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Peripheral benzodiazepine receptor ligands in rat liver mitochondria: effect on 27-hydroxylation of cholesterol

Virginia Tsankova [b], Mario Visentin [a], Lavinia Cantoni [a], Maria Carelli [a], Maria Teresa Tacconi [a,*]

[a] *Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, 20157 Milano, Italy*
[b] *Higher Medical School, Faculty of Pharmacy, Sofia, Bulgaria*

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Abstract

The effect of peripheral benzodiazepine receptor ligands: PK11195 (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)isoquinoline-3-carboxamide), Ro 5-4864 (4-chlorodiazepam), hemin, *N*-methyl protoporphyrin IX and protoporphyrin IX on liver mitochondrial 27-hydroxylation of cholesterol was studied by adding them together with [4-^{14}C]cholesterol. *N*-Methyl protoporphyrin IX, PK11195 and protoporphyrin IX stimulated mitochondrial 27-hydroxylation of [4-^{14}C]cholesterol in vitro, the first two being the most potent (2–3-fold increase). Ro 5-4864 and hemin were not active. 27-Hydroxylation of [4-^{14}C]cholesterol was reduced to below control levels (respectively 40 and 56% decrease compared to control, $P < 0.01$) when PK11195, *N*-methyl protoporphyrin IX or protoporphyrin IX were allowed to equilibrate in vitro with mitochondria for 20 min at 37°C. Hepatic protoporphyria was induced using 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (100 mg/kg, i.p.) to study the effect of in vivo accumulation of large amounts of dicarboxylic porphyrins, i.e. endogenous peripheral benzodiazepine receptor ligands, on cholesterol 27-hydroxylation. DDC treatment caused an increase in total porphyrin content in liver homogenate (10-fold) and mitochondria (2-fold). Mitochondrial 27-hydroxylation of [4-^{14}C]cholesterol was depressed after treatment (60% decrease, $P < 0.01$). We suggest that peripheral benzodiazepine receptor ligands act on liver mitochondrial 27-hydroxylation of cholesterol by a mechanism coupled to these receptors and that the time of exposure of peripheral benzodiazepine receptors to ligands is a major factor. The modulation of 27- hydroxycholesterol production may have a physiological role in liver and possibly in other tissues.

Keywords: Benzodiazepine receptor, peripheral; Porphyrin; PK11195; Cholesterol; Metabolism; Liver

1. Introduction

The liver plays a key role in regulating the body's cholesterol balance. Cholesterol can be: (1) incorporated into cell membranes; (2) converted to cholesteryl esters; (3) metabolized to the bile acids and later secreted into the bile; (4) directly secreted into the bile. Factors regulating the flux of cholesterol by each of these pathways have yet to be clarified (Johnson et al., 1990).

In steroidogenic tissues, ligands which bind with high affinity to peripheral benzodiazepine receptors mediate the flux of cholesterol into the inner mitochondrial membrane (Anholt et al., 1986; Krueger and Papadopoulos, 1990). In liver, peripheral benzodiazepine receptor numbers are considerable, mainly located on the mitochondrial fraction (O'Beirne et al., 1990).

In the inner membrane of rat liver mitochondria, cholesterol undergoes oxidation of the sterol side-chain to 27-hydroxycholesterol, which is further metabolized to bile acids. Bile acids can be formed from cholesterol through different pathways. Although microsomal 7α-hydroxylation of cholesterol is the major pathway for synthesis of cholic and chenodeoxycholic acids, 27-hydroxylation of cholesterol is an alternative, quantitatively important route for the formation of bile acids, particularly for the synthesis of chenodeoxycholic acid (Princen et al., 1991; Axelson et al., 1992; Björkhem, 1992). The enzyme, sterol 27-hydroxylase (EC 1.14.13.15), is a mixed function monooxygenase, cytochrome P-450-dependent and localized on the

* Corresponding author. Tel.: 39.2 39014.1; fax: 39.2 3546277.

