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The inhibition of rat adipocyte ecto-5′-nucleotidase by xanthines is not related to lipolysis

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The effects of alkylxanthines on fat cell metabolism especially the stimulation of lipolysis are regarded as the result of the combination of adenosine receptor antagonism and cAMP-phosphodiesterase inhibition. Since alkylxanthines have been reported to inhibit 5′-nucleotidase in several tissues [1, 2] and Newby *et al.* [3] showed that the enzyme of adipocytes is predominantly a cell surface enzyme we investigated if alkylxanthines inhibited the cleavage of exogenously added 5′-AMP and if there was any relation to the effect of the agents on lipolysis.

Methods

Isolation and incubations of rat adipocytes were performed in a Krebs–Ringer medium buffered with 4-(2-hydroxyethyl)-piperazine ethanesulfonic acid (10 mM; pH 7.4). the epididymal fat pads of male, fed Wistar rats (140–180 g) were digested with collagenase according to Rodbell [4]. Cytocrit determination was accomplished by the addition of aliquots of cells to hematocrit capillaries and subsequent centrifugation [5].

Ecto-5′-nucleotidase of the fat cells [3] was tested after preincubation with the agents under investigation. The velocity of the hydrolysis of exogenously added [2-^{3}H]-5′-AMP (20,000–30,000 cpm) and unlabelled 5′-AMP at the concentrations of 5, 50 and 200 μM was determined in duplicate with 500 μl aliquots of the treated cells. After 2, 3 or 10 min respectively the reaction was stopped and the remaining nucleotide precipitated by a $ZnSO_4$ (0.3 M)/$Ba(OH)_2$ (0.3 N) procedure [3]. After a minute centrifugation (10,000 *g*) the amount of label remaining in 500 μl of the clear supernatant was determined. Blank values were obtained by the simultaneous addition of $ZnSO_4$ and cells to the substrate. After subtraction of these blank values the velocity of 5′-AMP hydrolysis was expressed as nmol substrate hydrolysed per μl lipid per min. The selective inhibitor of 5′-nucleotidase α,β-methylene ADP was added to the substrate at a concentration of 50 μM. Lipolysis of the same pretreated cells was evaluated by the determination of medium glycerol content according to [7].

Results and discussion

Theophylline dose-dependently inhibited the hydrolysis of 5′-AMP (200 μM) by rat adipocytes (Fig. 1). The maximal inhibition of 40% was achieved by 5 mM theophylline. PGE_2 (1 μg/ml) failed to significantly alter the enzyme inhibition by the methylxanthine. In contrast, this concentration of the prostaglandin completely antagonized the lipolysis stimulated by theophylline (Fig. 1).

Time course experiments of 5′-AMP (200 μM) hydrolysis at a cytocrit of 1% were linear for at least 30 min in the absence and the presence of theophylline (1.7 mM). The methylxanthine without delay caused a 30% inhibition of the hydrolysis of the nucleotide at all tested time points.

The kinetics of the hydrolysing activity are shown in Fig. 2 in the form of a Hanes plot (S/v vs S) for one representative

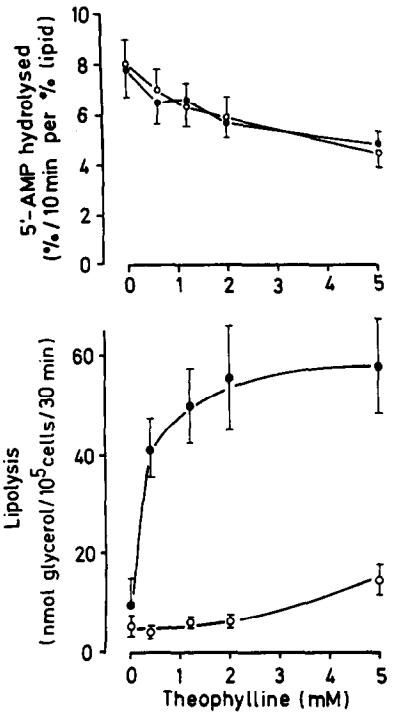

Fig. 1. Effects of theophylline on 5′-nucleotidase (upper panel) and on lipolysis (lower panel) in isolated adipocytes in the absence (●) or presence (○) of prostaglandin E_2 (1 μg/ml). Data on inhibition of 5′-AMP (200 μM) hydrolysis are the means ± standard error of 4 experiments. Mean cell cytocrit was 1.1% in nucleotidase experiments. Data on lipolysis are the means ± standard error of 3 experiments. The mean cell concentration was 2.04×10^5 cells/ml.

