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EFFECT OF CAFFEINE ON UREA BIOSYNTHESIS AND SOME RELATED PROCESSES, KETONE BODIES, ATP AND LIVER AMINO ACIDS

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Abstract—An increase in urea synthesis has been found in rats administered large doses of caffeine. A parallel increase in urea biosynthesis was also found in hepatocytes isolated from caffeine-treated rats, which confirms a greater capacity for urea synthesis induced by caffeine. This increase appeared only after some days of caffeine treatment; during the first days there was no increase in serum urea levels or in *in vitro* studies of urea synthesis in isolated hepatocytes. However, no appreciable changes were found in either cytosolic or mitochondrial redox states, or in ATP levels in *in vivo* and *in vitro* studies. A parallelism was observed between the decreased amino acid levels in caffeine-treated rats and in isolated hepatocytes incubated with different concentrations of caffeine. Several possible mechanisms to explain these findings are considered in the discussion.

Caffeine, which is present in coffee, tea, carbonated beverages, medical products and a variety of foodstuffs, is one of the most widely consumed pharmacologic agents in the world. The use of caffeine is partly due to its stimulating effect on the central nervous system, its ability to increase alertness and work capacity [1].

Several relatively well known aspects of the physiological, behavioural, teratogenic, carcinogenic and mutagenic effects of caffeine, as well as the effects on the cellular repair process of DNA, and part of the mechanisms involved in these processes have been described (for review see Ref. 2). However, despite its wide consumption and potential toxicity, the effects of caffeine on the intermediary metabolism of liver have not been well established.

Recently, we have demonstrated, in an experimental model of the Lesch–Nyhan syndrome, that large doses of caffeine can elicit self-destructive behavior in rats [3–5] similar to that observed in Lesch–Nyhan patients. We have also found that caffeine is an effective agent for increasing the serum urea levels [4, 6]. The alterations observed in the free amino acid pool of rat cerebral cortex treated with caffeine [4] were similar to those obtained by Kikuchi *et al.* [7] in brains of both acute and chronic uremic rats. Here we show the *in vivo* effect of caffeine on urea biosynthesis and related processes. In *in vitro* studies, the effect of different caffeine concentrations on the rate of urea synthesis in isolated hepatocytes has been shown. The levels of other metabolic parameters such as cofactors, ketone bodies, redox state, ATP and amino acids in isolated rat hepatocytes are also reported. The possible pharmacological and physiological significance of these findings are discussed.

MATERIALS AND METHODS

Chemicals. Standard analytical grade reagents were obtained from Sigma Chemical Co. (St Louis, MO) and Boehringer (Mannheim, F.R.G.). ^{14}C-glutamate was obtained from Amersham (Bucks, U.K.) and *N*-acetyl-^{14}C-glutamate was synthesized enzymatically [8].

Treatment of animals. The animals (male Wistar rats of 195–215 g) were fed a standard diet (15% protein) (Letica, S.A., Barcelona, Spain). Caffeine was added to the drinking water in gradually increased amounts so that the animals became accustomed to it: 2 g/l. the first 3 days, 4 g/l. the next 3 days, and finally 8 g/l. the last 4 days. Sucrose 100 g/l. was added to the caffeine solutions to mask the bitter taste. Pair-fed control rats were given the same diet and the sucrose solution without caffeine. Another control group was fed the standard diet *ad lib.* During the experimental period, the rats were housed in plastic boxes, one animal per box at 22°, 60% humidity, on a 12 hr light/dark cycle.

Enzymatic activities in liver. Urea cycle enzymes were assayed as described in [9], glutamine synthetase as in Meister [10], and glutaminase as in Kvamme *et al.* [11].

Rat liver cells. Isolated hepatocytes were prepared according to Berry and Friend [12], with the modifications described in Viña *et al.* [13]. Cells were suspended in Krebs–Henseleit saline, equilibrated with $O_2 + CO_2$ (95:5) and sealed with rubber stoppers. Incubations were in 25 ml Erlenmeyer flasks containing 2 ml of cell suspension (about 4×10^6 cells) and 2 ml of additions (10 mM NH_4Cl + 3 mM

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ornithine for urea biosynthesis assays in isolated hepatocytes of control, pair-fed and caffeine-treated rats and appropriate substrates and medium for the *in vitro* assays in cells isolated from control rats, and incubated with different concentrations of caffeine). $HClO_4$ was added at the end of the incubation period and metabolites were determined in neutralized supernatants. The dry weight was determined and a factor of 3.7 was used to convert dry to wet weight [14].

