Hoffer & Stephen, 1955; Merrill & Lemley-Stone, 1957), but not in rats (Friedman & Byers, 1959; Duncan & Best, 1960) or dogs (Grande & Amatuzio, 1960). According to some reports, nicotinic acid inhibits cholesterol synthesis in rat liver (Garattini, Paoletti, Bizzi, Grossi & Vertua, 1961; Gamble & Wright, 1961); according to others (Merrill, 1958; Hardy, Gaylor & Baumann, 1960), it has a stimulatory effect.

In view of these species differences, it seemed to us that clinically relevant information might be obtained from experiments on animals more closely related to man. We have therefore studied the effect of large doses of nicotinic acid on cholesterol metabolism in two Rhesus monkeys.

METHODS

Two immature male Rhesus monkeys (*Macaca mulatta*), weighing 2.32–2.40 kg, were studied. They were housed in separate cages in a room kept at 23°C and were fed *ad libitum* with pellets of MRC Diet no. 41 B (Bruce & Parkes, 1946) supplemented with apples and carrots. Both monkeys had been eating this diet for more than 6 months before the present study. The daily intake of cholesterol, estimated from the daily intake of pellets and their cholesterol content, was 26–39 µmol (10–15 mg). To facilitate the collection of faeces, the floor of the cage was replaced by wire netting of 2.5 cm (1 in) mesh through which the faeces dropped into a metal tray covered with sawdust.

**Procedure**

Initially, observations were made on the effect of single and multiple doses of nicotinic acid on serum lipids. Each monkey then received an intravenous injection of 50 µCi of [4-14C]-cholesterol emulsified in its own plasma by the method of Whereat & Staple (1960), and blood samples were taken at 1 h, and then daily for the first week and then at weekly intervals. Faeces were collected at the end of each week and were stored in polythene bags at −15°C until analysed.

Biopsies of liver were taken under pentothal sodium anaesthesia, given in an initial dose of 75 µmol/kg (20 mg/kg) followed by a further injection if necessary. To minimize variations in the glycogen content of the liver, 3 g of glucose in 10 ml of NaCl (150 mmol/l) was injected intravenously 1 h before the operation and all biopsies were taken at the same time of day (14.00 hours). The liver was exposed by a midline incision below the xiphisternum and a wedge of tissue weighing 50–100 mg was removed. The liver was sutured with catgut and the abdominal incision closed in separate layers. The wedge of tissue was sliced by hand with a razor blade immediately after removal from the liver. The slices were placed in ice-cold weighed tubes containing the buffered solution of [14C]acetate in which the specimens were incubated.

Nicotinic acid was given by subcutaneous injection of a sterile neutral solution of sodium nicotinate in 1 ml of water.

**Incubation of liver slices**

The incubation mixture used for measurement of cholesterol synthesis *in vitro* contained: Krebs–Ringer bicarbonate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1964), modified by equalizing the concentrations of sodium and potassium ions; potassium acetate (8.0 mmol/l);
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[1-\(^14\)C]sodium acetate (0.01 mmol/l, 5 μCi/flask); 5–10 mg of liver slices; the total volume was 0.5 ml. The incubation flasks were gassed with O\(_2\)+CO\(_2\) (95:5) for 1 min immediately before the incubation. All incubations were carried out in quadruplicate for 3 h at 37°C with shaking.

Preliminary experiments with rat-liver slices (5–10 mg incubation) showed that incorporation of [\(^14\)C]acetate into digitonin-precipitable sterols was maximal when the concentration of acetate in the incubation mixture was 8.0 mmol/l and that the rate of incorporation was constant for the first 3 h of incubation.

At the end of the incubation, 5.2 μmol (2 mg) of non-radioactive cholesterol in 0.4 ml of NaOH (2.5 mol/l) and 2 ml of ethanol was added to the incubation mixture. The cholesterol was extracted, precipitated as the digitonide and assayed for radioactivity as described by Moutafis & Myant (1968). Incorporation of acetate into cholesterol was calculated from the amount of \(^14\)C incorporated into digitonin-precipitable sterols and the specific radioactivity of the [\(^14\)C]acetate in the incubation mixture. The values for incorporation were corrected for losses of [\(^14\)C]cholesterol during the extraction procedure by measuring the recovery of the cholesterol added to the incubation mixture.

