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CL316243 induces phosphatidylinositol 3,4,5-triphosphate production in rat adipocytes in an adenosine deaminase-, pertussis toxin-, or wortmannin-sensitive manner

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Subtitle: CL316243 effects in modulator-treated or -untreated cells

Summary

The effect of β_3-adrenoceptor (β_3-AR) agonists on adipocytes treated or not treated with signaling modulators has not been sufficiently elucidated. Using rat epididymal adipocytes (adipocytes) labeled with [^{32}P]orthophosphate, we found that treatment with the selective β_3-AR agonist CL316243 (CL; 1 μM) induces phosphatidylinositol (PI) 3,4,5-triphosphate (PI[3,4,5]P_3) production and that this response is inhibited by adenosine deaminase (ADA, an adenosine-degrading enzyme; 2 U/ml), pertussis toxin (PTX, an inactivator of inhibitory guanine-nucleotide-binding protein; 1 μg/ml), or wortmannin (WT, a PI-kinase inhibitor; 3 μM). The results showed that CL induced PI(3,4,5)P_3 production in intact adipocytes and that this production was affected by signaling modulators. Taken together, our findings indicate that CL produces PI(3,4,5)P_3 in an ADA-sensitive, PTX-sensitive, or WT-sensitive manner and will advance understanding of the effect of β_3-AR agonists on adipocytes.

Key words: Adipocytes, CL316243 (disodium (*R*,*R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate), phosphatidylinositol 3,4,5-triphosphate

Treatment of adipocytes with β_3-adrenoceptor (β_3-AR) agonists, including CL316243 (CL), induces various responses, including glucose transport and protein-kinase B phosphorylation (PKB activation) in a signaling modulator-sensitive manner (Ohsaka *et al.* 1998; Zmuda-Trzebiatowska *et al.* 2007). Polymorphic studies have indicated that signaling molecules associated with β_3-AR can be targeted to improve adipocyte dysregulation (Arner and Hoffstedt 1999). However, the effect of β_3-AR agonists on adipocytes, including those treated with signaling modulators, has not been fully elucidated.

Phosphatidylinositol (PI) 3,4,5-triphosphate (PI[3,4,5]P_3) is a component of the PI 3-kinase-related pathway and has been shown to activate PKB (Walker *et al.* 1998). Additionally, expression of a PI(3,4,5)P_3-responsive kinase in rat epididymal adipocytes (adipocytes) induces an insulin (INS)-responsive glucose transport-related response (Standaert *et al.* 1997). PI 3-kinase activation is induced in adipocytes treated with CL as well as INS (Ohsaka *et al.* 2014). In a previous study, PI(3,4,5)P_3 was produced by INS treatment (0.1 μM), which peaked at about 1 min, in adipocytes in a signaling modulator (e.g., wortmannin [WT])-sensitive manner (Takasuga *et al.* 1999). However, it is unclear whether CL treatment produces phosphoinositides in intact adipocytes. The effect of CL treatment for 1 min on PI(3,4,5)P_3 production is unknown.

Adenosine is released from adipocytes and degraded in adenosine deaminase (ADA;

2 U/ml, <10 min)-treated adipocytes (Shirakura and Tokumitsu 1990). Adipocyte membranes treated with an adenosine-receptor agonist modulate the inhibitory guanine-nucleotide-binding (G) protein G_i (which can be affected by pertussis toxin [PTX, Mitchell *et al.* 1989]) (Soeder *et al.* 1999). In addition, WT treatment (from 0.1 to <10 μM) inactivates the kinases that produce PI 3- and PI 4-monophosphates (Okada *et al.* 1994). Previous studies showed that adipocytes treated with ADA (2 U/ml, 30 min), PTX (0.2 μg/ml, 180 min), or WT (0.1 μM, 10 min) exhibit altered responses to β_3-AR agonists (0.01−100 μM, 10−30 min) (Chaudhry *et al.* 1994; Ohsaka *et al.* 1997, 1998; Zmuda-Trzebiatowska *et al.* 2007), including ADA- or PTX-sensitive alteration of β_3-AR agonist-induced adenosine 3′,5′-cyclic monophosphate (cAMP) accumulation. It is unclear whether CL produces $PI(3,4,5)P_3$ in an ADA-, PTX-, or WT-sensitive manner.

To investigate the effect of β_3-AR agonists on signaling modulator-treated or -untreated adipocytes, we examined whether treatment with CL (1 μM; Lederle Laboratories, Wayne, NJ) for 1 min produces $PI(3,4,5)P_3$ and whether this response is affected by treatment with ADA (2 U/ml, 10 min; Sigma-Aldrich Co., St. Louis, MO), PTX (1 μg/ml, 60 min; Kaken Pharmaceutical Co., Tokyo, Japan), or WT (3 μM, 1 min; Kyowa Hakko Kogyo Co., Tokyo, Japan).

Adipocytes (10^6 cells/ml) were prepared from rat epididymal adipose tissues as described previously (Ohsaka *et al.* 2014); animal experiments, which were approved by the

institutional review board, were conducted in accordance with the guidelines established by the Japanese Association for Laboratory Animal Science (JALAS) (JALAS 1987). The adipocytes were incubated for 2 h in phosphate-free Krebs-Ringer bicarbonate buffer containing 3% bovine serum albumin (Sigma-Aldrich Co.) in the presence of carrier-free [^{32}P]orthophosphate (DuPont NEN, Boston, MA) at 37°C. [^{32}P]-labeled adipocytes treated with or without agents were immediately separated from the medium by centrifugation at 500 x g for 20 s, and phospholipids were extracted and separated by thin-layer chromatography (TLC) in a solution of chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14, v/v) as described previously (Arcaro and Wymann 1993). The radioactivity of PI(3,4,5)P_3 was detected and quantified using a Fuji BAS2000 Bioimaging Analyzer (Fuji Photo Film Co., Tokyo, Japan).

