Biochemical Pharmacology, Vol. 32, No. 7, pp. 1309–1312, 1983.
Printed in Great Britain.

0006–2952/83/071309–04 $03.00/0

SHORT COMMUNICATIONS

Modulation of hepatic microsomal Ca^{2+}-stimulated ATPase and drug oxidase activities of guinea pigs by dietary cholesterol

(*Received* 8 *March* 1982; *accepted* 27 *August* 1982)

We have previously demonstrated that cholesterol and other substances, including certain steroid hormones, bind to biomembranes and thus evoke functional changes of integral proteins [1–5]. It was of interest to study whether such phenomena do occur *in vivo* as well. As a first approach, we studied the effects of cholesterol upon the activities of the hepatic microsomal Ca^{2+}-stimulated ATPase and on the drug oxidase enzyme system.

Material and methods. Guinea pigs weighing 200–250 g at the beginning of the experiment were utilized in this study. They were fed a standard diet consisting of 70% whole-meal flour, 25% skimmed milk powder and 5% dried brewer's yeast. Animals were also fed with green vegetables. The animals were given in admixture with their standard diet 0.2% cholesterol for 9 weeks and water *ad lib.* Cholesterol (Merck) was dissolved in ether, mixed with the food pellets, and the ether was allowed to evaporate. A group of animals was given the cholesterol diet plus 30 mg of ascorbic acid, dissolved in drinking water, per 100 g body wt per 12 hr. Controls were fed the standard diet. Five animals were used in each group for the determinations and the results were treated statistically. A two-tailed Student's *t*-test was used to determine the significance of differences between experimental groups. Liver microsomes were prepared as described by Burke and Bridges [6] and suspended in 1.15% KCl–5 mM Tris–HCl (pH 7.0) at a concentration of 10 mg protein/ml. The protein content was determined by the Lowry method as described by Miller [7] using bovine serum albumin (Sigma) as standard. The microsomal lipids were quantitified by $CHCl_3$:CH_3OH extraction [8]. The extract was chromatographed on silica gel G with appropriate standards using $CHCl_3$:CH_3OH:CH_3COOH:H_2O (20:12:3:1 v/v) as the developing system. The content of lipid phosphorus was measured [9] in the scraped spots previously digested in 70% $HClO_4$ at 180°, and the cholesterol content by gas chromatography using cholestane as an internal standard. The microsomal marker, glucose-6-phosphatase, was measured by the method of Swansen [10].

Calcium-stimulated, magnesium-dependent ATPase activity of the liver microsomes was assayed in the following incubation medium: 50 mM Tris–HCl, 20 mM HEPES (pH 7.0), 2 mM $MgCl_2$, 2 mM ATP and 20 μM $CaCl_2$ in a total volume of 1 ml. The reaction was started by the addition of 0.25 mg of microsomal protein and was stopped after 30 min with 0.2 ml of 50% trichloroacetic acid. The liberated P_i was measured by the method of Fiske and Subbarow [11]. The Ca^{2+}-stimulated ATPase activity was defined as the difference between the inorganic phosphate liberated during incubation in the presence and absence of calcium. Calcium uptake by the microsomal membranes was measured under conditions similar to those used for estimating Ca^{2+}-ATPase activity except for the addition of 0.1 μCi per tube of $^{45}CaCl_2$ (Radiochemical Centre, Amersham, U.K.). The incubation was run for 30 min at 37° and terminated by rapid filtration under suction through Millipore filters (type HAWP, 0.45 μ pore dia.) using a Millipore 3025 sampling manifold. The filters were washed 3 times by adding 5 ml of ice-cold 0.25 M sucrose–5 mM Tris–HCl (pH 7.0) and transferred into counting vials. They were then shaken in 1 ml of 1% sodium dodecyl sulfate and mixed with 10 ml dioxane-based scintillation fluid [12]. Cpm were converted to nmoles by the known specific radioactivity. The amount of radioactivity constantly adsorbed on membranes at zero time (instantaneous deposition of radioactivity from the labeled calcium onto microsomal membranes) was subtracted in each case from all samples. Calcium uptake was estimated by addition of 5 mM ammonium oxalate to the incubation medium. ATP-dependent Ca^{2+}-uptake was calculated by subtracting Ca^{2+} binding of microsomes in the absence of ATP in the medium from the total Ca^{2+} uptake in its presence (binding in the absence of ATP was completed by 2 min).

Biphenyl-4-hydroxylase activity was determined as described by Prough and Burke [13]; aniline hydroxylase activity as described by Imai *et al.* [14]; NADPH cytochrome *c* reductase according to Phillips and Langdon [15] and *p*-nitroanisole *O*-demethylase according to Netter and Seidel [16].

