Insulin stimulates hormone-sensitive cyclic GMP-inhibited cyclic nucleotide phosphodiesterase in rat brown adipose cells

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Abstract **The presence and regulation of a hormone-sensitive cyclic GMP-inhibited cyclic nucleotide phosphodiesterase (cGI PDE) in rat brown adipose cells was investigated. cDNA clones for two cGI PDE isoforms, cGIP1 and cGIP2, have been isolated. Using a rat cGIP1 (RcGIP1) cDNA probe, RcGIP1 mRNA (~5.3 kb) was detected in Northern blots of both brown and white adipose RNA. cGI PDE was detected in both microsomal and plasma membrane fractions of brown and white adipose cells by Western blotting using anti-RcGIP1 peptide antibody. When cells were incubated with insulin before membrane preparation, cGI PDE activity in the microsomal fraction was increased by 2- to 2.5-fold within 10 min. Isoproterenol also stimulated the activity of cGI PDE in the microsomal fraction by 1.5-fold. In cells incubated with both insulin and isoproterenol, microsomal cGI PDE activity was similar to that in microsomal fractions isolated from cells incubated with insulin alone. These results suggest that the hormonal regulation of cGI PDE, presumably a cGIP1 isoform, in rat brown adipose cells is similar to that in white adipose cells.**

Key words: cGMP-inhibited cyclic nucleotide phosphodiesterase; Insulin; Isoproterenol; Brown adipose cell (rat)

1. Introduction

Two types of adipose tissues with quite different functions are present in mammals: white adipose tissue whose main function is energy storage and brown adipose tissue (BAT), which is the major site of non-shivering and diet-induced thermogenesis. BAT is thought to play an important role in energy balance by dissipating excess energy intake as heat. Several rodent models of genetic obesity that are associated with development of diabetes mellitus, such as the Zucker rat and ob/ob mouse, have been reported to be associated with defective BAT function [1,2]. Recently, the cDNA for uncoupling protein (UCP) which is uniquely expressed in BAT mitochondria has been cloned [3] and used to identify brown adipose cells in white adipose tissues. In morphological studies, UCP has been identified in human BAT [4,5]. Therefore, understanding hormonal regulation of BAT may be useful in understanding the pathogenesis of human obesity and insulin resistance in non-insulin dependent diabetic patients [6–9].

Thermogenesis in BAT activated by nonadrenaline is thought to be mediated via a β-adrenergic pathway and cyclic AMP. Adenylyl cyclases serve as biological transducers, which generate cAMP in response to extracellular stimuli and cyclic nucleotide phosphodiesterases (PDE) represent the only known mechanism for the degradation of cAMP [10]. Seven mammalian PDE gene families have been identified and classified on the basis of their most salient characteristics, i.e. their different substrate affinities, responses to specific effectors, sensitivities to specific inhibitors, biochemical and physical properties, and regulatory control mechanisms [10]. In intact cells, type III (or cGI) PDEs are activated by insulin and catecholamines or other agents that increase cellular cAMP concentration [11]. The regulation of cGI PDE in rat white adipose cells and 3T3-L1 adipocytes has been well characterized [12–18]. In white adipose cells, insulin decreases intracellular cAMP concentration and exerts its anti-lipolytic action, at least in part, via activation of cGI PDE. Recently, two distinct but related cDNAs for cGI PDEs (cGIP1 and 2) have been cloned from rat(R) adipose tissue and human(H) myocardial cDNA libraries. RcGIP1, thought to be hormone sensitive, is relatively highly expressed in white adipose cells [19,20]. It has also been reported that the inhibition of PDEs increases oxygen consumption and glucose uptake in brown adipose cells [21,22]. This is the first report on the regulation of cGI PDE, most likely a cGIP1 isoform, in rat isolated brown adipose cells by insulin and isoproterenol.

2. Experimental

2.1. Materials

Male Sprague–Dawley rats, purchased from Charles River Breeding Laboratories Inc. (Boston, MA), were fed standard NIH chow ad libitum for at least 5 days prior to use. Insulin was a gift from Dr. R.B. Chance (Eli Lilly, Indianapolis, IN). Fraction V bovine serum albumin was purchased from Intergen (Purchase, NY) and class II crude collagenase was obtained from Worthington Biochemical (Freehold, NJ). Pepstatin, leupeptin and AEBSF were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Cilostamide was kindly supplied by Otsuka Pharmaceutical Co. (Tokushima, Japan). Rabbit polyclonal antiserum P2T2, prepared against a peptide (RRSSGASGLLTSEHHSR, amino acids 424–440) sequence in the regulatory domain of RcGIP1 [19], was a gift from Dr. E. Degerman (University

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Abbreviations: BAT, brown adipose tissue; cAMP, cyclic adenosine monophosphate; cGI PDE, cyclic GMP-inhibited cyclic nucleotide phosphodiesterase; ADA, adenosine deaminase; PAGE, polyacrylamide gel electrophoresis; T3, triiodothyronine; UCP, uncoupling protein.

