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INFLUENCE OF SOME BIOLOGICAL PYRIMIDINES ON THE SUCCINATE CYCLE DURING AND AFTER CEREBRAL ISCHEMIA

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Abstract—Some cortical metabolites (glycogen, glucose, glucose-6-phosphate, pyruvate, lactate, α-ketoglutarate, succinate, fumarate, malate, citrate, glutamate, glutamine, alanine, NH_4^+) were studied in rat brain after 5 min of complete compression ischemia, as well as after 15 min of recirculation following 5 min of ischemia. These two conditions (ischemia and post ischemic restitution) were induced in control animals and in rats pretreated 1 hr before by an intraperitoneal injection of 120 mg·kg^{-1} of some biological pyrimidines (uridine, cytidine and uridine disphosphate glucose).

At the cerebral level total complete ischemia induced the: (a) drop of substrates and of glycolytic intermediates, consistent with the increase of lactate and redox state; (b) increase of succinate and alanine; (c) decrease of malate and fumarate; and (d) depletion of α-ketoglutarate. Some of these events may be regarded as the expression of the activation of the succinate cycle which contributed by approx. 10 per cent to the release of anaerobic energy during cerebral ischemia. Pretreatment with the tested pyrimidines did not modify this cerebral biochemical pattern.

During post-ischemic recovery, cerebral parameters tended to normalize, except for a further increase in alanine production (as an expression of the activation of the alanine aminotransferase reaction) with conversion of pyruvate into α-ketoglutarate available for the ammonia-detoxicating processes (amination to glutamate and amidation to glutamine). During post-ischemic recovery, pretreatment with cytidine was poorly active. Pretreatment with uridine decreased glucose, glucose-6-phosphate and pyruvate cerebral concentrations, while succinate and alanine were increased. This latter effect was also present in the case of pretreatment with uridine diphosphate glucose. However, this substance increased the cerebral concentration of glycogen and decreased those of fumarate and malate. The different biochemical actions of uracyl derivatives are discussed with regard to their biological effects.

Experimental and clinical observations have long shown that some biological pyrimidines (uridine, cytidine and uridine diphosphate glucose) are related to cerebral function. In experiments carried out in isolated cat brain by Geiger and Yamasaki [1] it has been shown that the addition of cytidine and uridine to the perfusion blood keeps the brain in a good functional state. Moreover, the addition of nucleosides, after 1 hr of perfusion, shifts carbohydrate metabolism toward normal, while decreasing brain lactate. Other investigators [2–4] have evaluated in several animal species the effect induced by nucleosides, nucleotides and heterocyclic pyrimidinic bases on: (a) bipolar electrocorticograms utilizing electrodes placed on the olfactory bulb and posterior forebrain of each hemisphere; (b) transport through the blood–brain barrier of cytosine, uracyl and their nucleosides and nucleotides; (c) the interaction with barbiturate narcosis, or with brain stem analeptic- and penicillin-induced seizures. These investigations, performed using different experimental techniques, have shown that uracyl derivatives are the most active in potentiating barbiturate narcosis and in antagonizing analeptic- or penicillin-induced seizures. These results have been confirmed in the clinic [5, 6]. On the other hand, it has been demonstrated that uridine diphosphate glucose antagonizes the convulsant effect of hyperbaric oxygen, while concurrently controlling cerebral ATP [7]. These functional events at brain level have also been ascribed [4, 8] to the structural similarity between barbiturates and pyrimidines. Indeed, the group –CO–NH–CO– is present in all pyrimidine nucleosides and nucleotides. However, only uridine and its related compounds possess clear anticonvulsant properties. These observations have suggested that pyrimidine nucleosides and nucleotides play a role in the regulation of cerebral nervous system function [4]. However, the action of pyrimidines on cerebral energy metabolism, strongly affected by both anesthetics [9–16] and analeptics [17], has not yet been investigated.

