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EFFECTS OF DIPROPYLACETATE ON THE GLYCINE CLEAVAGE ENZYME SYSTEM AND GLYCINE LEVELS

A POSSIBLE EXPERIMENTAL APPROACH TO NON-KETOTIC HYPERGLYCINEMIA

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Abstract—The effect of chronic administration of the anticonvulsivant drug di-*n*-propylacetate (DPA) on the glycine cleavage enzyme system was studied. Glycine concentrations were monitored in blood, liver, brain and spinal cord of 10-day-old rats. DPA treatment decreases glycine cleavage activity by approximately 50% in liver, and 35% in brain. The decreased cleavage activity correlates with an increase of glycine levels in blood, liver and brain.

Failure to cleave glycine characterizes a metabolic disorder known as non-ketotic hyperglycinemia, which is associated with elevated concentrations of glycine in biological fluids. The inhibitory effect of DPA may provide an experimental approach to study the biochemical and pathogenic mechanisms of non-ketotic hyperglycemia.

Non-ketotic hyperglycinemia (NKH)† is an inborn error of metabolism resulting from a defect in the glycine cleavage enzyme system (G.C.S.) [1]. This enzyme system is a major pathway for the catabolism of glycine in vertebrates [2]. NKH is characterized biochemically by elevated concentrations of glycine in blood, urine and cerebrospinal fluid [3]. The clinical symptoms usually appear within the first few days after birth and progress rapidly thereafter [4]. The accumulation of glycine in the central nervous system [5] can impair the normal development of glycine receptors [6–8]. Alternatively, the decreased activity of the glycine cleavage system may produce a decrease of one-carbon units [2, 7], which may be required for biosynthetic processes in the brain [9]. However, the major consequences of reduced G.C.S. activity in the neurochemical and metabolic processes of the central nervous system still remain unclear.

Several experimental models have been used to study the pathogenesis of N.K.H. Hyperglycinemia was induced in rats by repeated injections of glycine and serine [7, 9]. High glycine levels in cerebrospinal fluid and tissues were found, but the enzymatic defect found in NKH was not reproduced.

Recently, it has been reported that elevated amounts of glycine were found in the blood and urine of humans and rats receiving the antiepileptic drug di-*n*-propylacetate (sodium valproate, DPA) [10, 11]. The DPA-induced hyperglycinemia was due to an inhibition of the liver G.C.S. We have studied the effect of chronic administration of DPA on the concentration and the cleavage of glycine by G.C.S. in livers and brains of rats treated with DPA during the first 10 days after birth. The results presented in this paper suggest that administration of DPA may be useful in providing an experimental approach for further biochemical studies on non-ketotic hyperglycinemia.

MATERIALS AND METHODS

Chemicals

(1-^{14}C)-Glycine and (U-^{3}H)-strychnine were purchased from Amersham. (G-^{3}H)-di-*n*-propylacetate was supplied by the Centre de Neurochemie, Strasbourg, France. Di-*n*-propylacetate was a gift from Laboratorios Labaz, Barcelona, Spain. All other chemicals were of reagent grade and were used without further purification.

Treatment of the animals

Suckling Wistar rats, from the same litter, were injected intraperitoneally with 150 mM DPA, pH 7 (5 μmoles/g body wt). Adult Wistar rats weighing 200–300 g, were injected intraperitoneally with DPA in the same proportion. Control rats were treated with equal volumes of 0.9% (w/v) NaCl. Suckling rats were maintained in an incubator (Tectron, Selecta, Spain) at 37° and 70% relative humidity for the duration of the experiment. After 0–3 hr, the animals were sacrificed by decapitation.

In chronic administration experiments, several doses of DPA were tested in order to keep the maximal number of hyperglycinemic rats alive during the first 10 days after birth. Rats were finally injected intraperitoneally twice daily, with increasing doses of 150 mM DPA, pH 7, according to age as indicated below.

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† Abbreviations used: DPA, di-*n*-propylacetate; G.C.S., glycine cleavage enzyme system; NKH, non-ketotic hyperglycinemia.