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of Lund Medical School, Sweden) and IgG was purified by protein G from P2T2. All radiolabelled compounds were purchased from Du-Pont-New England Nuclear (Boston, MA).

2.2. Preparation of rat brown adipose cells

Rats (6 weeks old) were anesthetized with a gas mixture of 70% CO_2, 30% 0_2 and killed by decapitation. Excised interscapular brown adipose tissues were cleaned, minced and digested with the combination of collagenase and DNase I as described previously [22]. After the final washing, brown adipose cells were suspended and incubated in Krebs Ringer bicarbonate Hepes buffer (KRBH buffer, 120 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM $NaHCO_3$, 30 mM Hepes, pH 7.4) containing 5% serum albumin, 2.5 mM glucose and 200 nM adenosine. White adipose cells were prepared from rat epididymal fat pads as described previously [12].

2.3. Preparation of P1 and P2 fractions from brown adipose cells

Two ml of cells (3.6×10^6 cells/ml) were incubated with additions as indicated in KRBH buffer containing 5% albumin, 2.5 mM glucose and 200 mM adenosine at 37°C for specified periods. To remove adenosine where indicated, 1 U/ml of ADA was added to the incubation solution. Cells were usually incubated for 15 min with insulin and 10 min with isoproterenol. The cell suspensions were then centrifuged ($100 \times g$, 60 s) and the buffer was discarded. Cells were suspended in 10 ml of TES/sucrose buffer (10 mM TES, pH 7.0, 250 mM sucrose, 5 μg/ml pepstatin, 1 μg/ml leupeptin and 0.5 mM AEBSF) at room temperature, homogenized (4°C in a glass Teflon homogenizer), and centrifuged in a Sorvall SS-34 rotor (9500 rpm, 10,000 × g, 15 min). The pellet containing plasma membranes, mitochondria and nuclei (P1 fraction) was suspended in 200 μl of TES/sucrose buffer, pH 7.4, and homogenized (dounce homogenizer). The supernatant was further centrifuged in a Beckman 70.1 Ti rotor at 65,000 rpm (300,000 × g) for 45 min and the microsomal pellet (P2 fraction) was suspended in 150 μl of TES/sucrose buffer, pH 7.4. Protein concentration was measured using BCA reagent (Pierce, Rockford, IN) with bovine serum albumin as standard.

2.4. cGI PDE assay

The activity of PDE in membrane fractions was assayed in duplicate with 0.5 μM [^{3}H]cyclic AMP as substrate as previously described [12]. The specific activity of cGI PDE was calculated from the difference between activities in the presence and absence of 0.5 μM cilostamide, a specific inhibitor of cGI PDE [23].

2.5. Northern and Western blotting

Total RNA was extracted from isolated rat brown and white adipose cells by the method described previously [24]. Approximately 10 μg of total RNA was subjected to electrophoresis in 1% agarose/formaldehyde gels (25 V, 20 h). Gels were stained with ethidium bromide and RNA was then transferred to nylon membranes and fixed by using a UV cross-linker (Stratalinker, from Stratagene, LaJolla, CA). The nylon membranes were prehybridized and then hybridized overnight at 65°C with ^{32}P-labelled partial rat cGIP1 cDNA clone (nt 818–3391) [19] labelled by the random priming technique (Stratagene). Following a brief washing at room temperature with 2.0 × SSC (1 × SSC is 0.15 M NaCl/0.15 M sodium citrate, pH 7.0) containing 0.1% SDS, membranes were washed with 1.0 × SSC containing 0.1% SDS for 20 min at 65°C, and then washed with 0.5 × SSC containing 0.1% SDS for 20 min at 65°C before exposure to Kodak X-ray film with an intensifying screen for 3 days at −70°C.

For Western blotting, 50 μg of membrane fractions, solubilized in sample buffer (2.3 M urea, 1.5% SDS, 15 mM Tris-HCl, pH 6.8 and 100 mM dithiothreitol), were subjected to SDS-PAGE (8% gel) and transferred to a nitrocellulose membrane, which was incubated with anti-RcGIP1 peptide IgG (P2T2). Bound IgG was labelled with [^{125}I]protein A and exposed to Kodak X-ray film for 2 days at −70°C.

