

## Methylxanthine Drugs Are Human Pancreatic Lipase Inhibitors

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Methylxanthines such as caffeine, theobromine and theophylline are intensively consumed as food components by large proportion of human population all over the world. This class of compounds show various biological activities and have been found to act as broad specificity inhibitors towards numerous enzymes. However, their action on digestive enzymes have not been yet investigated. In this paper we aimed to evaluate the effects of methylxanthines on the human pancreatic lipase activity *in vitro*. Emulsions of short- and long-chain triglycerides (tributylin and tripalmitate, respectively) were used as substrates. The concentrations of methylxanthines in the reaction mixtures covered the range between 0.015 mmol/L to 15 mmol/L. We demonstrated that all three tested substances, caffeine, theophylline and theobromine inhibited the hydrolysis of tributyrin and tripalmitate catalysed by human pancreatic lipase in dose-dependent fashion. The highest lipase inhibition ratios during tripalmitate and tributyrin hydrolysis were 25.74% and 79.54% respectively in the presence of caffeine, 29.89% and 62.79% respectively with theophylline and 21.08% and 67.74% respectively in the presence of theobromine. All the tested methylxanthines exert stronger inhibition in the short-chain triglyceride lipolysis comparing to long-chain substrates. Their mechanism of action involves most likely the interaction with enzyme protein but not substrate emulgation. In case of tripalmitate lipolysis all the methylxanthines showed mixed type of inhibition. Interestingly, during tributyrin lipolysis theophylline behaved as classical noncompetitive inhibitor.

### INTRODUCTION

Human pancreatic lipase (HPL) is the most important lipolytic enzyme involved in the digestion of dietary triglycerides (TG). HPL is a 50 kDa polypeptide composed of 449 amino acid residues and N-glycosylated on Asn166 with a high mannose carbohydrate moiety [Miled *et al.*, 2000]. The HPL molecule contains two main structural domains: a large N-terminal domain with the  $\alpha/\beta$  hydrolase motif and the active site with a catalytic triad formed by Ser 152, Asp 176, His 263, and a smaller C-terminal domain [Colin *et al.*, 2008]. The active site is covered by a large surface loop called a 'lid' domain (Cys 237 - Cys 261) [Winkler *et al.*, 1990; Ranaldi *et al.*, 2010]. The HPL activity towards water-soluble substrates is low, but the enzyme is highly active when operating at the interface between water and hydrophobic triglyceride layer. This phenomenon is known as interfacial activation [Steiner & Williams, 2002]. Physiological concentrations of bile acids inhibit lipolysis by covering the oil-water interface and preventing the adsorption of HPL at that interface [Brockman, 2000]. This inhibition by bile salts can be overcome by the presence of colipase, a specific lipase-anchoring protein secreted by pancreas. Colipase forms a specific 1:1 complex with lipase and facilitates its adsorption at the bile acid layered

lipid-water interface [Miled *et al.*, 2000, Freie *et al.*, 2006]. *In vitro*, the highest specific HPL activity in excess of colipase and with 4 mmol/L sodium deoxycholate, at pH 8.0 was recorded for tributyrin as a substrate [Miled *et al.*, 2000].

Methylxanthines, including caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (3,7-dimethylxanthine), are natural purine alkaloids produced by many plant species. These compounds are present in large quantities in a variety of food, beverages and pharmaceutical products. Caffeine is consumed every day by more than 80% of the world population [Papaioannou *et al.*, 2005]. It is a constituent of coffee, tea [Mandel, 2002], cocoa beans, guarana, colas [Harland, 2000], and included in many medicines, particularly over-the-counter drug mixtures for cold and cough [Baselt, 2004] as well as in weight loss supporting products [Greenway *et al.*, 2004]. Human body metabolises caffeine to theobromine and theophylline [Grosso & Bracken, 2005] and these derivatives are also present in cocoa, guarana, tea and chocolate products [Caudle & Bell, 2000]. Average daily consumption of theophylline and theobromine, accounted only for drinking tea and cocoa, is estimated to 0.51 mg and 39 mg, respectively [WHO, 1995]. Theophylline is also a frequently used bronchodilating agent with a narrow serum therapeutic range [Møller *et al.*, 2000]. Theobromine, however, at the therapeutic dose of 500 mg/kg has been used in the treatment of cardiac oedema and angina pectoris [Eteng & Ettarh, 2000].

