Abstract—Effects of Phthalazinol (EG 626), a cyclic AMP phosphodiesterase inhibitor, a thromboxane A₂ antagonist and an antiatherosclerotic agent was examined regarding lipolytic enzyme activities in rat epididymal adipose tissue. The effect of Pyridinolcarbamate (Anginin) was concomitantly examined. There was a significant decrease in serum triacylglycerol levels in rats given EG 626 (100-500 mg/kg), p.o. for 1-3 weeks. In adipose tissue from EG 626 treated rats, the basal and adrenalin induced lipolysis, and cholesterylester hydrolase activity were markedly enhanced, while the phosphodiesterase activity was decreased. Anginin treatment had no effect either on the serum lipid levels or the cholesterylester hydrolase activity. An elevation in cholesterylester hydrolase activity and lipolysis by EG 626 was observed both in vivo and in vitro. Incubation of the adipose tissue with 0.229 mM of EG 626 or 0.603 mM of theophylline induced a lipolysis equivalent to that seen with 2.7×10⁻² mM of adrenalin. These results indicate that EG 626 exerted marked effects on lipolysis and cholesterylester hydrolase activity, probably through inhibition of phosphodiesterase. Possible contributions of the enhanced cholesterylester hydrolase activities to the antiatherogenic effect were discussed.

Phthalazinol (EG 626) is a phthalazin derivative with the chemical structure shown in Fig. 1. Pharmacological studies have shown that EG 626 has an antagonistic effect on
thromboxane A₂ (1, 2) and a potent inhibitory effect on cyclic-AMP phosphodiesterase activity (3, 4). EG 626 prevents the contraction of rabbit aortic strips induced by thromboxane A₂ (1). Shimamoto (2) observed that myocardial infarction induced in rabbits by an intracoronary injection of thromboxane A₂ could be prevented by pretreating the animals with EG 626. The inhibitory effect on cyclic-AMP phosphodiesterase has been demonstrated in various organs including the aorta and platelets (3). EG 626 has further been shown to possess an inhibitory effect on platelet aggregation (5), a preventive effect on arterial cholesterol accumulation (6) and on the decrease of cyclic-AMP in the atheromatous lesion in the aorta of the cholesterol-fed rabbit (7).

Rat adipose tissue contains multiple acyl hydrolase activities—lipoprotein lipase (LPL), hormone-sensitive triacylglycerol lipase, diacylglycerol hydrolase, monoacylglycerol hydrolase (8-11), cholesterylester hydrolase (12, 13). Cholesterylester hydrolase activity was first demonstrated in human adipose tissue by Arnaud and Boyer (12). Pittman et al. found that the enzyme from rat adipose tissue was activated to about the same degree as the triacylglycerol lipase from adipose tissue by cyclic-AMP dependent protein kinase (13). Cholesterylester hydrolase from rat adrenal is also phosphorylated to an active form by cyclic-AMP dependent protein kinase (14). Therefore, it was of interest to examine the effect of EG 626, a potent phosphodiesterase inhibitor, on the two enzymes: hormone sensitive triacylglycerol lipase and cholesterylester hydrolase in adipose tissue.

In attempts to elucidate the mechanism of the antiatherogenic effect of EG 626 in lipid metabolism, we examined in vivo and in vitro effects of EG 626 on the lipolysis and cholesterylester hydrolase activity in adipose tissue which contained triacylglycerols and cholesterol in dynamic equilibrium with plasma lipids. Effects of Pyridinolcarbamate (Fig. 1) (15) were simultaneously examined.

MATERIALS AND METHODS

Animal treatment: Male Wistar rats weighing about 150 g were maintained on the usual laboratory chow diet and were given orally EG 626 (100–500 mg/kg) or Anginin (200 mg/kg) suspended in 1% carboxymethyl cellulose during the indicated term. Control rats were given only the vehicle. The animals were fasted for 12 hr and given the last dose of drugs 5 hr before sacrifice.

Analysis of serum lipids: Serum cholesterol and triacylglycerols were determined according to the method of Zak and Dickenman (16), and Block and Jarret (17), respectively.