Serum lipids

Total cholesterol in the serum was measured by the fluorimetric method of Carpenter, Gotsis & Hegsted (1957). For measurement of serum triglycerides, 0.2 ml of serum was added to 10 ml of hot chloroform–methanol (2:1, v/v) and the mixture was filtered. A known proportion of the filtrate was submitted to thin-layer chromatography by the two-stage method of Skipski, Smolowe, Sullivan & Barclay (1965). The triglyceride band was eluted and saponified, and the glycerol assayed enzymically by the method of Garland & Randle (1962). For measurement of serum phospholipids, a known proportion of the chloroform–methanol extract was dried and the residue ashed in the presence of perchloric acid and carborundum. The phosphorus in the ashed sample was measured by the method of Chen, Toribara & Warner (1956). The following further methods were employed: serum free fatty acids, Novák (1965); serum nicotinic acid, Carlson (1966); alkaline phosphatase, King (1951); aspartate transaminase, Karmen (1955).

Faecal steroids

Radioactivity in the neutral and acidic steroids excreted in the faeces was assayed by the method of Moutafis & Myant (1969). Daily faecal excretion of acidic steroids and of neutral steroids derived from exchangeable cholesterol (endogenous neutral steroids) was calculated by dividing the radioactivity in each steroid fraction by the specific radioactivity of serum cholesterol at the mid-point of the sampling period. The faecal clearance rate of serum cholesterol was calculated by dividing the daily faecal excretion of total endogenous steroids by the serum cholesterol concentration. In some cases, faecal bile acids were measured by the gas–liquid chromatography method of Grundy, Ahrens & Miettinen (1965), 3α,7α-dihydroxy-12-keto-5β-cholanoic acid being used as an internal standard (Simons & Myant, 1974).

Materials

[4-\(^14\)C]Cholesterol and [1-\(^14\)C]acetate were obtained from The Radiochemical Centre, Amersham, Bucks. The [4-\(^14\)C]cholesterol was purified by thin-layer chromatography with
benzene-ethyl acetate (5:1, v/v) as solvent. Nicotinic acid as its sodium salt was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Effect of single injections of nicotinic acid on serum lipids

The effects of a single subcutaneous injection of nicotinic acid 2·0 mmol/kg (250 mg/kg) are shown in Fig. 1. Similar results were obtained in two other experiments on the two monkeys. Serum nicotinic acid concentration rose to about 1·2 mmol/l (150 mg/l) at 30 min

Fig. 1. Effect of a single subcutaneous injection of nicotinic acid [2·0 mmol (250 mg) kg⁻¹] on serum cholesterol, phospholipid, free fatty acid, triglyceride and nicotinic acid concentrations. Each point is the mean of values obtained from the two monkeys injected at 10·00 hours on the same day.
and fell to a negligible level within 24 h. Serum free fatty acid concentration fell to less than half the resting value 30 min after the injection and then rose to the pre-injection level 24 h later. Serum triglyceride concentration fell to half the resting level 6 h after the injection and returned to the pre-injection level during the next 18 h.

Fig. 2. Effect of subcutaneous injections of nicotinic acid [2·0 mmol (250 mg) kg⁻¹ twice daily] on serum cholesterol concentration, hepatic synthesis of cholesterol, serum enzymes, faecal excretion of steroids of endogenous origin and faecal clearance rate of plasma cholesterol in monkey I. Nicotinic acid (NA) was given during the periods shown in the upper panel.

Serum triglyceride concentration was measured serially during a 24 h period in three monkeys not given an injection of nicotinic acid but kept under the same conditions of feeding and housing as those used for the experiment shown in Fig. 1. Blood samples were taken at 10.00 hours and then 3 h, 6 h and 24 h later. There was no significant difference between the mean
serum triglyceride concentration at 10.00 hours \([344 \pm 13(\text{SD}) \mu\text{mol}/\text{l} \ (30.6 \pm 1.2 \text{ mg}/100 \text{ ml})]\) and the mean concentration at 3, 6 and 24 h \((378 \pm 13, 316 \pm 19 \text{ and } 383 \pm 13 \mu\text{mol}/\text{l} \text{ respectively})\).

Serum cholesterol and phospholipid concentrations showed slight but consistent decreases 6 h after the injection, rising towards the pre-injection values during the next 18 h.

