Effects of Isoprothiolane and Phytosterol on Lipogenesis and Lipolysis in Adipocytes from Rats of Dietary Fat Necrosis

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ABSTRACT. To study effects of isoprothiolane and phytosterol on dietary fat necrosis, 3 groups of rats were fed hardened-tallow (HT) diet. Two groups of rats received either isoprothiolane (50 mg/kg) or phytosterol (20 mg/kg) orally once a day consecutively for 10 weeks. One group of rats received standard diet (CE-2) as a control. Fat necrotic lesions were observed in epididymal and perirenal adipose tissues from all rats in the 3 groups fed HT diet. Rats with fat necrosis were characterized by visceral type obesity and saturation in fatty acid composition of triglyceride in adipose tissue. The highest glucose conversion to total lipids was seen in adipocytes from the rats given phytosterol. There was no lipolytic response to epinephrine stimulation (1–100 μ M) in adipocytes from the rats given only HT diet, while similar response of adipocytes from the 2 groups treated with either drug to those from the rats fed standard diet was observed. The levels of total saturated fatty acids of phospholipid in adipose tissue from the rats given either drug were lower than that of the rats given only HT diet. These data suggest that either drug alters fatty acid composition of phospholipid in fat cell membrane and enhances lipolysis of the cells.—**KEY WORDS:** adipocyte, fat necrosis, isoprothiolane, phytosterol, rat.

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Adlay (*Coix Lachryma-jobi*) [15] and by-product of soybean oil extraction [10] have been used for the treatment in bovine fat necrosis. However, these should be administered over a long period of time, *i.e.*, 4 months, and the therapeutic effects were refractory. Isoprothiolane (diisopropyl 1,3-dithiolan-2-ylidenemalonate) [11, 17] and phytosterol [11] are more effective than adlay on this disease. The efficacy of these drugs was evaluated by morphological changes of necrotic fat masses. Nevertheless the mechanism of the therapeutic effect of each drug has not been well elucidated. Recently, we found that an administration of isoprothiolane or phytosterol altered serum lipoprotein level and fatty acid composition of phospholipid in adipose tissue in the rats given saturated fat diet [7].

In the present work, fat necrosis was successfully induced in the rats by feeding of more volume saturated fat diet than that of the previous study [7], and the effects of

isoprothiolane and phytosterol on glucose utilization and lipolytic activity in rat adipocytes were evaluated.

MATERIALS AND METHODS

Drugs: Isoprothiolane and phytosterol were supplied by Nihon Nohyaku Co., Ltd., Tokyo, Japan and Kaken Pharmaceutical Co., Ltd., Tokyo, Japan, respectively.

Materials: Bovine insulin (23.4 IU/mg) and epinephrine from Sigma Chemical Co., Ltd., bovine serum albumin (BSA, fraction V) from Armour Pharmaceutical Co., collagenase (type I) from Worthington Biochemical Corp., and D-[U- 14 C] glucose from Amersham Japan Ltd., were used.

Animals and diet: Male Sprague-Dawley rats, 5 weeks of age, were used. Hardened-tallow (HT) diet and stock diet (CE-2) were prepared by Nihon Clea Co., Ltd., Tokyo, Japan. Composition of HT diet has been reported previously [7]. Composition of

fatty acids in experimental diets is listed in Table 1. CE-2 diet contained 24.8% crude protein, 4.4% crude fat, 3.5% crude fiber and 51.6% nitrogen-free extract.

Experimental design: Rats were randomly divided into 4 groups of 6 rats each. Rats in group 1 through 3 were fed HT diet and those in group 4 were fed CE-2 diet for 10 weeks. All animals had free access to the respective feed and water. Isoprothiolane was suspended in 2% arabic gum solution to give a concentration of 2.5% (W/V) and was administered orally to the rats of group 1 in a dose of 50 mg/kg daily for 10 weeks. Phytosterol was also suspended in 2% arabic gum solution to give a concentration of 1% (W/V) and was administered orally to the rats of group 2 in a dose of 20 mg/kg daily for 10 weeks. Rats in group 3 received equal volume of arabic gum solution with HT diet. Rats in group 4 were fed CE-2 diet as a control through the same experimental period. Animals were killed between 10 AM and 11 AM by decapitation. Epididymal and perirenal adipose tissues and livers were immediately excised and weighed. The ratios of these tissues to body weight were estimated.