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matrix side of the inner mitochondrial membranes (Andersson et al., 1989). Cholesterol is mainly situated on the outer mitochondrial membrane. A transport mechanism to the inner membrane is required (Liscum and Dahl, 1992) to give access of the sterol to the enzyme for further oxidation.

We found that *N*-methyl protoporphyrin IX and PK11195 (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)isoquinoline-3-carboxamide) significantly increased cholesterol translocation into liver mitochondria (Tsankova et al., 1995). *N*-Methyl protoporphyrin IX, which mostly increased cholesterol translocation, is one of the *N*-monosubstituted protoporphyrin IX formed in liver by interaction between the heme prosthetic group of cytochrome P-450 and reactive derivatives of many structurally diverse chemicals (De Matteis and Cantoni, 1979; Ortiz de Montellano et al., 1983; Grab et al., 1988; Coffman et al., 1982). 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) is one of the drugs forming *N*-methyl protoporphyrin IX and its mechanism of action has been widely investigated (Smith and De Matteis, 1980). *N*-Methyl protoporphyrin IX produced in the endoplasmic reticulum migrates inside the mitochondria where it inhibits the enzyme, ferrochelatase (EC 4.99.1.1), leading to pronounced hepatic protoporphyria (De Matteis et al., 1987).

Protoporphyrin IX and *N*-methyl protoporphyrin IX are potent inhibitors of [^{3}H]PK11195 binding to peripheral benzodiazepine receptors when they are added to rat liver membranes in vitro. PK11195 binding is also inhibited when measured in liver membranes obtained from porphyric, DDC-treated rats (Cantoni et al., 1992).

The aim of this study was to investigate the effects of different peripheral benzodiazepine receptor ligands on the production of 27-hydroxycholesterol in control rat liver mitochondria and in mitochondria from DDC-treated protoporphyric rats.

2. Materials and methods

2.1. Materials

[4-^{14}C]Cholesterol (specific activity 52 mCi/mmol) was obtained from the Radiochemical Centre (Amersham, Bucks, UK). Ro 5-4864 was purchased from Fluka (Buchs, Switzerland), PK11195 from Sigma Chemical Co. (St. Louis, MO, USA). Protoporphyrin IX, hemin and *N*-methyl protoporphyrin IX were obtained from Porphyrin Products (Logan, UT, USA). 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) was obtained from Eastman Kodak, Rochester, NY, USA and was purified before use through recrystallization from an ethanol-water solution. All other materials were obtained from standard sources.

The standard 27-hydroxycholesterol was a generous gift from Dr. N.B. Javitt, New York University Medical Center.

2.2. Porphyrin solutions

A solution of hemin was prepared by dissolving 1.3 mg of hemin in 2.5 ml of 0.1 M NaOH containing 12 mg Tris; the volume was brought to 8 ml with distilled water and the pH was adjusted to 7.4 with 1 N HCl. Solutions of protoporphyrin IX and *N*-methyl protoporphyrin IX were prepared as follows: 1.5 mg protoporphyrin IX or 0.5 mg *N*-methyl protoporphyrin IX was dissolved in 100 μl of 1 M KOH; 100 μl of 1 M Tris-chloride buffer (pH 7.8) and 3.7 ml of distilled water were added and the pH of the solutions was adjusted to 7.4 with 1 N HCl. The solutions were filtered using a sterile 0.2 μm filter (Sartorius, SM 17597). Aliquots of filtered solutions were used to calculate the exact concentration (Falk, 1964; De Matteis et al., 1982). Porphyrin solutions were always used within 4 h of preparation.

2.3. Animals

Adult male Sprague-Dawley rats (200–250 g) were obtained from Charles River Italia (Calco, Italy) and were allowed free access to food and water. In the experiments in which protoporphyria was induced, rats (fasted for 24 h) were given one i.p. injection of DDC (100 mg/kg) dissolved in peanut oil (10 ml/kg) and were killed by decapitation 5 h later. Control animals were given oil alone. The livers were removed, rinsed in ice-cold saline and used immediately for preparation of the mitochondrial fraction.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council directive 86/609, OJ L 358,1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985.)