Day	1	2	3	4	5	6	7	8	9	10
DPA dose (μmoles/g/injection)	0.5	1.0	1.5	1.5	1.5	2.0	2.5	2.5	3.0	3.0

Control rats were injected with the same volume of 0.9% (w/v) NaCl. Rats were decapitated at 6 hr following the last injection. One half of the brain or liver from each rat was used to determine glycine, and the other half was used to measure glycine cleavage activity.

To study the distribution of DPA in plasma and brain, rats were injected intraperitoneally with 1 μmole/g body wt of 150 mM (G-^{3}H)-DPA, pH 7, with a specific activity of 0.33 Ci/mole. Following the injection, all the rats were fasted and the suckling rats maintained in an incubator at 37° and 70% relative humidity. After 15–90 min, the animals were sacrificed by decapitation.

Analytical methods

Glycine concentrations: rats were killed by decapitation, exsanguinated and the brain, liver and spinal cord rapidly removed. Tissues were freeze-clamped in liquid nitrogen and homogenised 1/10 (w/v) in 6% (w/v) perchloric acid, the homogenates spun down, and glycine measured spectrofluorimetrically, as described by Sardesai and Provido [12]. All determinations were determined by gas-chromatography, as described by Perry *et al.* [13]. The brains of animals injected with (G-^{3}H)-DPA were trimmed to remove blood capillaries, homogenised 1/4 (w/v) in ice-cold distilled water, and centrifuged at 12,000 *g*, 10 min. Aliquots of 100 μl of the supernatant (or plasma) were added to 10 ml of scintillation liquid and the radioactivity measured. All determinations were conducted in duplicate and the radioactivity (c.p.m.) corrected by external standard.

Glycine cleavage activity: Brain and liver were homogenised 1/20 (w/v) in 0.32 M sucrose, 10 mM Tris–HCl, pH 7.4. Brain mitochondria were prepared by the method of Clark and Nicklas [14]; liver mitochondria were prepared according to Schneider [15] and then resuspended in 50 mM Tris–HCl, 1 mM dithiothreitol, pH 8, at a concentration of 8 mg protein/ml of liver mitochondrial suspension or 20 mg/ml of brain mitochondrial suspension. Protein was determined by the fluorimetric method of Resch *et al.* [16], calibration being made by the method of Lowry *et al.* [17] with bovine serum albumin as the standard.

Activity of the glycine cleavage enzyme system was determined by measuring the formation of $^{14}CO_2$ from (1-^{14}C)-glycine by a modification of the method of Bruin *et al.* [18]. The reaction mixture contained in a final volume of 0.1 ml: 4.8 μmoles Tris–HCl buffer, pH 8; 0.19 μmoles nicotinamide-adenine-dinucleotide; 0.19 μmoles dithiothreitol; 0.19 μmoles pyridoxal-phosphate; 0.029 μmoles tetrahydrofolate; 1 μmole (1-^{14}C)-glycine with a specific activity of 1 Ci/mole and about 0.3 or 0.5 mg of protein homogenate (0.16 or 0.36 mg of mitochondrial protein) from liver or brain respectively. The reaction was carried out in Microtest tissue culture plates at 37°. The enzymatic extract was preincubated for 10 min with pyridoxal-phosphate, dithiothreitol and nicotinamide-adenine-dinucleotide. The reaction was initiated by addition of glycine and tetrahydrofolate, followed by mixing with a slight flow of nitrogen. The reaction was stopped 30 min later by addition of 20 μl of 20% (w/v) perchloric acid. $^{14}CO_2$ was collected in Millipore AP2501000 filters, impregnated with 20% (w/v) KOH, which were over the reaction well, and measured by scintillation counting. All the experiments were corrected for a blank obtained without the enzyme suspension. G.C.S. activity of each preparation was measured in quadruplicate.

^{3}H-Strychnine binding: Synaptic membrane fractions from spinal cord were obtained according to the method of Young and Snyder [19]. 0.3 mg of membrane protein were incubated with 20 nM ^{3}H-strychnine at 0° for 10 min, and bound from free ligand was separated by centrifugating the samples as described by Benavides *et al.* [8]. All binding measurements were conducted in quadruplicate, and for each measurement parallel-triplicate samples were assayed in the presence of 1 mM glycine to correct for non-specific binding.