Preparation of isolated adipocytes: Krebs-Ringer bicarbonate buffer added with 25 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was used in this experiment. Isolated fat cells were prepared

by shaking at 37°C for 60 min in 6 ml of Hepes-Krebs-Ringer bicarbonate (H-KRB) buffer, pH 7.4, containing 2 mg of collagenase, 4% BSA and 3 mM glucose, according to the method of Rodbell [14]. Cells were then filtered through 250 μ m nylon mesh. After three times washings with collagenase-free buffer, they were suspended in H-KRB buffer containing 2% BSA. Adipocyte were counted under a microscope with a Bürker-Türk counting chamber.

Experiment on glucose metabolism: Duplicate aliquots of the fat cell suspension ($0.2\text{--}6.6 \times 10^4$ cells/0.2 ml) were incubated in plastic scintillation vials of 20 ml volume at 37°C with H-KRB buffer, pH 7.4, containing 2% BSA, 0.25 μ Ci of [U- 14 C]glucose, 0.56 μ mol of unlabelled glucose and insulin concentration ranging from 0 to 1 mU/ml. The final volume was adjusted to 1 ml. After 2 hr of incubation, the reaction was stopped by addition of 0.5 ml of 8 N H₂SO₄ to the medium. Another plastic vials containing filter papers absorbed with 0.3 ml of Hyamine hydroxide were connected on the incubated vials by bottom opened plastic tubes to trap evolved CO₂. CO₂ was collected during additional 30 min incubation and scintillation fluid was added to the vials containing filter papers. The contents of the incubation vials were extracted by the method of Dole *et al.* [4]. Aliquots of the lipid extracted were transferred into counting vials and used for the measurement of 14 C incorporation into total lipids. Blanks were run in parallel with each experiment by incubating cells in the presence of acid (8 N H₂SO₄) in order to correct all the measured parameters for nonmetabolic 14 C labeling. 14 C lipids and CO₂ were counted in a scintillation solution consisting of 0.4% 2,5-diphenyloxazole and 0.004% 1,4-bis[2-(5-phenyloxazolyl)] benzene in toluene. Radioactivity was determined in a Aloka liquid scintillation system (Model LSC-900, Aloka, Tokyo).

Table 1. Composition of fatty acids of the experimental diets (wt. % of total fatty acids)

Fatty acids	CE-2	HT
14:0	0.3	3.3
16:0	12.6	27.2
16:1	2.2	2.7
17:0	0.1	1.3
18:0	3.6	36.7
18:1	25.5	24.6
18:2	51.4	3.8
18:3	4.4	0.4
TSFA ^{a)}	16.5	68.6

a) Total saturated fatty acids.

Experiment on lipolysis: Adipocytes ($0.2\text{--}6.6 \times 10^4$ cells/0.2 ml) were incubated in plastic tubes at 37°C for 1 hr with H-KRB buffer, pH 7.4, containing 4% BSA and 3 mM glucose in the absence or presence of epinephrine ($1\text{--}1000 \mu\text{M}$). The final volume was adjusted to 1 ml. Glycerol blanks were prepared in incubation medium containing no cells. At the end of the incubation period, the medium was cooled in ice and filtered through $25 \mu\text{m}$ nylon mesh to get cell free buffer. The glycerol released from adipocytes in the buffer was measured enzymatically by Glycerol and Neutral fat UV Test Kit (Boehringer Mannheim, Germany).

Fatty acid analysis: Lipids from diet and epididymal adipose tissue were extracted by the method of Folch *et al.* [5]. Triglyceride and phospholipid were separated by thin-layer chromatography on 0.5 mm thick silica gel 60 G (Merk, Germany) plates using the solvent system consisting of petroleum ether: diethyl ether: acetic acid (80:20:1). Fatty acid methyl esters of triglyceride and phospholipid were prepared with 5% (W/V) methanolic hydrogen chloride at 70°C for 2.5 hr and analyzed by gas liquid chromatography (Model GC-3BF, Shimadzu Co., Kyoto, Japan) equipped with a $2.1 \text{ m} \times 3 \text{ mm}$ i.d. glass column containing 10% diethylene glycol succinate (DEGS) on Chromosorb W (80–100 mesh). The column temperature was 190°C . The peak areas were calculated by an integrator (Chromatopac,

Model C-R6A, Shimadzu Co.). The identification of fatty acid methyl esters was accomplished using GLC Reference Standards for Fatty Acid Methyl Ester kit (Nu-chek-Prep Inc., United States).