3. Results and discussion

3.1. Expression of cGI PDE mRNA and protein in rat brown adipose cells

A partial RcGIP1 cDNA clone hybridized strongly with ~5.3 kb mRNA species in RNA from both white and brown adipose cells (Fig. 1), indicating that cGIP1 mRNA in brown adipose cells is the same size as that in white adipose cells. Proteins in both brown and white adipose cells of ~150 kDa and ~41 kDa (Fig. 2) reacted in Western blots with an anti-RcGIP1-peptide antibody raised against a peptide corresponding in sequence to amino acids 424–440 in the deduced sequence from RcGIP1 [19,25]. Reaction with material ~82 kDa was non-specific as it did not disappear when the antibody was incubated with the immunizing peptide before use. The sizes of cGI PDE in brown and white adipose cells in the present experiments are somewhat different from those reported in white adipose cells from which 135-kDa and 44-kDa ^{32}P-labelled proteins were precipitated by anti-cGI PDE antibody [15,17]. The reason for this apparent discrepancy in molecular size is unknown.

Fig. 1. Northern blots hybridized with probes to detect RcGIP1 mRNA in rat brown and white adipose cells. Total RNA (10 μg/lane) was extracted from isolated rat brown and white adipose cells, subjected to electrophoresis in a 1% agarose/formaldehyde gel, transferred to a nylon membrane, hybridized with ^{32}P-labelled cDNA probe (RcGIP1, nt 818–3391 [19]) and washed as described in section 2. B, brown adipose cells; W, white adipose cells.

3.2. Stimulation of cGI PDE in brown adipose cells by insulin

The concentration-dependency of insulin-stimulation of cGI PDE (cilostamide-sensitive PDE) activity in brown adipose cell P1 (plasma and mitochondrial membrane) and P2 (microsomal) fractions is shown in Fig. 3. Insulin (1 nM) increased cGI PDE activity in P2 fractions by approximately 2-fold (Fig. 3c). Insulin also enhanced cilostamide-sensitive cGI PDE activity in P1 fractions (Fig. 3c). Insulin did not significantly change cilostamide-insensitive PDE activities in P1 or P2 fractions (Fig. 3a,b).

3.3. Stimulation of cGI PDE in brown adipose cells by isoproterenol

It has been reported that cAMP-dependent protein kinase phosphorylates and activates cGI PDE in white adipocytes, probably related to 'feed back' regulation of cAMP concentrations [15,17]. As seen in Fig. 4 in the presence of extracellular

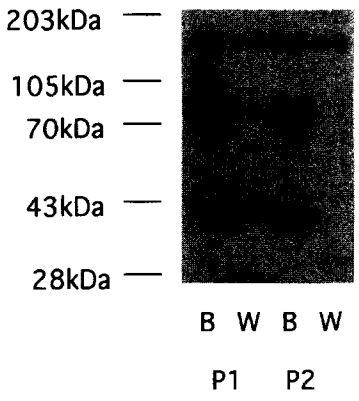

Fig. 2. Subcellular distribution of cGI PDE in brown and white adipose cells. P1 and P2 fractions were prepared from isolated rat brown and white adipose cells and protein (50 μg of each) was subjected to Western blotting with IgG (P2T2) purified from rabbit polyclonal antiserum raised against a peptide sequence within the regulatory domain of RcGIP1 as described in section 2. B, brown adipose cells; W, white adipose cells; P1, P1 fraction; P2, P2 fraction.

adenosine, isoproterenol stimulates cGI PDE in P2 fractions of brown adipose cells. Maximal stimulation was 1.5-fold at 100 nM isoproterenol.

Noradrenaline and insulin are two major regulators of BAT function; thermogenic activity is regulated by noradrenaline and glucose uptake is stimulated by both insulin and noradrenaline in vivo [26,28] and in vitro [21,22]. As seen in Fig. 5, when cells were treated with both insulin and isoproterenol, cGI PDE activity was no greater than that observed in the presence of insulin alone. cGI PDE activity was not apparently changed in the presence of adenosine deaminase, which would have removed any adenosine released during incubation of brown adipose cells.