Cholesterol was depleted from microsomes isolated from animals fed cholesterol by incubation of microsomes with egg-lecithin liposomes. Microsomal membranes (2.5 mg protein) were incubated for 4 hr with either 1.15% KCl–5 mM Tris–HCl (pH 7.0), or with egg-lecithin liposomes (3 mg) prepared as previously described [17] at 37° in a final volume of 3 ml under continuous magentic stirring.

Table 1. Phospholipid (PL) and cholesterol (C) content of hepatic microsomes isolated from guinea pigs fed on a standard commercial, a C and a C plus ascorbic acid supplemented diet

Diet	PL (μmoles/mg protein)	C (μmoles/mg protein)	Molar ratio (C/PL)
Standard	0.371 ± 0.022	0.079 ± 0.007	0.212
C	0.437 ± 0.027*	0.209 ± 0.018*	0.478
C + ascorbic acid	0.456 ± 0.035†	0.115 ± 0.012†	0.252

Values given are means ± S.D. for five animals.

* Statistically significant compared with microsomes isolated from animals fed on standard diet ($P < 0.01$).

† Statistically significant compared with microsomes isolated from animals fed on standard and cholesterol diet ($P < 0.01$).

Penicillin-G (100 U/ml) and streptomycin sulfate (100 μg/ml) were added. Aliquots (2.5 ml) were withdrawn and layered over 30 ml of 20% sucrose–5 mM Tris–HCl (pH 7.0) and centrifuged for 90 min at 95,000 *g* in a SW-27 rotor of a Spinco L5-75 ultracentrifuge. The precipitate of this centrifugation (free of liposomes) was used for protein, cholesterol, calcium uptake and enzymatic determinations as mentioned earlier.

Results and discussion. The results for the cholesterol and phospholipid content of hepatic microsomes isolated from guinea pigs fed on cholesterol or cholesterol ascorbic acid supplemented diet are summarized in Tables 1 and 2. The cholesterol content of microsomes isolated from animals fed on cholesterol has risen approximately 3 times by the end of the feeding period (9 weeks), while the phospholipid content increases about 20% during the same period. The ratio of cholesterol to phospholipid increases 2.3 times while the Ca^{2+}-stimulated ATPase activity decreases by 70%. As seen in Table 1, addition of ascorbic acid to the cholesterol-supplemented diet prevents to a large extent the increase of cholesterol content in microsomes while the phospholipid content remains at the same level. The cholesterol to phospholipid ratio reduced from 0.478 to 0.252 (a 50% reduction) while the cholesterol effect upon the Ca^{2+}-stimulated ATPase activity is reduced from 70 to 30%, that is, a 40% elevation of the Ca^{2+}-ATPase activity as compared to that in microsomes isolated from animals fed on cholesterol alone. The sp. act. of glucose-6-phosphatase was not altered in animals fed on a cholesterol diet.

The ATP-dependent uptake of calcium (Table 2) has been measured at a relatively low (20 μM) calcium conen, comparable to that prevailing intracellularly. Microsomes isolated from animals fed on cholesterol exhibit a lower calcium uptake sp. act. than those isolated from control animals. Reduction of the calcium uptake, but to a lesser degree, was also shown in microsomes isolated from animals fed on cholesterol plus ascorbic acid. A close relationship between reduction of microsomal calcium uptake and depression of microsomal Ca^{2+}-stimulated ATPase activities appears to exist.

Thus, a comparison of calcium uptake and enzyme activity shows a 50 and 25% loss of calcium uptake activity in the first and second group of animals, respectively, whereas a 60 and 30% loss of Ca^{2+}-ATPase activity is observed in the above groups of animals respectively.

Since calcium uptake activity is coupled to Ca^{2+}-stimulated ATPase activity which serves to transport Ca^{2+} at the expense of ATP hydrolysis [18], the present results suggest that cholesterol decreases the capacity of microsomes to retain calcium by decreasing the activity of the Ca^{2+}-stimulated ATPase.

The ability of exogenous cholesterol to incorporate itself into the phospholipid bilayer of the hepatic microsomes in *in vitro* experiments, causing changes in the activity of the drug oxidation system, has been reported previously [3]. Table 2 clearly shows that such phenomena can also occur *in vivo*. Thus, elevation of the cholesterol content of the microsomes resulted in a statistically significant increase ($P < 0.01$) of the activities of the drug-metabolizing enzymes tested, namely, biphenyl-4-hydroxylase, aniline hydroxylase, NADPH cytochrome *c* reductase and *p*-nitroanisole-*O*-demethylase. The addition of ascorbic acid to the cholesterol diet tends to shift the activity of the drug oxidation system back to the normal level (animals fed on standard diet). This study indicates that stimulation of the activity of drug oxidase, following cholesterol feeding, is strongly related to the cholesterol content of the microsomal membranes.