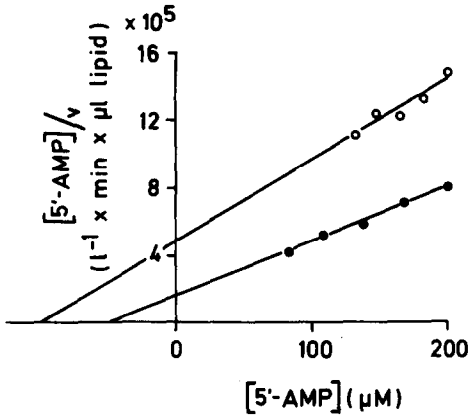

Fig. 2. Kinetics of 5′-AMP hydrolysis by isolated rat adipocytes in the absence (●) and the presence (○) of theophylline (1.7 mM). The results of one representative experiment are given as a Hanes plot [8]. The kinetic parameters were obtained by linear regression analysis. The mean data ± S.E.M. of seven experiments are given in the text.

experiment (one of seven) [8]. The data obtained from these experiments revealed that theophylline (1.7 mM) nearly tripled the K_m of the enzyme from 56.6 ± 8.9 µM (control) to 161.1 ± 27.4 µM. The V_{max} was reduced by about 15% from 0.274 ± 0.024 (control) to 0.238 ± 0.032 nmol/min × µl lipid. Thus the methylxanthine predominantly caused a competitive inhibition of 5′-AMP hydrolysis.

Since theophylline treatment of isolated rat adipocytes may produce manifold metabolic effects, e.g. fatty acid accumulation in medium and cells and depression of cellular ATP [9], it seemed reasonable to investigate whether the extent of enzyme inhibition is dependent on the time of contact of the methylxanthine to the cells. Theophylline (2 mM) independent of the duration of the preincubation (0–60 min) reduced the 5′-AMP hydrolysing activity by about 30% at a substrate concentration of 200 µM.

The specificity of the measured 5′-AMP hydrolysing activity of rat adipocytes was tested by simultaneous addition of the selective 5′-nucleotidase inhibitor α,β-methylene ADP [6]. The hydrolysis of 5′-AMP at substrate concentrations of 5, 50 and 200 µM was reduced by the inhibitor by 87%, 77%, and 69% respectively. The combination of α,β-methylene ADP and theophylline (2 mM) revealed no additive inhibitory effect at either substrate concentration. These data and the similar K_m of the hydrolysing activity of our cells with the fat cell nucleotidase characterized by Newby *et al.* [3] allowed the conclusion to name the examined activity as adipocyte ecto-5′-nucleotidase that was inhibited by the methylxanthine.

Table 1 shows the effects of various xanthines and substituted xanthines on the fat cell ecto-nucleotidase as compared to their effect on lipolysis. The enzyme activity was measured after a 25–35 min preincubation with the agents. 5′-AMP hydrolysis at two different substrate concentrations (5 + 50 µM) was expressed as percent of control activity. The concentration of xanthine and its derivatives was 1 mM except for 8-phenyltheophylline which was used at 20 µM (a nearly saturated aqueous solution). At 5 µM 5′-AMP, all xanthines with the exception of 8-phenyltheophylline, caused at least slight inhibition of the nucleotidase. The most pronounced inhibition (50%) was achieved by 3-methylxanthine, followed by theophylline and theobromine. At 50 µM 5′-AMP, the extent of inhibition was reduced, maximal inhibition of about 25% was accomplished by 3-methylxanthine. The rank order of inhibitory efficacy was similar to that obtained at the lower substrate concentration. Hypoxanthine, 3-isobutyl-1-methylxanthine (IBMX) and 8-phenyltheophylline failed to inhibit the enzyme at 50 µM of substrate. No correlation between 5′-nucleotidase inhibition and lipolysis became apparent. Most active in stimulating lipolysis were IBMX, theophylline, and 8-phenyltheophylline. Of these agents only theophylline inhibited the nucleotidase at 50 µM 5′-AMP. The most potent inhibitor of 5′-AMP hydrolysis 3-methylxanthine was only a moderate stimulator of lipolysis. Xanthine, which inhibited the nucleotidase to a level comparable to theobromine or caffeine, was even slightly antilipolytic.