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Methylxanthines exert a strong impact on the operation of central and peripheral nervous system [Mandel, 2002], cerebral blood flow and metabolism, cardiovascular system, respiratory and renal systems and gastrointestinal system [Greenway *et al.*, 2004; Haller *et al.*, 2004; Ruhl & Everhart, 2005; Westerterp-Plantenga *et al.*, 2006]. There are several recent lines of evidence suggesting that methylxanthines stimulate thermogenesis, promote weight and fat loss [Greenway *et al.*, 2004], as well as inhibit various enzymes, such as PDE, PLA<sub>2</sub> [Mandel, 2002] and chitinase [Rao *et al.*, 2005].

Nevertheless, neither of the three mentioned methylxanthines have been suspected to inhibit digestive enzymes, so far. Therefore, the aim of this study was to assess the potential inhibitory effects of caffeine, theophylline and theobromine on human pancreatic lipase, measured on short-chain and long-chain triglyceride substrates.

## MATERIAL AND METHODS

### Methylxanthines

Caffeine, theophylline and theobromine (purum, anhydrous, HPLC grade) were obtained from Sigma Chemicals, Inc. (Steinheim, Germany). The alkaloid stock solutions (40 mL) were prepared in double distilled water at the final concentrations of 0.05, 0.50, 5.0 and 50 mmol/L, with pH adjusted to 8.0 and subsequently deoxygenated with gaseous nitrogen.

### Enzyme

Human pancreatic lipase (Triacylglycerol acylhydrolase, EC 3.1.1.3) was purchased from Sigma Chemicals, Inc. (St. Louis, USA). The enzyme stock solution (5 mg/mL, which corresponds to 300 U/mL, according to the product technical data sheet) was prepared in doubly distilled water with pH adjusted to 8.0 with 0.01 mol/L NaOH.

### Substrates

Tributyryl, a short-chain triglyceride, and tripalmitate, a long-chain triglyceride, were obtained from Sigma Chemicals, Inc. (Steinheim, Germany). Bile acid mixture (Sigma Chemicals, Inc., Germany) and gum arabic (POCH S.A. Gliwice, Poland) were used for substrate emulsification. The optimal proportion of both emulsifiers was determined experimentally prior to lipase activity assays in order to provide complete substrate emulsification and possibly the highest enzyme activity. This step was necessary because in the absence of colipase the bile acids, even in physiological concentrations, negatively influence the pancreatic lipase activity [Brockman, 2000; Miled *et al.*, 2000].

Tripalmitate emulsion (TPE) was prepared by sonication of 24.22 g tripalmitate, 100 mL deoxycholate/NaCl solution (1.6 g/100 mL sodium deoxycholate, and 0.187 g/100 mL NaCl in doubly distilled water, pH 8.0) and 100 mL of gum arabic solution (8 g gum arabic, in 100 mL of doubly distilled water, pH 8.0).

Tributyryl emulsion (TBE) was prepared by sonication of 9 mL tributyrin, 100 mL deoxycholate/NaCl solution (1.6 g/100 mL sodium deoxycholate, and 0.187 g/100 mL NaCl in doubly distilled water, pH 8.0) and 91 mL gum arabic solution (8.8 g gum arabic in 100 mL of doubly distilled water, pH 8.0).

Sonication parameters were the same for both substrates: amplitude was adjusted to 100%, cycle 60, 20 min, on ice (Sonicate Labsonic P, B. Braun Biotech. International) to produce liposome size of 2–3  $\mu\text{m}$  in diameter. The liposome size of the emulsion was determined using an optical microscope (Nikon microscope Elipse E 400, Nikon Corporation, Japan) as described by Mun *et al.* [2007]. Finally, substrate emulsions (100 mL) were deoxygenated with gaseous nitrogen for 20 min.

