DDC-INDUCED HEPATIC PROTOPORPHYRIA AND CHANGES IN SERUM AND IN LIVER LIPIDS CONTENT IN RAT: IMPACT OF PERIPHERAL BENZODIAZEPINE RECEPTOR

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ABSTRACT
Administration of DDC (3,5-diethoxycarbonil-1,4-dihydro-2,4,6-trimethylpyridine) (100 mg/kg, i.p.) to male rats produced significant increase in total porphyrins thus giving rise to the biochemical picture of porphyria(s). Porphyrins, accumulated in hepatic porphyria are putative endogenous ligands for peripheral benzodiazepine receptor. A role of peripheral benzodiazepine receptors in cholesterol transport and metabolism is suggested. Hepatic protoporphyrin is associated with hepatomegaly, cholestasis and alterations in the microsomal cytochromes. We found that DDC administration in rat induced lipid disturbances and increased significantly total cholesterol in serum and in liver, as well as total phospholipids in liver. 27-Hydroxycholesterol content was significantly increased in serum and liver by DDC, while phenobarbital had no effect. We assume that the observed lipid disturbances are connected to the accumulation of porphyrins. These effects are probably modulated by porphyrin binding to peripheral benzodiazepine receptors.


Keywords: DDC-induced porphyria, cholesterol, 27-hydroxycholesterol, peripheral benzodiazepine receptor

Introduction
The liver is the main site for porphyrin biosynthesis. Physiological concentrations of porphyrins are kept by the coordinated functioning of the pathways of heme biosynthesis and degradation. However, these pathways might be altered by a number of chemicals. The dihdropropyridine DDC (3,5-diehotoxcarbonil-1,4-dihydro-2,4,6-trimethylpyridine) has a porphyrogenic activity in a variety of animal species (6, 19, 20). It decreases the activity of hepatic ferrochelatase (EC 4.99.1.1.) thus causing the accumulation of protoporphyrins (mainly protoporphyrin IX and N-methyl protoporphyrin) in the liver and giving rise to the biochemical symptoms of porphyria. Porphyrins are putative endogenous ligands for peripheral benzodiazepine receptor (PBR) (21, 25). Convincing evidence that porphyrins interact with PBR in vivo is provided by Cantoni et al. (3). The authors found a reduction in [³H] PK11195 binding in the liver of DDC-treated rats as a result of a specific N-methylprotoporphyrin IX and protoporphyrin IX accumulation (3).

Hepatic protoporphyrin is associated with hepatomegaly, cholestasis, alterations in the microsomal cytochromes and tumor formation (4, 11, 22). The observed hepatotoxicity which follows DDC administration in rat might be a result of different drug effects, mediated or not by their porphyrogenic activity (22).
Materials and Methods
All solvents used were HPLC grade (Merck, Darmstadt, Germany). 27-Hydroxycholesterol (27-OH cholesterol) was a generous gift from Dr. Norman B. Javitt, New York University Medical Center. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) was obtained from Eastman Kodak, Rochester, NY, USA and purified before use through recrystallization from an ethanol-water solution. Cholesterol, cholesterol oxidase, NADPH, Triton X-100, isocitrate dehydrogenase were purchased from Sigma (St. Louis). All other materials were obtained from standard sources.

Determination of 27-hydroxycholesterol
The production of 27-hydroxycholesterol was assayed by the method of Petrack and Lotario (17). Lipid extracts from plasma, liver homogenate and mitochondria were prepared as described by Folch et al. (9). The organic phase derived from the lipid extractions was evaporated under nitrogen and the residue was dissolved in a final volume of 1 mL of methanol/phosphate buffer (100 mM, pH 7.5) 10:90 (vol/vol). Cholesterol oxidase (2 units) was added and the tubes were incubated for 20 min at 37°C. The reaction was terminated with 1.5 mL methanol. Testosterone propionate (5 μg/mL hexane) was added as internal standard. The mixture was extracted twice with 3 mL hexane and the organic layer was evaporated under nitrogen. The residue was dissolved in 100 μL of 5% isopropanol in dodecane. Aliquots of 100 μL were analysed via normal phase HPLC on an Alltech spherisorb silica column (4.6 x 250 mm), using an isocratic mobile phase of hexane-isopropanol 95:5 and flow rate of 1 mL per min. Absorbance was monitored at 240 nm.