2.4. Preparation of rat liver mitochondria

The livers were homogenized in 5 mM Tris-chloride buffer, pH 7.2, containing 0.25 M sucrose (1 g liver in 5 ml buffer) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $800 \times g$ for 10 min, the supernatant was recovered and centrifuged again at $9000 \times g$ for 10 min. The resulting pellets were washed, suspended in 1.0 mM Tris-chloride buffer, pH 7.2, containing 0.28 M sucrose and 0.1 mM EDTA, and spun at $1500 \times g$ for 10 min. The supernatants were spun at $9000 \times g$ for 10 min, yielding an enriched mitochondrial preparation. All preparations were done on ice and the solutions were kept at 4°C.

Contamination of mitochondria by the microsomal fraction was less than 2% as calculated by comparison of specific activities (Tsankova et al., 1995) of NADPH cytochrome c reductase activity, a marker enzyme for endoplasmic reticulum (Nervi et al., 1980).

2.5. Measurement of 27-cholesterol hydroxylation

Mitochondria corresponding to 10 mg of protein were incubated with 1.3 μCi [4-^{14}C]cholesterol (specific activity 52 mCi/mmol), 3.5 μmol NADPH in 50 mM Tris-acetate buffer, pH 7.4, in 1.5 ml final volume with and without peripheral benzodiazepine receptor ligands. The concentration of labeled cholesterol (0.0025 μmol/mg protein) was much lower (1 : 35) than the concentration of endogenous mitochondrial cholesterol (0.087 μmol/mg protein). Incubation was for 40 min at 37°C. Boiled mitochondria were used as controls. The reaction was stopped by the addition of 3 ml ethanol 96% (v/v). After acidification and ether extraction, the dried extract was applied to a TLC plate (silica gel G, with concentration zone, Merck) and developed with benzene-ethylacetate 1 : 1 (v/v) (Lidström-Olsson and Wikvall, 1986). The spots corresponding to cholesterol and 27-hydroxycholesterol, identified using authentic standards run in parallel, were visualized with iodine, scraped and counted for radioactivity in a Beckman Liquid Scintillation Counter LS 1701.

In some experiments, mitochondria were preincubated with equimolar (1 μM) concentrations of *N*-methyl protoporphyrin IX, protoporphyrin IX or PK11195 for 20 min at 37°C and [4-^{14}C]27-hydroxycholesterol production from [4-^{14}C]cholesterol was measured directly after the preincubation or after repeated washing of the mitochondria. The washings were done by resuspending mitochondria in 50 mM Tris-chloride buffer, pH 7.4, and centrifuging at 9000 × *g* for 10 min.

2.6. Other tests

Proteins were measured according to Lowry et al. (1951). The cholesterol content was determined (Omodeo Salé et al., 1984) after lipid extraction (Folch et al., 1957). Mitochondrial cytochrome P-450 was assayed as described (Omura and Sato, 1964).

2.7. Statistics

The analysis of variance (ANOVA) was used for statistical analysis; Dunnett's test for multiple comparison was used when *F* was significant.

3. Results

3.1. Effect of peripheral benzodiazepine receptor ligands on 27-hydroxycholesterol production in rat liver mitochondria in vitro

To investigate whether peripheral benzodiazepine receptor ligands affect mitochondrial cholesterol 27-hydroxylation, liver mitochondria were incubated with equimolar concentrations (1 μM) of PK11195, Ro 5-4864, hemin, protoporphyrin IX and *N*-methyl protoporphyrin IX in the presence of substrate [4-^{14}C]cholesterol and 27-hydroxycholesterol production was measured (Fig. 1). [4-^{14}C]27-hydroxycholesterol production was more than doubled when *N*-methyl protoporphyrin IX or PK11195 was added simultaneously with [4-^{14}C]cholesterol (respectively by 148 and 120%, $P < 0.01$). Protoporphyrin IX was less effective (88% increase, $P < 0.01$). Ro 5-4864 and hemin were not active.