RESULTS

Effect of DPA on the glycine cleavage system in liver and brain mitochondria

To determine if DPA affected glycine catabolism in rats, mitochondria were isolated from liver and brain, and the glycine cleavage activity measured in the presence of DPA. The results of this experiment indicate that dipropylacetate inhibits the glycine cleavage system in rat liver and brain mitochondria. The kinetic characteristics of this inhibition were studied and are presented in Fig. 1. Under our experimental conditions, the G.C.S. has a V_{max} of 5.4 nmoles/min × mg protein and a K_m of 8.2 mM in liver mitochondria (a); in brain mitochondria (b), the V_{max} was of 0.23 nmoles/min × mg protein and the K_m was of 2.2 mM. DPA inhibits G.C.S. in a competitive manner (V_{max} remains unchanged) and with apparent K_ms of 36.2 mM (liver mitochondria) and 4.73 mM (brain mitochondria) at 2 mM DPA. From these data, the K_i calculated for DPA is 0.59 mM and 2 mM in liver and brain mitochondria respectively. This inhibitory effect has been previously reported by Kochi *et al.* [20] in liver mitochondria.

Effect of a single administration of DPA on the glycine levels in blood, liver, brain and spinal cord from adult and suckling rats

In order to establish if the inhibition of G.C.S.

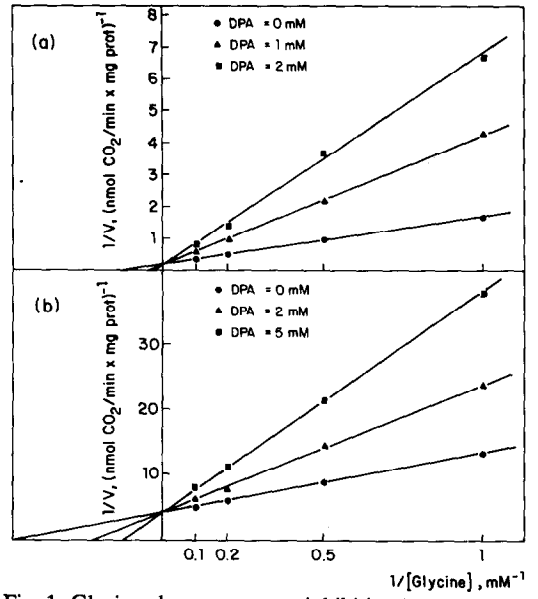

Fig. 1. Glycine cleavage system inhibition by DPA in liver (a) and brain (b) mitochondria. Lineweaver–Burk plots of the DPA effect. Glycine cleavage activity was assayed as described in Methods at several concentrations of 1-^{14}C-glycine and DPA. Each point represents the mean of five experiments. Lines were calculated by the least squares method. Correlation coefficients were higher than 0.96 in all cases.

activity observed in mitochondria (as shown above) correlated with an increase in the level of glycine, DPA was injected intraperitoneally into rats at a dose of 5 μmoles/g body wt.

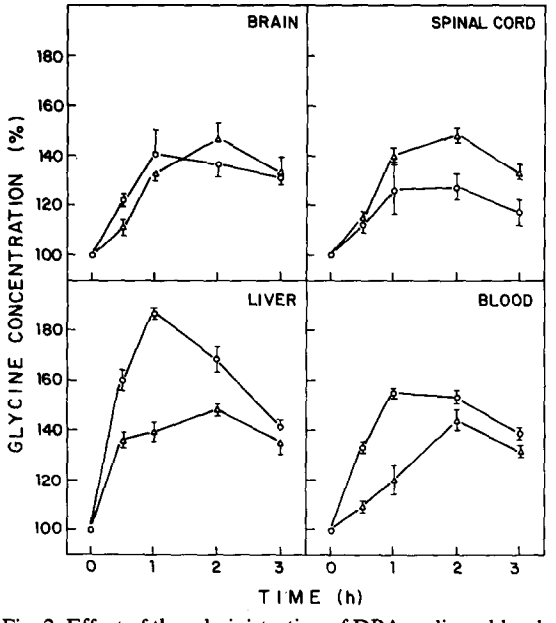

Fig. 2. Effect of the administration of DPA on liver, blood, brain and spinal cord glycine concentrations in adult (—○—) and 7-day-old (—△—) rats. Rats were injected intraperitoneally with NaCl (control) or DPA. After 0–3 hr, rats were killed and tissue used to determine glycine concentrations. 100% represents the mean glycine concentration in control rats. Each result is the mean ± S.E. of three different extracts measured in duplicate.