Liver porphyrin content assay
Total liver porphyrin content was determined fluorometrically with mesoporphyrin as an internal standard in a 10% liver homogenate prepared in 0.25 M sucrose as described by Abbritti and De Matteis (1). All procedures were carried out in dim light.

Other tests
Protein concentration was measured according to Lowry et al. (15), using BSA as the standard. The cholesterol and lipids content were determined after the lipid extraction (9). Determinations of individual lipid classes (triglycerides (TG), cholesterol (CHOL) and phospholipid (PL) was performed (16).

Statistical analysis
Statistical analysis was done using ANOVA test; Student’s test was used when F was significant. A p value of less than 0.05 was considered significant.

Results and Discussion
DDC treatment caused considerable and statistically significant increase in the total porphyrin content in liver homogenate (6.98±0.12 compared to control 0.53±0.12 nmol/g tissue) (Fig. 1). In liver mitochondria, the total porphyrins were increased approximately twice after DDC treatment (0.29±0.06 vs 0.167±0.06 nmol/g tissue). Analysis of the hepatic porphyrins through HPLC separation showed that in control and treated rats, protoporphyrin IX was the prevalent porphyrin (3).

Fig. 1. Effect of DDC on total porphyrin content in rat liver
Animals were given peanut oil (10 ml/kg) or DDC (100 mg/kg i.p. once) as described in Materials and Methods. Six animals per group were used. Values are means ± SD. P<0.01 vs control group, according to Student’s t-test

Table 1 summarizes the data obtained for lipid content in serum and rat liver, treated with DDC and phenobarbital. Total cholesterol in serum was significantly increased by DDC-treatment (69.11±4.99 vs 57.35±3.75 mg/dl). Phenobarbital also exerted a pronounced effect, increasing the serum total cholesterol concentration (79.69±7.49 vs 57.35±3.75 mg/dl). On the other hand, phenobarbital reduced significantly the serum TG (two fold), while no statistically significant differences were observed in serum TG levels in porphyrin animals.

DDC treatment increased total cholesterol in liver homogenate to 2.63±0.037 vs 2.38±0.102 mg/g tissue. On the other hand, phenobarbital treatment did not cause significant changes (2.45±0.026 mg/g tissue). Phospholipids (PL) content was increased to 29.05±2.43 vs 24.71±1.21 mg/g tissue in livers of porphyrin animals compared to the untreated controls. No significant changes were found in total PL content in liver homogenate after phenobarbital treatment (23.39±0.88 vs 24.71±1.21 mg/g tissue).

The 27-hydroxycholesterol concentration in serum and liver mitochondria were also measured after DDC and phenobarbital treatment. As shown in Table 1, DDC stimulated 27-hydroxycholesterol production. The 27-hydroxycholesterol concentration was significantly increased in serum (0.206±0.009 vs 0.014±0.002 μg/mL) as well as in liver mitochondria (0.013±0.0006 vs 0.003±0.0005 μg/mg protein). Phenobarbital treatment did not change 27-hydroxycholesterol concentration in serum and liver compared to the untreated controls.

The results show that DDC treatment causes a massive accumulation of total porphyrins in liver homogenate (by 10-fold increase) and in liver mitochondria (approximately a 2 fold increase). Our data are in agreement with previous studies, where an accumulation of porphyrins in liver of rats, treated with DDC (100 mg/kg, i.p.) were reported (6, 19, 20).
Effect of DDC and phenobarbital on total cholesterol, triglycerides, phospholipids and 27-hydroxycholesterol content in serum and liver homogenate

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<tr>
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<th>Serum</th>
<th>Liver homogenate</th>
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<tr>
<td></td>
<td>Cholesterol (mg/dL)</td>
<td>TG (mg/dL)</td>
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<tr>
<td>Control</td>
<td>57.35±3.75</td>
<td>228.17±7.08</td>
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<tr>
<td>DDC</td>
<td>69.11±4.99*</td>
<td>219.65±8.5</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>79.69±7.49*</td>
<td>100.25±10.38**</td>
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Values are means ± SD of eight animals per group.