It has therefore seemed interesting to evaluate the possible interference of several biological pyrimidines (uridine, cytidine and uridine diphosphate glucose) on the physiopathological deficit induced by total ischemia and in post-ischemic recovery. Under these conditions definite modifications take place in organic phosphates, glycolytic metabolites [13, 14, 17–24], citric acid cycle intermediates [25, 26] and related amino acids [24, 27–30]. In this work we have therefore studied the effect of uridine, cytidine and uridine diphosphate glucose (UDP-glucose) on the changes induced by total ischemia (5 min) and post-ischemic recovery (15 min) on some glycolytic metabolites, Krebs cycle intermediates and related amino acids.

MATERIAL AND METHODS

The experiments were performed on male Sprague–Dawley rats, weighting 340 ± 20 g, which were fasted overnight before the experiments. The trials were carried out according to the procedure described by Ljunggren *et al.* [22, 31] under light anesthesia, with control of blood pressure and body temperature. In brief, the

Table 1. Rat cerebral cortex. Concentration (μmoles·g^{-1}) of carbohydrate substrates, intermediates and end-products in control conditions and at the end of an ischemic period of 5 min durations.

Metabolite or ratio	Controls ($n = 10$) (a)	After the ischemic period and i.p. pretreatment with: Saline solution ($n = 8$) (b)	Uridine ($n = 8$) (c)	Cytidine ($n = 8$) (d)	Uridine diphosphate glucose ($n = 8$) (e)
Glycogen	2.05 ± 0.07*	<0.13	<0.15	<0.13	<0.19
Glucose	3.28 ± 0.10*	<0.10	<0.13	<0.13	<0.10
Glucose-6-P	0.108 ± 0.005*	0.012 ± 0.001	0.022 ± 0.003*	0.018 ± 0.003	0.018 ± 0.002
Pyruvate	0.120 ± 0.005*	0.014 ± 0.001	0.019 ± 0.003	0.023 ± 0.002*	0.022 ± 0.003
Lactate	1.95 ± 0.07*	11.85 ± 0.16	11.58 ± 0.58	12.18 ± 0.12	12.70 ± 0.83
α-Ketoglutarate	0.160 ± 0.009*	<0.013	<0.013	<0.016	<0.016
Succinate	0.514 ± 0.013*	1.315 ± 0.030	1.397 ± 0.029	1.281 ± 0.025	1.359 ± 0.075
Malate	0.390 ± 0.009*	0.271 ± 0.012	0.264 ± 0.011	0.281 ± 0.017	0.290 ± 0.011
Citrate	0.352 ± 0.016*	0.124 ± 0.005	0.126 ± 0.005	0.128 ± 0.006	0.131 ± 0.009
Fumarate	0.096 ± 0.004*	0.068 ± 0.002	0.072 ± 0.004	0.067 ± 0.004	0.072 ± 0.003
Glutamate	12.32 ± 0.15	11.94 ± 0.11	12.14 ± 0.08	11.86 ± 0.09	11.83 ± 0.20
Glutamine	5.28 ± 0.11	4.95 ± 0.17	5.27 ± 0.08	4.83 ± 0.15	4.96 ± 0.20
Alanine	0.526 ± 0.012*	0.786 ± 0.002	0.822 ± 0.014	0.768 ± 0.010	0.800 ± 0.028
NH_4^+	0.357 ± 0.030*	1.203 ± 0.052	1.145 ± 0.020	1.264 ± 0.056	1.216 ± 0.064
[Lactate]/[Pyruvate]	16.55 ± 1.09*	904.68 ± 99.73	706.99 ± 106.72	572.12 ± 55.99*	627.75 ± 62.81
[Citrate]/[α-Ketoglutarate]	2.23 ± 0.06*	>10.95	>10.9	>10.5	>9.92
[Succinate]/[Fumarate]	5.37 ± 0.12*	19.35 ± 0.72	19.95 ± 1.33	19.65 ± 1.19	19.09 ± 1.00
([Glutamate] × 10^{-2}) ([α-Ketogl.] × [NH_4^+])	2.26 ± 0.05*	>8.90	>9.10	>7.94	>8.10

