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ANTILIPOLYTIC ACTION OF SULOCTIDIL* IN RAT ADIPOSE TISSUE

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Abstract—(1) Suloctidil (1-(4-isopropylthiophenyl)-2-*n*-octylamino-propanol) inhibited the *in vitro* lipolysis due to the action of isoproterenol, ACTH, and dibutyryl cAMP in rat epididymal adipose tissue fragments. Fifty per cent inhibition occurred at a 2×10^{-4} M suloctidil concentration. The drug was not affecting cAMP and ATP levels. (2) Suloctidil inhibited non-competitively the hormonally stimulated activity of a crude preparation of triglyceride lipase, with a $K_{i_{app}}$ of 2.5×10^{-4} M. No effect was observed on lipoprotein lipase. (3) It is suggested that the antilipolytic action of the drug lies, at least partly, beyond the adenylate cyclase step, i.e. directly on the hormone sensitive triglyceride lipase. This effect is compared with that of nicotinic acid, clofibrate, procaine, and β-adrenergic blocking agents.

Hormone stimulated lipolysis in adipose tissue is mediated by cyclic 3',5'-adenosine monophosphate (cAMP). cAMP activates a protein kinase, which subsequently phosphorylates a triglyceride lipase, resulting in its activation [1]. This lipase in turn catalyses the stepwise hydrolysis of triglycerides to yield glycerol and free fatty acids.

A number of agents have been shown to inhibit lipolysis by interfering with some step in the lipolytic process [2, 3]. Previous work has shown that suloctidil (1-(4-isopropylthiophenyl)-2-*n*-octylamino-propanol) inhibits *in vitro* norepinephrine- and theophylline-induced lipolysis in rat epididymal fat. *In vivo*, this drug also displays hypolipidemic activity in the Rhesus monkey. In addition, suloctidil is a vascular antispasmodic agent endowed with antithrombogenic effects and with antiplatelet and blood viscosity-lowering activity [4]. In this study, the mechanism of action of suloctidil in inhibiting hormone-stimulated lipolysis in rat adipose tissue fragments was examined.

MATERIALS AND METHODS

Epididymal fat bodies were obtained from Wistar rats (140–160 g) which has been fed *ad lib.* on a standard laboratory chow (U.A.R., Villemoisson, France).

In vitro lipolysis. Forty-eight fragments were collected from four rats and distributed at random in twelve flasks. In each flask four fragments weighing approximately 160 mg altogether were incubated with shaking in 2 ml Krebs–Ringer bicarbonate medium (pH 7.4) containing 4% (w/v) albumin for 2 hr at 37° in a gas phase of 95% O_2 + 5% CO_2. The net release of glycerol was used as an index of lipolysis. Medium glycerol was measured enzymatically [5] and expressed in μmoles released/2 hr/100 mg fresh tissue.

cAMP levels. Adipose tissue fragments were preincubated in 2 ml Krebs–Ringer bicarbonate medium, containing 4% (w/v) albumin and 1 mM theophylline. After 30 min, the fragments were transferred into fresh medium containing the lipolytic agent. After 10 min (i.e. at a time corresponding to peak cAMP levels following the induction of lipolysis), the fragments were frozen at −70° in 2 ml trichloroacetic acid 5% (w/v) and then homogenized by ultrasonication. After centrifugation for 10 min at 1100 *g*, the supernatant was acidified with 0.1 ml 1 N HCl and extracted five times with 2.5 volumes diethyl ether. After lyophilisation the extract was dissolved in water and cAMP was determined by the protein binding assay of Gilman [6].

ATP levels. ATP levels were determined in adipose tissue fragments under conditions identical to those used for the measurement of lipolysis. At the end of the 2 hr incubation, the tissue fragments were frozen at −70° in 1 ml M perchloric acid. After ultrasonication, 50 μl of the internatant was used for ATP determination according to the luciferin-luciferase method of Bihler and Jeanrenaud [7]. ATP standards were also prepared in 1 M perchloric acid.