BAT is the major site of thermogenesis and very active in newborn babies, hibernating animals, and cold-adapted animals. Thermogenesis in BAT is regulated by hormones, such as noradrenaline, insulin and thyroid hormone [28]. Heat production in BAT is directly regulated by the secretion of noradrenaline from sympathetic nerve terminals and the mass of BAT is increased when animals are adapted to cold. The β3-adrenergic receptor agonist, D7114, stimulates proliferation of brown adipose cells in primary culture [29] and the effect of cold exposure on cell proliferation in vivo is inhibited by denervation of BAT [30]. It has also been reported that the amount of the energy released from BAT is related to the amount of UCP in BAT and gene expression of UCP is regulated by both T3 and cAMP [31]. Noradrenaline and insulin are thought to have synergistically positive effects on long-term regulation of thermogenesis in BAT. With respect to short-term regulation, insulin stimulates lipogenesis and glucose transport, and noradrenaline stimulates lipolysis, β-oxidation of fatty acids, glucose oxidation and glucose transport, and suppresses lipogenesis [32]. Thus, in regulation of lipogenesis and lipolysis, insulin and noradrenaline are counter regulatory, and at least some of the effects of insulin in BAT as in white adipose cells may involve activation of an insulin-sensitive cGIP1. Recently, the presence of cGIP1 mRNA in BAT was demonstrated by in situ hybridization [33], consistent with our findings of virtually identical cGIP1 mRNA species from white and brown adipose tissue.

Maximal activation of white adipose cell cGI PDE was observed at ~0.1 and ~100 nM insulin and isoproterenol, respectively [17]. As shown in our studies (Figs. 3 and 4), the sensitivity of cGI PDE to insulin stimulation in brown adipose cells is one tenth of that in white adipose cells; i.e. insulin concentrations of ~1 nM were required for maximal cGI PDE activation. The maximally effective concentration of isoproterenol was

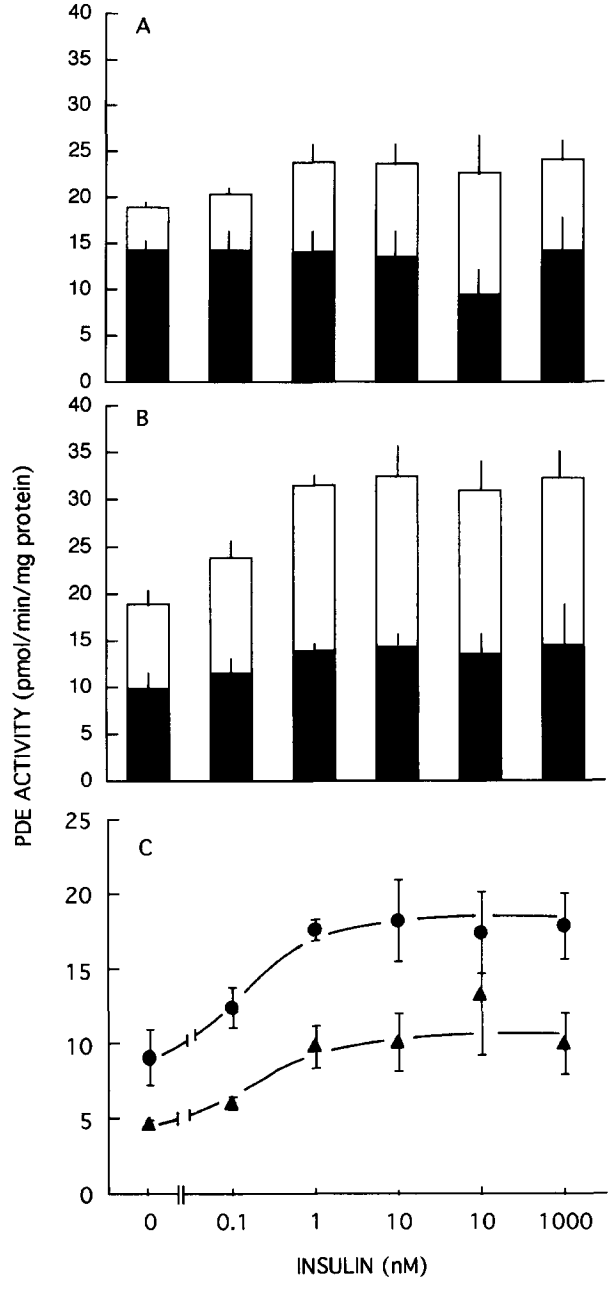

Fig. 3. Stimulation of cGI PDE (cilostamide-sensitive PDE) activity in P1 and P2 fractions from brown adipose cells by insulin. Cells were incubated with various concentrations of insulin prior to preparation of membrane fractions as described in section 2. Open columns show cilostamide-sensitive PDE (cGI PDE) activity and closed columns show cilostamide-insensitive PDE activity. (A) PDE activity in P1 fractions. (B) PDE activity in P2 fractions. (C) cGI PDE activity: closed circles, cGI PDE activity in P2 fractions; and closed triangles, cGI PDE activity in P1 fractions. Data are mean ± S.E.M. of three individual experiments.