The values are given as means ± S.E.M. *Refers to differences ($P < 0.01$) between the ischemic group pretreated with saline solution (b) and the other groups (a, c, d, e).

rats were maintained artificially ventilated on 70% N_2O–30% O_2. The body temperature was kept close to 37°, the arterial PCO_2 at 35–40 mmHg and the arterial PO_2 above 100 mmHg.

The atlanto-occipital membrane was exposed and a double-barrelled needle was inserted into the cisterna magna for pressure monitoring and for infusion of an artificial CSF [22]. When the operative procedure had been completed, the animals were maintained at steady-state for 30 min. Complete cerebral ischemia was then induced for 5 min by means of an increase of the CSF pressure above the arterial blood pressure [23, 31]. At the end of the ischemic period the brain was frozen *in situ* for subsequent biochemical analyses. In the 'post-ischemic' group, the CSF pressure was reduced to normal at the end of the 5 min period of ischemia, and recirculation was allowed for 15 min before the tissue was frozen. At the set time the removed frozen cerebral tissue was immediately immersed into liquid nitrogen for 10–15 min. The cortical portion of the frozen tissue was dissected and quickly (3–4 sec) powdered in a precooled automatic apparatus (Microdismembrator, Braun) using frozen perchloric acid. The subsequent steps were carried out in a precooled box at 0–5° until a neutral perchlorate-free extract was obtained. The neutralized perchlorate-free extract was then used according to Lowry and Passonneau [32] for immediate enzymatic analysis [21, 25] of glycogen, glucose, glucose-6-phosphate, pyruvate, lactate, α-ketoglutarate, succinate, malate, citrate, fumarate, glutamate, glutamine, alanine and ammonia.

Cortical metabolites were measured in the rat brain after 5 min of complete compression ischemia, as well as after 15 min of recirculation, following 5 min of ischemia. These two conditions (ischemia and post-ischemic recovery) were induced in control animals and in animals pretreated 1 hr before by an intraperitoneal injection of 120 $mg \cdot kg^{-1}$ of uridine, cytidine or uridine diphosphate glucose.

For the statistical analysis, the Dunnett's test was applied ($P < 0.01$) after checking the homogeneity of variance by the Bartlett's test.

RESULTS

The 5 min ischemia period caused a drop in glycogen, glucose, glucose-6-phosphate and pyruvate levels, consistent with the rise in lactate (Table 1). Some Krebs cycle intermediates (malate, citrate and fumarate) were decreased, while one (α-ketoglutarate) was depleted and another (succinate) was increased. In spite of the fall in the cerebral concentration of most Krebs cycle intermediates, the increase in succinate led to an 18 per cent rise in the size of the citric acid cycle pool. As for the ammonia-detoxicating system, ischemia did not cause changes in glutamate and glutamine cerebral concentrations, while alanine and ammonia were increased (Table 1). The ratios [lactate]/[pyruvate], [citrate]/[α-ketoglutarate], [succinate]/[fumarate] and [glutamate]/([α-ketoglutarate] × $[NH^+_4]$) were increased during ischemia (Table 1). Since these ratios are indirectly related to NADH/NAD^+ or $FADH_2$/FAD ratios, their increase can also be hypothesized. It should however be pointed out that no conclusions can be drawn from these indirect calculations on actual changes in redox systems, which are known to be influenced by physicochemical events (such as, e.g. intracellular H^+ concentration). I.p. pretreatment with

Table 2. Rat cerebral cortex. Concentration ($\mu moles \cdot g^{-1}$) of carbohydrate substrates, intermediates and end-products in control conditions and 15 min after an ischemic period of 5 min duration.

