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Regulation of Insulin-Sensitive Cyclic Nucleotide Phosphodiesterase in Adipocytes of Streptozotocin-Induced Diabetic Rats[†]

Kyung Sun Park[‡], Myung Soon Lee and Kyung Hwan Kim*

Department of Pharmacology, Yonsei University Wonju College of Medicine, Wonju 220-701
**Department of Pharmacology, Yonsei University College of Medicine, Seoul 150-752*

ABSTRACT

Possible changes in the role of insulin-sensitive cyclic nucleotide phosphodiesterase(PDE) in mediating the antilipolytic action of insulin were investigated in adipocytes from streptozotocin-induced diabetic rats. Isolated adipocytes prepared from epididymal adipose tissue were incubated, with or without insulin, at 37℃ for 15 min following pretreatment with various drugs or toxins, and three (plasma membranes, microsomal membranes, and cytosol) fractions prepared by differential centrifugation were then assayed for cAMP phosphodiesterase activity. The PDE activities only in the crude microsomal (P2) fractions were activated by insulin both in diabetic and control rats. The basal PDE activities in P2 fractions of adipocytes from diabetic rats were higher than those from control rats, although the maximal effects observed at 2 nM of insulin, 100 μM of isoproterenol or the combination of both were not significantly different from each other. The insulin-stimulated PDE activities in P2 fractions of adipocytes from diabetic rats were not changed by PIA, a A_1 adenosine receptor agonist, whereas they were decreased to the basal PDE activities in those from control rats. In addition, the adipocytes from diabetic rats showed an increased sensitivity to pertussis toxin compared to those from controls. There were no differences between diabetic and control rats in the sensitivity of adipocytes to cholera toxin. These data indicate that the impaired signalling through inhibitory receptors such as adenosine receptors in adipocytes from streptozotocin-induced diabetes relates to the loss or the decreased function of G_i proteins, and leads to the increased activity of the insulin-dependent PDE at the basal states.

Key Words: Insulin-sensitive cAMP phosphodiesterase, Adipocytes, Streptozotocin, Experimental diabetes

INTRODUCTION

When adipocytes (Kono *et al.*, 1975; Smoke and Solomon, 1989; Makino *et al.*, 1992; Manganiello *et al.*, 1992) or hepatocytes (Heyworth *et al.*, 1983; Pyne *et al.*, 1987) are incubated with insulin, it stimulates low K_m cyclic nucleotide phosphodiesterase (insulin-sensitive PDE). It has been reported that in case of adipocytes, the enzyme appears to the associated with the endoplasmic reticulum (Anderson *et al.*, 1989) and its activation is important for insulin action. Since the first step of insulin action is its binding to specific receptors on the plasma membranes, a transmembrane signal is

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[‡] To whom correspondence should be addressed.

required for the subsequent steps to occur (Czech, 1977). The detailed molecular mechanisms of insulin action are still not clear. However, several lines of evidence suggest that insulin may stimulate insulin-sensitive PDE in adipocytes by promoting its phosphorylation (Degerman *et al.*, 1990; Manganiello *et al.*, 1991; Smith *et al.*, 1991).

Insulin-associated activities, such as glucose utilization, glucose transport, and antilipolytic action are known to be impaired in streptozotocin-induced diabetic rats, although increased insulin binding to its receptor has been reported. The effects of streptozotocin-induced diabetes on the insulin-sensitive PDE activities in adipocytes are still controversial. Solomon *et al.* have shown that insulin-sensitive PDE activities are decreased in epididymal fat pads from streptozotocin-induced rats (Solomon, 1975; Solomon *et al.*, 1981). On the contrary, there are several reports indicating that the enzyme activities are even higher and shows an increased sensitivity to insulin in diabetic rats than control rats (Zumstein *et al.*, 1980; Makino *et al.*, 1983).

The present study was undertaken to clarify possible changes in the role of insulin-sensitive PDE in mediating the antilipolytic actions of insulin in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Chemicals

[8-^{3}H]cAMP (specific activity: 26 Ci/mmol) was purchased from Amersham International plc (Green End Aylesbury, Buckinghamshire, UK); Bovine serum albumin (BSA), fatty acid-free BSA, crude bacterial collagenase, HEPES, EDTA, cAMP, isoproterenol, (−)-N^6-(R-phenylisopropyl) adenosine (PIA), 5'-nucleotidase, leupeptin, pepstatin, PMSF and benzamide from Sigma Chemicals (St. Louis, MO, USA); DEAE-Sephadex from Pharmacia (Uppsala, Sweden); Phosphodiesterase (PDE) and streptozotocin from Boehringer Mannheim (Mannheim, FRG); Okadaic acid, H-7, cholera and pertussis toxins from RBI (Natick, MA, USA); Ketostix® and Diastix® from Miles Sankyo Co. (Tokyo, Japan); GF/B glass microfiber filter from Whatman International Ltd. (Maidstone, UK); Insulin Actrapid® Pork from Green Cross Co. (Seoul, Korea); All other chemicals were of analytical grade.