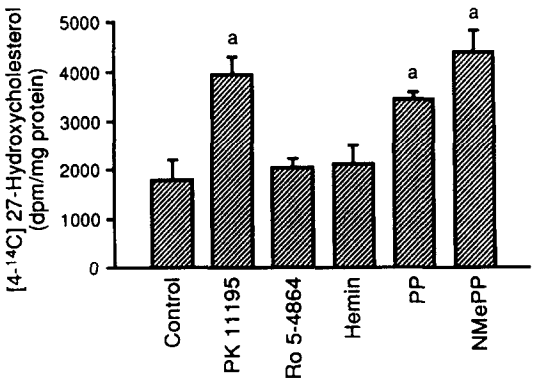

Fig. 1. Effect of equimolar (1 μM) concentrations of peripheral benzodiazepine receptor ligands on the formation of [4-^{14}C]27-hydroxycholesterol by rat liver mitochondria. Mitochondria were incubated with 1.3 μCi [4-^{14}C]cholesterol, 3.5 μmol NADPH in 50 mM Tris-acetate buffer, pH 7.2, with and without peripheral benzodiazepine receptor ligands (1 μM) for 40 min at 37°C and the [4-^{14}C]27-hydroxycholesterol produced was measured. Results are means ± S.E.M. of duplicates from two experiments ($n = 4$). PP = protoporphyrin IX; NMePP = *N*-methyl protoporphyrin IX. [a] $P < 0.01$ vs. controls.

Fig. 2 shows the concentration-response curves for peripheral benzodiazepine receptor ligands tested in this study. PK11195, Ro 5-4864, hemin, protoporphyrin IX and

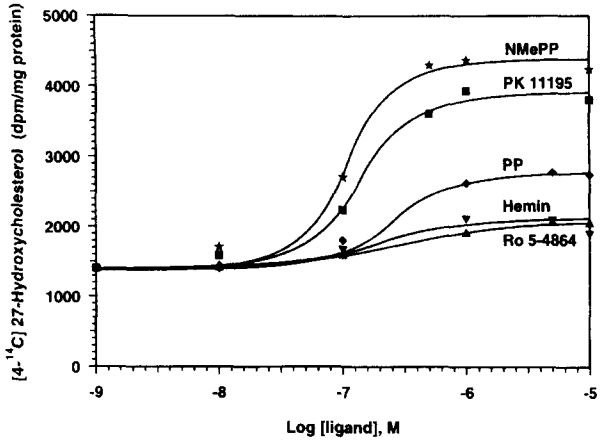

Fig. 2. [4-^{14}C]27-Hydroxycholesterol production in liver mitochondria: concentration-response curves for peripheral benzodiazepine receptor ligands. Mitochondrial [4-^{14}C]27-hydroxycholesterol production from [4-^{14}C]cholesterol was measured in the presence of peripheral benzodiazepine receptor ligands, used at concentrations from 10^{-5} to 10^{-9} M. Incubation was as described in the legend to Fig. 1. Curves were generated by computer-assisted non-linear regression analysis, according to a least-squares curve-fitting program. PP = protoporphyrin IX; NMePP = *N*-methyl protoporphyrin IX.

N-methyl protoporphyrin IX were used in a concentration range from 10^{-5} to 10^{-9} M. *N*-Methyl protoporphyrin IX and PK11195 were the most potent, stimulating [4-^{14}C]27-hydroxycholesterol production in a concentration-dependent manner, reaching about 3 times the level measured in the absence of ligands. Protoporphyrin IX was effective only at a high concentration (1.7-fold stimulation). Ro 5-4864 and hemin did not stimulate 27-hydroxylation of [4-^{14}C]cholesterol.