It is known that the hepatic cholesterol content and hence the hepatic cholesterol synthesis are controlled predominantly by dietary cholesterol [19] and that liver microsomal membrane cholesterol, which contributes up to 7% of the

Table 2. Ca^{2+} uptake, Ca^{2+}-ATPase and drug oxidation enzyme system of hepatic microsomes isolated from guinea pigs fed on a standard, a cholesterol (C) and a C plus ascorbic acid supplemented diet

Diet	Ca^{2+} uptake	Ca^{2+}-ATPase	Biphenyl-4-hydroxylase	Aniline hydroxylase	NADPH cytochrome *c* reductase	*p*-Nitroanisole-*O*-demethylase
Standard	87.4 ± 9.3	171.7 ± 18.5	1.15 ± 0.10	0.24 ± 0.02	22.1 ± 2.4	0.49 ± 0.04
C	41.8 ± 5.6*	67.8 ± 7.8*	2.40 ± 0.14*	0.44 ± 0.04*	49.5 ± 3.6*	0.96 ± 0.06*
C – ascorbic acid	66.5 ± 7.2†	123.1 ± 11.7†	1.62 ± 0.11†	0.32 ± 0.03†	30.8 ± 2.7†	0.75 ± 0.05†

Enzyme activities were estimated as: nmoles P_i/mg protein/hr; nmoles 4-hydroxybiphenyl/mg protein/min; nmoles *p*-aminophenol/mg protein/min; nmoles cytochrome *c* reduced/mg protein/min; nmoles *p*-nitrophenol/mg protein/min; Ca^{2+} uptake = nmoles/mg protein/30 min.

* Statistically significant compared with microsomes isolated from animals fed standard diet ($P < 0.01$).

† Statistically significant compared with controls and cholesterol-fed animals ($P < 0.01$). Values given are means ± S.D. for five animals.

Table 3. Effect of egg-lecithin liposomes, *in vitro*, on Ca^{2+} uptake, Ca^{2+}-ATPase and drug oxidation enzyme systems of hepatic microsomes isolated from guinea pigs fed on a cholesterol diet

Microsomes	Ca^{2+} uptake (nmoles/mg protein/30 min)	Ca^{2+}-ATPase	Biphenyl-4-hydroxylase	Aniline hydroxylase	NADPH cytochrome *c* reductase	*p*-Nitroanisole-*O*-demethylase
Untreated microsomes (control) (cholesterol content: 0.456 ± 0.022 μmoles/mg protein)	40.8 ± 2.9	75.4 ± 5.6	2.10 ± 0.11	0.46 ± 0.04	45.7 ± 3.2	0.91 ± 0.05
Egg-lecithin-treated microsomes (cholesterol content: 0.302 ± 0.018 μmoles/mg protein)	57.6 ± 3.3*	112.7 ± 8.2*	1.60 ± 0.09*	0.30 ± 0.03*	29.1 ± 2.3*	0.41 ± 0.04*

Enzyme activities were estimated as in Table 2.
* Statistically significant compared with control ($P < 0.01$).
Values given are means ± S.D. of duplicate estimations on microsomes isolated from three animals.

total lipid, has both metabolic and structural functions [20]. The mechanism of transport of excess cholesterol and the increase of phospholipid observed in prolonged cholesterol feeding experiments are not yet well understood. The evoked effects on the membranous enzymes, however, are in line with our *in vitro* experiments. Minor amounts of exogenous cholesterol, incorporated in the structure of the biomembranes produces marked functional changes of the integral membranous enzymes [1–4]. In subsequent work, with the aid of the more water-soluble cholesterol glucoside, we found that at small concns the glucoside behaves in an identical manner to cholesterol, i.e. it evokes an equally intense rise in the activity of ouabain-sensitive ATPase. At higher concns of the glucoside, however, the activity of the enzyme starts dropping exponentially [5].