**Effect of repeated injections of nicotinic acid on serum lipids**

When nicotinic acid in a dose of 1.2 mmol/kg (150 mg/kg) was injected daily for 7 days at
10.00 hours, the serum nicotinic acid concentration at 30 min after each injection was the same each day. Serum triglyceride concentration fell to about the same extent after each injection, returning more or less to the pre-injection level within 24 h. Serum cholesterol concentration fell in a stepwise manner, decreasing from a mean of about 3.88 mmol/l (150 mg/100 ml) to about 3.11 mmol/l (120 mg/100 ml) by the end of the seventh day.

![Graph showing the specific radioactivity of serum cholesterol after a single intravenous injection of [14C]cholesterol into two monkeys (○, ●). Nicotinic acid (2.0 mmol kg⁻¹ twice daily) was given during the period shown by the black rectangle. The exponential portion of each curve was extrapolated back to cut the vertical axis at C_b. The broken lines intersecting with the vertical axis at C_a were obtained by subtracting the extrapolated lines from the values observed during the first 4 weeks.](image)

**Fig. 4.** Specific radioactivity of serum cholesterol after a single intravenous injection of [14C]cholesterol into two monkeys (○, ●). Nicotinic acid (2.0 mmol kg⁻¹ twice daily) was given during the period shown by the black rectangle. The exponential portion of each curve was extrapolated back to cut the vertical axis at C_b. The broken lines intersecting with the vertical axis at C_a were obtained by subtracting the extrapolated lines from the values observed during the first 4 weeks.

When the dose of nicotinic acid was increased to 2·0 mmol (250 mg)/kg twice daily and was continued for periods of 15–28 days, serum cholesterol concentration fell to 67% of the control value, returning to the pre-treatment value 3 or 4 weeks after the treatment was stopped. The serum triglyceride concentration measured immediately before the first of the two daily injections also fell to 64% of the control value.

In a single experiment, serum nicotinic acid concentration was measured in each monkey on repeated occasions during a course of daily injections continued for 31 days. The value obtained 30 min after each injection showed no change throughout the whole course of injections,
suggesting that under these conditions there is neither accumulation of nicotinic acid in the body nor an increasing ability to metabolize or excrete it.

\textit{Lack of side-effects of nicotinic acid}

An adult man given nicotinic acid in a single oral dose of 80 $\mu$mol/kg body weight experiences marked discomfort, with flushing of the skin, palpitations, headache and generalized itching. However, in none of the experiments described above did either monkey show any signs of discomfort when 2.0 mmol (250 mg) of nicotinic acid/kg was given by subcutaneous injection. When 2.0 mmol (250 mg)/kg was given twice daily for 3–4 weeks, there was a moderate increase in the serum activity of aspartate transaminase in both monkeys, but no increase in that of alkaline phosphatase (Fig. 2 and Fig. 3). A liver biopsy taken from each monkey at the end of a 3 week course of nicotinic acid [2.0 mmol (250 mg)/kg twice daily] appeared normal when examined by light-microscopy.

\textbf{TABLE 1. Faecal excretion of bile acids and endogenous neutral steroids in control periods and during treatment with nicotinic acid}

Assumed average molecular weights were: total endogenous steroids, 388; bile acids, 390; neutral steroids, 386.

\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
 & Total endogenous steroids (pmol day$^{-1}$ kg$^{-1}$) & Bile acids (pmol day$^{-1}$ kg$^{-1}$) & Neutral steroids (pmol day$^{-1}$ kg$^{-1}$) & Faecal clearance (ml day$^{-1}$ kg$^{-1}$) \\
\hline
Monkey & Control$^{(1)}$ & Treated & Control$^{(1)}$ & Treated & Control$^{(1)}$ & Treated & Control$^{(2)}$ & Treated \\
\hline
1 & 98.1$\pm$5.4 & 93.9$\pm$1.0 & 62.3$\pm$3.9 & 61.3$\pm$1.3 & 35.8$\pm$2.8 & 32.6$\pm$0.8 & 23.5$\pm$1.3 & 34.5$\pm$3.6 \\
2 & 81.3$\pm$5.2 & 83.6$\pm$5.2 & 51.3$\pm$3.8 & 55.6$\pm$4.4 & 30.0$\pm$0.8 & 28.0$\pm$0.5 & 19.3$\pm$1.1 & 29.5$\pm$3.1 \\
\hline
\end{tabular}

$^{(1)}$ Control values for steroid excretion are means $\pm$ SEM for the periods before and after treatment with nicotinic acid.