When the logs of the inhibition constants (K_i) of the ligands competing for [^{3}H]PK11195 binding in liver membranes (Cantoni et al., 1992) were plotted against the logs of the concentrations necessary to attain half-maximum stimulation of the corresponding ligand (log ED_{50}), a significant correlation was found ($r = 0.963$). This suggests that the stimulatory effect of peripheral benzodiazepine receptor ligands on cholesterol 27-hydroxylation might be related to ligand occupancy of peripheral benzodiazepine receptors.

To further characterize the interaction between ligand occupancy of peripheral benzodiazepine receptors and the effect on cholesterol 27-hydroxylation, 1 μM *N*-methyl protoporphyrin IX, protoporphyrin IX or PK11195 was preincubated for 20 min at 37°C with mitochondria to allow equilibration of the ligand with the receptor. The preincubation time was chosen on the basis of a previous report that, in liver membranes, [^{3}H]PK11195 binding demonstrates rapid association kinetics, reaching equilibrium within 20 min (Cantoni et al., 1992). After the preincubation, [4-^{14}C]cholesterol was added and [4-^{14}C]27-hydroxycholesterol production was measured and compared with that in control mitochondria preincubated in the absence of ligands (Fig. 3, panel A). Pre-exposure of mitochondrial peripheral benzodiazepine receptors to the ligands modified their effect on cholesterol 27-hydroxylation: PK11195 was no longer stimulatory and the levels of [4-^{14}C]27-hydroxycholesterol produced were the same as in control mitochondria. *N*-Methyl protoporphyrin IX or protoporphyrin IX reduced the levels of [4-^{14}C]27-hydroxycholesterol produced (respectively to 40 and 56% compared to the control, $P < 0.01$).

The effect of stimulation of 27-hydroxycholesterol production by the peripheral benzodiazepine receptor ligands was partly restored when mitochondria were washed after the preincubation (Fig. 3, panel B): when *N*-methyl protoporphyrin IX and PK11195 were used, [4-^{14}C]27-hydroxycholesterol production was again increased significantly (respectively to 70 and 37% compared to control, $P < 0.01$), while with protoporphyrin IX the increase was not significant. These results indicate that the time of exposure of peripheral benzodiazepine receptors to the ligands can influence the effect on 27-hydroxylation.

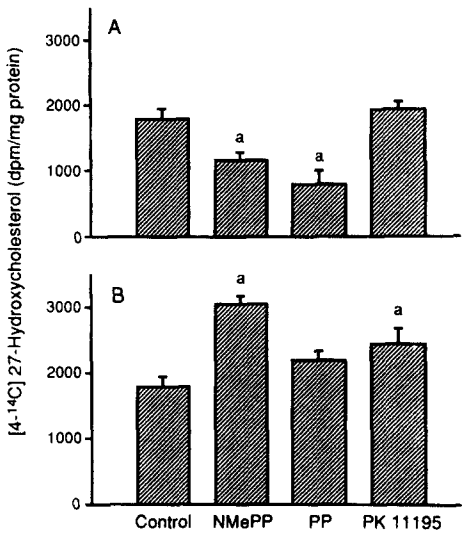

Fig. 3. Effect on [4-^{14}C]27-hydroxycholesterol production of in vitro pre-exposure of liver mitochondria to *N*-methyl protoporphyrin IX, protoporphyrin IX and PK11195. Mitochondria were preincubated with 1 μM *N*-methyl protoporphyrin IX, protoporphyrin IX and PK11195 for 20 min at 37°C, before beginning the reaction. [4-^{14}C]27-Hydroxycholesterol was measured in preincubated mitochondria before (panel A) and after (panel B) washing to remove unbound ligands. Control values were obtained from samples preincubated without addition of ligands. Values are means ± S.E.M. of duplicates from three experiments ($n = 6$). PP = protoporphyrin IX; NMePP = *N*-methyl protoporphyrin IX. [a] $P < 0.01$ vs. control.