Metabolite or ratio	Controls ($n = 10$) (a)	After post-ischemic recovery and i.p. pretreatment with: Saline solution ($n = 10$) (f)	Uridine ($n = 8$) (g)	Cytidine ($n = 8$) (h)	Uridine diphosphate glucose ($n = 8$) (i)
Glycogen	2.05 ± 0.07*	0.36 ± 0.03	0.22 ± 0.03	0.29 ± 0.04	0.66 ± 0.04*
Glucose	3.28 ± 0.10*	5.25 ± 0.25	4.51 ± 0.13*	4.78 ± 0.07	4.59 ± 0.13
Glucose-6-P	0.108 ± 0.005*	0.211 ± 0.011	0.157 ± 0.008*	0.181 ± 0.008	0.226 ± 0.007
Pyruvate	0.120 ± 0.005*	0.168 ± 0.005	0.141 ± 0.004*	0.152 ± 0.007	0.177 ± 0.008
Lactate	1.95 ± 0.07*	3.32 ± 0.09	3.08 ± 0.13	3.25 ± 0.12	3.13 ± 0.09
α-Ketoglutarate	0.160 ± 0.009	0.181 ± 0.007	0.168 ± 0.006	0.172 ± 0.008	0.161 ± 0.011
Succinate	0.514 ± 0.013*	0.704 ± 0.009	0.982 ± 0.023*	0.862 ± 0.059*	0.990 ± 0.050*
Malate	0.390 ± 0.009	0.445 ± 0.006	0.402 ± 0.015	0.426 ± 0.021	0.379 ± 0.022*
Citrate	0.352 ± 0.016*	0.491 ± 0.020	0.409 ± 0.025	0.442 ± 0.033	0.503 ± 0.011
Fumarate	0.096 ± 0.004	0.086 ± 0.002	0.079 ± 0.004	0.081 ± 0.005	0.069 ± 0.004*
Glutamate	12.32 ± 0.15*	11.09 ± 0.11	11.49 ± 0.15	10.87 ± 0.28	11.56 ± 0.17
Glutamine	5.28 ± 0.11*	6.04 ± 0.13	6.71 ± 0.22	6.43 ± 0.18	6.51 ± 0.14
Alanine	0.526 ± 0.012*	1.525 ± 0.028	1.725 ± 0.023*	1.635 ± 0.039	1.686 ± 0.022*
NH^+_4	0.357 ± 0.030	0.308 ± 0.029	0.204 ± 0.009	0.263 ± 0.016	0.290 ± 0.015
[Lactate]/[Pyruvate]	16.55 ± 1.09	19.98 ± 1.00	21.87 ± 0.36	21.59 ± 1.06	17.95 ± 0.90
[Citrate]/[α-Ketoglutarate]	2.23 ± 0.06	2.72 ± 0.09	2.43 ± 0.13	2.57 ± 0.15	3.20 ± 0.20
[Succinate]/[Fumarate]	5.37 ± 0.12*	8.32 ± 0.12	12.57 ± 0.45*	10.64 ± 0.46*	14.57 ± 1.04*
([Glutamate] × 10^{-2})/([α-Ketogl.] × $[NH^+_4]$)	2.26 ± 0.05	2.10 ± 0.13	3.43 ± 0.23*	2.48 ± 0.17	2.58 ± 0.17

The values are given as means ± S.E.M. *Refers to differences ($P < 0.01$) between the post-ischemic group pretreated with saline solution (f) and the other groups (a, g, h, i).

uridine, cytidine or uridine diphosphate glucose did not modify cerebral parameters, as evaluated at the end of the 5 min of complete ischemia (Table 1).