$^{(2)}$ Control values for faecal clearance rate of plasma cholesterol are means $\pm$ SEM for the period before treatment.

\textit{Effect of nicotinic acid on the specific radioactivity of serum cholesterol}

In each monkey, the specific radioactivity of the serum cholesterol was measured for 27 weeks after labelling the plasma with $[^{14}\text{C}]$cholesterol. Twelve weeks after the injection of labelled cholesterol (i.e. on day 82), nicotinic acid was given daily for 4 weeks. The specific radioactivity of the serum cholesterol fell rapidly during the first 4 weeks after labelling and then declined exponentially with a half-life of 23 days in one monkey and 25 days in the other. When nicotinic acid was given, there was a temporary decrease in the slope of the curve in both monkeys (Fig. 4). When nicotinic acid was discontinued, the specific radioactivity resumed its exponential decline with the same half-life as that observed before nicotinic acid treatment.

As shown in Fig. 4, when the exponential portion of the curve was extrapolated back to zero time and subtracted from the experimental values for the first 4 weeks, the values so obtained lay on a straight line when plotted semilogarithmically. Hence the behaviour of the injected $[^{14}\text{C}]$cholesterol justified analysis of the specific radioactivity curve in terms of a model.
consisting of two connected pools of exchangeable cholesterol (Gurpide, Mann & Sandberg, 1964; Goodman & Noble, 1968), one with rapid turnover (pool A of Goodman & Noble, 1968) and the other with slow turnover (pool B of Goodman & Noble, 1968). Average values for the two monkeys, calculated from the slopes of the two lines and their intercepts on the vertical axis, were: mass of pool A, 1001 µmol (386 mg)/kg; half-life of pool A, 2.25 days; production rate of pool A, 324 µmol (125.5 mg)/day or 137 µmol (53 mg) day⁻¹ kg⁻¹; mass of pool B, 2788 µmol (1076 mg)/kg.

Effect of nicotinic acid on faecal excretion of steroids

The faecal excretion of bile acids and endogenous neutral steroids was measured from day 0 to day 140 after labelling the plasma with [¹⁴C]cholesterol. Nicotinic acid, in a dose of 2.0 mmol (250 mg)/kg twice daily, was given from day 82 to day 110. The results are shown in Table 1 and Figs. 2 and 3. During the control periods before and after nicotinic acid treatment, faecal excretion of total endogenous steroids averaged 212 µmol (82.3 mg)/day [90 µmol (35 mg) day⁻¹ kg⁻¹] in the two monkeys, of which 63–64% was in the bile acid fraction. During nicotinic acid treatment there was no significant change, either in the faecal excretion of total endogenous steroids or in the ratio of bile acids to endogenous neutral steroids in the faeces. In each monkey, bile acids were measured by gas–liquid chromatography in three samples of faeces from monkey 1 and in two from monkey 2. The values obtained by the g.l.c. method differed from those obtained by the isotopic balance method by less than 12%.

The faecal clearance rate of the plasma cholesterol averaged 23.5 ml day⁻¹ kg⁻¹ in monkey 1 and 19.3 ml day⁻¹ kg⁻¹ in monkey 2 during the control period before nicotinic acid treatment. When nicotinic acid was given, there was an increase in faecal clearance rate in both monkeys (Fig. 2 and Fig. 3). Faecal clearance rate was calculated by dividing the daily faecal excretion of total endogenous steroids by the serum cholesterol concentration.
Effect of nicotinic acid on hepatic synthesis of cholesterol and fatty acids

In both monkeys, synthesis of cholesterol and fatty acids was measured in liver biopsies taken before, during and after each of three courses of nicotinic acid treatment in a dose of 2.0 mmol (250 mg)/kg twice daily. The first course of treatment lasted from day 82 to day 110, the second from day 200 to day 214 and the third from day 413 to day 441. In all treatment periods, the biopsy was taken 2 h after the last injection of nicotinic acid. The results are shown in Figs. 2 and 3 and in Table 2. At the end of each course of treatment, cholesterol synthesis was lower than during the control period before treatment and in both monkeys the mean rate of synthesis during treatment was significantly lower than the mean rate during the control periods without treatment ($P<0.05$). There was also a decrease in fatty acid synthesis during treatment, but the decrease was not statistically significant ($P>0.05$).