Histopathological examination: Epididymal and perirenal adipose tissues were fixed in 10% formalin solution and embedded in paraffin. Tissue sections were stained with H.E. Frozen sections of adipose tissues were stained with Sudan III.

Statistical analysis: Differences among treated groups and control group were compared using an analysis of variance by Duncan's new multiple range test [19].

RESULTS

Animal condition: Mean body weight of rats in the 3 groups fed HT diet was significantly lower than that of rats fed CE-2 diet (Table 2). The ratios of epididymal and perirenal adipose tissues to body weight were greater in the 3 groups fed HT diet than those of the control group. In the rats given isoprothiolane, the ratio of liver to body weight was higher than other groups.

Pathological findings: In the gross examination of epididymal and perirenal adipose tissues, the flecks of white material were observed in 8 rats fed HT diet (4 rats in group 1, 2 rats in group 2 and 2 rats in group 3). In all of these rats, microscopic examination of sections showed necrotic change of fat cells (Fig. 1). The lesion consisted of the

Table 2. Body weight and the ratio of epididymal and perirenal adipose tissue and liver to body weight in rats

Groups	Diet and medication	Body weight (g)	Ratio of tissue to body weight $\times 100$		
			Epididymal adipose tissue	Perirenal adipose tissue	Liver
1	HT + Isoprothiolane	368.7 ± 9.6^a	1.84 ± 0.14^a	2.93 ± 0.16^a	3.89 ± 0.14^a
2	HT + Phytosterol	396.3 ± 12.0^a	1.59 ± 0.15^a	2.26 ± 0.22^b	3.31 ± 0.07^b
3	HT	408.7 ± 12.8^a	1.71 ± 0.12^a	2.77 ± 0.20^{ab}	3.56 ± 0.06^{bc}
4	CE-2	452.7 ± 17.3^b	1.15 ± 0.08^b	1.48 ± 0.19^c	3.60 ± 0.07^c

Data represent the mean $\pm$ SE of six rats per group. Means within a column not sharing a common superscript differ significantly ($P < 0.05$).

Fig. 1. Necrotic change of fat cells in the rats fed hardened-tallow diet. Infiltration of multinucleated foreign body giant cells (G), histiocytes (H) and epithelioid cells (E) into the affected fat cells is seen. HE stain, $\times 240$.

infiltration of multinucleated foreign body giant cells, histiocytes and epithelioid cells into the affected fat cells. Some epithelioid cells had the processes of cytoplasm extended toward the center of fat cell vacuole. Necrotic change involved one or more adjacent fat cells and the affected cells were interspersed among normal cells. The flecks of white materials observed grossly in adipose tissues of some rats were microscopically massive necrosis of fat cells. Severity of lesions was similar among the 3 groups fed HT diet. In the frozen sections of the adipose tissue stained with Sudan III, the multiple crystals aggregated in star-shaped clusters appeared in fat cells of the rats fed HT diet. Necrotic lesion and crystals were not detected in the rats fed CE-2 diet (Fig. 2).

[U- 14 C]Glucose metabolism: Less oxida-

Fig. 2. Fat cells in the rats fed CE-2 diet. Necrotic lesion was not detected. HE stain, $\times 240$.

tion for $[U-^{14}C]$ glucose to CO_2 was found in adipocytes from the 2 groups given either drug than that in the other 2 groups regardless of the presence of insulin (Fig. 3). Stimulation of insulin for glucose oxidation in adipocytes from the 3 groups fed HT diet was not noted as compared with the control group. The highest $[U-^{14}C]$ glucose conversion to total lipids in adipocytes from the rats given phytosterol was seen regardless of the presence of insulin (Fig. 4). There was no significant difference between adipocytes from the rats given only HT diet and isoprothiolane.