We found that total serum cholesterol content was significantly increased to 120% and to 139%, respectively by both DDC- and phenobarbital treatment compared to the untreated controls. In liver homogenate DDC treatment increased total cholesterol and total phospholipids to 110% and to 117%, respectively, while phenobarbital treatment did not caused significant changes.

These results confirmed an increase in cholesterol serum levels after DDC-treatment observed in different animal species. Taddei reported that DDC-treatment caused a considerable increase in liver size and a marked elevation of serum cholesterol and total lipids in rabbits (22). The finding of hypercholesterolaemia together with increased porphyrin formation in rabbits suggested the existence of a close relationship between porphyrin accumulation and lipid metabolism disturbances (2, 7, 10, 22). On the other hand, phenobarbital reduced significantly the serum TG (two fold reduction), while no statistical significant differences were observed in serum TG levels in porphyrinic animals. PL content was increased to 118% in livers of porphyrinic animals compared to the untreated controls.

The mechanisms underlying the observed effects are not clear. Some of them might be result of alterations of physiological processes caused by porphyrin accumulation and consequently by modulation of peripheral benzodiazepine receptor (PBR). The hypothesis of impact of PBR-modulation on disturbances in cholesterol and lipid metabolism is strongly supported by other authors. In fact, it was reported that PK11195, a selective PBR ligand increases total plasma cholesterol and triglycerides in rats in vitro (5, 12, 13). The authors found that PBR ligands antagonized the antiatherogenic effect of diazepam and zopiclone (ligands to central benzodiazepine receptors) (8, 13).

Porphyrrins are putative endogenous ligands for PBR (4). DDC-induced protoporphyrin is a suitable experimental model for investigating the effects of large endogenous modifications of PBR ligands, since it causes an accumulation of dicarboxilic porphyrins (7, 10). Convincing evidence that porphyrins interact in vivo with PBR is provided by Cantoni et al., 1993 (3). An in vivo increase in liver porphyrins following DDC-treatment in rats, significantly affect [3H]PK11195 binding to PBR. The basal concentration on protoporphyrin IX in normal rat liver is about 0.3 μM (3). It increases to 7 μM after DDC-induced porphyria. Since Ki value in vitro is 4.5 μM it is unlikely that under normal conditions endogenous porphyrins might interacts with PBR. However, in conditions where it accumulates, like in porphyrias, this concentration is fully compatible with the possibility that it acts in vivo as an endogenous PBR ligand.

In previous studies we found that porphyrins increase the oxidative metabolism of cholesterol to 27-hydroxycholesterol in vitro by mechanism coupled to PBR (23, 24). To further support the idea for PBR modulation after DDC-treatment we have compared the effect of both DDC-treatment (endogenous accumulation of porphyrins, PBR ligands) and phenobarbital (central benzodiazepine receptor agonist) on cholesterol metabolism to 27-hydroxycholesterol in serum and liver mitochondria in vivo. DDC caused marked increase in the amount of 27-hydroxycholesterol both in serum and in liver mitochondria, while phenobarbital did not show statistically significant effect on 27-hydroxycholesterol levels.

The availability of cholesterol to the enzyme 27-hydroxylase (EC1.14.13.15) is a limiting factor for its further metabolism to 27-hydroxycholesterol in vivo (14). The cholesterol transport from outer to the inner membrane in mitochondria is facilitated by PBR in different tissues (18, 23). Most probably the observed increase in 27-hydroxycholesterol production is coupled by modulation of PBR by porphyrins, while phenobarbital which is coupled to central and not to the peripheral benzodiazepine receptors has not effect.

Conclusions

We found that DDC-treatment caused a considerable elevation of serum cholesterol and total lipids in rat serum and liver in vivo. These findings, together with increased porphyrin formation, suggested the existence of a close relationship between porphyrin accumulation and lipid disturbances. We suppose that the observed effects are coupled to PBR.
modulation by an excessive accumulation of porphyrins during the treatment.

REFERENCES