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Studies on 3,7-Dimethyl-1-(5-oxo-hexyl)-xanthine (BL 191). II.[1] Effect of BL 191 on Lipolysis in Rat Epididymal Adipose Tissue

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Effects of 3,7-dimethyl-1-(5-oxo-hexyl)-xanthine (BL 191) on lipolysis in rat epididymal adipose tissue were investigated by comparison with those of theophylline.

BL 191 blocked the activity of phosphodiesterase in the 105000×*g* supernatant prepared from rat epididymal adipose tissue, and the type of inhibition was noncompetitive with the K_i value of 2.1 mM. Furthermore, BL 191 elicited a dose-dependent lipolytic response, as did theophylline, and the response increased 1,3-fold and about 3-fold over the basal activity at 2 mM and 5 mM, respectively. BL 191 as well as theophylline potentiated the lipolytic action of epinephrine and increased adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP) levels in adipose tissue. BL 191, in a concentration producing no effects by itself (10^{-4}M), together with epinephrine (10^{-6}M) caused a 38-fold increase in cyclic 3′,5′-AMP levels and an about 5-fold increase in lipolytic effects.

It has been reported that methyl xanthines like caffeine and theophylline stimulate lipolysis in rat epididymal adipose tissue and fat cells[3] and that the increased lipolysis is correlated with the rise in adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP) levels in adipose tissue.[4] Moreover, it has been found that the inhibitory effects of methyl xanthines on cyclic 3′,5′-nucleotide phosphodiesterase (PDE) are roughly proportional to their lipolytic stimulation.[5] Recently, we reported that the inhibitory effect of 3,7-dimethyl-1-(5-oxo-hexyl)-xanthine (BL 191) on PDE prepared from rat cerebral cortex was approximately similar to that of theophylline.[1] The present study was designed to investigate the relation between the lipolytic stimulation and PDE inhibition by BL 191 in rat epididymal adipose tissue, and the results suggest that BL 191 causes an increase in the lipolytic activity the degree of which depends on the level of cyclic 3′,5′-AMP.

Experimental

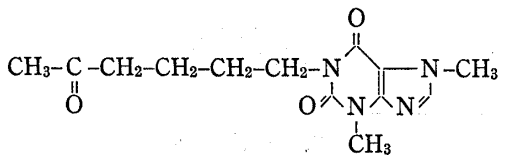

Fig. 1. Chemical Structure of BL 191

Drugs——BL 191,[6] a vasoactive agent developed by Hoechst Aktiengesellschaft, was examined in comparison with theophylline,[7] a known PDE inhibitor. The chemical structure of BL 191 is shown in Fig. 1.

Determination of Lipolysis in Adipose Tissue——Epididymal adipose tissues were removed from male Sprague-Dawley rats weighing 240—280 g. The tissues weighing 100—150 mg were incubated for 2 hr at 37° in a glass-stoppered test tube containing 4% bovine serum albumin (fraction V, Sigma Co.) and 1.9 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, that was previously equilibrated with a mixture of 95% O_2 and

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5% CO_2. Incubations were carried out with or without epinephrine, theophylline, and BL 191 in a volume of 0.1 ml each. After incubation, FFA was extracted and determined according to the method of Itaya and Ui.[8] The lipolytic value was estimated by determining the amount of FFA (μFq) released from rat adipose tissue per gram per hour.

Enzyme Preparation——Sprague-Dawley male rats weighing 240—280 g were decapitated, and epididymal adipose tissues were isolated and homogenized in chilled 50 mM Tris buffer (pH 7.4) containing 0.32M sucrose which was 4 times the volume of the tissues. The homogenate was centrifuged for 10 min at $1000 \times g$ at 0° to discard the nuclei fraction. The supernatant further centrifuged at $105000 \times g$ for 60 min at 0° was served as the enzymic preparation.

Assay of PDE Activity in Rat Epididymal Adipose Tissue——PDE assay was performed according to a modified method[1] of Pöch, *et al.*[9] using ^{3}H-cyclic 3′,5′-AMP in a concentration of 3.6×10^{-4}M as the substrate. Enzyme activity was determined as the rate of hydrolysis of cyclic 3′,5′-AMP after incubation for 40 min at 37°.

Protein Determination——Protein contents were determined by the method of Lowry, *et al.*[10] using bovine serum albumin as standard.