The present data demonstrate that xanthines are weak inhibitors of cell surface 5′-nucleotidase in isolated adipocytes. The accumulation of 5′-AMP rather than adenosine was observed in adipocytes after the stimulation of lipolysis with norepinephrine and theophylline [10]. Therefore, if our data on cell surface 5′-nucleotidase could be ascribed also to the intracellular form of the enzyme the inhibition of the conversion of AMP to adenosine might activate adenylate cyclase, and thereby lipolysis. However, the present data do not favour this idea. Firstly, the concentration dependency of the stimulation of lipolysis by theophylline differs considerably from that of the inhibition of the nucleotidase. Lipolysis was nearly maximally stimulated with 1 mM theophylline, whereas a significant inhibition of the 5′-nucleotidase could be observed only at concentrations of the methylxanthine above 1 mM, if the

Table 1. Effects of xanthine and xanthine derivatives on lipolysis and activity of cell surface 5′-nucleotidase of rat adipocytes at two different substrate concentrations

Drug		% of basal 5′-nucleotidase activity		Lipolysis (nmol/10^5 cells × 60 min)
		5 µM 5′-AMP	50 µM 5′-AMP	
Hypoxanthine	(1 mM)	81.8 ± 1.3	95.7 ± 1.7	14.06 ± 1.01
Xanthine	(1 mM)	67.4 ± 2.5	83.4 ± 2.3	12.28 ± 0.95
3-Methylxanthine	(1 mM)	52.5 ± 1.7	73.9 ± 1.4	45.57 ± 3.35
Theophylline	(1 mM)	55.5 ± 1.2	78.8 ± 2.0	115.54 ± 8.36
Theobromine	(1 mM)	55.6 ± 1.2	84.7 ± 2.8	58.74 ± 5.23
Caffeine	(1 mM)	65.2 ± 2.4	85.1 ± 3.2	71.35 ± 7.06
IBMX	(1 mM)	87.1 ± 1.9	108.6 ± 2.9	128.08 ± 8.75
8-Phenyltheophylline	(20 µM)	95.4 ± 1.4	104.3 ± 3.0	103.51 ± 8.09

Data are the means ± standard errors of 9 experiments. The mean cell concentration was 284,000 ± 16,000 cells/ml, and the mean cytocrit was 2.8%. Basal lipolysis was 21.94 ± 2.81 nmol glycerol/10^5 cells × 60 min. Control cells hydrolysed 0.0577 ± 0.0051 nmol/min × µl lipid at 50 µM 5′-AMP and 0.0128 ± 0.0011 nmol/min × µl lipid at 5 µM 5′-AMP.

activity was measured at a 5'-AMP concentration of 200 μM. Secondly, the potency of alkylxanthines to stimulate lipolysis did not correlate with their inhibitory potency on the nucleotidase (Table 1). IBMX and 8-phenyltheophylline were at least equipotent to theophylline in stimulating lipolysis but showed no inhibition of the 5'-nucleotidase at a substrate concentration equivalent to the K_m of the non-inhibited enzyme. Xanthine itself significantly inhibited 5'-AMP hydrolysis but was devoid of lipolytic efficacy. Thirdly, the stimulation of lipolysis could be completely antagonized with PGE_2 (1 μg/ml) (Fig. 1), whereas the prostaglandin did not alter the inhibition of the nucleotidase by the methylxanthine (Fig. 1). Therefore, on the basis of the present experiments it seems unlikely that inhibition of 5'-nucleotidase may contribute to the lipolytic action of xanthines.