Animals

Male Sprague-Dawley rats weighing 170 to 230 g, which had been acclimatized in the animal care facilities of the university for more than a week, were used in all experiments. Rats were allowed free access to food and tap water, under a light-dark cycle with the light on from 6 a.m. to 6 p.m..

Induction of experimental diabetes

In all experiments, animals were divided into two groups. Animals received streptozotocin (50 mg/kg body wt., dissolved in the citrate buffer containing 50 mM citric acid and 50 mM sodium citrate, pH 4.5) or an equivalent volume of vehicle under light ether anesthesia into a tail vein (Rerup, 1970). The animals were then sacrificed one week following streptozotocin treatment. Levels of glucose and ketone body were checked in the urine using Ketostix® and Diastix®, respectively, every 2 or 3 days and around 10 a.m. on the day of sacrifice, and rats whose blood glucose concentrations were over 300 mg/dl and ketone body was negative were selected for the diabetic group. The two parameters of control rats were all negative. Under the light ether anesthesia 0.5 ml of blood was collected from the inferior vena cava using a syringe with heparin and stored in the refrigerator for the determination of the blood glucose by the glucose oxidase method (Raabo and Terkildsen, 1960).

Preparation of isolated fat cells

Isolated fat cells were prepared according to the method of Rodbell (1964). Rats were killed by cervical dislocation around 10 a.m. to avoid any circadian variations and epididymal fat pads were quickly removed and pooled in Buffer A (118 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 3 mM pyruvate, 25 mM HEPES, pH 7.4). These fat fads were then incubated with crude collagenase (3 mg/g fat tissues /3 ml Buffer A) at 37℃ for 1 hour in a Dubnoff shaking incubator (110 cycles/min). The isolated fat cells were gently filtered through a fine mesh silk screen. The cell suspensions were centrifuged in a IEC clinical centrifuge at 1,000 rpm for 10 sec

and the infranatants were removed. After four volumes of Buffer A were added to them, the tubes were centrifuged under the same conditions. This washing procedure was repeated a total of four times.

Treatment with insulin and other drugs

The above prepared fat cells (5×10^4 cells) were incubated at 37℃ for 15 min in a 7.5 ml portion of Buffer A containing 20 mg/ml BSA. The stabilized cells were treated with insulin (INS) for 15 min following pretreatment with isoproterenol (ISO), PIA, okadaic acid (OKA) or H-7 (H7) for 15 min. To see the effects of cholera toxin (CT) or pertussis toxin (PT), fat cells were incubated with the toxin at 37℃ for 60 min, washed two-fold using Buffer A containing 20 mg/ml BSA, and then treated with above-mentioned drugs.

Following the incubation, the fat cells were washed once with Buffer B (0.25 M Sucrose, 1 mM $MgCl_2$, 19 mM HEPES-NaOH, pH 7.5, 10~15℃) containing protease inhibitors (0.1 mM PMSF, 2 mM benzamide, 0.1 μg/ml pepstatin, 0.1 μg/ml leupeptin). The washed cells were then homogenized using Polytron (setting at 7, 10 sec) and the resulting homogenates were centrifuged at 6,500 rpm (3,900 x g) for 3 min in Sorvall RC-5B centrifuge at room temperature (McKeel and Jarett, 1970; Anderson *et al.*, 1989). The supernatant was separated from the pellet (P1 fraction) and centrifuged at 20,000 rpm (37,000 x g) for 30 min. The final pellet (P2 fraction) was separated from the supernatant (S2 fraction). The P1 and P2 fractions were resuspended in 200 μl of cold Buffer C (50 mM HEPES-NaOH, 5 mM $MgCl_2$, pH 7.4). P1, P2, and S2 fractions were stored in 50 μl-aliquots in a −70℃ deep freezer.