3.2. Effect of hepatic accumulation of porphyrins on mitochondrial 27-hydroxycholesterol production

Protoporphyrin IX and *N*-methyl protoporphyrin IX were allowed to accumulate in vivo with DDC (100 mg/kg, i.p.) (Smith and De Matteis, 1980). The existence of protoporphyria was assessed by measuring total hepatic porphyrins, which were increased 10-fold at the time of killing, 5 h after treatment (Table 1). This is when the maximum increase in hepatic porphyrin concentration is seen (De Matteis et al., 1973). Liver mitochondria from

Table 1
Effect of DDC on total porphyrin, cholesterol content and total phospholipids in liver homogenate and mitochondria

Treatment	Liver homogenate		Liver mitochondria	
	Oil	DDC	Oil	DDC
Total porphyrins (nmol/g liver) (8) [a]	0.53 ±0.014	6.98 ±0.02 [b]	0.167 ± 0.02	0.291 ± 0.051 [b]
Cholesterol mg/g liver (8) [a]	1.551 ± 0.272	2.315 ± 0.119 [b]	0.272 ± 0.09	0.315 ± 0.05
Total phospholipids mg/g liver (8) [a]	29.54 ±0.12	30.23 ±0.39	1.63 ±0.31	1.68 ±0.24

Animals were given peanut oil (10 ml/kg) or DDC (100 mg/kg i.p. once). They were fasted 24 h before treatment and until killed (5 h after treatment).
[a] Number of animals in parentheses. Values are means ± S.D. [b] $P < 0.01$ vs. oil group, Student's *t*-test.

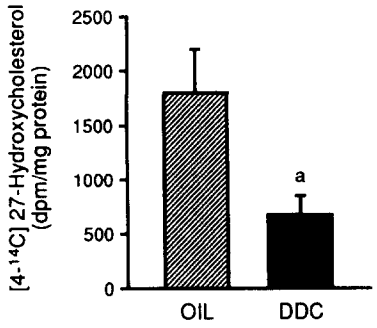

Fig. 4. Effect of DDC treatment on [4-^{14}C]27-hydroxycholesterol production in liver mitochondria. The animals were given peanut oil (10 ml/kg) or DDC (100 mg/kg i.p. once), and were fasted 24 h before treatment and until the time of killing (5 h after treatment). Mitochondria from oil and DDC-treated animals were incubated with 1.3 μCi [4-^{14}C]cholesterol and 3.5 μmol NADPH in 50 mM Tris-acetate buffer, pH 7.2, 1.5 ml final volume. After 40 min incubation at 37°C, the [4-^{14}C]27-hydroxycholesterol produced was measured. The values are means ± S.E.M. for eight animals per group. [a] $P < 0.01$ vs. controls.

treated animals also showed an increased total porphyrin content (60% more than control, $P < 0.01$). Cholesterol increased in total liver homogenate (149% of control, $P < 0.01$) but not in mitochondria. No changes were found in the total phospholipid composition in liver homogenate and mitochondria.

27-Hydroxylation of [4-^{14}C]cholesterol was measured in mitochondria from treated animals (Fig. 4). The production of [4-^{14}C]27-hydroxycholesterol was 60% lower than in the controls ($P < 0.01$). It seems unlikely that this effect was due to a DDC-induced mitochondrial cytochrome P-450 deficiency, since absorbance of the Fe^{2+}-CO complex of mitochondrial cytochrome P-450 was similar in controls and DDC-treated animals (data not reported).