The effect of ascorbic acid on the metabolism of cholesterol in guinea pigs, which, like man, are dependent on an exogenous supply, is probably due to an increased activity of the cholesterol 7α-hydroxylase (a mixed-function oxidase, catalyzing the rate-limiting step in the pathway of bile acid synthesis [21]). This action of ascorbic acid is related in some way to its effects on the microsomal cytochrome P_{450} content [22, 23]. A clear connection with respect to the changes in concns of cholesterol and other lipids in the plasma of guinea pigs in feeding experiments with ascorbic acid has been observed [23]. The *in vivo* alterations in the calcium uptake activity and in the microsomal enzymatic activity due to an increase in the cholesterol to phospholipid ratio are also observed in the *in vitro* treatment of microsomes with egg-lecithin liposomes, which are capable of withdrawing cholesterol from a given membrane [24]. Thus, liposomes formed by sonication of egg-lecithin in water and incubated *in vitro* with microsomes isolated from animals fed on cholesterol decreased appreciably the cholesterol content of microsomes. Consequently, the calcium uptake and the Ca^{2+}-stimulated ATPase activities were enhanced, while the activity of the drug oxidase enzyme system was depressed (Table 3). This finding clearly indicates that the cholesterol effect on calcium uptake and on the activity of membranous enzymes could be reversed by removing the excess cholesterol from the microsomes.

Under normal physiological conditions, the cell maintains a constant level of cholesterol, the physiological role of which could be considered as a stabilizing factor contributing to the overall integrity of cell membranes [25]. However, there are some situations where cholesterol levels may be altered, for example familiar hypercholesterolaemia, cancer or nutritional disorders. An increased cholesterol level in membranes could interfere with the specific function of membrane-bound enzymes (as shown in this study for the Ca^{2+}-stimulated ATPase) with detrimental effects on cell metabolism.

Acknowledgements—We are indebted to Professors S. G. A. Alvisatos and G. Skalkeas, University of Athens Medical School, for the valuable criticism and help they provided during the preparation of this manuscript.

Department of Physiology
University of Athens
Medical School
Athens 609, Greece

GEORGE DELICONSTANTINOS
KATERINA ANASTASOPOULOU
PANAYIOTIS KARAYIANNAKOS

REFERENCES

1. S. G. A. Alivisatos, G. Deliconstantinos, A. Papaphilis and G. Theodosiadis, *Biochim. biophys. Acta* **643**, 642 (1981).
2. S. G. A. Alivisatos, G. Deliconstantinos and G. Theodosiadis, *Biochim. biophys. Acta* **643**, 650 (1981).

3. G. Deliconstantinos, *Experientia* **37**, 81 (1981).
4. A. Papaphilis and G. Deliconstantinos, *Biochem. Pharmac.* **29**, 3325 (1980).
5. S. G. A. Alivisatos and G. Deliconstantinos, in *Cell Function and Differentiation* (Ed. A. Evagellopoulos), p. 479, Alan R. Liss, New York (1982).
6. M. D. Burke and J. W. Bridges, *Xenobiotica* **5**, 357 (1975).
7. G. L. Miller, *Analyt. Chem.* **31**, 964 (1959).
8. J. Folch, M. Lee and G. H. M. Stanley, *J. biol. Chem.* **226**, 479 (1957).
9. G. R. Bartlett, *J. biol. Chem.* **234**, 466 (1959).
10. M. A. Swansen, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 2, p. 541. Academic Press, New York (1955).
11. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 373 (1925).
12. G. A. Bray, *Analyt. Biochem.* **1**, 279 (1960).
13. R. A. Prough and M. D. Burke, *Archs Biochem. biophys.* **170**, 160 (1975).
14. Y. Imai, A. Ito and B. Sato, *J. Biochem.* **60**, 417 (1966).
15. A. M. Phillips and G. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
16. K. J. Netter and G. Seidel, *J. Pharmac. Exp. Ther.* **146**, 61 (1964).
17. G. Deliconstantinos, G. Gregoriadis, G. Abel, J. Marvyn and D. Robertson, *Biochem. Soc. Trans.* **5**, 1327 (1977).
18. L. Moore, T. Chen, H. K. Knap, Jr and E. J. Landon, *J. biol. Chem.* **250**, 4562 (1975).
19. R. G. Anderson, J. L. Goldstein and M. S. Brown, *Nature, Lond.* **270**, 695 (1977).
20. D. Papahajopoulos, M. Cowden and H. Kimelberg, *Biochim. biophys. Acta* **330**, 8 (1973).
21. E. Ginter, *Int. J. Vitam. Nutr. Res.* **16**, Suppl., 53 (1977).
22. S. T. Omaye and J. D. Turnbull, *Life Sci.* **27**, 441 (1980).
23. F. Yokota, Y. Igarachi and R. Suzue, *Atherosclerosis* **38**, 249 (1981).
24. G. Gregoriadis and C. Davis, *Biochem. biophys. Res. Commun.* **89**, 1287 (1979).
25. R. A. Demel and B. deKruyff, *Biochim. biophys. Acta* **457**, 109 (1976).