Measurements of metabolites. Rats were killed by decapitation and the livers were quickly removed and frozen in liquid nitrogen. Aliquots were used for measurement of amino acids, *N*-acetylglutamate (NAGA), ATP, lactate, pyruvate, acetoacetate and β-OH-butyrate. These parameters were also measured in isolated hepatocytes incubated with different concentrations of caffeine. Amino acids and NAGA levels were measured by HPLC, as previously described [4, 15, 16]. The other parameters were measured as described: ATP in Lamprecht and Trantschold [17], lactate in Gutmann and Wahlefeld [18], pyruvate in Czok and Lamprecht [19], acetoacetate in Mellamby and Williamson [20] and β-OH-butyrate in Williamson and Mellamby [21]. Serum caffeine was determined according to Blanchard *et al.* [22], serum urea as in Bachmann [23], urea in isolated hepatocytes as in Gutmann and Bergmeyer [24], and protein was determined by the method of Lowry *et al.*, [25]. Cytosolic and mitochondrial redox states were calculated according to Williamson *et al.*, [26].

Statistical. All results have been submitted to statistical analysis and the degree of significance calculated by Student's *t*-test.

RESULTS

Body weight, food and fluid intake decreased gradually. During the last 3 days the food and fluid intake were stabilized. Both pair-fed and caffeine-treated rats lost 12% of the initial body weight in 10 days, probably due to a decrease in food intake (Fig. 1A). Caffeine intake increased slightly as can be calculated from the data described in Materials and Methods (Treatment of animals), and those shown in Fig. 1A (Fluid intake). No change was observed in serum urea levels of caffeine-treated rats during the first days; however, between the 3rd and 6th days of treatment, the urea levels increased and at 6 days were similar to those found after 10 days of treatment (Fig. 1B). The increase in serum caffeine concentrations after 3 days of treatment could perhaps be explained by a change in the drinking and feeding behaviour of the rats, or by an accumulation of caffeine in tissues and serum [3], or both, which might result in the increases in serum urea levels observed.

Interestingly, in the caffeine-treated rats the greater increases in activity of urea cycle enzymes were for carbamoylphosphate synthetase I (ammonia) (CPS I), argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). The most directly involved in the control of the overall rate of the urea cycle are CPS I and ASS [27]. The ornithine carbamoyltransferase (OTC) and arginase activities were not changed (Table 1). *N*-acetylglutamate (NAGA), natural cofactor or CPS I [28] also increased in caffeine-treated rats. There were no important changes among the three animal groups in the levels in liver or ornithine, citrulline, aspartate or arginine (Table 1). Thus, according to previous results [29, 30] the rate of hepatic urea synthesis is not regulated through changes in the levels of these substrates.

To confirm the increased urea synthesis, we studied the capacity of hepatocytes to synthesize urea. We found that isolated hepatocytes from caffeine-treated rats synthesize urea at a higher rate, compared with both control and pair-fed rats, which were essentially the same. Thus, the increase in urea synthesis, together with other metabolic changes reported, may be induced by caffeine *per se*. Glutamine levels and glutamine synthetase activity were not changed in the liver of caffeine-treated rats, and glutaminase activity and glutamate levels were decreased slightly (Table 2).

Lactate increased in pair-fed and caffeine-treated rats with respect to the controls (Table 3). Pyruvate, acetoacetate and β-OH-butyrate decreased slightly in pair-fed and caffeine-treated groups, and no change was observed in ATP levels (Table 3). The marked fall observed in the cytosolic redox state of pair-fed and caffeine-treated rats, with respect to the *ad lib.* was probably due to their lower food intake or perhaps to higher carbohydrate intake. No changes were observed among the three groups in the mitochondrial redox state.

Table 4 shows the levels in liver of the amino acids that changed in caffeine-treated rats with respect to the pair-fed. No changes were observed in the other amino acids tested. Leucine (ketogenic amino acid), isoleucine, phenylalanine and tyrosine (glucogenic and ketogenic amino acids), and alanine, glutamate, histidine and valine (glucogenic amino acids) decreased in caffeine-treated rats with respect to pair-fed. The amino acid levels in liver of rats fed *ad lib.* were, in general, decreased compared with the other two groups (Table 4).