Determination of cAMP PDE activity

cAMP PDE activity was determined by a modification of the method of Thompson and Appleman (1971). The total volume of the incubation medium was 300 μl which contained about 100,000 dpm of [^{3}H]cAMP, 0.01 mM cAMP, 0.1 mg/ml BSA, 5 mM $MgCl_2$, 50 mM HEPES-NaOH (pH 7.5). Reaction was started by adding membrane fractions into the tubes and carried out at 30℃ for 10 min. Reaction was terminated by adding 50 μl of 0.2 N HCl into the tubes, and the reaction mixtures were neutralized with 50 μl of 0.2 N NaOH. Into tubes were added 50 μl of 5'-nucleotidase, and they were incubated at 37℃ for 15 min. [^{3}H]adenosine formed was separated from [^{3}H]cAMP using DEAE-Sephadex mini column (0.5×2 cm), and the radioactivity from [^{3}H] adenosine was counted in a Beckman liquid scintillation counter.

Determination of protein concentrations

Protein concentrations were determined by the method of Bradford using BSA as standard (Bradford, 1976).

Data analysis

Comparisions between groups were carried out using the Student *t*-test.

RESULTS

Distribution of PDE activity in subcellular fractions

Adipocytes from diabetic and control rats were homogenized and the homogenates were subject to differential centrifugation to compare the subcellular localizations of PDE activities (McKeel and Jarett, 1970; Anderson *et al.*, 1989). Table 1 shows typical analysis of the subcellular fractions. The increases in PDE activities in response to insulin were mainly found in the crude microsomal fractions (P2 fractions), which contained approximately to 10 to 13% of the total activity. There were no significant differences between diabetic and control groups in the total PDE activities of P2 fractions.

Effects of cAMP regulators on insulin-sensitive PDE activity

In order to see if there are any changes in receptors linked to adenylyl cyclase, the effects of cAMP regulators on PDE activities of adipocytic P2 fractions were compared (Fig. 1)(Makino and Kono, 1980; Smith and Manganiello, 1988; Robinson *et al.*, 1989; Degerman *et al.*, 1990). In basal states, PDE activities of P2 fractions of adipocytes from diabetic rats were 1.3 to 1.6-fold higher compared with those of control rats. Insulin (2 nM) in-

Table 1. Distribution of PDE activity in subcellular fractions

Fraction	Treatment	PDE activity (pmoles/min/mg protein)		Total PDE activity (pmoles/min)			
		Controls	Diabetics	Controls		Diabetics	
P1	None	33.6±1.9	45.5±6.3	1.97	9.9%	4.80	14.8%
	Plus insulin	32.0±1.6	46.7±2.2	2.52	9.7%	5.99	16.6%
P2	None	26.5±1.6	43.4±1.6	2.40	12.1%	3.30	10.1%
	Plus insulin	44.3±2.8	59.4±3.8	3.06	11.8%	4.67	13.0%
S2	None	2.3±0.1	3.3±0.1	15.55	78.1%	24.45	75.1%
	Plus insulin	2.8±0.4	3.4±0.4	20.30	78.4%	25.35	70.4%

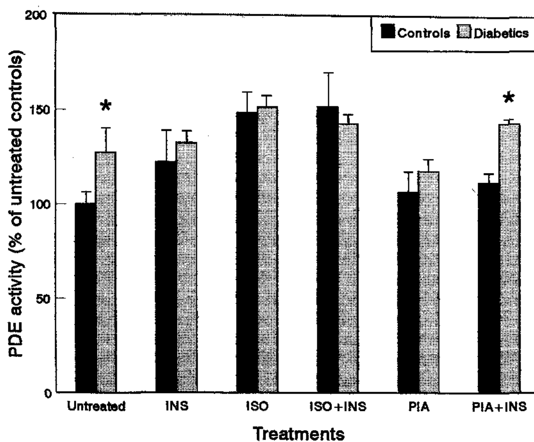

Fig. 1. Effects of insulin and adenylyl cyclase regulators on PDE activities of adipocytes P2 fractions from diabetic and control rats. Adipocytes were incubated with 2 nM insulin for 15 min following pretreatment with 10^{-4} M isoproterenol or 10^{-4} M phenylisopropyladenosine for 15 min. Values are given as means±standard errors of triplicate determinations.