Assay of Cyclic 3′,5′-AMP in Adipose Tissue——The levels of cyclic 3′,5′-AMP in adipose tissue were determined along with the examination of the degree of lipolysis in adipose tissue induced by incubation for 15 min at 37° in the same medium as was used for the determination of lipolysis. Ten to 20 mg of adipose tissues frozen with liquid nitrogen were homogenized in 0.5 ml of 6% trichloroacetic acid. After centrifugation at 4000 rpm for 15 min at 0°, 5 ml of water-saturated ether was added to the supernatant which had been separated. After shaking, the upper ether layer was discarded. This procedure was repeated twice more. Then, the lower water layer was dried by the aeration with nitrogen gas, dissolved in 1 ml of 0.05M acetate buffer, pH 6.2, and served as the sample for the determination of cyclic 3′,5′-AMP. Cyclic 3′,5′-AMP assay was performed by the method of Steiner, *et al.*[11] with the Schwarz/Mann radioimmunoassay kit.

Results

The Relationship between the Inhibitory Effect of BL 191 on PDE and Lipolysis in Rat Epididymal Adipose Tissue

As shown in Table I, BL 191 inhibited the PDE activity in the $105000 \times g$ supernatant from rat epididymal adipose tissue in a dose-related manner. PDE activity was inhibited by 30% and 71% when 1 mM and 10 mM BL 191 were added to the standard incubation medium, respectively. BL 191 increased the FFA release into the medium in parallel with the PDE inhibition, namely, FFA increased up to 141% and 336% by addition of BL 191 at 2 mM and 10 mM, respectively, into the same medium.

Table I. Effects of BL 191 on PDE and FFA Output of Isolated Adipose Tissue

Concentration of BL 191 (M)	PDE activity (*n* moles cAMP hydrolysed/mg protein/hr)	Inhibition of PDE (%)	FFA output (μEq/g tissue/hr)
0	189	—	2.2±0.3(6)[a]
1×10^{-4}	198	0	2.0±0.2(6)
3×10^{-4}	180	5	2.3±0.1(6)
1×10^{-3}	132	30	2.5±0.3(6)
2×10^{-3}	96	49	3.1±0.1(6)
5×10^{-3}	68	64	6.0±0.8(6)
1×10^{-2}	54	71	7.4±1.0(6)

PDE in the $105000 \times g$ supernatant prepared from the rat epididymal adipose tissue was incubated with BL 191 in the assay medium containing 425 μg of protein at 37° for 40 min. Each value is mean of three experiments. Figures in parentheses represent the number of samples.
a) means±S.E.M.

8) K. Itaya and M. Ui, *J. Lipid Res.*, **6**, 16 (1965).
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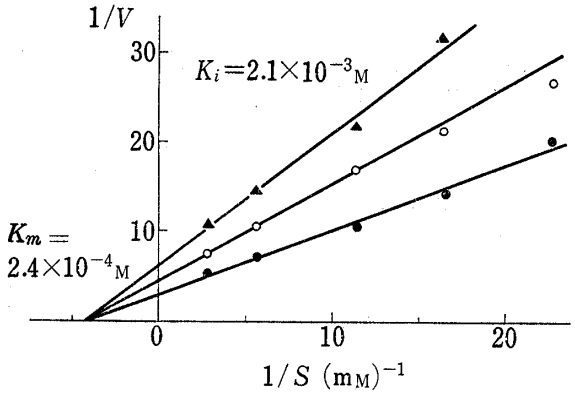

Fig. 2. Lineweaver-Burk Plot of PDE Activity in Rat Epididymal Adipose Tissue and the Inhibitory Effect of BL 191

Assays were performed as described in Experimental. Each point represents the average of three determinations. V is expressed as μ moles of cyclic 3′,5′-AMP hydrolysed per hr per mg of protein. The concentration of cyclic 3′,5′-AMP as the substrate ranged from 45 μM to 360 μM.
—●—: No inhibitor
—○—: BL 191 10^{-3}M
—▲—: BL 191 2×10^{-3}M

Lineweaver-Burk Plots of PDE Activity in Rat Epididymal Adipose Tissue

The kinetic study of PDE inhibition of BL 191 in epididymal adipose tissue was performed using Lineweaver-Burk plots when cyclic 3′,5′-AMP in concentrations ranging from 0.45×10^{-4}M to 3.6×10^{-4}M was used as the substrate, and the results are presented in Fig. 2. The K_m and K_i of BL 191 were 2.4×10^{-4}M and 2.1×10^{-3}M, respectively. And, the type of PDE inhibition of BL 191 in the 105000 $\times g$ supernatant from rat epididymal adipose tissue was non-competitive.