### Measurements of pancreatic lipase activity

Lipase activity assay was performed as described by Naher [1974] in the absence (control) or presence of methylxanthines, using excessive amounts of tripalmitate and tributyrin emulsions as substrates. Free fatty acids were automatically titrated using a pH-stat (ino-Lab level 2, WTW, Germany) with degassed 0.01 mol/L NaOH at the constant pH value of 8.0. The reactions were performed for 10 min with constant stirring in a thermostated vessel (37°C) containing 20 mL of substrate emulsion, 9 mL of doubly distilled water at pH 8.0 (control) or alkaloid solution and 1 mL of lipase solution. The final concentration of tripalmitate and tributyrin in the reaction mixture was 0.1 mol/L and the final concentrations of methylxanthines in the reaction mixture were 0.015 mmol/L, 0.15 mmol/L, 1.5 mmol/L and 15 mmol/L. To investigate the effects of caffeine, theophylline and theobromine on pancreatic lipase activity the following experimental approaches were employed:

1. Methylxanthines were added to the substrate emulsion just before starting the reaction.
2. The substrate emulsion (TPE and TBE) was incubated with methylxanthines for 10 min before addition of the enzyme.
3. The enzyme was incubated with methylxanthines for 10 min before addition of the substrate (TPE and TBE).

Incubations with methylxanthines were performed with the magnetic stirrer at 37°C, which was the optimal temperature for lipase activity. All samples were analysed in quadruplicate. One lipase unit (LU) was defined as 1  $\mu\text{mol}$  of fatty acid titrated per minute. To determine the endogenous level of fatty acids present prior to the action of lipase, the amount of free fatty acids of each substrate emulsion was also measured.

### Determination of inhibition types

The inhibition kinetics were studied using a non-linear regression and Hanes-Woolf plots ( $[S]/v_0$  against  $[S]$ ). Initial velocity  $v_0$  and the value of Michaelis-Menten constant  $K_m$  were determined by the pH-stat method at different substrate concentrations in the presence and absence of constant methylxanthine concentration. The concentrations of the substrates varied from 0.25 mmol/L to 125 mmol/L for tributyrin and from 0.5 mmol/L to 50 mmol/L for tripalmitate. The final concentration of caffeine, theophylline and theobromine in the reaction mixture was 1.5 mmol/L. All experiments were performed in triplicate.

### Statistical analysis

The results are presented as means $\pm$ SEM. The statistical significance of the differences was determined by one-way ANOVA and by Fisher's exact test using the Statgraphics Plus software (Manugistics Inc., USA).

## RESULTS AND DISCUSSION

**Dose-response inhibitory effect of methylxanthines on short-chain and long-chain triglycerides hydrolysis**

The hydrolytic activity of human pancreatic lipase towards long-chain and short-chain triglycerides depended on the dose of methylxanthines added to the reaction mixture. For tripalmitate lipolysis, the lowest concentration (0.015 mmol/L) of caffeine, theophylline and theobromine inhibited the hydrolysis by 7.98%, 18.06% and 6.46%, respectively, and by 23.78%, 18.94% and 21.75% for tributyrin lipolysis. A thousand fold increase in methylxanthines concentration resulted in a severe drop in lipase activity. As a result, the lipase inhibition ratio of tripalmitate hydrolysis was 21.08% in the presence of theobromine, 25.74% for caffeine and 29.89% for theophylline. During tributyrin hydrolysis the value of the inhibition ratio varied from 62.79% for theophylline, 67.74% for theobromine, to 79.54% for caffeine (Figure 1). These results suggest that the inhibitory effect of methylxanthines on human pancreatic lipase may be put in the order as follows: 1,3-dimethylxanthine (theophylline) > 1,3,7-trimethylxanthine (caffeine) > 3,7-dimethylxanthine (theobromine) for the long-chain triglyceride, and 1,3,7-trimethylxanthine (caffeine) > 3,7-dimethylxanthine (theobromine) > 1,3-dimethylxanthine (theophylline) for the short-chain triglyceride hydrolysis. It is likely that the inhibitory efficacy of methylxanthines depends on the number of methyl groups on the purine ring and their different positions. The presence of  $-CH_3$  group at the N1 atom seems to facilitate the inhibition of the long-chain triglyceride hydrolysis,

