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HEPATIC PROTOPORPHYRIA IS ASSOCIATED WITH A DECREASE IN LIGAND BINDING FOR THE MITOCHONDRIAL BENZODIAZEPINE RECEPTORS IN THE LIVER

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Abstract—Protoporphyrin IX (PP) and *N*-methylprotoporphyrin IX (*N*-MePP) added *in vitro* to liver membranes reduced dose-dependently the affinity of [^{3}H]PK 11195 for the mitochondrial benzodiazepine receptors (MBRs), the latter being about 20 times more potent (K_i 4.5 and 0.25 μM). Preincubation of these two porphyrins with liver homogenates for 120 min at 4° resulted in significant inhibition of [^{3}H]-PK 11195 binding even after repeated washings of the membranes due to the residual presence in the membranes of about 35 and 5% of PP and *N*-MePP, respectively. Thus, the hypothesis that an *in vivo* increase in the hepatic porphyrin content modifies the binding of the isoquinoline PK 11195 to the MBRs was investigated in an experimental model of protoporphyria. PP and *N*-MePP were allowed to accumulate *in vivo* through treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (100 mg/kg i.p., once), and rats were killed 5 h after treatment when hepatic porphyrin accumulation was marked (10-fold increase), PP predominating. In the liver, treatment reduced the affinity (K_d) of [^{3}H]PK 11195 for MBRs (from 3.56 to 15.37 nM, $P < 0.01$) and the maximum number of binding sites (B_{max}) (55% decrease, $P < 0.05$); the affinity (K_i) of RO 5-4864 for [^{3}H]PK 11195 binding sites was also reduced (from 23.9 to 72.99 nM, $P < 0.05$). No significant differences were found in the brain cortex. Liver and brain diazepam binding inhibitor levels and plasma corticosterone levels were unchanged. The reduction in [^{3}H]PK 11195 binding to MBRs in the liver of DDC-treated rats thus appears to be attributable to a specific effect of the DDC-induced formation of the two protoporphyrins; this conclusion suggests that in hepatic protoporphyria processes modulated by MBRs may be altered.

The importance of porphyrins as physiological compounds has been based up to now mainly on the fact that they are the precursors of heme. In the liver, which is their principal site of synthesis, levels of porphyrins are kept in the physiological range by the coordinated functioning of the pathways of heme biosynthesis and degradation. However, these pathways can be altered by several chemicals which cause accumulation of the various intermediate products, giving rise to the biochemical picture of porphyria(s). In humans this is also observed in individuals with a genetic predisposition to these syndromes [1, 2].

Recently, evidence has been obtained from *in vitro* studies that porphyrins, especially the physiological dicarboxylic porphyrins like protoporphyrin IX (PP§), are putative endogenous ligands for a binding site on the outer mitochondrial membrane, known as the peripheral-type or mitochondrial benzodiazepine receptor (MBR) [3]. The MBRs are found in virtually all mammalian tissues and are distinct from the central benzodiazepine receptors located on neurons. They have different roles in distinct cellular functions (for a review see Ref. 4), and bind drugs like the benzodiazepine RO 5-4864 and the isoquinoline PK 11195 as well as endogenous compounds like diazepam binding inhibitor (DBI) and related peptides [5].

If the porphyrins are endogenous ligands for MBRs, an adaptive response of MBRs located on liver mitochondria might be induced *in vivo* in porphyrias by accumulating porphyrins.

The main purpose of this study was to demonstrate *in vivo* that the increase in the porphyrin content of the liver induced by treatment with a porphyrogenic compound was correlated to a significant modification of the binding of the isoquinoline PK 11195 to the MBRs.

We utilized 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) which, in experimental animals, causes accumulation of PP [2]. The mechanism through which DDC and similarly acting drugs like griseofulvin cause liver PP accumulation is complex since they have to convert the heme of cytochrome P450 into *N*-methylprotoporphyrin (*N*-MePP), a powerful inhibitor of the mitochondrial enzyme ferrochelatase (protoheme ferrolyase, EC 4.99.1.1); the inhibition of this enzyme blocks the metabolism of PP and, at the same time, causes a secondary, compensatory stimulation of δ-aminolaevulinate

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§ Abbreviations: PP, protoporphyrin IX; *N*-MePP, *N*-methylprotoporphyrin IX; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; MBR, mitochondrial benzodiazepine receptor; DBI, diazepam binding inhibitor; PBS, phosphate-buffered saline.

synthase (EC 2.3.1.37). The combination of these two effects results in marked accumulation of PP (see Refs 6 and 7 for two reviews). Both PP and the modified porphyrin, *N*-MePP, were reported to bind to MBRs *in vitro* [8].