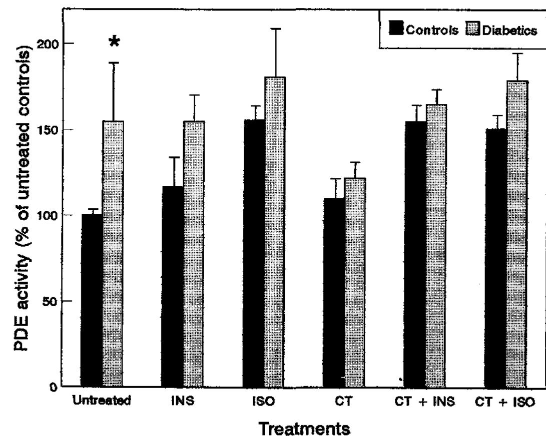

Fig. 2. Effects of cholera toxin on PDE activities of adipocyte P2 fractions from diabetic and control rats. Adipocytes were incubated with 2 nM insulin for 15 min following pretreatment with cholera toxin (0.1 μg/ml) for 1 hr. Values are given as means±standard errors of triplicate determinations.

creased PDE activities of P2 fractions 1.0 to 1.4-fold in diabetic rats, and 1.2 to 1.7-fold in control rats. One micromolar concentration of isoproterenol increased the activities of P2 fraction 1.2-fold in diabetic rats, and 1.5 to 1.6-fold in control rats. The PDE activities of P2 fractions when activated by both insulin and isoproterenol were not significantly different from those when activated only by isoproterenol. However, it was interesting to see that adipocytes of diabetic rats reacted differently to PIA, A_1 adensoine receptor agoinst, in the presence of insulin from those of control rats (Schwabe *et al.*, 1973; Elks *et al.*, 1987). In other words, the PDE activities stimulated by insulin were not changed by PIA, whereas they were decreased to the basal activities in control rats.

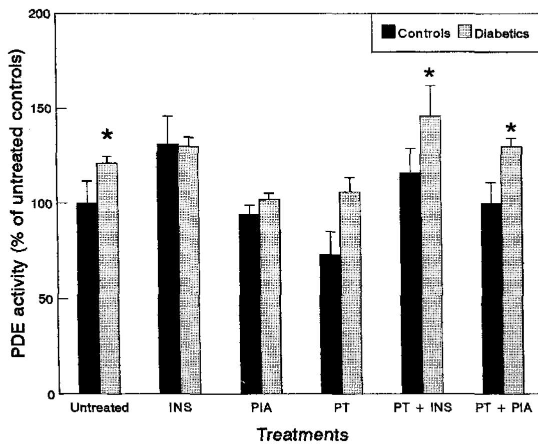

Fig. 3. Effects of pertussis toxin on PDE activities of adipocyte P2 fractions from diabetic and control rats. Adipocytes were incubated with 2 nM insulin for 15 min following pretreatment with pertussis toxin (0.1 μg/ml) for 1 hr. Values are given as means $\pm$ standard errors of triplicate determinations.

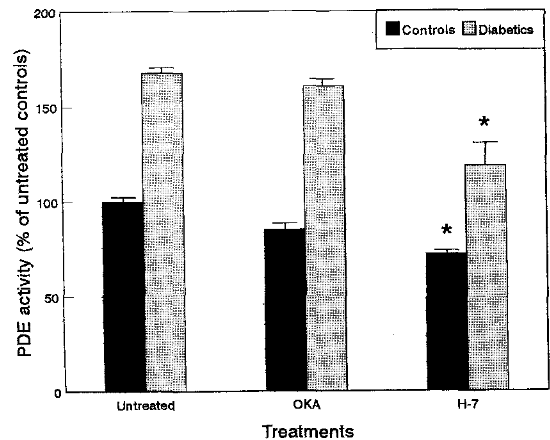

Fig. 4. Effects of okadaic acid and H-7 on PDE activities of adipocyte P2 fractions from diabetic and control rats. Adipocytes were incubated with 2 nM insulin for 15 min following pretreatment with 10^{-7} M okadaic acid or 10^{-4} M H-7 for 15 min. Values are given as means $\pm$ standard errors of triplicate determinations.

Effects of cholera and pertussis toxins on insulin-sensitive PDE activity

In order to see if there are any changes in G proteins, we first compared the effects of cholera toxin on PDE activities of adipocytic P2 fractions from diabetic and control rats (Fig. 2) (Owens *et al.*, 1985). Adipocytes were incubated with 2 nM insulin for 15 min following pretreatment with 100 ng/ml cholera toxin for 1 hour. The PDE activities following pretreatment with cholera toxin in both groups showed no significant differences in PDE activities of P2 fractions compared with those when incubated without cholera toxin.