Lipolysis of adipocytes: In the absence of epinephrine, more glycerol was released from adipocytes of the 3 groups fed HT diet than that of the control group (Fig. 5). There was no response to epinephrine stimulation over the concentration range 1–100 μM in adipocytes from the rats given only HT diet. Similar response of adipocytes

Fig. 3. Effect of insulin on D-[U-¹⁴C]glucose oxidation by adipocytes from the rats given HT diet + isoprothiolane (□), HT diet + phytosterol (△), HT diet (○) and CE-2 diet (●). Data represent the mean ± SE of 12 experiments. Means not sharing a common superscript differ significantly ($P < 0.05$).

Fig. 4. Effect of insulin on the conversion of D-[U-¹⁴C] glucose to total lipids by adipocytes from the rats given HT diet + isoprothiolane (□), HT diet + phytosterol (△), HT diet (○) and CE-2 diet (●). For further details see Fig. 3.

from the 2 groups given isoprothiolane or phytosterol to those from the control group was observed.

Composition of fatty acid: The fatty acid composition of triglyceride in adipose tissue is listed in Table 3. The levels of saturated and monounsaturated fatty acids, *e.g.*, 14:0, 16:0, 16:1, 18:0 and 18:1, were higher in the 3 groups fed HT diet than those in the control group. There was no difference, however, among 3 groups fed HT diet. The differences of fatty acid composition among 3 groups fed HT diet were shown in phospholipid of adipose tissue. In the rats given isoprothiolane, the level of 16:0 was lower and the levels of 18:1 and 18:2 were higher than those of the rats given only HT diet (Table 4). Higher level of 14:0 and lower level of 16:0 were seen in the rats given

phytosterol as compared with the rats given only HT diet. As a result, two groups of the rats given isoprothiolane or phytosterol had the lower levels of total saturated fatty acids of phospholipid than that of the rats given only HT diet.

DISCUSSION

In Japan, fat necrosis is most frequent in Japanese Black cattle of high quality beef with abundant marbling. It was suggested that disordered endocrine function of the pancreas and subsequent abnormality of lipid metabolism were related to this disease [10].

In rats, it is reported that the feeding of high levels of saturated long-chain fatty acids or their esters have resulted in abdo-

Fig. 5. Effect of epinephrine on glycerol release by adipocytes from the rats given HT diet + isoprothiolane (□), HT diet + phytosterol (Δ), HT diet (○) and CE-2 diet (●). For further details see Fig. 3.

Table 3. Fatty acid composition of triglyceride in rat adipose tissue (wt. % of total fatty acids)

Fatty acids	Groups (Diet and medication)			
	1 (HT + Isoprothiolane)	2 (HT + Phytosterol)	3 (HT)	4 (CE-2)
14:0	3.4±0.3 ^a	3.2±0.2 ^{ab}	2.7±0.1 ^b	1.0±0.0 ^c
14:1	tr.*	0.1±0.1	0.2±0.1	—**
15:0	0.3±0.0 ^{ab}	0.4±0.0 ^a	0.1±0.0 ^a	0.3±0.0 ^b
15:1	tr.	0.1±0.0	0.1±0.0	0.1±0.0
16:0	31.4±0.8 ^a	30.2±1.5 ^a	28.7±0.8 ^a	23.9±0.5 ^b
16:1	6.8±0.2 ^a	6.9±0.2 ^a	6.3±0.4 ^a	4.3±0.4 ^b
17:0	0.9±0.1 ^a	1.0±0.0 ^a	0.9±0.1 ^a	0.2±0.0 ^b
17:1	0.6±0.1 ^{ab}	0.7±0.0 ^a	0.7±0.0 ^a	0.3±0.1 ^b
18:0	13.3±0.2 ^a	13.0±0.3 ^a	13.2±0.5 ^a	3.5±0.1 ^b
18:1	37.9±0.9 ^a	38.6±1.0 ^a	39.8±0.6 ^a	27.3±0.4 ^b
18:2	5.3±0.2 ^a	5.7±0.7 ^a	6.7±0.3 ^a	36.3±1.1 ^b
18:3	—	—	0.3±0.1	2.9±0.1
TSFA ⁺	49.3±1.0 ^a	47.9±1.7 ^a	45.9±1.0 ^a	28.8±0.6 ^b

Data represent the mean ± SE of six rats per group. Means within a row not sharing a common superscript differ significantly ($P < 0.05$).