Treatment of rats *in vivo* with a porphyrogenic dose of DDC reduced the affinity for MBRs and the number of these binding sites in the liver.

MATERIALS AND METHODS

Materials. PP was obtained from Porphyrin Products (Logan, UT, U.S.A.). A sample of *N*-MePP dimethylester mixed isomers was a kind gift from Dr F. De Matteis (MRC Toxicology Unit, Carshalton, U.K.) [9]. DDC was obtained from Eastman Kodak (Rochester, NY, U.S.A.) and purified before use through extensive recrystallization from ethanol–water solution. RO 5-4864 was purchased from Fluka (Buchs, Switzerland); PK 11195 was from Pharmuka (Gennervilliers, France). DBI and DBI antibodies were a kind gift from Dr A. Guidotti (Fidia-Georgetown Foundation for the Neurosciences, Washington DC, U.S.A.). All other materials were obtained from standard sources.

Porphyrin solutions. PP solution was prepared as described by Hronis and Traugh [10]. The solution was then filtered with a sterile 0.2 μm filter (Sartorius, SM 17597). A portion of this filtered solution was used to calculate the exact concentration [11].

After hydrolysis, *N*-MePP was dissolved in dimethyl sulfoxide and diluted with this solvent until appropriate. The exact concentration was calculated as described by De Matteis *et al.* [12]. Porphyrin solutions were always used within 4 hr of preparation.

Animal treatment. Male Crl:CD(SD)BR rats, weighing 180–200 g and fasted for 24 hr, were given an intraperitoneal injection of DDC (100 mg/kg) dissolved in peanut oil (10 mL/kg) and were killed 5 hr later by decapitation. Control animals were given oil alone. Liver and cortex were removed, rinsed in ice-cold saline, blotted dry and immediately frozen on dry ice. Tissues were kept at −80° until utilized.

[^{3}H]PK 11195 binding assay. The frozen tissues (liver, cerebral cortex) were homogenized in 50 vol. of ice-cold phosphate-buffered saline (PBS phosphate buffer, 50 mM, pH 7.4, with 120 mM NaCl) using an Ultra Turrax TP-1810 (2 × 20 sec) and centrifuged at 50,000 *g* for 10 min. The pellet was then washed another four times by resuspension in fresh buffer and centrifugation as before. The last pellet was resuspended just before the binding assay in 50 vol. of PBS for the cerebral cortex and liver.

[^{3}H]PK 11195 binding was assayed in a final incubation volume of 1 mL consisting of membrane suspension (0.5 mL, 100 vol. final dilution), [^{3}H]PK 11195 (0.5 mL NEN, sp. act. 83 Ci/mmol, final concentrations 0.5–12 nM) and displacing agents or the drug solvent (0.02 or 0.01 mL). The assay mixtures were incubated at 4° for 120 min.

Incubation was stopped by rapid filtration *in vacuo* through GF/B fiber filters (Brandell MR 48) which were then washed with 12 mL of cold PBS and counted in 8 mL of Filter Count (Packard) in an LKB liquid scintillation spectrometer Mod. Rackbeta 1214, with a counting efficiency of 55%.

The IC_{50} values were calculated using the Allfit program; kinetic parameters (K_d, B_{max}, K_i) with the Ligand program [13, 14]. All programs were run on an AT IBM PC.

Liver and cortex porphyrin content. Total liver porphyrin content was determined fluorometrically with mesoporphyrin as internal standard in a 10% liver homogenate prepared in 0.25 M sucrose as described by Abbritti and De Matteis [15]. The same method was used to measure porphyrin content of membrane preparations used for binding experiments and of the cortex except that volumes were modified to concentrate the samples.

Liver PP content was determined by HPLC separation [16] of a porphyrin extract in methanol obtained as described by Kennedy *et al.* [17].

All these procedures were carried out in dim light.

DBI content. Peptide extraction, DBI radioimmunoassays and reverse phase HPLC were performed according to the methods described by Ferrarese *et al.* [18].

Plasma corticosterone. Corticosterone was measured in plasma according to Guillemin *et al.* [19].