Effects of Epinephrine, BL 191, and Theophylline on Lipolysis in Rat Epididymal Adipose Tissue *in Vitro*

Effects of epinephrine, BL 191, and theophylline on lipolytic activity, which were determined from the rate of FFA release into the incubation medium, are shown in Fig. 3. The lipolytic action of epinephrine was dose-dependent and reached a plateau at 5×10^{-6}M, increasing about 4.3-fold over the control value which was determined without epinephrine. BL 191 also stimulated the lipolytic response in rat adipose tissue in a dose-related manner, as did theophylline. However, the maximal lipolysis stimulated by theophylline was observed at the concentration of 3×10^{-3}M and was 3.5-fold over the control value which was determined without theophylline, while the effect of BL 191 was maximal not at 5×10^{-3}M but at 10^{-2}M and was about 3.2-fold over the control value which was determined without BL 191.

The enhancing effects of BL 191 or theophylline on the lipolytic activity of epinephrine are shown in Fig. 4. BL 191 at 10^{-4}M by itself did not stimulate lipolytic activity as compared with the control activity. However, the lipolytic activity evoked by epinephrine in concentrations ranging from 10^{-7}M to 10^{-5}M was further stimulated in combination with BL 191 at 10^{-4}M. The lipolytic activity stimulated by the combination of epinephrine and BL 191 was

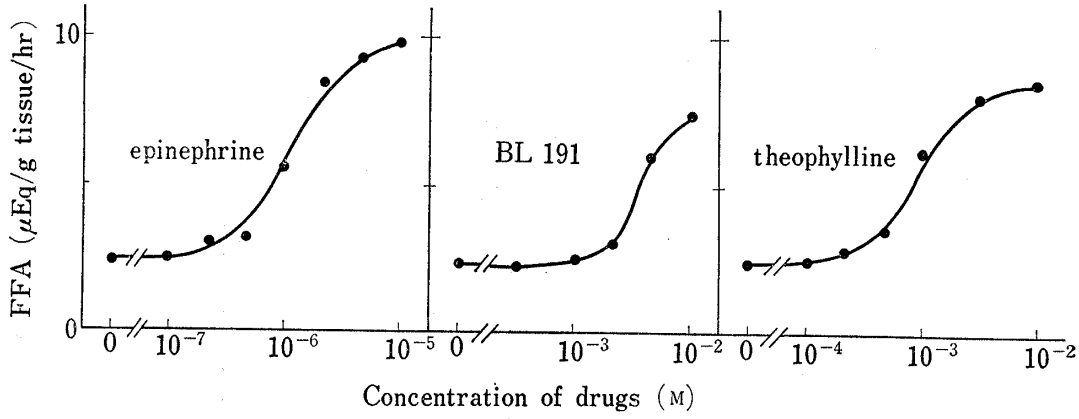

Fig. 3. Effects of Epinephrine, BL 191 and Theophylline on Lipolytic Activity in Rat Adipose Tissue

Minces of epididymal fat pads were incubated in Krebs-Ringer bicarbonate buffer with 4% bovine albumine as described in Experimental. Lipolytic activity is expressed as the rate of FFA output. Each point represents mean value of six experiments.

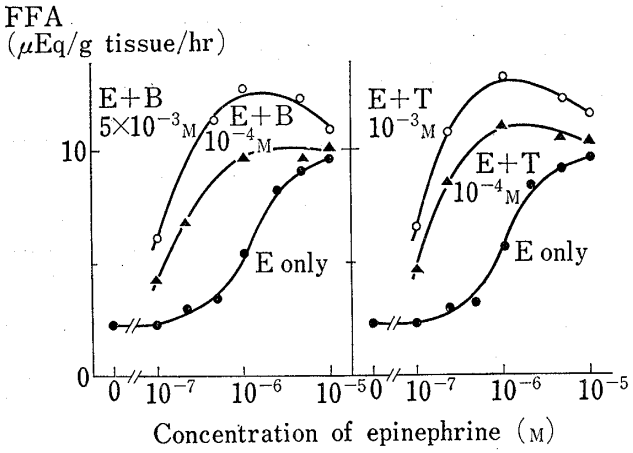

Fig. 4. Effects of Epinephrine (E) and of Its Combination with BL 191 (B) or Theophylline (T) on Lipolytic Activity in Rat Adipose Tissue

Epididymal fat pads were incubated as described in the legend for Fig. 4. Each point represents mean value of four to six experiments.