Next, the effects of pertussis toxin (Olansky *et al.*, 1983; Elks *et al.*, 1983; Goren *et al.*, 1985; Northup and Hollenberg, 1985) on PDE activities of adipocytic P2 fractions from diabetic and control rats were compared (Fig. 3). Adipocytes were incubated with 2 nM insulin and 10^{-4} M PIA for 15 min following pretreatment with 100 ng/ml pertussis toxin for 1 hour. The PDE activities of P2 fractions from diabetic rats were significantly increased by pertussis toxin pretreatment, whereas they were not changed in control rats.

Effects of H-7 and okadaic acid on insulin-sensitive PDE activity

Okadaic acid, a potent inhibitor of Type 1 and Type 2A protein phosphatase, was employed to see if there are any differences in the role of phosphatase in insulin action on insulin-sensitive PDE between diabetic and control rats (Fig. 4)(Shibata *et al.*, 1991*a*). The PDE activities were little affected by okadaic acid in both groups when adipocytes were incubated with 0.1 μM okadaic acd for 15 min.

H-7 is a potent inhibitor of protein kinases including protein kinase A (Shibata *et al.*, 199*b*). Therefore, in the experiment shown in Fig. 4, we compared the effects of H-7 on the insulin-stimulated PDE activities between diabetic and control rats. H-7 was shown to decrease PDE activities of P2 fractions approximately 30% in both groups.

DISCUSSION

cAMP is an important intracellular second messenger mediating the hormonal regulation of lipolysis in adipocytes. Incubation of rat adipocytes with insulin or lipolytic hormones which activate adenylyl cyclase, such as catecholamines, results in activation of particulate low K_m PDE (insulin-sensitive PDE) with little or no effect on the activity in the superanatant. It has been reported that, in the presence of a maximally effective concentration of insulin (0.1~3 nM), the activation is maximal within 10~12 min in contrast to 2~3 min with isoproterenol and remains relatively constant for the next 20~25 min (Smith and Manganiello, 1988).

In the present study, we have shown that the basal PDE activities in crude microsomal fractions of adipocytes from diabetic rats were increased compared with those from control rats (Fig. 1). The maximal effects stimulated by insulin or isoproterenol were not significantly different from each other. Makino *et al.* (1983) have shown that in diabetic rats the dose-response curve of insulin shifted to the left, suggesting an increased sensitivity in fat cells from streptozotocin diabetic rats. It is interesting to note that Solomon *et al.* (1981) reported that one effect of streptozocin-induced diabetes was a specific reduction in the activity of the low K_m membrane-bound cAMP phosphodiesterase in adipose and liver tissue.

As shown in Fig. 1, the insulin-stimulated PDE activities in P2 fractions of adipocytes from diabetic rats were not lowered by PIA, a A_1 adenosine receptor agonist, whereas they were decreased to the basal PDE activities in those from control rats. This effect is consistent with our observation that adipocytes from diabetic rats showed an increased sensitivity to pertussis toxin compared those from controls (Fig. 3). There were no differences between diabetic and control rats in the sensitivity of adipocytes to cholera toxin (Fig. 2).

Several lines of evidence suggest that rat adipocytes are subject to tonic inhibitory regulation by endogenously produced adenosine. Since the removal of endogenously adenosine inhibition in rat adipocytes with either adenosine deaminase (Schwabe *et al.*, 1973) or pertussis toxin (Olansky *et al.*, 1983) drastically increases cAMP concentrations and protein kinase A activity, and since insulin-sensitive PDE in adipocytes can also be regulated by cAMP, the effects of pertussis toxin could be secondary to elevated cAMP levels. Gawler *et al.* (1989) have shown that streptozotocin-induced diabetes lead to the loss of expression of G_i in rat liver. Green and Johnson (1991) have reported that immunoassayable quantities of G_{i1}, G_{i2}, G_{i3} and G_S were all normal in adipocytes from diabetic rats, but Gpp(NH)p was a much less potent inhibitor of (−)-[^{125}I]N^6-hydroxyphenylisoprophyladenosine ([^{125}I]HPIA) binding to adipocytic membranes from diabetic rats than those from control rats, suggesting that there was less functional G_i in the diabetic rats. Thus, our data indicate that the impaired signalling through inhibitory receptors such as adenosine receptors in adipocytes from streptozotocin-induced diabetes is probably due to the loss or the decreased function of G_i proteins.