Administration of DPA to adult rats resulted in a rise of blood, liver, brain and spinal cord glycine levels (Fig. 2). The highest glycine levels were reached at 1–2 hr after injection, decreasing thereafter to the concentrations observed in control rats. The control values were reached 5 hr after DPA treatment (data not shown).

Since clinical symptoms of non-ketotic hyperglycinemia appear during the neonatal stage of the development, we tested the effect of DPA (5 μmoles/g body wt) on glycine concentrations in 7-day-old rats. Increased glycine levels were found in suckling rats given DPA (Fig. 2). The observed increase was lower in liver, of the same order in brain, and slightly higher in spinal cord than that seen in adult rats. Furthermore, peak glycine concentration in the liver and brain of suckling rats occurs approx. 1 hr later than in adult animals.

In order to test if the differences mentioned above were due to changes in the metabolism of DPA, adult and 7-day old rats were injected with ^{3}H-DPA (Fig. 3). High levels of radioactivity were detected in plasma early after administration of ^{3}H-DPA, those levels being greater in adult than in suckling

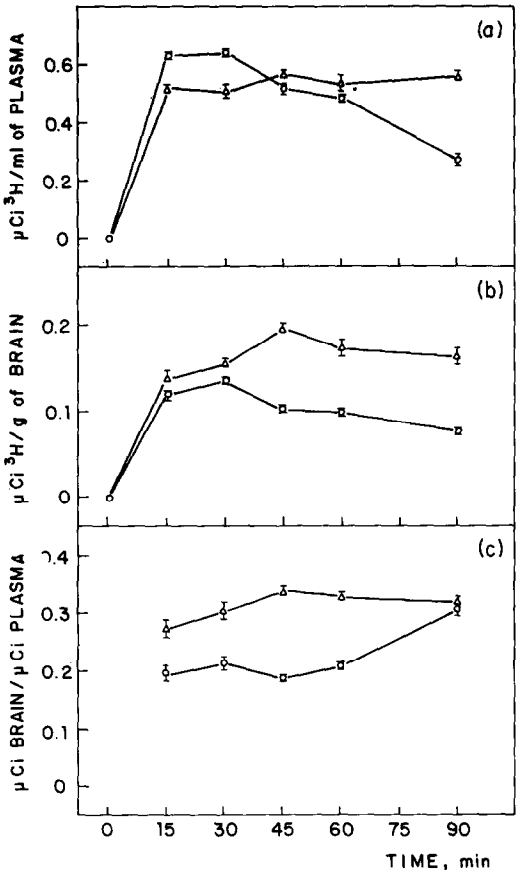

Fig. 3. Distribution of radioactivity from (G-^{3}H)-DPA in plasma (a) and brain (b), and μCi brain/μCi plasma ratio (c) in adult (—○—) and 7-day-old (—△—) rats. Rats were injected intraperitoneally with (G-^{3}H)-DPA. After 15–90 min, rats were sacrificed and radioactivity was measured in brain and plasma, as described in Methods. Each point represents the mean ± S.E. of four different plasmas (a) or brain extracts (b) measured in duplicate.