maximal when the concentration of epinephrine was 10^{-6} M, and at higher concentrations of epinephrine the lipolysis did not increase. When BL 191 at 5×10^{-3} M, which alone increased the lipolytic activity about 3-fold over the control activity, was added together with epinephrine in the same concentrations described above, the maximal lipolysis in adipose tissue was obtained when the concentration of epinephrine was 10^{-6} M, and the lipolysis gradually decreased by higher concentrations of epinephrine (5×10^{-6} M, 10^{-5} M). With theophylline at 10^{-4} M and 10^{-3}M, similar enhancing effect upon the lipolytic activity of epinephrine in concentrations ranging from 10^{-7} M to 10^{-5} M was observed.

Effects of Epinephrine, BL 191, and Theophylline on Cyclic 3′,5′-AMP in Rat Adipose Tissue

The change in cyclic 3′,5′-AMP contents in rat adipose tissue *in vitro* after the addition of epinephrine, BL 191, or theophylline is illustrated in Table II. In the preliminary experiment, it was noted that the most suitable incubation time for the measurement of cyclic 3′,5′-AMP contents in adipose tissue was 15 min which was different from that for lipolysis determination. Therefore, the contents of cyclic 3′,5′-AMP in adipose tissue were determined after the 15-min incubation. When epinephrine at 10^{-6} M, BL 191 at 5×10^{-3} M, or theophylline at 10^{-3} M alone was added to the incubation medium, cyclic 3′,5′-AMP contents in adipose tissue increased 3- to 4-fold in each case as compared with the contents before the addition of the drug. At 10^{-4} M, BL 191 or theophylline by itself increased neither cyclic 3′,5′-AMP contents nor lipolytic activity in adipose tissue. However, by addition of epinephrine at 10^{-6} M together with BL 191 or theophylline (10^{-4} M), the contents of cyclic 3′,5′-AMP increased 33-fold and 38-fold and the lipolytic activity 4.5-fold and 5.1-fold, respectively, as compared with the values which were determined in the absence of epinephrine (10^{-6} M). Increases in the contents of cyclic 3′,5′-AMP and lipolysis produced by BL 191 at 5×10^{-3} M or theophyl-

Table II. The Effects of Epinephrine, BL 191, and Theophylline on the Level of Cyclic AMP and FFA Output in Rat Epididymal Adipose Tissue

Drugs in the medium (M)	Cyclic 3′,5′-AMP (*n* moles/g tissue)	FFA (μEq/g tissue/hr)
None	0.12±0.02(4)[a]	2.2±0.3(18)[a]
Epinephrine 10^{-6}	0.49±0.13(3)	5.6±0.9(6)
Theophylline 10^{-4}	0.08±0.03(3)	2.5±0.4(6)
10^{-3}	0.38±0.17(3)	6.2±0.4(6)
BL 191 10^{-4}	0.12±0.02(3)	2.0±0.2(6)
5×10^{-3}	0.44±0.08(3)	6.0±0.8(6)
Epi. 10^{-6}+Theo. 10^{-4}	4.55±0.23(3)	11.3±0.5(4)
Epi. 10^{-6}+Theo. 10^{-3}	6.05±0.46(3)	13.4±1.2(4)
Epi. 10^{-6}+BL. 10^{-4}	3.98±0.20(3)	9.9±0.9(4)
Epi. 10^{-6}+BL. 5×10^{-3}	6.50±0.24(3)	12.8±1.5(4)

Tissues were prepared and incubated as indicated in Experimental. Numbers in parentheses indicate numbers of experiments.
a) means±S.E.M.

line at 10^{-3} M by itself were further enhanced by the addition of epinephrine at 10^{-6} M, and cyclic 3′,5′-AMP contents increased 54-fold or 50-fold and lipolysis 5.8-fold or 6.1-fold, respectively.

Discussion

As seen from the results of the present study discerned by Lineweaver-Burk plots, the K_m for PDE activity in the 105000 $\times g$ supernatant prepared from rat epididymal adipose tissue was found to be 2.4×10^{-4} M, this being almost the same as the value (1.8×10^{-4} M) for PDE activity in the 2000 $\times g$ supernatant from the same tissue reported by Pöch, *et al.*[12] Furthermore, it is apparently indicated in this experiment that BL 191 inhibits the activity of PDE in adipose tissue. The addition of BL 191 at 1 mM and 5 mM to the incubation medium inhibited PDE activity 30% and 64%, respectively, as compared with the activity in the absence of BL 191, thereby indicating the K_i value of 2.1 mM and the non-competitive type of inhibition.