* Less than 0.1%.

** Not detected.

+ Total saturated fatty acids.

minal fat necrosis [3, 6]. Although lipid metabolism in monogastric animals is essentially different from that in ruminant animals, histopathological changes and fatty acid composition of necrotic fat lesions are

similar to those in cattle [16]. In this study, dietary fat necrosis was induced histopathologically in all rats of 3 groups by feeding HT diet. The rats with fat necrosis showed lower body weight and greater ratios of

Table 4. Fatty acid composition of phospholipid in rat adipose tissue (wt. % of total fatty acids)

Fatty acids	Groups (Diet and medication)			
	1 (HT + Isoprothiolane)	2 (HT + Phytosterol)	3 (HT)	4 (CE -2)
12:0	0.2±0.1 ^a	0.2±0.1 ^a	0.7±0.1 ^b	0.1±0.1 ^a
13:0	0.1±0.0 ^a	0.2±0.1 ^a	1.5±0.6 ^b	0.1±0.1 ^a
13:1	0.1±0.1 ^a	0.4±0.1 ^a	1.8±0.8 ^b	0.7±0.1 ^{ab}
14:0	5.8±2.7 ^a	14.6±3.5 ^b	7.0±2.2 ^a	3.9±0.4 ^a
14:1	0.1±0.0 ^a	0.2±0.1 ^a	0.7±0.1 ^b	0.8±0.1 ^b
15:0	1.4±0.2 ^a	1.6±0.4 ^{ac}	2.5±0.3 ^{bc}	3.1±0.4 ^b
15:1	0.5±0.1 ^a	0.9±0.3 ^{ac}	1.3±0.2 ^{bc}	1.6±0.2 ^b
16:0	26.3±2.0 ^a	24.0±1.3 ^a	37.9±1.6 ^b	38.5±1.5 ^b
16:1	4.6±0.3 ^a	6.0±0.9 ^{ab}	4.7±0.3 ^a	7.3±0.6 ^b
17:0	1.9±0.2	2.8±0.9	2.1±0.2	2.1±0.2
17:1	1.2±0.2	2.1±1.1	1.4±0.5	1.4±0.2
18:0	19.2±1.9 ^a	14.6±1.0 ^b	16.6±1.6 ^{ab}	13.2±1.1 ^b
18:1	26.7±1.3 ^a	22.0±2.2 ^{ab}	17.7±1.1 ^b	22.4±1.7 ^{ab}
18:2	6.0±1.8 ^a	3.1±0.4 ^{ab}	0.5±0.2 ^b	1.9±1.2 ^b
18:3	0.7±0.2	0.8±0.3	0.4±0.4	0.5±0.3
19:0	1.6±0.5	2.4±0.7	0.9±0.1	1.4±0.5
20:0	1.3±0.2	2.3±0.5	1.7±0.6	0.6±0.4
20:2	0.2±0.2	0.1±0.1	—*	—
20:3	0.9±0.2	0.8±0.2	—	—
21:0	1.1±0.3	1.1±0.4	0.5±0.2	0.4±0.4
TSFA ⁺	58.9±2.4 ^a	63.6±2.5 ^a	71.4±0.9 ^b	63.2±2.9 ^a

Data represent the mean ± SE of six rats per group. Means within a row not sharing a common superscript differ significantly (P<0.05).

* Not detected.

+ Total saturated fatty acids.

abdominal adipose tissues to body weight than those in control rats. This indicates that feeding of high levels of saturated fat produces imbalance of nutrition and visceral type obesity in rats. The obesity at the growing stage also has been considered as a predisposition to fat necrosis in cattle [18]. In histopathological examination, isoprothiolane and phytosterol were ineffective in reducing severity of necrotic fat lesions, indicating that these two drugs may not have the effects to prevent the formation of causative materials which trigger a proliferation of interstitial cells and macrophages in adipose tissues.