Hormone-sensitive triglyceride lipase activity (EC 3.1.1.3). Lipase activity in adipose tissue homogenates was assayed by the second method of Khoo and Steinberg [8] which is based on the hydrolysis of tri [1-^{14}C]oleylglycerol and the extraction of free [1-^{14}C]oleate by liquid-liquid partition. The homogenate was prepared from epididymal adipose tissue of rats fasted 24 hr prior to sacrifice. Fat pads were preincubated for 2 hr in 4 ml Krebs–Ringer bicarbonate buffer containing 4% bovine serum albumin. Epinephrine in a final concentration of 5×10^{-5} M was added 15 min before the end of this preincubation period. The adipose tissue was homogenized in a glass tissue grinder containing 5 vol. of 0.25 M sucrose with 1 mM EDTA. The homogenate was centrifuged at 100,000 *g* for 1 hr. The internatant was used as enzymatic source for lipase activity determination. The assay system contained in a total vol. of 0.8 ml: 1

* Solocton®, Continental Pharma, S.A., Brussels, Belgium.

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or 0.5 μmole of tri[1-^{14}C]oleylglycerol (0.125 μCi per μmole of triolein) sonicated in 2.5 mg gum arabic; 20 mg albumin; 30 μmoles EDTA; 0.2 ml of enzyme preparation, and 40 μmoles phosphate buffer (pH 6.8). After 1 hr at 30°, the reaction was stopped with 3 ml chloroform–methanol–benzene (2:2.4:1, v/v/v) enriched with 0.3 μmole unlabelled oleic acid. After adding 0.1 ml N NaOH and vortexing for 15 sec, the mixture was centrifuged at 2500 rpm for 10 min. This modification [8] of the liquid–liquid partition system of Belfrage and Vaughan [9] allowed the collection of free labelled oleic acid in the supernatant. The radioactivity present in 1.8 ml of supernatant was counted with 10 ml liquid scintillation medium (toluene 6.6 ml, Triton X-100 3.4 ml, Permablend III from Packard Instrument Co. (Downers Grove, U.S.A.) 55 mg).

Lipoprotein lipase activity (*EC 3.1.1.34*). Acetone–ether powders from the epididymal adipose tissue of rats fed *ad lib.* were prepared as described previously [10]. The enzyme activity was extracted with 0.025 M NH_4OH–NH_4Cl buffer (pH 8.6) (2 ml/g original fresh adipose tissue). After 30 min centrifugation, the 4000 *g* supernatant was used directly as the enzyme source. Lipoprotein lipase was assayed according to a minor modification of the method of Bensadoun *et al.* [11].

The assay system contained in a total vol. of 0.8 ml: 1 μmole of tri[1-^{14}C]oleylglycerol (0.125 μCi per μmole) sonicated in 2.5 mg gum arabic; 25 mg albumin; 0.08 ml rat serum for activation; 60 μmoles NaCl for low ionic strength; 6 μmoles $CaCl_2$; 0.2 ml of enzyme preparation and 120 μmoles Tris buffer (pH 8.6). The labelled free fatty acid split off from triolein after 1 hr at 30° was separated from the substrate by the modification [8] of the liquid–liquid partition system already described in the preceding paragraph.

Reagents. Suloctidil 5×10^{-4} M was slowly dissolved by 16 hr stirring at room temperature, in 10% bovine serum albumin (w/v), under the protection of streptomycin (50 μg/ml) and penicillin-G (100 U/ml). Both antibiotics were also present in control incubations and were without effect on the investigated parameters.