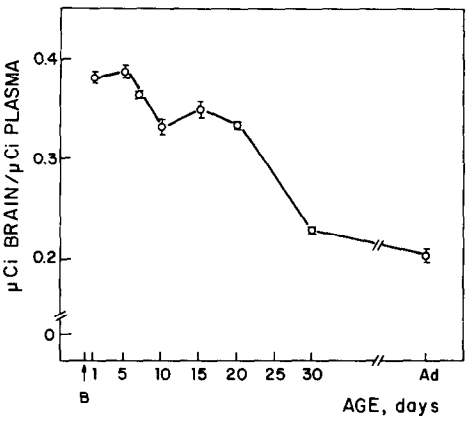

Fig. 4. Changes in the μCi brain/μCi plasma ratio during the postnatal development of rats treated with (G-^{3}H)-DPA. Rats were injected intraperitoneally with (G-^{3}H)-DPA. After 45–90 min, the animals were sacrificed and radioactivity was measured in brain and plasma, as described in Methods. Results are means ± S.E. (N = 3–5).

rats. These levels decreased sharply in adult rats, but remained high in 7-day-old rats. A similar time-course was observed in brain. As calculated from these data, the μCi ^{3}H in brain/μCi ^{3}H in plasma ratio is greater in 7-day-old rats for nearly all the observation period. This ratio was also measured 45 min after administration of ^{3}H-DPA, during postnatal development of the rats. The data, presented in Fig. 4, show a significant decrease in the ratio beginning at 20 days after birth.

These results indicate a decrease in the permeability of DPA across the blood–brain barrier and/or a faster removal of DPA in adult rats compared to 7-day-old rats. These facts could explain the differences found in the increase of glycine concentrations between adult and 7-day-old rats after administration of DPA.

Effect of chronic administration of DPA on the glycine cleavage system and glycine concentrations

Non-ketotic hyperglycinemia is characterized by the presence of elevated amounts of glycine in the biological fluids, that is due to a defect in the glycine cleavage enzyme system. The results presented above indicate that DPA inhibits the activity of the G.C.S. The decrease in G.C.S. activity is correlated with an increase in glycine levels. Our results suggest therefore that DPA may be useful to provide an experimental approach for bicohemical studies on NKH.

For this purpose, rats were chronically treated with DPA during the first 10 days after birth. Table 1 shows the effect of chronic administration of DPA on glycine cleavage activity, glycine concentrations and specific ^{3}H-strychnine binding to spinal cord synaptic membranes from 10-day-old rats, treated with DPA from birth. DPA reduces glycine cleavage activity in liver and brain and appears to inhibit liver activity more than brain activity. The increased glycine concentrations seen in brain are of the same

Table 1. Glycine cleavage activity, glycine and propionate concentrations, and specific (U-^{3}H)-strychnine binding to spinal cord synaptic membranes in 10-day-old rats chronically treated with DPA from birth

		Control	DPA-treated	P <
Body wt (g)		18.50 ± 0.58	10.85 ± 0.69	0.0005
Glycine cleavage activity (%)	Liver	[a]100 ± 2	54 ± 2	0.0005
	Brain	[b]100 ± 6	68 ± 5	0.0005
Glycine concentrations	Blood (mM)	0.520 ± 0.015	0.916 ± 0.055	0.0005
	Liver (μmoles/g wet tissue)	1.710 ± 0.115	3.483 ± 0.217	0.0005
	Brain (μmoles/g wet tissue)	1.172 ± 0.082	1.583 ± 0.075	0.0025
	Spinal cord (μmoles/g wet tissue)	3.235 ± 0.125	3.703 ± 0.186	0.025
Specific-^{3}H-strychnine binding (pmoles ^{3}H-strych bound/mg prot)	*Spinal cord* Synaptic membranes	1.859 ± 0.056	1.796 ± 0.118	NS
Propionate (μM)	Plasma	10.73 ± 0.52	< 4	NS
Dipropylacetate	Plasma (mM)	—	0.496 ± 0.008	
	Brain (μmoles/g tissue)	—	0.165 ± 0.005	

[a] 100% = 402.78 ± 7.66 pmoles/min × mg prot.
[b] 100% = 63.59 ± 3.71 pmoles/min × mg prot.
Rats were injected intraperitoneally twice daily with NaCl or DPA. The dose of DPA is described in Methods. In binding experiments, the ligand concentration used was 20 nM. Results are means ± S.E. of 15 rats of three different litters.

order as that of the reduced enzyme activity (30%). However, in spinal cord the increase in the concentration of glycine is much lower than brain. Blood glycine, which was 1.5 and 1.7 times the control value around the 5th and the 8th day of postnatal life respectively (data not shown), is about 1.8 times the control value at 10 days after birth. Liver glycine concentrations are doubled in relation to those found in control rats. The increase of glycine in liver is higher than that found in brain. This fact again reflects a reduction in glycine cleavage activity, that is greater in liver than in brain.