The glucose metabolism studies showed decreased glucose oxidation in the 2 groups treated with either drug and increased glucose conversion to total lipids in the rats given phytosterol. Stimulation for glucose metabolism by insulin is markedly diminished in adipocytes from rats fed high-fat

diet [9] and in large adipocytes from older rats [13] compared to small adipocytes from younger animals. This blockade in fatty acid synthesis is apparently due to diminished activities of acetyl CoA carboxylase and fatty acid synthetase and may reflect a biochemical feedback mechanism that operates to restrict further enlargement of these already fragile cells [13]. Therefore, the present result suggests that adipocytes from the rats given phytosterol still retain greater capacity to accumulate triglyceride than those from the rats of other groups. Since the size of the adipocytes was not measured in this study, it is unknown whether the adipocytes from the rats given phytosterol are smaller than those from the rats of other groups. However, this possibility is suggested by the finding that the rats given phytosterol showed the lowest ratios of adipose tissues to body weight in the 3 groups fed HT diet.

The response of adipocytes to lipolytic action of epinephrine was not demonstrated in the rats given only HT diet over the concentration range 1–100 μ M. On the other hands, similar response of adipocytes from the 2 groups treated with either drug to those from the rats fed standard diet was observed. Amount of glycerol release in adipocytes from 2 groups treated with either drug was larger than that of the rats given only HT diet. The mobilization of fatty acids from the adipose tissues has been lower both in rats and cattle with fat necrosis as compared with that in normal animals [16]. It was reported that lipolysis [2] and glucose utilization [1] in adipose tissue of rats fed a diet rich in saturated fatty acids were lower than those in adipose tissue of rats fed a diet rich in polyunsaturated fatty acids and those alterations correlated with changes in adipose tissue lipid composition. We found that the 2 groups treated with each drug had the lower levels of total saturated fatty acids of phospholipid in adipose tissue than that of the rats given only HT diet. In the previous study [7], although each drug altered fatty acid composition of phospholipid in adipose tissue of rats fed HT diet, each did not decrease the levels of total saturated fatty acids of phospholipid. These different results may be attributed to the differences of fatty acid composition of HT diet and age of animals. The mechanism of epinephrine-induced lipolysis has been explained in terms of the cyclic AMP theory, in which epinephrine stimulates adenylate cyclase in the fat cell membrane and increases the cyclic AMP content of the cells resulting in ultimate stimulation of hormone-sensitive lipase [8]. Moreover, it is assumed that adipocyte phospholipid may have an important role in hormone-induced lipolysis [12]. Therefore, our results suggest that accelerated lipolytic response of the adipocytes from the 2 groups treated with either drug may be associated with the alterations in

fatty acid composition of phospholipid in adipose tissue. Further investigations are needed in bovine fat necrosis.

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要 約

食餌性脂肪壊死ラットの脂肪細胞の脂肪合成と脂肪分解に対するイソプロチオランおよびファイトステロールの効果：片本 宏・栗原伸二・島田保昭(大阪府立大学農学部家畜外科学教室)——食餌性脂肪壊死症に対するイソプロチオランおよびファイトステロールの効果を調べるため、3群のラットを硬化牛脂を含む(HT)飼料で飼育し、その内2群のラットにイソプロチオラン(50 mg/kg)またはファイトステロール(20 mg/kg)を毎日経口的に10週間連続して投与した。また標準(CE-2)飼料で飼育したラットを対照とした。HT飼料で飼育した3群すべてのラットの精巢上体および腎周囲脂肪組織において、脂肪壊死の病変が観察された。脂肪壊死を発症したラットの特徴として内臓脂肪増加型肥満および脂肪組織のトリグリセライドの脂肪酸組成の飽和化が認められた。グルコースの脂質への取り込みは、ファイトステロールを投与したラットの脂肪細胞で最も高かった。HT飼料のみを与えたラットの脂肪細胞において、エピネフリン刺激(1-100 μ M)に対する脂肪分解の反応性は見られなかったが、両薬剤を投与したラットの脂肪細胞では標準飼料で飼育したラットの脂肪細胞と同様な反応性が見られた。両薬剤を投与したラットの脂肪組織リン脂質の総飽和脂肪酸比率はHT飼料のみを与えたラットのものに比較して低かった。これらの結果から両薬剤は脂肪細胞の膜リン脂質の脂肪酸組成に変化を与え、脂肪細胞における脂肪分解を促進することが示唆された。