Materials were obtained from the following sources: Suloctidil (1-(4-isopropylthiophenyl)-2-*n*-octylaminopropanol) from Continental Pharma S.A. (Brussels, Belgium); *n*-octylamine from Aldrich-Europ (Beerse, Belgium); clofibrate (2-(*p*-chlorophenoxy)-2-methylpropionic acid ethyl ester) from Ayerst Laboratories (New York, U.S.A.); pindolol (1-(Indol-4-yloxy)-3-(isopropylamino)-2-propanol) from Sandoz (Basel, Switzerland); propanolol (1-(Isopropylamino)-3-(1-naphtyloxy)-2-propanol hydrochloride) from ICI (Macclesfield, England); procaine hydrochloride (*p*-aminobenzoyl-diethylaminoethanol) from Merck (Darmstadt, Germany); bovine serum albumin Pentex poor in fatty acid from Miles Research Co. (Kankakee, U.S.A.); glycerokinase and glycerol-3-phosphate dehydrogenase from Boehringer (Mannheim, Germany); tri[1-^{14}C]oleylglycerol from the Radiochemical Center (Amersham, England); lyophilized firefly lanterns and isoproterenol from Sigma Chemical Co. (St. Louis, U.S.A.). ACTH (synthetic corticotropin 1–24) was a gift from Ciba-Geigy (Basel, Switzerland). All other chemicals were of the highest analytical grade available.

RESULTS

Suloctidil significantly and dose-dependently inhibited lipolysis in epididymal adipose tissue maximally stimulated with 2.5×10^{-6} M isoproterenol or 1×10^{-6} M ACTH. A 2×10^{-4} M drug concentration inhibited by 50 per cent the lipolysis stimulated in the presence of both hormones. Higher concentrations of suloctidil could not be tested due to the limitations of solubility. Suloctidil, when given alone, exerted a variable inhibitory effect (Tables 1 and 4) on the low basal glycerol release. *n*-Octylamine, which is part of the molecule of suloctidil, was without effect on basal or stimulated lipolysis (Table 1).

The inhibitory effects exerted by 1×10^{-4} M or 2×10^{-4} M suloctidil on isoproterenol or ACTH-stimulated lipolysis were as pronounced as those of nicotinic acid, clofibrate, and procaine-HCl used at similar concentrations. Propanolol (1×10^{-4} M) and pindolol (3×10^{-5} M), two well known β-blockers, provoked a more important reduction of hormone stimulated lipolysis.

Isoproterenol (2.5×10^{-6} M) and 1×10^{-6} M

Table 1. Comparison of the inhibition of lipolysis by suloctidil and other drugs in rat adipose tissue fragments incubated with or without isoproterenol or ACTH

Drug	No hormone	Isoproterenol 2.5×10^{-6} M	ACTH 1×10^{-6} M
Control	0.21 ± 0.02 (24)	1.59 ± 0.12 (18)	1.24 ± 0.11 (12)
1×10^{-5} M Suloctidil	0.18 ± 0.03 (6)	1.31 ± 0.07 (6)*	1.01 ± 0.09 (6)*
1×10^{-4} M Suloctidil	0.16 ± 0.03 (24)	1.13 ± 0.03 (18)*	0.73 ± 0.13 (12)*
2×10^{-4} M Suloctidil	0.15 ± 0.05 (4)	0.84 ± 0.09 (4)*	0.66 ± 0.10 (4)*
1×10^{-4} M *N*-Octylamine	0.24 ± 0.05 (4)	1.35 ± 0.21 (4)	1.21 ± 0.09 (4)
1×10^{-4} M Nicotinic acid	0.17 ± 0.04 (12)	1.16 ± 0.09 (8)*	1.07 ± 0.09 (8)*
1×10^{-4} M Clofibrate	0.17 ± 0.02 (12)	1.07 ± 0.13 (8)*	1.03 ± 0.06 (8)*
2×10^{-4} M Procaine	0.23 ± 0.04 (8)	1.08 ± 0.04 (4)*	1.02 ± 0.04 (4)*
3×10^{-5} M Pindolol	0.64 ± 0.15 (8)*	0.75 ± 0.08 (4)*	0.97 ± 0.06 (4)*
1×10^{-4} M Propanolol	0.02 ± 0.02 (4)*	0.16 ± 0.03 (4)*	0.43 ± 0.07 (4)*

Incubations were performed at 37° for 2 hr. Results are expressed as net glycerol release in μmoles/2 hr/100 mg fresh tissue. Each value represents the mean ± S.E.M. Number of experiments in parentheses. The statistical analysis was performed on paired data according to Student's *t*-test (*$P < 0.05$: lipolysis significantly lower than the corresponding value obtained without drug).