Assay of adipose tissue cholesterylester hydrolase activity (E.C. 3.1.1.13): The epididymal adipose tissue (1 g) was homogenized by Polytron (PT 10, Kinematica, GMBH, Lausanne, Switzerland) in 10 vol. of 0.03 M phosphate buffer (pH 7.4) and centrifuged for 10 min at 1000 g. The clear supernatant was utilized for assay of cholesterylester hydrolase. Cholesterylester hydrolase activity was measured in terms of release of [1-¹⁴C]-oleic acid from cholesteryl-[1-¹⁴C]-oleate (54 mCi/nmol, NEN, Boston, Mo., U.S.A.). The substrate solution was prepared according to the method of Brecher et al. (18) by sonicating labelled compounds with egg yolk lecithin in 0.01 M Tris (pH 7.4)/0.1 M NaCl/0.01% sodium azide.
The reaction mixture for cholesterylester hydrolase assay contained 50–100 µl the supernatant, 20 µl the substrate solution (cholesteryl-[1-14C]-oleate, about 5 × 10^4 dpm/3.5 nmol) and 100 µl 0.1 M phosphate buffer (pH 6.5) (total 0.2 ml). The reaction was carried out for 1.5 hr at 37°C, and stopped by the addition of 0.6 ml 0.3 M NaOH. An extraction solvent (CHCl₃: MeOH : Benzene, 0.5 : 1.2 : 1) containing 0.1 mM oleic acid (3 ml) was then added and the mixture shaken vigorously. The radioactivity (released [1-14C]-oleic acid) in the aqueous layer was counted using a liquid scintillation counter (Aloka LSC, 602, Nihon Musen, Tokyo).

Measurement of adrenalin induced lipolysis: The method used was as described elsewhere (19). In short, a 100 mg of epididymal adipose tissue was preincubated for 15 min at 37°C in 3 ml Krebs-Ringer Henseleit bicarbonate buffer (pH 7.4)/0.54 mg glucose/ml. Each piece was transferred to a tube with 1 ml of the same buffer containing various amounts of adrenalin (Merck, Darmstadt, Germany) and incubated for 2 hr at 37°C under a gas phase of 95% O₂–5% CO₂. The reaction was stopped by quickly chilling the tube in ice and the tissue was removed. Glycerol released in the medium was assayed using the enzyme spectrophotometric technique (20).

Assay of adipose tissue 3',5' cyclic-AMP phosphodiesterase (E.C. 3.1.4.17) activity (21): About 200 mg of the epididymal adipose tissue excised from rats after a 12 hr fast was homogenized in 5 vol. of 0.03 M phosphate buffer (pH 7.5) and the homogenate was centrifuged for 20 min at 1000 g. The supernatant was assayed for phosphodiesterase activity. The reaction mixture for phosphodiesterase assay contained 10 µl enzyme solution and 30 µl substrate solution containing 0.3 µCi [8-3H]-cyclic AMP (30 mCi/µmol, The Radio-Chemical Center, Amsterdam), 0.09 nmol cyclic-AMP (Kohjin Co. Tokyo), 3 µg snake venum (Sigma, St. Louis, Mo.) and 0.2 µmol MgCl₂ (Wako Chemicals, Tokyo) in 50 mM Tris-HCl buffer (pH 7.4). The reaction was carried out for 10 min at 37°C and stopped by the addition of 10 µl 2NHCl in the cold. The mixture (5 µl) was applied on a TLC plate (Polygram cell DEAE 300, 20×20 cm, Macherey-Nagel Co., Düren, Germany) and the plate was developed with 1 M ammonium acetate: 95% EtOH (30 : 75). Spots were visualized under a ultra violet lamp (2536Å) and identified with standards; cyclic-AMP, adenosine (Kohin, Co., Tokyo) and 5'-AMP (Boehringer Mannheim Co., Mannheim, Germany). Each spot was scraped into a counting vial and the radioactivity was counted.

RESULTS

Physical parameters of the experimental animals: Administration of EG 626 (100–500 mg/kg) for less than 3 weeks did not affect growth, while 3 weeks of Anginin treatment (200 mg/kg) slightly enhanced the growth of these rats. There was no liver enlargement such as was seen in the case of Clofibrate (22, 23). When EG 626 and Anginin were administered less than 2 weeks, the weight of the epididymal adipose tissue tended to decrease.