In our previous paper,[1] it was shown that BL 191 inhibited PDE activity in the 105000 $\times g$ supernatant from rat cerebral cortex non-competitively with the K_i value of about 3 mM and that the type and degree of PDE inhibition of BL 191 were approximately the same as those of theophylline. In addition, the results of our study reported here show that both of the type and degree of PDE inhibition by BL 191 in adipose tissue were not different from those in cerebral cortex tissue. These results may suggest that BL 191 exerts approximately the same inhibitory effects on PDE in adipose tissue as theophylline.

When BL 191 at 5 mM, which alone inhibited adipose tissue PDE by 64%, was added to the incubation medium, the lipolytic activity was stimulated about 3-fold and this effect of BL 191 was found to be slightly less potent than that of theophylline. Recently, Krishna, *et al.* advocated the process of the mobilization of FFA illustrated in Chart 1,[13] and suggested that an increase in lipolytic response might be associated with an increased activity of adenyl cyclase or a decreased PDE activity and that the hydrolysis of triglycerides to FFA might be regulated through cyclic 3′,5′-AMP levels in adipose tissue.[4,14] In the present experiment, BL 191 at 5 mM induced about 4-fold increase in cyclic 3′,5′-AMP levels in adipose tissue, and the degree of this effect of BL 191 was approximately the same as that of theophylline. From this result, it may be said that BL 191, as well as theophylline, enhances lipolysis through an increased level of cyclic 3′,5′-AMP by inhibition of PDE.[13]

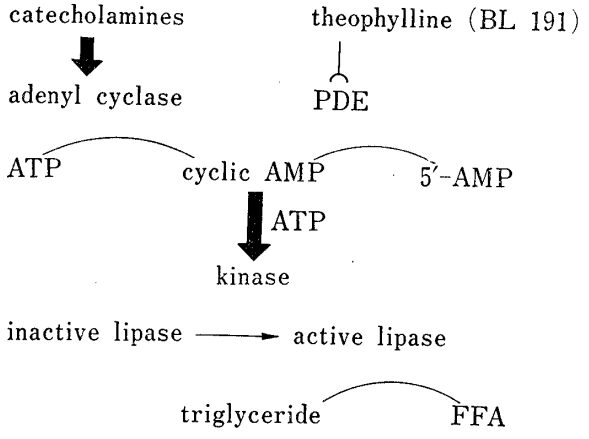

Chart 1. The Theory of FFA Mobilization[13]
↓: stimulation, ⊥: inhibition

As shown in Table II, when BL 191 at 10^{-4} M, a concentration producing no effects on either cyclic 3′,5′-AMP levels or lipolytic activity, was added together with epinephrine at 10^{-6} M, both cyclic 3′,5′-AMP levels and lipolytic activity increased. Moreover, as Fig. 4 shows, the addition of epinephrine at 10^{-6} M alone induced the submaximal stimulation of lipolysis, whereas this concentration of epinephrine together with BL 191 produced the maximal lipolysis. According to the report by Butcher, *et al.* on the relation between cyclic 3′,5′-AMP levels and lipolytic activity in rat adipose tissue,[14] epinephrine in concentrations up to 55 μM increased

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the tissue level of cyclic 3′,5′-AMP linearly but lipolytic activity reached a plateau at 5.5 μM. As regards these problems, Weiss, *et al.*[15] suggested that the response to norepinephrine is limited not by the capacity of the lipase system but by that of adenyl cyclase, and that maximal effects are attained when adenyl cyclase is fully activated. On the contrary, Moskowitz and Fain[16] maintained that the limiting step lies in the lipase system, based on the experimental result that there was no added increase in lipolysis in the presence of cyclic 3′,5′-AMP in concentrations more than a certain limit because of the small intracellular pool of cyclic 3′,5′-AMP.

At any rate, it is apparent from the results of this study that BL 191, like other xanthine derivatives, stimulates lipolysis according to such a process as is given in Chart 1. However, the mode of action cannot be explained or speculated only from the results reported here. Further investigations are necessary.

Acknowledgement Grateful acknowledgement is made to Prof. H. Ozawa of Tohoku University Pharmaceutical Institute for his constant interest and guidance in this investigation, and to Mr. S. Sawada in our laboratory for his technical assistance.

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16) J. Moskowitz and J.N. Fain, *J. Biol. Chem.*, **245**, 1101 (1970).