DISCUSSION

These observations show that a substantial fall in serum cholesterol concentration can be induced in Rhesus monkeys by prolonged treatment with nicotinic acid, without significant side-effects. As in human subjects with hyperlipoproteinaemia (Carlson, Orö & Östman, 1968), single doses of nicotinic acid caused a fall in the serum triglyceride concentration within a few hours, preceded by a fall in the serum free fatty acid concentration.

During nicotinic acid treatment, there was a decrease in hepatic synthesis of cholesterol. This would be expected to lead to a flattening of the decay curve for specific radioactivity of serum cholesterol (Fig. 4), since the fall in specific radioactivity after intravenous $[^{14}C]$cholesterol is due partly to replacement of circulating radioactive cholesterol by non-radioactive molecules newly synthesized in the liver and other tissues. However, if nicotinic acid caused a shift of cholesterol from the tissues into the plasma this also could diminish the slope of the curve, since tissue cholesterol acquires a higher specific radioactivity than serum cholesterol within 2 or 3 months of an intravenous injection of $[^{14}C]$cholesterol in monkeys (Myant, 1971).

Nicotinic acid caused no detectable change in faecal excretion of endogenous steroids. In this respect the two monkeys differed from the hyperlipidaemic subjects investigated by Miettinen (1971), in whom nicotinic acid increased the faecal excretion of neutral steroids. However, in both monkeys there was an increase in faecal clearance of total steroids during treatment, suggesting that nicotinic acid in some way enables the animal to maintain a normal output of steroids at a decreased serum cholesterol concentration.

The observed decrease in hepatic synthesis of cholesterol raises the question as to how far this effect can account for the fall in serum cholesterol concentration. If the model suggested by Goodman & Noble (1968) is a true representation of the metabolism of exchangeable cholesterol in Rhesus monkeys, the production rate of pool A should equal the rate at which cholesterol is removed from the body as bile acids and neutral steroids. Under steady-state conditions this should equal the rate at which exchangeable cholesterol enters the body by synthesis and absorption from the intestine. In fact, the mean production rate of pool A in the two monkeys was about 50% higher than the mean faecal excretion of total endogenous steroids. A difference of about this magnitude is also apparent from the values reported by Lofland, Clarkson, St Clair & Lehner (1972) for squirrel monkeys. Part of this discrepancy may have been due to degradation of neutral steroids in the lower intestine, thought to occur.
in some human subjects (Grundy, Ahrens & Salen, 1968). C. D. Moutafis & N. B. Myant (unpublished observations) have also found that up to 12% of the radioactivity lost from Rhesus monkeys given intravenous [4-14C]-cholesterol leaves the body by a route other than the faeces, probably as neutral steroids removed with hair that is continually shed. Hence, values for faecal excretion of total endogenous steroids should be regarded as minimum estimates of the rate of renewal of exchangeable cholesterol by synthesis and absorption of dietary cholesterol. Since the diet supplied not more than 39 μmol (15 mg) of cholesterol/day, the rate of synthesis of exchangeable cholesterol is unlikely to have been less than 174 (213–39) μmol/day and may well have been as high as 285 μmol/day (the mean production rate of pool A minus 39 μmol/day).

During the first course of nicotinic acid shown in Fig. 2 and Fig. 3 the serum cholesterol concentration fell by an average of 155 μmol/100 ml in 10 days. This is equivalent to an average decrease of 233 μmol in the total amount of cholesterol present in the plasma (volume assumed to be 5% of total body weight). The minimum rate of synthesis estimated above is such that partial inhibition of synthesis by nicotinic acid could easily account for a decrease of 23 μmol/day in the amount of circulating cholesterol, in the absence of any increase in faecal excretion of steroids. This would still be true if some cholesterol enters the plasma from the tissues, a possibility that cannot be excluded by the present observations. Since the liver is a major source of exchangeable cholesterol of endogenous origin in monkeys (Dietschy & Wilson, 1968), the inhibition of hepatic synthesis demonstrated in vitro may have been responsible for much of the effect of nicotinic acid on serum cholesterol concentration observed in the present work.

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