4. Discussion

In this study we have demonstrated that the peripheral benzodiazepine receptor ligands, *N*-methyl protoporphyrin IX, PK11195 and protoporphyrin IX, can modulate 27-hydroxylation of cholesterol, the first side-chain oxidative reaction in the bile acid biosynthesis pathway, in rat liver intact mitochondria in vitro. Cholesterol 27-hydroxylation is catalyzed by a mitochondrial cytochrome P-450-dependent enzyme (sterol 27-hydroxylase) located in the mitochondrial inner membrane (Okuda, 1994). This enzyme has a broad spectrum specificity, catalyzing the 27-hydroxylation of cholesterol and many oxysterols (Andersson et al., 1989; Okuda, 1994; Javitt, 1990).

There are close similarities between liver mitochondrial sterol 27-hydroxylase and the mitochondrial cholesterol side-chain cleavage cytochrome P-450 enzyme (P-450$_{scc}$) in adrenals. The deduced amino acid sequence of a cDNA clone encoding the rabbit sterol 27-hydroxylase shares similarity (34% homology) with the mitochondrial cytochrome P-450$_{scc}$ (Andersson et al., 1989). Both enzymes use a similar electron transfer system (Okuda, 1994) and cholesterol as a substrate for steroid and bile acid biosynthesis, respectively. In steroidogenic tissues and cells, cytochrome P-450$_{scc}$ converts cholesterol to pregnenolone, which is the first step in steroid biosynthesis. In these tissues peripheral benzodiazepine receptor ligands stimulate intramitochondrial cholesterol transport to inner membranes by binding to peripheral benzodiazepine receptors, thus promoting cholesterol availability to P-450$_{scc}$ (Mukhin et al., 1989; Krueger and Papadopoulos, 1990). In adrenals the access of cholesterol to this enzyme is the limiting step for its further metabolism (Simpson et al., 1978).

We have previously shown that peripheral benzodiazepine receptor ligands stimulated liver intramitochondrial cholesterol translocation from the outer to the inner membranes (Tsankova et al., 1995) and the rank order of potency was *N*-methyl protoporphyrin IX > PK11195 > protoporphyrin IX ≫ Ro 5-4684 ≫ hemin. Under experimental conditions similar to those used to stimulate cholesterol translocation (i.e. addition of ligands immediately before [4-^{14}C]cholesterol) peripheral benzodiazepine receptor ligands increased 27-hydroxycholesterol production with an identical rank order of potency. The correlation between the constants of inhibition (K_i) for each ligand for competing for [^{3}H]PK11195 binding and their potencies for stimulating 27-hydroxycholesterol production provides evidence that the ligands' effect is a consequence of their binding to peripheral benzodiazepine receptors. The findings of this study suggest that, in liver, the availability of cholesterol to the enzyme, sterol 27-hydroxylase, might be the limiting factor for its further metabolism.

The absence of effect of Ro 5-4864, a classical agonist of peripheral benzodiazepine receptors, is in agreement with previous data that showed a lack of activity of this compound on cholesterol translocation in liver mitochondria (Tsankova et al., 1995). This is not surprising since the potency of Ro 5-4864 as a peripheral benzodiazepine receptor ligand varies markedly in different organs and species (Awad and Gavish, 1987), whereas the effect of PK11195 is relatively constant. Furthermore, in certain tissues, Ro 5-4864 and PK 11195 act as agonist and antagonist, respectively, to peripheral benzodiazepine receptors (Mizoule et al., 1985).

We found that the time of contact of mitochondria with the ligands was an important factor in 27-hydroxycholesterol production: pre-exposure of ligands to the receptor abolished (PK11195) or inhibited (protoporphyrin IX, *N*-methyl protoporphyrin IX) the stimulatory effect on 27-hydroxylation. This effect cannot be ascribed to exhaustion of available cholesterol, since the stimulatory activity was restored by washing the mitochondria. It also seems unlikely that washing removed considerable amounts of 27-hydroxycholesterol, which might inhibit the activity of 27-hydroxylase; in fact, 27-hydroxycholesterol is poorly soluble in water. The stimulatory effect of ligands may

possibly be best observed in the initial phase of the incubation, when there is a concentration gradient of endogenous labeled cholesterol from the outer to the inner mitochondrial membranes. This is the state in the experiments without preincubation. This gradient will decrease with time, eventually reaching equilibrium: from this time on ligands might only alter the diffusion rate of cholesterol across the membranes. The preincubation time of 20 min might correspond to the interval of time during which this equilibrium was reached, thus unfavourably affecting the metabolism of labeled cholesterol (which was added in much smaller amounts than the endogenous substrate). Washing might restore the initial gradient of cholesterol.