On the other hand, in other experiments isolated rat hepatocytes were incubated in the presence of several caffeine concentrations, ranging from 0 to 4 mM. Although the results obtained cannot be directly comparable to the *in vivo* studies, they do, nevertheless, help to give a better comprehension of these processes. In these studies no changes were observed in urea synthesis in isolated hepatocytes incubated with the different concentrations of caffeine mentioned above (not shown); *N*-acetylglutamate and ATP levels increased slightly (15% and 20%, respectively) at higher concentrations of caffeine (4 mM). Pyruvate, lactate and β-OH-pyruvate also increased at higher concentrations of caffeine (1.5-, 1.7- and 1.4-fold, respectively, at 2 and 4 mM caffeine); no changes were observed in acetoacetate levels. Cytosolic and especially mitochondrial redox states decreased (13% and 36%, respectively) with increasing concentrations of caffeine (at 4 mM). Amino acid levels in isolated hepatocytes were affected differently. Leucine (ketogenic amino acid), isoleucine, phenylalanine and tyrosine (glucogenic and ketogenic amino acids), and glu-

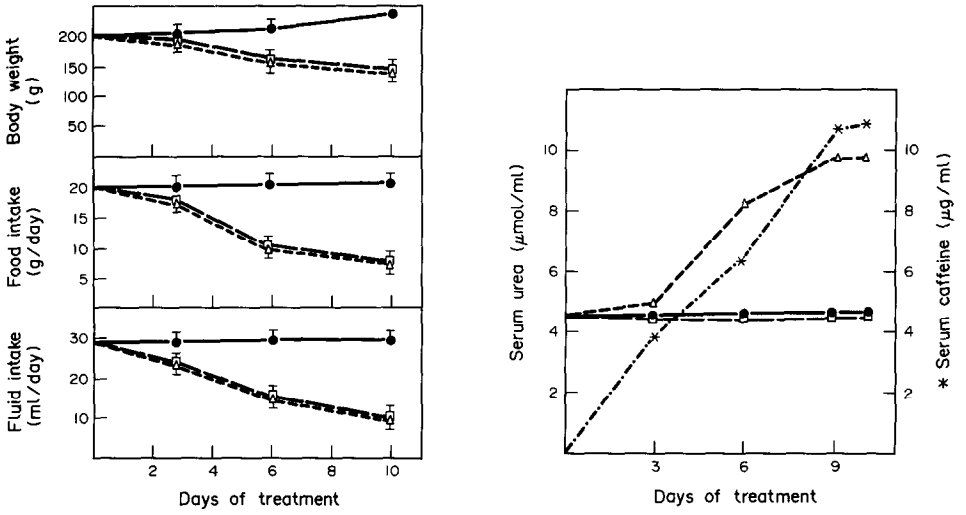

Fig. 1. (A) Variations in body weight, and food and fluid intake, with time of caffeine treatment. (●) *ad lib.* (□) pair-fed, and (△) caffeine-treated rats. (B) Serum urea levels (in (●) *ad lib.*, (□) pair-fed and (△) caffeine-treated rats), and (★) serum caffeine levels in caffeine-treated rats. The results are expressed as mean ± SD of 8–10 rats.

Table 1. Activities of urea-cycle enzymes, and levels of their intermediates and cofactors in *ad lib.*, pair-fed and caffeine-treated rats

	Ad lib. (A)	Pair-fed (B)	Caffeine (C)	P value (C) vs (B)
CPS I	1.9 ± 0.2	2.2 ± 0.3	3.2 ± 0.2	<0.001
OTC	87 ± 15	98 ± 12	105 ± 11	N.S.
ASS	1.0 ± 0.1	1.0 ± 0.2	1.5 ± 0.1	<0.001
ASL	1.6 ± 0.1	1.8 ± 0.1	2.3 ± 0.1	<0.001
Arginase	325 ± 38	353 ± 42	373 ± 36	N.S.
NAGA	33 ± 8	25 ± 7	41 ± 6	<0.001
Ornithine	312 ± 91	224 ± 28	219 ± 30	N.S.
Citrulline	60 ± 7	80 ± 8	91 ± 12	<0.05
Aspartate	510 ± 58	614 ± 51	704 ± 55	<0.002
Arginine	71 ± 6	70 ± 7	72 ± 5	N.S.
Urea synthesis in isolated hepatocytes	3.1 ± 0.3	2.9 ± 0.4	4.9 ± 0.3	<0.001

Enzyme activities are expressed as follows: CPS I and OTC, as μmol of citrulline formed per hr/mg protein; ASS, ASL and arginase, as μmol of urea formed per hr/mg protein. NAGA, ornithine, citrulline, aspartate and arginine levels are expressed as nmol/g liver, and urea synthesis in isolated hepatocytes as μmol/min/g wet wt. Results are given as mean ± SD of 8–10 rats.