RESULTS

Liver and cortex porphyrin content

DDC treatment increased the total porphyrin

Table 1. Effect of DDC treatment on liver and brain porphyrin, DBI content and plasma corticosterone levels

	Cortex		Liver	
	Vehicle	Treated	Vehicle	Treated
Total porphyrins (10) (nmol/g tissue)	0.064 ± 0.01	0.059 ± 0.01	0.80 ± 0.23	10.20 ± 3.10*
PP (10) (nmol/g liver)	—	—	0.32 ± 0.09	7.20 ± 2.56*
DBI (4) (pmol/mg protein)	25.200 ± 2.20	26.700 ± 3.10	84.50 ± 8.50	73.70 ± 5.10
Plasma corticosterone (10) (μg/100 mL)	Vehicle: 35.5 ± 15.2		Treated: 45.2 ± 12.1	

* $P < 0.01$ by Student's *t*-test.
Values are means ± SD.
Number of rats is given in brackets.
DDC (100 mg/kg) was dissolved in peanut oil and injected i.p.
Animals were fasted for 24 hr before treatment and until killed (5 hr after treatment).

Table 2. Effect of DDC treatment on [^{3}H]PK 11195 binding

	Cortex		Liver	
	Vehicle	Treated	Vehicle	Treated
Bound (pmol/g tissue)	4.53 ± 1.2	3.53 ± 1.0	36.80 ± 14.7	10.40 ± 10.7*
B_{max} (pmol/g tissue)	52.50 ± 11.4	50.10 ± 14.1	160.30 ± 81.0	72.10 ± 21.0*
K_d (nM)	9.10 ± 1.5	12.90 ± 6.1	3.50 ± 0.7	15.37 ± 2.4*
K_i of RO 5-4864 (nM)	35.10 ± 16.0	43.30 ± 25.0	23.90 ± 5.4	72.99 ± 37.6*

* $P < 0.05$ by Student's *t*-test.
Values are means ± SD of six animals per group.
DDC (100 mg/kg) was dissolved in peanut oil and injected i.p.
Animals were fasted for 24 hr before treatment and until killed (5 hr after treatment).

content of the liver more than 10-fold (Table 1). Analysis of the hepatic porphyrin pattern through HPLC separation (data not reported) showed that in control and treated rats the prevalent porphyrin was PP, followed by coproporphyrin and uroporphyrin; traces of heptacarboxylic and hexacarboxylic porphyrin were detectable. After treatment, the absolute amount of PP increased about 20 times (Table 1). This explains the rise in the total hepatic porphyrin content.

In the membrane preparations used for *ex vivo* [^{3}H]PK 11195 binding, the total porphyrin content was about 30% of that found in whole liver homogenate (data not reported).

DDC did not affect brain cortex porphyrin content (Table 1).

[^{3}H]*PK* 11195 *binding*

Ex vivo. Table 2 summarizes [^{3}H]PK 11195 binding in the brain cortex and liver from rats treated with vehicle or DDC. In the brain cortex no significant effects were seen on [^{3}H]PK 11195 binding or on the inhibition of this binding by the agonist RO 5-4864. In the liver the amount of [^{3}H]PK 11195 bound at 1 nM was significantly reduced after DDC treatment. This decrease was mainly due to a reduced affinity (K_d) of the ligand (from 3.56 to 15.37 nM, $P < 0.01$) although there was also a reduction in the maximum number of binding sites (B_{max}) (55% decrease, $P < 0.05$). The affinity (K_i) of RO 5-4864 for [^{3}H]PK 11195 binding sites was significantly reduced in livers from animals treated with DDC (from 23.9 to 72.99 nM, $P < 0.05$).

The reduction of [^{3}H]PK 11195 binding induced by DDC was not modified by addition *in vitro* of ATP 1 mM, which did not affect [^{3}H]PK 11195 binding *per se* in control or DDC-treated rats (data not shown).

In vitro. Figure 1 shows the effect of PP and *N*-MePP on [^{3}H]PK 11195 binding in the livers of control rats. The two porphyrins reduced dose-dependently the affinity of [^{3}H]PK 11195 binding, *N*-MePP being about 20 times as potent as PP (K_i 0.25 μM for *N*-MePP and 4.5 μM for PP). In the presence of concentrations higher than 5 μM of PP, the B_{max} tended to be lower, suggesting a complex inhibition (mixed type).