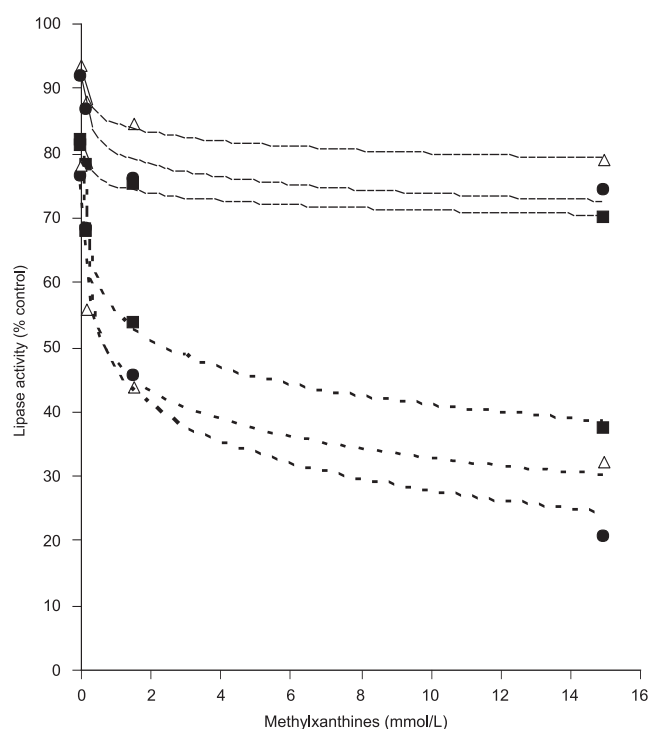


FIGURE 1. Dose-response effects of methylxanthines (● caffeine; ■ theophylline; △ theobromine) on the human pancreatic lipase activity (100% is the activity measured in the absence of an inhibitor). --- inhibition of pancreatic lipase against tributyrin; — inhibition of pancreatic lipase against tripalmitate

while methylation at the N7 site – to facilitate the short-chain triglyceride hydrolysis inhibition. The importance of the methyl group position as an inhibitory effect determinant was observed by Sugawara *et al.* [2005], who showed that caffeine, theophylline and theobromine blocked human organic anion transporter 1 (hOAT1) in the following order: theophylline > theobromine > caffeine. Nafisi and his collaborators [Nafisi *et al.*, 2002] reported that the blockage of the purine N7 atom by  $-CH_3$  group resulted in lower capability of caffeine for Mg(II) and Ca(II) cation chelation, as compared to theophylline.

**Preincubation effects**

The inhibitory effect of methylxanthines depended on the incubation system used (Table 1). All the methylxanthines showed the highest inhibitory effect when preincubated with the enzyme (incubation system 3). This indicates that methylxanthines are able to bind with the protein. This is consistent with the results of Sugawara *et al.* [2005] and Westerterp-Plantenga *et al.* [2006] who described the interaction of caffeine, theophylline and theobromine with human organic anion transporter 1 or with adenosine receptors, and with phosphodiesterases that hydrolyse cyclic AMP or cyclic GMP.

The lack of preincubation period of caffeine, theophylline or theobromine with the enzyme (incubation system 1) resulted in a significant ( $p < 0.05$ ) decrease in the inhibitory effect of methylxanthines. This indicates that to inhibit lipase efficiently, the methylxanthines have to interact directly with the protein, prior to the enzyme-substrate complex formation. Without preincubation with the enzyme, the affinity of methylxanthines for pancreatic lipase restricts the enzyme-triglyceride interaction by about 30%. Furthermore, since the triglycerides are in excess in the reaction mixture, this would favour enzyme-substrate interactions rather than enzyme-methylxanthines interactions.

TABLE 1. Effect of the highest doses of caffeine, theophylline and theobromine on the activity of human pancreatic lipase determined in different incubation systems.

Incubation system*	Conditions	Lipase activity			
		Tributyrin		Tripalmitate	
		units**	%#	units**	%#
1	cf+E+S	779.8 <sup>b</sup> ±2.0	70.95	13.50 <sup>b</sup> ±0.18	82.76
2	(cf+S)+E	971.5 <sup>a</sup> ±3.4	88.36	15.91 <sup>a</sup> ±0.08	97.54
3	(cf+E)+S	224.5 <sup>c</sup> ±3.4	20.46	12.07 <sup>c</sup> ±0.09	74.26
1	tf+E+S	806.3 <sup>b</sup> ±0.6	73.36	13.16 <sup>b</sup> ±0.21	80.69
2	(tf+S)+E	839.1 <sup>a</sup> ±1.7	76.35	14.69 <sup>a</sup> ±0.06	90.08
3	(tf+E)+S	408.3 <sup>c</sup> ±2.7	37.21	11.40 <sup>c</sup> ±0.08	70.11
1	tb+E+S	794.4 <sup>b</sup> ±0.8	72.28	13.53 <sup>b</sup> ±0.08	82.94
2	(tb+S)+E	827.9 <sup>a</sup> ±3.2	75.33	14.79 <sup>a</sup> ±0.06	90.69
3	(tb+E)+S	354.1 <sup>c</sup> ±2.9	32.26	12.83 <sup>c</sup> ±0.05	78.92

\* See materials and methods for details. \*\* One unit of enzyme activity is defined as 1  $\mu$ mol of the fatty acids titrated per minute. Results represent mean  $\pm$  SEM (n=4). Activities within one particular group marked within different letters are significantly different by Fisher's comparison test ( $p < 0.05$ ). # Percent relative to the control. cf, caffeine (15 mmol/L); tf, theophylline (15 mmol/L); tb, theobromine (15 mmol/L); E, human pancreatic lipase; S, substrate (0.1 mol/L).