To confirm the function of isolated adipocytes, we examined whether treatment with INS (0.7 μM, 1 min; Sigma-Aldrich Co.) produces PI(3,4,5)P_3 in a WT-sensitive manner and whether ADA or PTX treatment alters CL (1 μM, 10 min)-induced cAMP accumulation; cAMP accumulation was determined as described previously (Shirakura and Tokumitsu 1990). Treatment of the adipocytes with INS induced WT-sensitive PI(3,4,5)P_3 production (Fig. 1e), and ADA or PTX treatment altered CL-induced cAMP accumulation (Fig. 1f).

PI(3,4,5)P_3 is produced from PI 4,5-bisphosphate (PI[4,5]P_2) in a PI 3-kinase p85 regulatory subunit-containing immunocomplex (Kelly and Ruderman 1993). In G-protein

subunit β (Gβ) antibody immunoprecipitates, CL treatment increases the PI 3-kinase p85 subunit level and phosphorylates the 3'-position of the inositol ring (Ohsaka *et al.* 2014). In addition, PI(3,4,5)P_3 is dephosphorylated by a PI(3,4,5)P_3 phosphatase *in vitro*, and this dephosphorylation is enhanced by PI(4,5)P_2 (Campbell *et al.* 2003). As shown in Fig. 1a–e, CL treatment produced PI(3,4,5)P_3. This response is presumed to be regulated by a PI or phospho-PI kinase(s) and/or a phospho-PI phosphatase(s). Production of PI(3,4,5)P_3, which is produced by INS, was induced in CL-treated intact adipocytes.

PI(3,4,5)P_3 is able to activate PKB isoforms (Walker *et al.* 1998), including PKB-α/-β (which can be phosphorylated). Additionally, treatment of adipocytes with PI(3,4,5)P_3 activates the protein-kinase C (PKC) isoform PKC-ζ (Standaert *et al.* 1997). The number of glucose transporters (GLUT4) in the plasma membrane (PM) is increased by expression of PKB-α (Tanti *et al.* 1997) or PKC-ζ (Standaert *et al.* 1997). CL-induced PI(3,4,5)P_3 production may regulate activation of PKB-α and/or -β (expression of which is observed in adipocytes [Walker *et al.* 1998]) and PKC-ζ and may induce PM GLUT4 expression.

Adipocytes have the G_i-coupled A1 adenosine-receptor (Burnstock 2014). The G-protein subunit Gα of G_i1 or G_i2 is modified by PTX (Mitchell *et al.* 1989); PTX inhibits receptor signaling-induced G-protein dissociation of the Gα and Gβγ subunits (see [23] and the references therein). ADA or PTX treatment did not induce CL-induced PI(3,4,5)P_3

production (Fig. 1a–c). The CL-induced PI(3,4,5)P_3 production may be regulated by adenosine-sensitive molecules including the A1 adenosine-receptor and by a PTX-sensitive G_i isoform(s).

Adipocytes express the PI 3-kinase isoforms p85/p110-α/p110-β (see Ohsaka *et al.* 2014 and Discussion section therein) and PI 4-kinase (Okada *et al.* 1994). The p85/p110-β isoform is activated by Gβγ *in vitro* (Hazeki *et al.* 1998). WT inactivates these kinases. In Fig. 1d, CL-induced PI(3,4,5)P_3 production was inhibited by WT; such an inhibitory effect was not observed when another response (lipolysis) was measured (data not shown). Adipocyte membranes treated with β_3-AR agonists modulate G proteins (Soeder *et al.* 1999), including G_i (which can regulate p85 complex formation in Gβ antibody immunoprecipitates [Ohsaka *et al.* 2014]). CL-induced PI(3,4,5)P_3 production may be regulated by WT-sensitive PI kinases, including a PI 3-kinase isoform of p85/p110.

In this study, CL induced the production of PI(3,4,5)P_3 in intact adipocytes, and this production was affected by signaling modulators. Our findings indicate that CL produces PI(3,4,5)P_3 in an ADA-sensitive, PTX-sensitive, or WT-sensitive manner and will advance understanding of the effect of β_3-AR agonists on adipocytes.

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Figure captions

Figure 1. Effect of CL alone or in combination with ADA, PTX or WT on PI(3,4,5)P_3 production in adipocytes. [^{32}P]orthophosphate-labeled adipocytes that were treated or not treated with ADA (2U/ml, 10 min) or PTX (1 μg/ml, 60 min) were incubated for 1 min with or without CL (1 μM; a−c). In addition, [^{32}P]orthophosphate-labeled adipocytes treated with or without WT (3 μM, 1 min) were incubated for 1 min with or without CL (d and e) or INS (0.7 μM; e). Furthermore, adipocytes treated or not treated with ADA or PTX were incubated for 10 min with or without CL (f). The radioactivity of PI(3,4,5)P_3 (PIP_3) that was separated by TLC and the accumulation of cAMP in adipocytes were determined. Values are presented as means ± standard deviation of 3 or 4 experiments. Autoradiographic images represent typical results. The results were analyzed using analysis of variance with Scheffe's test or unpaired Student's *t*-test. †$P < 0.05$ vs. cells treated without agents (b−e). *$P < 0.05$ vs. cells

treated with CL alone (d and f) or INS (e).

Figure 1