Figure 2 shows the effect of membrane washings on

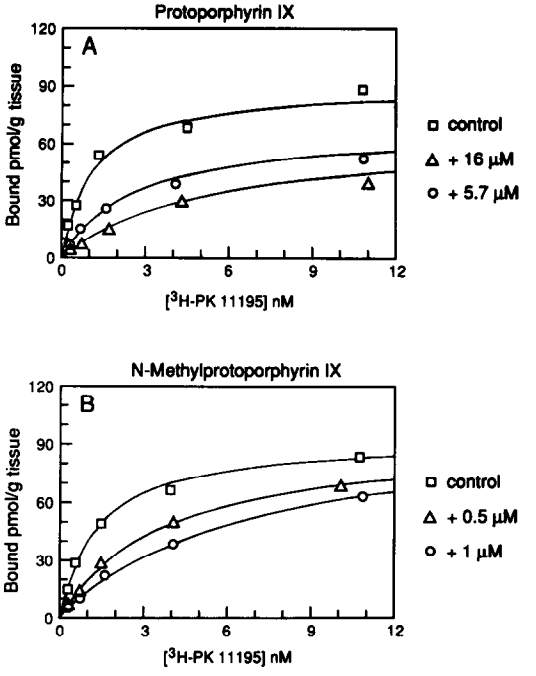

Fig. 1. *In vitro* effect of PP and *N*-MePP on [^{3}H]PK 11195 binding in rat liver. Liver membrane preparations from control rats were incubated with different concentrations of [^{3}H]PK 11195 (0.5–12 nM) in the absence or presence of two concentrations of PP (panel A) or *N*-MePP (panel B). Representative saturation curves as shown. The kinetic parameters K_d (nM) and B_{max} (pmol/g tissue) of [^{3}H]PK 11195, calculated using the Ligand program, were: Panel A: control: 1.09 and 90.3; PP 5.7 μM: 2.46 and 67.4; PP 16 μM: 5.07 and 64.6. Panel B: control: 1.32 and 94; *N*-MePP 0.5 μM: 3.86 and 95.6; *N*-MePP 1 μM: 6.84 and 104.1. The K_d values in the presence of inhibitors were significantly different from control values ($P < 0.01$), assessed using an F test, according to the "extra-sum of squares" principle [13].

[^{3}H]PK 11195 inhibition by the two protoporphyrins. Liver homogenates from control rats were incubated for 120 min at 4° in the absence or presence of two concentrations of *N*-MePP (1 and 10 μM) or PP (5.7

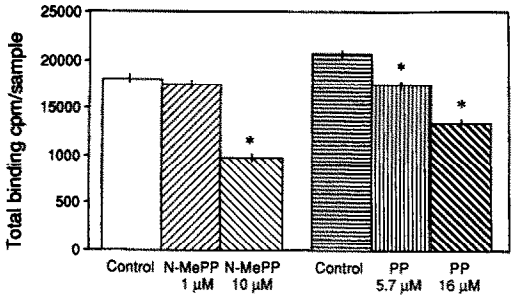

Fig. 2. Effect of membrane washing on [^{3}H]PK 11195 binding inhibition by PP and *N*-MePP. Columns represent mean values ± SD of total [^{3}H]PK 11195 binding (1 nM) in membrane preparation from rat liver. PP or *N*-MePP was added during 120 min preincubation at 4°, and membranes were then washed four times and tested for [^{3}H]PK 11195 binding, as described in Materials and Methods. * Significantly different from controls, $P < 0.05$, Student's *t*-test.

and 16 μM) in order to reach equilibrium between drug added and receptors. After incubation the membranes were centrifuged and washed four times by resuspension as done for *ex vivo* experiments, then used for the [^{3}H]PK 11195 binding assays.

Inhibition of 1 nM [^{3}H]PK 11195 binding was still significant even after the washing procedure (Fig. 2). The inhibition was about 50% when 10 μM *N*-MePP were added and 15 and 34% after addition of 5.7 and 16 μM PP, respectively. Comparison with percentages of inhibition obtained by the addition of drugs together with [^{3}H]PK 11195 in competition experiments (Fig. 1) shows that the amount of *N*-MePP remaining in the membranes was 5% of that added before the washing procedure, while for PP this percentage increased to 35%.

DBI content

Tissue concentrations of DBI were not significantly modified by DDC treatment in the brain cortex or liver (Table 1).

Plasma corticosterone

Plasma corticosterone concentrations were not significantly modified by DDC treatment (Table 1).

DISCUSSION

The major finding of this study is that binding of [^{3}H]PK 11195 to hepatic MBRs was reduced after acute treatment with DDC, a hepatic porphyrogenic compound [2]. The results are compatible with the theory that in this model of hepatic porphyria processes mediated by MBRs may be altered.

DDC causes the formation of *N*-MePP and the accumulation of mainly PP in the liver [2, 6, 7]. These dicarboxylic porphyrins were suggested to be endogenous ligands for MBRs, while porphyrins with a higher number of carboxylic groups do not appear to bind these receptors with high affinity [3, 8, 20]. We confirmed that both *N*-MePP and PP bind with high affinity to MBRs and *N*-MePP showed a 20 times higher affinity than PP for MBRs.