Fish & Thompson [1991] described similar observations in relation to tannic acid and red kidney bean lectin that have direct inhibitory effects on digestive enzymes. The authors reported the lack of significant changes in the  $\alpha$ -amylase activity when the enzyme was not preincubated with red kidney bean lectin or tannic acid. In our studies, the incubation of methylxanthines with the excessive amounts of substrates prior to exposure to lipase resulted in further reduction of the inhibition ratio (incubation system 2). In case of tributyrin hydrolysis, the decrease in the lipase activity was not higher than 25%, whereas in case of tripalmitate hydrolysis not higher than 10%. This suggests that caffeine, theophylline and theobromine interact with highly emulsified substrates without exerting any detrimental effect on the emulsification process. To our best knowledge, this is the first report that shows no effects of purine alkaloids on the lipid emulsification process.

### Studies of inhibition types

The detailed analysis of the lipolysis kinetic parameters (Michaelis-Menten constant and maximum velocity), carried on in the presence or absence of alkaloids, demonstrated that the methylxanthines differed in the inhibitory activity as well as in the mechanism of action. Independently on the substrate used, caffeine and theobromine significantly increased  $K_m$  and decreased  $V_{max}$  (Table 2). Therefore they were mixed type inhibitors of lipase, which means that they were able to bind both free enzyme and an enzyme-substrate complex. The same conclusions were presented by Gu *et al.* [2011], who studied the effect of cocoa extract on lipase activity. However, the authors attributed the inhibitory effect to procyanidins contents only and did not comment the role of theobromine in this process. In our study, the effect of the third methylxanthine, *i.e.* theophylline, was dependent on the substrate hydrolysed by lipase. In the tripalmitate lipolysis reaction, it acted similarly to caffeine and theobromine and evoked mixed type inhibition. On the other hand, during the tributyrin lipolysis, theophylline reduced the maximal reaction velocity, but the  $K_m$  values remained unchanged. This indicates that in short-chain substrate hydrolysis theophylline is a classic non-competitive inhibitor.

TABLE 2. Kinetic parameters of tripalmitate and tributyrin emulsions hydrolysis catalysed by human pancreatic lipase in the absence and presence of methylxanthine inhibitors. The inhibition kinetics were studied using Hanes-Woolf plots ( $[S]/V_o = f[S]$ ).

	Substrates			
	Tributyrin		Tripalmitate	
	$K_m$ (mmol/L)	$V_{max}$ ( $\mu$ mol/min)	$K_m$ (mmol/L)	$V_{max}$ ( $\mu$ mol/min)
Caffeine	1.08 <sup>b</sup>	833.3 <sup>c</sup>	2.05 <sup>c</sup>	14.62 <sup>b</sup>
Theophylline	0.64 <sup>a</sup>	909.1 <sup>b</sup>	3.83 <sup>d</sup>	14.66 <sup>b</sup>
Theobromine	1.09 <sup>b</sup>	909.1 <sup>b</sup>	1.81 <sup>b</sup>	14.70 <sup>b</sup>
Control	0.67 <sup>a</sup>	1111.1 <sup>a</sup>	1.50 <sup>a</sup>	16.61 <sup>a</sup>

Values within a column marked within different letters differ significantly by Fisher's comparison test ( $p < 0.05$ ).

### CONCLUSION

Based on the analysis presented in this report, we show for the first time ever that caffeine, theobromine and theophylline which are ubiquitous components of our diet can significantly impede the lipid hydrolysis catalysed by the pancreatic lipase, even at low doses. The extent of inhibition depends not only on the methylxanthine concentration but also on the lipid type. All the tested methylxanthines inhibit short-chain triacylglyceride lipolysis more efficiently than the long-chain substrates, and the mechanism of their action relies on the interaction with protein but not on substrate emulsification.

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