Table 2. Effect of suloctidil and other antilipolytic agents on cAMP levels in adipose tissue, stimulated or not by isoproterenol and ACTH, in the presence of 1 mM theophylline

Drug	No hormone	Isoproterenol 2.5×10^{-6} M	ACTH 1×10^{-6} M
Control	22 ± 1 (16)	948 ± 125 (26)	839 ± 67 (7)
1×10^{-4} M Suloctidil	24 ± 3 (16)	910 ± 114 (26)	931 ± 93 (7)
1×10^{-4} M *N*-Octylamine	19 ± 1 (4)	995 ± 96	982 ± 105 (4)
1×10^{-4} M Nicotinic acid	14 ± 1 (5)*	360 ± 105 (7)*	143 ± 18 (3)*
1×10^{-4} M Clofibrate	14 ± 1 (5)*	1071 ± 120 (7)	982 ± 235 (3)
2×10^{-4} M Procaine	20 ± 1 (4)	1261 ± 85 (4)*	571 ± 136*
3×10^{-5} M Pindolol	16 ± 2 (4)*	20 ± 10 (4)*	1032 ± 101
1×10^{-4} M Propanolol	17 ± 1 (4)*	28 ± 10 (4)*	965 ± 82

All incubations were performed in the presence of 1 mM theophylline, as detailed in the methods. Results are expressed as pmoles of cAMP accumulated after 10 min/100 mg fresh tissue. Each value respresents the mean ± S.E.M. (number of experiments in parentheses).

* Significant effect of the drug considered according to Student's *t*-test on paired data ($P < 0.05$).

ACTH, after a 10 min incubation period in the presence of 1×10^{-3} M theophylline, increased cAMP levels in adipose tissue fragments by about 40 times (Table 2). Suloctidil (1×10^{-4} M) had no significant effect on either the basal or hormone-elevated levels of cAMP. Thus, the mode of action of suloctidil was different from that of nicotinic acid, which reduced cAMP levels during stimulation by isoproterenol and ACTH (Table 2). The same Table shows that isoproterenol-stimulated cAMP levels decreased dramatically in the presence of the two β-blockers, pindolol (3×10^{-5} M) and propanolol (1×10^{-4} M) while increasing moderately (by 33%) with 2×10^{-4} M procaine. Clofibrate 1×10^{-4} M was without effect on this parameter (Table 2). This is not surprising since decreases in cAMP levels are obtained only when clofibrate is used at higher concentrations. Indeed, a 3.3 mM clofibrate concentration is needed to reduce cAMP levels in rat adipocytes stimulated with norepinephrine [12] (In this respect, human fat cell adenylate cyclase is inhibited by about 25 per cent by 2 mM clofibrate [13]).

In accordance with previous observations, maximal concentrations of isoproterenol and ACTH decreased ATP levels by approximately one third, after a 2 hr incubation period (Table 3). Neither suloctidil, nicotinic acid, nor clofibrate, used at the same 1×10^{-4} M concentration, was able to modify these ATP levels (Table 3). Thus, the absence of drug effects on cAMP levels did not result from variations in ATP.

The marked stimulation of lipolysis by 1×10^{-3} M and 5×10^{-3} M dibutyryl cAMP was also inhibited by 1×10^{-4} M suloctidil (Table 4). Therefore, the essential metabolic alteration in lipolysis appeared to be beyond the formation of cAMP by adenylate cyclase.

As shown in Table 5 suloctidil, above a 1×10^{-6} M concentration, caused a dose-dependent inhibition of triglyceride lipase activity, ranging from −15 per cent at 1×10^{-5} M to −43 per cent at 1×10^{-4} M (Table 5). This inhibition was of a non-competitive type with a $K_{i_{app}}$ of 2.5×10^{-4} M (Fig. 1). By contrast, *n*-octylamine, clofibrate and nicotinic acid were without significant effect.

The activity of lipoprotein lipase, the other major adipose tissue lipase, was not influenced by suloctidil, *n*-octylamine, nicotinic acid, or clofibrate (Table 5). As a control, the same preparation was inhibited by 88 per cent in the presence of 1 M NaCl (Table 5).