Serum lipid levels: Table 1 shows changes in the serum cholesterol and triacylglycerol levels. There was a significant and consistent decrease in the triacylglycerol level after administration of EG 626, while the serum cholesterol level was decreased only with a 3
week treatment of EG 626 (200 mg/kg). Anginin had no effect on either level. Serum non-esterified fatty acid was significantly lower in the 2 week EG 626 treated group (200 mg/kg) than in the control group (0.72±0.047 μmol/ml, P<0.05).

Adrenalin-induced lipolysis in the epididymal adipose tissue: Figure 2 shows the effect of EG 626 and Anginin on adrenalin-induced lipolysis of the epididymal adipose tissue. EG 626 treatment significantly enhanced the basal lipolytic activity (the activity in the absence of adrenalin), while Anginin had no effect. Incubation with a low concentration of adrenalin (2.7×10^-4 mM=0.05 μg/ml) did not alter the lipolytic activity in the adipose tissue of control rats, whereas it greatly enhanced lipolysis in tissues from EG 626 or Anginin treated rats. At a high concentration of adrenalin (2.7×10^-2 mM=5 μg/ml) lipolysis was fully activated, while EG 626 treatment apparently had no further effects on the maximum 

**TABLE 1. Effect of EG 626 and Anginin on serum lipid levels**

| Exp. | Term (days) | Drug     | Dose (mg/kg) | No. of Rats | Cholesterol (mg %) | Triacylglycerols (mean±SE) |
|------|-------------|-----------|--------------|-------------|---------------------|---------------------------|
| 1    | 6           | None      | 8            | 8           | 50±2                | 66±3                      |
|      |             | EG 626    | 100          | 8           | 49±2                | 56±2* (-15%)              |
|      |             |           | 500          | 8           | 55±2                | 41±2** (-38%)             |
|      |             |           | 100          | 7           | 51±3                | 44±4** (-33%)             |
| 2    | 14          | None      | 13           | 8           | 57±1                | 78±2                      |
|      |             | EG 626    | 200          | 13          | 59±3                | 62±1** (-21%)             |
|      |             | Anginin   | 200          | 6           | 58±1                | 80±4                      |
| 3    | 21          | None      | 8            | 8           | 51±1                | 60±2                      |
|      |             | EG 626    | 200          | 8           | 44±2** (-14%)       | 53±2* (-12%)              |
|      |             | Anginin   | 200          | 7           | 50±2                | 60±1                      |

* Significance p<0.05, ** Significance p<0.01

FIG. 2. Effect of EG 626 and Anginin on the lipolytic response to adrenalin in epididymal adipose tissue in vivo. After a 12 hr fast, the epididymal adipose tissue was excised and approximately 100 mg of the tissue was incubated for 2 hr at 37°C in 1 ml of Krebs-Ringer Henseleit bicarbonate buffer (pH 7.4) in the presence or absence (basal activity) of adrenalin. Glycerol released in the medium was assayed using the spectrophotometric technique (18).
lipolysis. Anginin treatment slightly enhanced this activity.

**Cholesterylester hydrolase activity of the epididymal adipose tissue:** Figure 3 shows the change in cholesterylester hydrolase activity of the epididymal adipose tissue due to EG 626 treatment. The treatment markedly enhanced the cholesterylester hydrolase activity, particularly in the short term experiment, while Anginin treatment had no effect.

**Phosphodiesterase activity of the epididymal adipose tissue:** As the increase in lipolysis and cholesterylester hydrolase activity in the adipose tissue from EG 626 treated rats suggested elevation of the cyclic AMP level, phosphodiesterase activity in the tissue was measured in tissues from the 3 week EG 626 treated rats (200 mg/kg) and the control rats. Phosphodiesterase activity in the treated rats (0.861±0.0411 [7] nmol adenosine/mg protein/10 min) was significantly lower than that in control rats (1.061±0.0092 [6] nmol adenosine/mg protein/10 min).