Specific binding of ^{3}H-strychnine to spinal cord synaptic membranes was measured at a ligand concentration of 20 nM, where saturation is achieved in control rats (K_d = 12.5 nM, [8]). Under these conditions, no effect on specific ^{3}H-strychnine binding was observed by administration of DPA, in contrast to the increase reported in rats given glycine and serine [8].

DISCUSSION

The results presented in this manuscript indicate that DPA inhibits the glycine cleavage system in brain and liver. This inhibition produces corresponding increases in the levels of glycine found in brain and blood.

Simila *et al.* [10] have reported hyperglycinemia and hyperglycinuria in patients receiving valproate. It has been suggested that these effects may be due to an inhibition of the liver glycine cleavage system by DPA [11]. We have also demonstrated this inhibitory effect on the rat brain glycine cleavage system. We observed an increase in the glycine concentrations of blood, liver and central nervous system of adult and suckling rats. The differences found in the increases of both brain and liver glycine levels between adult and 7-day-old rats, could be explained by measuring the uptake of radioactivity over time in brain and in plasma. These patterns could be due to a lower permeability of DPA across the blood–brain barrier and/or the ability of adult rat to better clear DPA. These two points are probably responsible for the faster return to normal glycine levels (control values) in DPA-treated adult rats. There was also a marked parallelism between the levels of radioactivity and the levels of glycine found in the livers and brains of DPA-treated rats. This fact suggests a correlation between DPA concentration and reduced activity of the G.C.S. *in vivo*, as indicated by the increased levels of glycine observed in brain and liver.

The chronic administration of dipropylacetate for 10 days after birth increases glycine levels in suckling rats to values twice that of the control concentrations. This increase is of the same magnitude as that observed in the blood of NKH-patients in relation to normal individuals. The hyperglycinemia induced by DPA cannot be attributed to an increase of propionate from the metabolism of dipropylacetate, as some authors have suggested [21, 22], since plasma concentrations of propionate remain unaltered (Table 1). The effects of chronic DPA administration on glycine concentrations appear to be due to inhibition of the enzyme system by DPA. Furthermore, none of these effects could be shown when similar concentrations of DPA were detected in plasma, after administration of a single DPA dose. This fact suggests that chronic administration of DPA may irreversibly inhibit glycine cleavage. These results are in agreement with those previously reported by Kochi *et al.* [20]. The brain glycine cleavage system defect, that biochemically characterizes non-ketotic hyperglycinemia, would produce the accumulation of glycine in the central nervous system, and could interfere with the synaptic stabilization process by blocking the glycine receptors [23, 24, 9]. Alternatively, the enzyme defect could reduce the synthesis of 5, 10-methylene-tetrahydrofolate (one carbon units), with the subsequent alteration of brain biosynthetic processes. The chronic administration of DPA induces a reduction in glycine cleavage activity in rat brain. However, the glycine concentration in the C.N.S. does not increase greatly. Furthermore, in DPA-treated rats one does not see an increase in specific ^{3}H-strychnine binding to spinal cord synaptic membranes, as has been described in rats given glycine and serine [8].

It has to be reported that administration of glycine, serine (according to the experimental model of NKH described by Hommes [7]), and DPA provoked rat mortality around the second hour after treatment. This is probably due to the high increase of glycine levels in the C.N.S. (unpublished results), resulting from inhibition of the G.C.S. by dipropylacetate.

In summary, the administration of DPA produced an alteration of glycine catabolism, with no apparent effect on glycine receptors. Alternatively, behavioural signs of NKH, such as seizures and severe effects on motility, were observed following chronic DPA administration. We suggest that DPA-treatment may provide a biochemical approach to study non-ketotic hyperglycinemia. This approach would be useful in studying alterations in brain biosynthetic processes due to a defect in the G.C.S. The alterations may explain partially the morbidity of NKH patients during postnatal development.

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