DISCUSSION

Suloctidil was shown to inhibit the lipolytic activity of isoproterenol, ACTH and dibutyryl cAMP in rat adipose tissue. We were therefore able to confirm and extend the finding of Roncucci *et al.* [4] showing that the drug depresses the lipolysis stimulated by norepinephrine and theophylline.

It should be mentioned that 1×10^{-4} M suloctidil also exerted an antilipolytic effect on human fat. In a pilot study, 1×10^{-4} M suloctidil markedly inhibited basal (−64%) as well as isoproterenol- (−77%) and theophylline- (−65%) stimulated lipolysis in frag-

Table 3. Effect of suloctidil, nicotinic acid and clofibrate on ATP levels in adipose tissue following stimulation of the tissue by isoproterenol or ACTH

Drug	No hormone	Isoproterenol 2.5×10^{-6} M	ACTH 1×10^{-6} M
Control	20.8 ± 2.2	13.0 ± 2.1	14.1 ± 0.5
1×10^{-4} M Suloctidil	16.8 ± 0.3	11.8 ± 2.1	16.7 ± 2.2
1×10^{-4} M Nicotinic acid	18.8 ± 0.8	13.4 ± 2.1	18.4 ± 1.1
1×10^{-4} M Clofibrate	19.2 ± 1.6	12.3 ± 1.2	20.3 ± 1.1

Incubation conditions were identical with those described in Table 1. Each value is expressed as nmoles ATP/100 mg fresh tissue and represents the mean ± S.E.M. of four experiments performed in duplicate.

Table 4. Effect of suloctidil on dibutyryl cAMP induced lipolysis

Addition	No drug	Suloctidil 1×10^{-4} M
Control	0.06 ± 0.01	0.03 ± 0.01*
1×10^{-3} M Dibutyryl-cAMP	1.16 ± 0.10	0.96 ± 0.05*
5×10^{-3} M Dibutyryl-cAMP	1.31 ± 0.04	1.11 ± 0.08*

Incubations were performed as indicated in Table 1. Each value is expressed as μmoles glycerol/2 hr/100 mg fresh tissue and represents the mean ± S.E.M. of five experiments.

* Significant inhibition by suloctidil according to Student's *t*-test on paired data ($P < 0.05$).

ments of mesenteric human adipose tissue (data not shown).

Suloctidil exerted no action on the steps involved in the elevation of cAMP levels as the agent had no effect on either basal or hormone-stimulated cAMP levels in adipose tissue (Table 2). It can be concluded that suloctidil acted distal to the production of cAMP. This is at variance with the mode of action of β-adrenergic blocking agents, prostaglandins, nicotinic acid and clofibrate, which prevent hormone-stimulated lipolysis, by inhibiting the activation of adenylate cyclase and the formation of cAMP (Table 2 and refs [3, 12]). Another group of antilipolytic agents, including α-adrenoceptor blocking agents, sulfonylurea drugs, the biguanide phenformin, naphtoquinones, tricyclic antidepressants of the dibenzazepin group, and local anesthetics (such as procaine) inhibit hormone-stimulated lipolysis under conditions of an unchanged or even increased cAMP concentration (Table 2 and refs [3, 14, 15]). Evidence for the ability of these drugs to interfere with adenylate cyclase activity and low K_m cAMP phosphodiesterase through calcium movements is still under debate.

At first view, it might be tempting to include suloctidil in this second group of agents. However, our results provide a simpler biochemical explanation on the manner in which suloctidil lowers lipolysis.

Suloctidil was able to reduce triglyceride lipase activity. This activity was measured in 100.000 *g* internatants from fat pads preincubated for 15 min with epinephrine prior to homogenization, i.e. on fully active lipase. Suloctidil acted non-competitively on this enzyme activity (Fig. 1) at concentrations which inhibited the lipolytic response of intact fat tissue (Table 1). Taken together, these results suggest that the antilipolytic action of suloctidil reflected a direct inhibition of triglyceride lipase.