Table 2. Activities of glutamine synthetase and glutaminase, and liver levels of their substrate and products for *ad lib.*, pair-fed and caffeine-treated rats

	Ad lib. (A)	Pair-fed (B)	Caffeine (C)	P value (C) vs (B)
Glutamine synthetase	1.8 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	N.S.
Glutaminase	4.5 ± 0.4	7.6 ± 0.6	6.8 ± 0.5	<0.01
Glutamine	6.1 ± 0.6	5.0 ± 0.7	5.1 ± 0.5	N.S.
Glutamate	3.2 ± 0.3	8.7 ± 0.4	6.4 ± 0.4	<0.001

Enzyme activities are expressed as follows: glutamine synthetase, as μmol of γ-glutamyl-hydroximate per hr/mg protein; glutaminase, as μmol of glutamate per hr/mg protein. Glutamine and glutamate are expressed as μmol/g liver. Results are given as mean ± SD of 8–10 rats.

Table 3. Ketone bodies, redox state and ATP levels in liver of *ad lib.*, pair-fed and caffeine-treated rats

	Ad lib. (A)	Pair-fed (B)	Caffeine (C)	P value (C) vs (B)
Pyruvate	0.30 ± 0.04	0.28 ± 0.03	0.25 ± 0.03	N.S.
Lactate	2.0 ± 0.3	3.3 ± 0.4	3.5 ± 0.4	N.S.
$NAD^+/NADH$ (cytosol)*	1351	765	643	N.S.
Acetoacetate	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	N.S.
β-OH-butyrate	0.20 ± 0.03	0.16 ± 0.02	0.17 ± 0.03	N.S.
$NAD^+/NADH$ (mitochondria)†	8.0	7.8	7.2	N.S.
ATP	2.9 ± 0.4	2.8 ± 0.5	2.8 ± 0.6	N.S.

* Cytosolic redox state = $NAD^+/NADH = [pyruvate]/[lactate] \times K_{LDH}$); † Mitochondrial redox state = $NAD^+/NADH = [acetoacetate]/[\beta\text{-OH-butyrate}] \times K_{\beta\text{-OH-BDH}}$). LDH and BDH, are lactic dehydrogenase and butyrate dehydrogenase, respectively. *† Calculated from Ref. 26. The results are expressed in μmol/g liver, and are the mean ± SD for 6–8 rats.

Table 4. Liver amino acid levels in *ad lib.*, pair-fed and caffeine-treated rats

Amino acids (μmol/g liver)	*Ad. lib.* (A)	Pair-fed (B)	Caffeine (C)	P value (C) vs (B)
Leucine (ketogenic amino acid)	0.53 ± 0.02	0.91 ± 0.03	0.65 ± 0.04	<0.001
Isoleucine	0.24 ± 0.01	0.39 ± 0.02	0.33 ± 0.03	<0.001
Phenylalanine	0.31 ± 0.04	0.90 ± 0.10	0.75 ± 0.10	<0.02
Tyrosine (glucogenic and ketogenic amino acids)	0.15 ± 0.01	0.15 ± 0.02	0.10 ± 0.01	<0.001
Alanine	2.0 ± 0.4	3.2 ± 0.5	2.0 ± 0.5	<0.001
Glutamate	3.2 ± 0.3	8.7 ± 0.4	6.4 ± 0.4	<0.001
Histidine	1.7 ± 0.1	3.5 ± 0.1	2.7 ± 0.1	<0.001
Valine (glucogenic amino acids)	0.73 ± 0.08	1.10 ± 0.10	0.72 ± 0.11	<0.001

Results are expressed in μmol/g liver, and are the mean ± SD of 6–8 rats.

tamate, histidine and valine (glucogenic amino acids) decreased (23%, 19% and 29%, respectively) with increasing concentrations of caffeine (at 2 and 4 mM). The changes observed in isolated hepatocytes of caffeine-treated rats were similar to those found in isolated hepatocytes incubated with caffeine, except for alanine, which was decreased in liver of caffeine-treated rats with respect to pair-fed (Table 4), whereas no change was observed in isolated hepatocytes incubated with caffeine. No changes were observed in the other amino acids tested either *in vivo* or *in vitro* (not shown).