Our findings suggest that the reduction in [^{3}H]PK 11195 binding to MBRs in the livers of treated rats is indeed attributable to specific DDC-induced formation of the two protoporphyrins. First of all, at the time after treatment at which animals were killed, porphyrin accumulation appeared to be maximal [21]. Among the porphyrins formed, PP was predominant, and this is indirect evidence of the formation of *N*-MePP [6, 7], the amount of which might be estimated approximately as being at least 10 times less than that of PP [6].

Unlike the significant increase in porphyrins, we did not observe any changes in liver DBI and plasma corticosterone concentrations, two other possible modulators of MBRs [5, 22], that might otherwise explain the changes in [^{3}H]PK 11195 binding.

Porphyrins accumulated in the cell membranes after treatment with DDC were not easily removed during membrane preparation, as confirmed by our results showing that the porphyrin content of the membranes used for [^{3}H]PK 11195 binding amounted to about 30% of that of whole liver homogenate. Verma and Snyder [8] have shown that the inhibition of [^{3}H]PK 11195 binding by PP *in vitro* is slowly reversed. Our experiments in which porphyrins were added to liver homogenate before the washing procedure used for membrane preparation indicate that 5 and 35% of the original amount of *N*-MePP and PP, respectively, remained in the membranes at the time of binding assay. These data may suggest that the reduction of MBR binding in the liver of rats treated with DDC reflects competition of endogenous porphyrins remaining in the tissue for the [^{3}H]PK 11195 binding sites. This is further supported by the observation that the affinity of the agonist, RO 5-4864, for liver MBRs was reduced in DDC-treated rats.

However, even if one assumes that all porphyrins remain in the membranes used for binding assays, the concentration of the porphyrins present in the incubation mixture from DDC-treated rats would be around 0.1 μM, i.e. too low to explain the observed modifications in [^{3}H]PK 11195 affinity. Moreover, after treatment *in vivo* with DDC we also found a reduction in the maximum number of [^{3}H]PK 11195 binding sites. This could hardly be related to porphyrins still remaining, since a tendency to cause complex inhibition of [^{3}H]PK 11195 binding *in vitro* is obtained only when PP is added at a concentration higher than 5 μM, i.e. considerably higher than that of membranes used for [^{3}H]PK 11195 binding. It seems, therefore, more likely that the observed modification in MBR binding is due to changes in receptor conformation during *in vivo* exposure to porphyrins.

Additional evidence for a direct role of porphyrins in the modification of hepatic binding of [^{3}H]PK 11195 to MBR was the finding that [^{3}H]PK 11195 binding to the brain cortex and porphyrin levels were not modified by DDC treatment. The absence of porphyrogenic effects in the brain of DDC is probably related to the fact that DDC needs to be metabolized by cytochrome P450 to cause *N*-MePP formation and PP accumulation; however, the level

of cytochrome(s) P450 in the brain is about 1% of the level in liver microsomes [23] and their association with drug metabolism is uncertain [24].

Hepatic protoporphyria is associated with hepatomegaly, cholestasis, alterations in the microsomal cytochromes and tumor formation [25, 26]. While the concentration of PP (0.3 μM) in the livers of control animals is about one tenth its K_i *in vitro* (4.5 μM), suggesting that in this organ PP is unlikely to act as a modulator of metabolic processes mediated by MBRs under physiological conditions, the affinity of PP for MBRs is in the range of the liver PP concentration (7 μM) attained after treatment with DDC. This suggests that alterations in mitochondrial hepatic or extrahepatic processes modulated by MBRs as a result of PP accumulation might be expected in this model of experimental porphyria and might contribute to the reported pathological DDC effects.

In the liver, the main function of MBRs is not known. It is possible that in this tissue also MBR may be involved in translocation processes across the mitochondrial membranes of either cholesterol, as was shown in steroidogenic cells [27], or intermediates of heme biosynthesis. Cholesterol and porphyrins already show some similarities in mechanisms of transport inside the cell since they both can use proteins of the Z class as carriers [28]. It was also reported that the receptor binding affinities of mesoporphyrin IX and deuteroporphyrin IX correlated with their potencies for inhibiting respiratory control [29] and the effects of these compounds on mitochondrial respiration are similar to those described for PP [30].

Impairment of mitochondrial energy generation has been suggested to explain some of the alterations in human porphyrias [31], and an inverse correlation between ATP and porphyrin concentration was found under some experimental conditions [32]. However, we found that the addition of ATP did not modify [^{3}H]PK 11195 binding in control animals and did not reverse the reduction of binding in DDC-treated rats.

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