The *n*-octylamine part of suloctidil was found to be inactive (Tables 1 and 5). It remains to be established which structural changes in the substituted basic phenethylamine molecule produce the full activity of suloctidil. Suloctidil is able to inhibit two other lipases: pancreatic lipase [16] and milk lipoprotein lipase [4]. However, our negative data on adipose tissue lipoprotein lipase (Table 5) show that the drug does not inhibit all lipolytic activities present in mammalian tissues.

Suloctidil shares this property of inhibiting lipolysis by direct action on adipose tissue triglyceride lipase with only a few compounds such as the antiphlogistic drug benzydamine [17] and the stilbene derivative clomiphene [18]. A direct inhibitory effect of tolbutamide on triglyceride lipase has been suggested [19] but also denied [14].

We tried to exclude the possibility that suloctidil possesses additional effects on the lipolytic system at the level of cAMP-dependent protein kinase(s). Unfortunately, we were unable to test the effect of the drug on the binding of cAMP to adipose tissue protein kinase(s) or on the phosphorylating capacity of the free catalytic subunit(s), since the albumin concentration necessary to dissolve suloctidil interfered with the assay procedures.

In conclusion, suloctidil does not act as a β-blocker nor as a local anesthetic. Its mode of action on lipolysis is also different from that of the two hypolipide-

Table 5. Effect of suloctidil, *N*-octylamine, clofibrate, nicotinic acid and NaCl on the activity of hormone sensitive triglyceride lipase and lipoprotein lipase

Addition	Activated triglyceride lipase	Lipoprotein lipase
Suloctidil 1×10^{-6} M	102 ± 3	99 ± 2
Suloctidil 1×10^{-5} M	85 ± 3	97 ± 3
Suloctidil 5×10^{-5} M	73 ± 1*	100 ± 2
Suloctidil 1×10^{-4} M	57 ± 2*	107 ± 4
N-Octylamine 1×10^{-6} M	97 ± 4	101 ± 3
N-Octylamine 1×10^{-5} M	98 ± 3	100 ± 3
N-Octylamine 1×10^{-4} M	87 ± 3	101 ± 2
Clofibrate 1×10^{-6} M	104 ± 2	95 ± 3
Clofibrate 1×10^{-5} M	103 ± 4	97 ± 2
Clofibrate 1×10^{-4} M	114 ± 3	97 ± 2
Nicotinic acid 1×10^{-5} M	100 ± 2	96 ± 3
Nicotinic acid 1×10^{-4} M	108 ± 3	98 ± 2
NaCl 1 M	—	12 ± 2*

The results are expressed relative to the value without the test substance, i.e. in % of control (Means ± S.E.M. of five experiments performed in triplicate).

* Significant inhibition by the drug considered according to Student's *t*-test on paired data ($P < 0.05$).

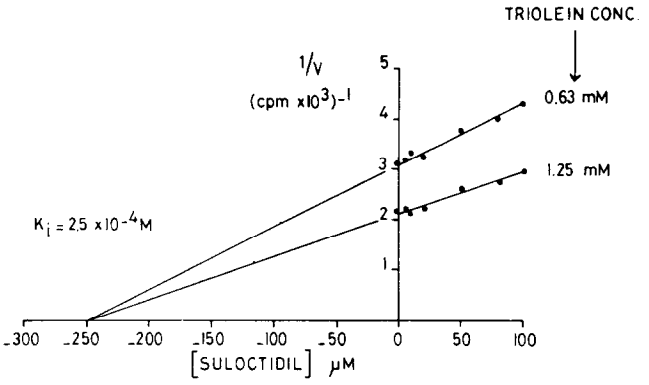

Fig. 1. Dixon's plot [20] illustrating the non-competitive inhibition exerted by suloctidil on activated triglyceride lipase extracted from rat adipose tissue (details of the preparation in the Methods). Incubations were performed for 1 hr at 30°. The two triolein concentrations used were 1.25 mM and 0.63 mM. Means of three separate experiments.

mic agents clofibrate [12] and nicotinic acid [3], which inhibit adenylate cyclase but have no effect on triglyceride lipase. It is clear, however, that much investigation would be needed to see if suloctidil might contribute to the treatment of hyperlipemia.

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