In comparison with the ischemic condition (Table 1), the 15 min resumption (Table 2) of circulation after ischemia decreased the cerebral concentration of lactate and increased those of some glycolysis intermediates up to values even higher than those found in controls (for example, the concentration of glucose-6-phosphate was doubled). Glucose was also found at concentrations higher than in controls. The Krebs cycle intermediates succinate and citrate exhibited values higher than controls (Table 2). As a consequence, after post-ischemic recovery, the size of the Krebs cycle pool was 26 percent higher than that of controls. With regard to the ammonia-detoxicating system, the 15 min of post-ischemic recovery increased glutamine and alanine while decreased ammonia. As for the indirect evaluation of the redox state, intermediate ratios (Table 2) tended to return to control values, even though these were not always attained.

Uridine pretreatment (Table 2) affected some cerebral biochemical parameters during the 15 min of post-ischemic recovery. In particular, the cerebral concentrations of glucose, glucose-6-phosphate and pyruvate were lower than in post-ischemic untreated rats. Rats pretreated with uridine, at the end of post-ischemic recovery showed higher levels of succinate and alanine than did untreated rats. In addition, the ratios [succinate]/[fumarate] and [glutamate]/([α-ketoglutarate] × [NH_4^+]) were higher than those found at the end of the post-ischemic period in untreated controls. The only effect of pretreatment with cytidine (Table 2) was an increased concentration of cerebral succinate.

In the brain of rats pretreated with uridine diphosphate glucose, at the end of post-ischemic recovery (Table 2) succinate and alanine showed higher values than in controls, while malate and fumarate concentrations were lower. The [succinate]/[fumarate] ratio was higher than that found at the end of the post-ischemic period in untreated rats. Cerebral glycogen increased at the end of the post-ischemic recovery in rats pretreated with uridine diphosphate glucose.

DISCUSSION

The present data are in agreement with previous observations [13, 14, 17–19, 21–24] according to which *cerebral ischemia* leads both to an increase in energy transduction through anaerobic glycolysis and to tissular hyperammonemia. This was not affected by pretreatment with the pyrimidines studied (Table 1). During ischemia, Krebs cycle intermediates exhibited different changes: α-ketoglutarate was depleted, malate and fumarate were decreased by 30 per cent, citrate by 65 per cent, while succinate was increased by 155 per cent. Simultaneously, alanine increased by 50 per cent. These empirical data can be accounted for by the fact that an aliquot of phosphoenolpyruvate (PEP) can be alternatively channelled (Fig. 1) towards the succinate cycle where, in the absence of molecular oxygen, succinate acts as a terminal acceptor of electrons [25, 33–36]. In this way, alanine induces a negative modulation on pyruvate kinase and a positive modulation on PEP carboxykinase [34], the alanine inhibition of pyruvate kinase being potentiated by increasing [H^+] [34]. The

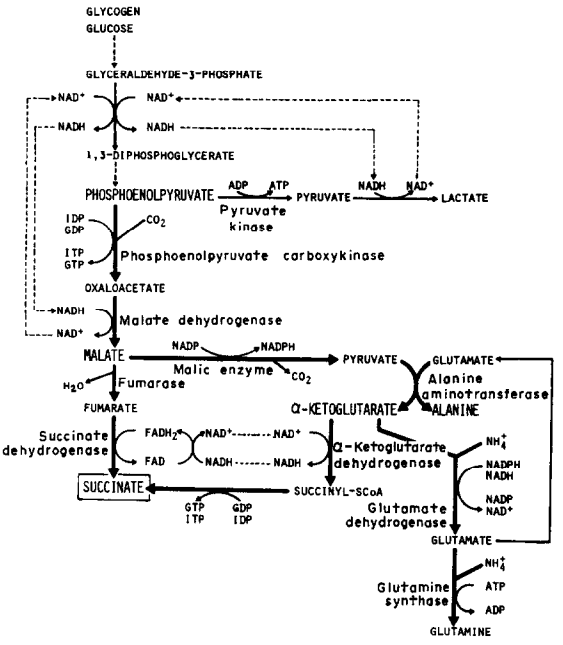

Fig. 1.