However, porphyrins are physiological compounds with a metabolic profile inside the mitochondria. They interact with the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane, a component of the complex which forms the peripheral benzodiazepine receptor assembly, containing an adenine nucleotide carrier and an 18 kDa protein (McEnery et al., 1992). This complex mediates various cellular functions, one of which was suggested to be the transport of porphyrins across mitochondrial membranes (Verma and Snyder, 1988; Pastorino et al., 1994; Taketani et al., 1994). If this is true, porphyrins might have effects different from those on translocation inside the mitochondria, reducing the availability of cholesterol, perhaps by influencing its subsequent transfer to the enzyme.

DDC-induced protoporphyria appears a suitable experimental model for investigating the effect of large endogenous modifications of the levels of peripheral benzodiazepine receptor ligands on 27-hydroxylation of cholesterol in vivo, since it causes the accumulation of two dicarboxylic porphyrins, *N*-methyl protoporphyrin IX and protoporphyrin IX. It was previously shown that, in this model, the binding of [^{3}H]PK11195 to peripheral benzodiazepine receptors was reduced and the effect appeared to be attributable to DDC-induced formation of the two protoporphyrins (Cantoni et al., 1992).

The results obtained with mitochondria from porphyric animals reproduced those obtained when mitochondria from control rats were pre-exposed to porphyrins, i.e. mitochondria of porphyric rats produce less 27-hydroxycholesterol. The concentrations measured in mitochondria from porphyric rats were in the same order of magnitude (0.4–0.6 μM) as those used when non-porphyric mitochondria were pre-exposed to porphyrins in vitro. The liver of DDC-treated rats contains two porphyrins at the same time. The preincubation of non-porphyric mitochondria with protoporphyrin IX and *N*-methyl protoporphyrin IX in combination, i.e. under conditions similar to those observed in mitochondria from porphyric rats, again inhibits 27-hydroxylation of cholesterol (1278 $\pm$ 140 dpm/mg protein vs. control 1800 $\pm$ 145 dpm/mg protein, $P < 0.01$).

These results suggest that when peripheral benzodiazepine receptors are exposed to porphyrins for a long time, not only is the maximum number of [^{3}H]PK11195 binding sites reduced (Cantoni et al., 1992), but there are also functional consequences, which might result in the inhibition of 27-hydroxylation of cholesterol.

This study opens an interesting new perspective regarding the possible role of peripheral benzodiazepine receptor ligands in the removal of excess cholesterol. This is suggested by several observations: the activity of sterol 27-hydroxylase is present not only in liver but also in extrahepatic tissues, like kidney (Postlind and Wikvall, 1989) and fibroblasts (Skrede et al., 1986) and is as high in bovine aortic endothelial cells as in HepG2 cells (Reiss et al., 1994); mRNA for synthesis of this enzyme was also found in macrophages (Björkhem et al., 1994) and ovary (Rennert et al., 1990).

On the other hand, in vivo studies with models of atherosclerosis showed that there are interactions between peripheral benzodiazepine receptor ligands and cholesterol metabolism. Diazepam (a ligand that binds to both central and peripheral benzodiazepine receptors) given chronically lowers plasma cholesterol in several animal species (Feszt et al., 1977; Wong et al., 1980) and inhibits cholesterol synthesis in normal rat liver (Bell, 1985). PK11195 increases total plasma cholesterol and triglycerides in rats (Horak et al., 1990) and antagonizes the antiatherogenic effect of diazepam (Cuparencu et al., 1990).

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