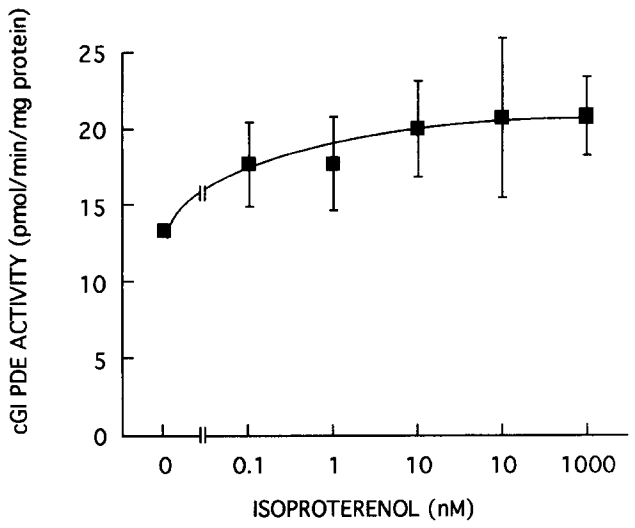

Fig. 4. Effects of isoproterenol on cGI PDE (cilostamide-sensitive PDE) activity in P2 fractions from brown adipose cells. Cells were incubated with various concentrations of isoproterenol for 10 min prior to preparation of membrane fractions as described in section 2. Data are shown as mean ± S.E.M. of three individual experiments, with S.E.M. of ± 0.4 for 0-isoproterenol.

similar in brown and white adipose cells. In contrast, however, to white adipose cells, brown adipose cells (Fig. 5) did not apparently respond synergistically to insulin and isoproterenol [14]. Thus, 'feed back' regulation of cAMP content in brown adipose cells via activation of cGI PDE by noradrenaline may be less than in white adipose cells.

In white adipose cells, activation of cGI PDE is an important component of the antilipolytic action of insulin [11]. BAT has an extremely high rate of glucose utilization when stimulated

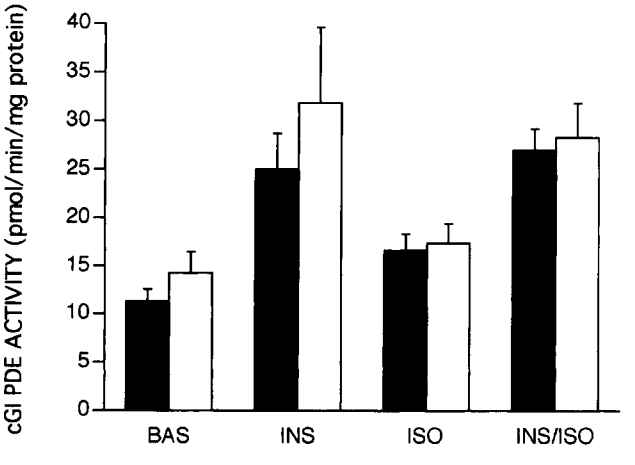

Fig. 5. Effects of isoproterenol on insulin-stimulated cGI PDE (cilostamide-sensitive) activity in P2 fractions from brown adipose cells. Cells were incubated with insulin and/or isoproterenol prior to preparation of membrane fractions as described in section 2. BAS, basal condition (cells were incubated in the buffer for 25 min); INS, cells were incubated in buffer for 10 min and then with 10 nM insulin for 15 min; ISO, cells were incubated in buffer for 15 min and then with 100 nM isoproterenol for 10 min; INS/ISO, cells were incubated with 10 nM of insulin for 15 min and then with 100 nM of isoproterenol for 10 min. In the absence of adenosine, 1 U/ml of ADA was added to the incubation media. Closed columns present results with adenosine; open columns, without adenosine. Data are mean ± S.E.M. of three individual experiments.

by insulin under physiological conditions [34]. To determine whether abnormal expression of cGI PDE might be involved in the pathogenesis of obesity, we investigated the mRNA and protein content of cGIP1 in brown and white adipose cells of six-week-old genetically obese Zucker rats. We did not find any differences between adult lean and obese rats (data not shown), suggesting that cGIP1 expression was not altered and perhaps not related to development of obesity (at 6 weeks) in this model system. Whether regulation of brown adipose tissue cGI PDE activity is abnormal or altered in these animals is not known.

In summary, we report here that the same insulin-sensitive cGI PDE (cGIP1) found in white adipose cells is expressed in brown adipose cells. Brown adipose cell cGI PDE is less sensitive to hormonal regulation than is white adipose cGI PDE. Both brown and white adipose cGI PDEs, by regulating cAMP, may be important in triacylglycerol metabolism.

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