α-ketoglutarate depletion may be related also to its utilization in the succinate cycle. Indeed, after the 5 min of ischemia, cerebral glutamate and glutamine did not undergo any modification and ammonia was increased by approx. 240 per cent (Table 1).

The overall inbalance of the redox state during ischemia is indirectly shown by the ratios reported in Table 1. In the succinate cycle, even assuming a redox balance between the α-ketoglutarate dehydrogenase and succinate dehydrogenase reactions, the change of the redox state induced by the malic enzyme reaction remains unbalanced. With regard to the energy yield, since half of the malate produced is converted to succinate via α-ketoglutarate, it must be concluded that succinate cycle reactions would lead to the formation of 3 ~ P moles/mole of glucose utilized: 2 ~ P from glucose → malate + 1 ~ P from α-ketoglutarate → succinate. The ratio succinate/lactate was 7.5:92.5 (Table 1), while that of the corresponding ~ P was 10.8:89.2, the succinate cycle contributing to anaerobic energy yield by 10% during the 5 min of ischemia.

After 15 min of *post-ischemic recovery* (Table 2) a few peculiarities were still present in the brain: (1) the further increase in alanine, related to the oxidation of excess lactate providing a large amount of pyruvate available for the formation of alanine and α-ketoglutarate; (2) the high concentration of glucose-6-phosphate, probably related to the high level of citrate inhibiting the phosphofructokinase reaction [38]; and (3) the high succinate level, in line with the tendency of Krebs cycle intermediates to increase as a consequence of (a) a slowing down of the flux in the cycle of tricarboxylic acids, as also shown by the slight reduction in cerebral O_2 consumption [39] and (b) an increase of anaplerotic reactions [25].

The action induced by pretreatment with cytidine was low, thus confirming previous observations [2–4, 8] that, among pyrimidine nucleosides, uracyl ones are

the most active. The cerebral alanine and succinate increase induced by pretreatment with uridine can be related to an increased availability of pyruvate, probably from the oxidation of excess lactate stored during ischemia. Pretreatment with uridine caused a drop in both glucose and glucose-6-phosphate cerebral levels and, therefore, an activation of the PEP → succinate branch of the succinate cycle may also be hypothesized. The action of uridine might be related to: (1) its interference with some cerebral enzymatic activities such as, e.g., alanine aminotransferase; (2) its availability in tissues for biochemical reactions, in which it is used as a substrate; (3) the production of a tissular biochemical situation where, as in ischemia, succinate would partially act as a terminal electron acceptor.

Pretreatment with uridine diphosphate glucose induced events in post-ischemic recovery which were partially similar to those of uridine, increasing succinate and alanine cerebral concentrations. On the other hand, pretreatment with uridine diphosphate glucose induced an increase of the glycogen cerebral concentration during the restitution phase. Unlike some other tissues, the brain can not synthesize glycogen by a reversal of glycolysis from lactate, the obstacle being the thermodynamically irreversible steps, mainly the phosphofructokinase and hexokinase reactions [40]. Uridine diphosphate glucose is primarily involved in glycogen synthesis [41–46] by means of the glycogen synthetase activation [47] or by supplying the substrate for the reaction, glucose-6-phosphate being a probable modulator of the rate of glycogen synthesis from uridine diphosphate glucose [48]. Finally, pretreatment with uridine diphosphate glucose induced low malate and fumarate concentrations during post-hypoxic recovery. This event may be related to a lower flux of the Krebs cycle or to a decrease in the rate of anaplerotic reactions.

On the whole, the effect of uridine and uridine diphosphate glucose on the cerebral biochemical pattern during post-ischemic recovery may be related to the succinate cycle which, on the other hand, appears to be activated by ischemia. Even though in this condition its anaerobic energy contribution is low (10 per cent), the succinate cycle appears important because of its connections with the ammonia-detoxicating processes and its possible interference with several substances of biological interest.

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