In summary, the present results show that a cell surface form of rat adipocyte 5'-nucleotidase is inhibited by alkylxanthines. The most potent inhibitors were 3-methylxanthine, theophylline and theobromine. The inhibition of the enzyme was predominantly competitive. The potency of alkylxanthines to inhibit the hydrolysis of 5'-AMP did not correlate with their lipolytic activity.

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Comparative effects of famotidine and cimetidine on trimethadione metabolism in the rat

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A histamine H_2-receptor antagonist, cimetidine, is known to inhibit the metabolism of many drugs that are metabolized by the hepatic mixed function oxidase system [1, 2]. There is evidence suggesting that some H_2-receptor antagonists (e.g. furan derivative ranitidine) do not share this property.

Famotidine (YM 11170) is a newly developed selective H_2-receptor antagonist having the chemical structure of amidine-substituted thiazole [3]. We have reported a useful method for estimation of hepatic drug-oxidizing capacity. Serum concentration ratios of dimethadione (DMO), which is the only metabolite of trimethadione (TMO), to unchanged TMO after oral administration of TMO are well correlated to drug-oxidizing enzyme activities in rats pretreated with chemicals such as hepatotoxic agents [4–8] and inducers [9] of the enzyme system.

In the present study, we compared the effects of cimetidine and famotidine on the metabolism of TMO, a marker drug of hepatic oxidizing activity in the rat.

Materials and methods

Male Wistar rats (Doken, Ibaraki, Japan) weighing 221–250 g were used throughout the study and allowed free access to water and food. In a single administration study, these rats were injected intraperitoneally (i.p.) with cimetidine (100 mg/kg) or famotidine (100, 150 or 200 mg/kg) 0.5 hr prior to the oral administration of TMO (100 mg/kg). In the short-term administration study, these rats were injected i.p. with cimetidine (100 mg/kg) or famotidine (200 mg/kg) twice daily for 5 days. In the pharmacokinetic study, blood samples were obtained from the jugular vein after oral administration of TMO. Procedures for preparing liver samples were described previously [8]. Microsomal cytochrome P-450 contents were determined by the method of Omura and Sato [10]. The activity of TMO (2–20 mM) *N*-demethylase was measured by the procedure of Cochin and Axelrod [11]. Experiments to determine the K_m values for cimetidine and famotidine were performed by using control rat hepatic microsome fraction as the enzyme preparation. Four concentrations (2, 5, 10 and 20 mM) of substrates were used for each determination of K_m. Kinetic constants were determined from graphically depicted data as described by Lineweaver and Burk. Serum TMO and DMO levels were determined by a gas–liquid chromatographic method as described previously [4]. Concentration–time curves for serum TMO and DMO levels were drawn on semilogarithmic scales. The half-life ($t_{\frac{1}{2}}$) and elimination rate constant (K_{el}) were calculated by linear regression analysis. The apparent volume of distribution (V_d) was calculated from the ratio of the administered dose to the concentration extrapolated to time zero.

Total body clearance (CL) was calculated according to the equation $CL = 0.693V_d/t_{\frac{1}{2}}$. Famotidine was obtained from Yamanouchi Pharmaceutical Co. (Tokyo, Japan). The results were statistically analysed by the Student's *t*-test.

Results and discussion

The effects of a single dose of cimetidine and famotidine on TMO metabolism in the rat *in vivo* are shown in Fig. 1. The serum DMO/TMO ratios at 2 hr after oral administration of TMO in cimetidine-treated rats were significantly decreased by 32.8% compared to the controls. However, famotidine did not significantly decrease this ratio from control levels in a dose of not only 100 mg/kg but also 150 and 200 mg/kg. In the pharmacokinetic study, the pretreatment of rats with cimetidine significantly prolonged the TMO serum $t_{\frac{1}{2}}$ (1.61 ± 0.14 vs 2.11 ± 0.10 hr, mean ± SEM, $P < 0.05$) and decreased CL (0.32 ± 0.03 vs 0.281 ± 0.051/kg/hr, $P < 0.05$), while in the rats pretreated with famotidine in a dose of 100, 150 or 200 mg/kg, these parameters were not changed. V_d values were