Biochemical Pharmacology, Vol. 32, No. 7, pp. 1312–1314, 1983.
Printed in Great Britain.

0006–2952/83/071312–03 $03.00/0

Effect of anticonvulsant drugs on the activity of acetyl CoA:arylamine *N*-acetyltransferase (EC 2.3.1.5) and hydroxyindole-*O*-methyltransferase (EC 2.1.1.4) from pineal gland

(*Received* 20 *August* 1982; *accepted* 12 *October* 1982)

There is evidence implicating the involvement of the pineal gland in seizure states [1, 2]. A number of reports attribute the anti-seizure activity of the pineal gland to the hormone melatonin [2–5]. In the light of these findings it is interesting that no reports have studied the effect of anticonvulsant drugs on the pineal gland and more specifically on the enzymes responsible for synthesis of melatonin.

The present study examined the influence of 12 anticonvulsant drugs on the activities of acetyl CoA:arylamine *N*-acetyltransferase (EC 2.3.1.5) (SNAT) and hydroxyindole-*O*-methyltransferase (EC 2.1.1.4) (HIOMT) *in vitro*. These two enzymes are responsible for production of melatonin from 5-hydroxytryptamine.

Methods. Rats of the Wistar strain (200–300 g) of both sexes were treated with isoprenaline HCl (25 mg/kg i.p.) 3 hr before being killed in order to induce SNAT levels in the pineal gland which was assayed as previously described [6]. This assay technique relied on the transfer of an acetyl group from [1-^{14}C]acetyl CoA [205 MBq/mmole (Amersham, U.K.)] to tryptamine HCl (Sigma, St. Louis, MO). Enzyme homogenates in 0.05 M phosphate buffer (pH 6.5) contained one substrate at a constant concn (3×10^{-3} M in the case of tryptamine and 1×10^{-4} M in the case of acetyl CoA) while the concn of the second substrate varied (2×10^{-6}–3×10^{-5} M in the case of acetyl CoA and 2×10^{-4}–3×10^{-3} M in the case of tryptamine). These homogenates were incubated at 20° for 1 hr in the presence and absence of the drugs used (Table 1). After incubation the reaction was terminated by the addition of 0.2 M borate buffer (pH 10) and [^{14}C]*N*-acetyltryptamine extracted into a toluene : isoamyl alcohol (97 : 3) mixture and quantitated.

Bovine pineal glands were collected shortly after death, homogenised in 0.15% KCl, centrifuged at 3000 *g* for 30 min to remove cell debris and the supernatant was lyophilised. This lyophilisate served as a source of HIOMT and prior to assay was dissolved in 0.05 M phosphate buffer (pH 7.9) (1 mg in 100 μl). This enzyme solution was assayed according to a previous report [7] in which *N*-acetylserotonin (Sigma) was *O*-methylated using *S*-adenosyl-L-[methyl-^{14}C]methionine [18.5 MBq/mmole (Amersham)] as a methyl donor. Enzyme incubates contained one substrate at a constant concn (4×10^{-4} M) while the concn of the second substrate varied (2×10^{-5}–3×10^{-4} M) and the reaction was monitored in the presence and absence of the various drugs (Table 1). Incubation was carried out at 42° for 1 hr, and extraction and quantitation were carried out as previously described.

The results were fitted to the Michaelis–Menten kinetic equation using computer-assisted iterative non-linear regression. The points generated for SNAT in the presence of sulthiame (STH) were reanalysed according to Dixon [8] in order to derive dissociation constants.

Results and discussion. None of the drugs tested affected HIOMT and only STH had an effect on SNAT (Fig. 1a and b). This effect was a mixed non-competitive inhibition with respect to tryptamine and the rate expression can be represented as follows [9]:

$$\frac{1}{V} = \frac{1}{V_s}\left(\frac{1 + \text{STH}}{K_{ii}}\right) + \frac{K_{ia}}{V_s}\left(\frac{1 + \text{STH}}{K_{is}}\right)\frac{1}{[\text{tryptamine}]} \quad (1)$$

where V is the velocity at a certain tryptamine concn [tryptamine], V_s is the maximum velocity. K_{ii} is the dissociation constant for STH from the E·tryptamine·STH complex, K_{ia} is the dissociation constant for the E·tryptamine complex and K_{is} is the dissociation constant for the E·STH complex.