DISCUSSION

We have shown that rats which were caffeine-treated for 10 days had an increased excretion of urea nitrogen without changes in renal function [4]. The present study was designed to extend and clarify these findings. The results show clearly that livers of caffeine-treated rats have a greater capacity to synthesize urea than either pair-fed or rats fed *ad lib.* as suggested by the increase in the activities of the key enzymes of the urea cycle (CPS I and ASS) [27] and concentration of NAGA, natural cofactor of CPS I [28]. The capacity of isolated hepatocytes for urea biosynthesis was also enhanced. However, no changes were observed in the hepatic levels of the intermediates, which indicates that these compounds have no direct role in the regulation of urea synthesis, as previously reported [29, 30].

Despite the widely different caffeine concentrations used in the *in vivo* and *in vitro* studies, there were no appreciable differences in cytosolic and mitochondrial redox states between the *in vivo* and *in vitro* studies, although both were decreased slightly as a function of caffeine concentration. No change was found in urea synthesis in isolated hepatocytes incubated with caffeine; however, *in vivo* studies showed that the capacity for urea synthesis was increased in caffeine-treated rats. No changes were observed in the ATP levels either in *in vivo* or in *in vitro* studies. These facts indicate that in caffeine-treated rats, urea synthesis was not affected appreciably by either cytosolic or mitochondrial redox states or by ATP levels. The effect of caffeine on the amino acid levels was similar *in vivo* and *in vitro*, since the same amino acids (except alanine) were

decreased despite the fact that the caffeine concentrations used *in vitro* were many-fold higher than the serum concentrations found in the treated rats. These results could indicate that the changes in amino acid levels induced by caffeine in rat liver occur rapidly.

The possible mechanisms involved in the caffeine-enhanced capacity for urea synthesis remain unclear. A direct activation by caffeine of the key enzymes seems unlikely, since no changes were found following direct incubation of isolated hepatocytes with high concentrations of caffeine. Another possibility to be considered would be an increase in endogenous protein catabolism and amino acid breakdown; however, the amino acid degradation would be faster than its production from proteolysis since we have found diminished levels of amino acids in the liver of caffeine-treated rats. Any increase in proteolysis, however, would begin after several days of treatment, since there were no changes in serum urea levels during the first days of treatment (Fig. 1B). Although no changes in protein synthesis were observed in livers of caffeine-treated rats [5], it is evident that the amounts of key urea cycle enzymes must be increased, particularly CPS I, which represents 15–20% of mitochondrial protein [31, 32]. It has been shown that there is a parallel between enzyme activity and the amount of enzymatic protein [32–34].

The first widely considered mechanism relates to cyclic AMP [35]; Sutherland's group has shown that xanthines inhibit phosphodiesterase. However, significant inhibition of phosphodiesterase requires millimolar concentrations of caffeine. Further, cyclic AMP was found to be increased in several metabolic conditions with increases in urea synthesis [36]. Recently Guinzberg *et al.*, [37] have shown that adenosine (which has been considered as a mediator of the caffeine effects; Ref. 2) produced a dose-dependent stimulation of ureagenesis in isolated rat hepatocytes. Caffeine removed the adenosine from its receptors at concentrations similar to those found in plasma after the consumption of one to three cups of coffee (at these concentrations caffeine occupies 50% of the adenosine receptors; Ref. 2); thus there could be increases in both the free adenosine levels and in their hepatic availability [1, 2, 38]. Moreover, adenosine increases the accumulation of cyclic AMP. The mechanism for the stimulation of urea synthesis by adenosine remains unknown, but could be due in part to the facts mentioned here.

Of the mechanisms considered, all remain to be confirmed, whether they act singly or collectively, and perhaps with others not yet considered. Studies for further clarification are in progress.

Finally, some pharmacological considerations might be mentioned because of the possible physiological implications (for review see Refs 1, 2, 38). More than 99% of an orally administered dose of caffeine is absorbed, with peak plasma levels occurring in 15–45 min. A 250 mg dose yields peak plasma concentrations between 5 and 25 μg/ml. Caffeine is excreted as various metabolites in urine and is also excreted unchanged into saliva, semen and breast milk, and is present in umbilical cord blood. The half-life of caffeine shows considerable variation between persons, with reported values ranging from 3 to 8 hours. However, caffeine half-life is prolonged in pregnancy, and is surprisingly slow in the fetus and newborn (reports of 65–110 hours). Further, the levels of caffeine in plasma and cerebrospinal fluid are nearly identical in newborns, and caffeine and theophylline are metabolically interconverted. These facts are important since, in addition to the general consumption, pregnant women are often habitual consumers of coffee, and theophylline is also used in neonatal apnea. It is worth emphasizing that the findings shown in this and other papers suggest the potential dangers and deleterious consequences to health.

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