**Effects of EG 626 on cholesterylester hydrolase activity and lipolysis in vitro:** As the administration of EG 626 exerted marked effects on lipolysis and cholesterylester hydrolase activity in the adipose tissue, the *in vitro* effect was examined. When the fat pad was incubated with EG 626 (1 mM) for 20 min at 37°C, the cholesterylester hydrolase activity was

![Figure 3](image-url)
elevated by 25% (Table 2) compared to the activity seen when incubation was carried out without addition of the drug. The activation by EG 626 was comparable to that seen with theophylline (1 mM) or adrenalin (2.7 × 10⁻² mM). Figure 4 shows the in vitro effect of EG 626 on lipolysis of adipose tissue. An incubation of the fat pad with EG 626 (0.05–1.0 mM), theophylline (1.0–1.0 mM) or adrenalin (2.7 × 10⁻⁴ and 2.7 × 10⁻² mM) gradually enhanced lipolysis with the increasing concentrations. The ratio of lipolytic activity in the presence of the drugs to the basal activity in their absence was linear to the log dose of the drugs. Equal lipolytic response to adrenalin (2.7 × 10⁻² mM) was produced with 0.229 mM EG 626 and 0.603 mM theophylline.

DISCUSSION

Adipose tissue is the major storage organ not only for triacylglycerols but also for cholesterol (24). The pools of triacylglycerols and cholesterol in adipose tissue are in dynamic equilibrium with plasma lipids through functions of lipolytic enzymes in the tissue. Adrenalin-induced lipolysis is reported to be mediated by cyclic AMP through activation of adrenergic β-receptors (25). In adipose tissue from our rats treated with EG 626, basal lipolysis was significantly enhanced compared with that of control rats, and in the treated rats only, lipolysis was greatly enhanced by a lower concentration of adrenalin. With a higher concentration of adrenalin, lipolysis was stimulated to the same extent in tissues from both the treated and control animals. Since phosphodiesterase activity was lower in adipose tissue following the EG 626 treatment, the enhancement in lipolysis, both basal and adrenalin-induced, and in the activity of cholesteryl ester hydrolase was probably mediated by the inhibitory effect of cyclic AMP phosphodiesterase. Cyclic AMP produced with incubation of a low concentration of adrenalin would therefore be less likely to decompose in the
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epididymal adipose tissue from the treated animals and could induce the lipolytic response. With a higher concentration of adrenalin, an excessive amount of cyclic AMP would be produced. Thus, with such a slight difference in the catabolic rate of the nucleotides, the rate of lipolysis was not influenced. Anginin treatment significantly enhanced adrenalin-induced lipolysis at the high and low concentrations but there was no effect on the basal lipolysis. As this compound apparently has no effect on phosphodiesterase, the mechanism of the enhancement by Anginin probably differs from that by EG 626.

Cholesterylester hydrolase activity was enhanced by EG 626 treatment to almost the same extent as the basal lipolysis except for the two week treatment, and the activation of the enzyme was observed on incubation of adipose tissue not only with EG 626 but also with other cyclic AMP modulators; adrenalin and theophylline. Accordingly, our observations suggest that the activation of cholesterylester hydrolase by EG 626 is the result of cyclic AMP dependent phosphorylation of the enzyme protein.

Nikkila et al. observed that there was a highly significant positive correlation between the plasma HDL cholesterol level and LPL activity in adipose tissue, and they proposed that the rate of catabolism of triacylglycerol rich lipoprotein might be one of the factors to determine the concentration of HDL in plasma (26). The physiological functions of adipose tissue cholesterylester hydrolase are obscure. It has been shown that adipose tissue can take up cholesterol from plasma lipids both in vivo and in vitro (27–29), and that the cholesterylester of lipoproteins is hydrolyzed during uptake by adipocytes (18). Thus, the enhancement of cholesterylester hydrolase activity in adipose tissue by treatment with EG 626 may produce a change in the distribution of cholesterol in HDL and LDL. In fact, though the mechanism is unclear, a long term administration of EG 626 (one year, 50 mg/kg, p.o.) resulted in an significant elevation in the ratio of HDL- to LDL-cholesterol in the rat. Thus, antiatherosclerotic effects of EG 626 would be exerted through alteration in plasma lipids as well as through alteration in arterial lipolytic enzyme activities. Effects of EG 626 on rat arterial enzyme activities were reported elsewhere (30).

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