Recent purification of insulin-sensitive PDE from several sources have provided new insights regarding the activation mechanisms. Evidence to date suggests that the activation of insulin-sensitive PDE might be mediated by phosphorylation reaction. Degerman *et al.* (1987) have shown that the pure insulin-sensitive PDE from the adipose tissue is a good substrate for protein kinase A. The enzyme was readily dephosphorylated with protein phosphatase type 1, 2A, and 2C. The predominance of protein phosphatase 2A in adipocytes may indicate insulin-sensitive PDE dephosphorylation by this phosphatase would be the most important in the intact adipocytes.

Okadaic acid, a potent inhibitor of Type 1 and 2A protein phosphatase, was employed to investigate possible changes in insulin action on insulin-sensitive PDE in adipocytes of streptozotocin-induced diabetic rats. Okadaic acid has been shown to greatly stimulate lipolysis. The insulin-sensitive PDE activities in adipocytes from both diabetic and control rats were little affected by okadaic acid under the conditions used in this experiment. It appears that this was probably caused by the relatively low concentration of okadaic acid employed in our experiment (Shibata *et al.*, 1991).

H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpipera-

zine) is a potent inhibitor of protein kinases including protein kinase A. In electroporated adipocytes, 1 mM H-7 was shown to completely inhibit lipolysis (Shibata *et al.*, 1991). There were no significant differences in responses to H-7. Thus, H-7 lowered insulin-sensitive PDE ac-tivation of P2 fractions approximately 30% in both groups.

In summary, we have shown that the basal PDE activities in P2 fractions of adipocytes from diabetic rats were increased compared with those from control rats, although the maximal effects were not significantly different from each other. The inhibition of insulin-stimulated PDE activities by PIA were impaired, and adipocytes from diabetic rats showed an increased sensitivity to pertussis toxin compared to those from controls. These data indicate that the impaired signalling through inhibitory receptors such as adenosine receptors in adipocytes from streptozotocin-induced diabetes relates to the loss or the decreased function of G_i proteins, and leads to the increased activation of the insulin-dependent PDE at the basal states.

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=국문초록=

Streptozotocin으로 당뇨병을 유발시킨 흰쥐의 지방세포에서 일어나는 Insulin-Sensitive Phosphodiesterase의 조절에 관한 연구

연세대학교 원주의과대학 약리학교실 및 연세대학교 의과대학 약리학교실*

박 경 선 · 이 명 순 · 김 경 환*

Streptozotocin으로 당뇨병을 유발시킨 흰쥐를 모델로 하여 당뇨병으로 인한 인슐린의 antilipolytic action을 매개하는 insulin-sensitive cyclic nucleotide phosphodiesterase의 역할의 변화 가능성을 연구하였다. 흰쥐의 epididymal adipose tissue로부터 분리한 지방세포를 여러 약물과 toxin으로 전처치한 다음, insulin을 처치 또는 처치하지 않고 37℃에서 15분 동안 incubation하였다. 그리고 나서 differential centrifugation으로 3 fractions로 분리한 다음 cAMP phosphodiesterase activity를 assay하였다. Insulin에 의한 PDE activities의 증가는 당뇨병군과 대조군 모두 crude microsomal (P2) fraction에서만 볼 수 있었다. P2 fraction을 2 nM insulin, 100 μM isoproterenol, 또는 두 약물을 함께 처치하여 나타난 maximal effect는 두 군 모두에서 유의한 차이가 없었다. 그러나 basal PDE activities는 당뇨병군이 대조군에 비해 증가한 것으로 나타났다. 당뇨병군의 P2 fraction의 insulin-sensitive PDE activities는 A_1 adenosine receptor agonist인 PIA에 의해서 영향을 받지 않은 반면, 대조군의 경우 PIA에 의해 basal PDE activities와 같게 감소하였다. 그리고 지방세포의 pertussis toxin에 대한 sensitivity는 당뇨병군이 대조군보다 더욱 민감하였다. 그러나 cholera toxin에 대한 sensitivity는 당뇨병군과 대조군 사이에 큰 차이를 볼 수 없었다. 이러한 결과로 보아 streptozotocin으로 당뇨병을 유발시킨 흰쥐의 지방세포에서, adenosine receptor와 같은 inhibitory receptor를 경유한 signalling의 감소는 G_i proteins의 소실 또는 기능의 감소와 관련이 있으며, 또한 basal state에서 insulin-dependent PDE의 활성을 증가시키는 것으로 사료된다.