Supplemental Data

Insulin-induced formation of macromolecular complexes involved in activation of cyclic nucleotide phosphodiesterase3B (PDE3B) and its interaction with PKB

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MATERIALS AND METHODS

cAMP PDE Assay

PDE3 activity was measured by modification of a published method [1]. Samples (usually 100 ul) were incubated for 10 min at 30 ^{0}C (total volume 0.3 ml) with 50 mM HEPES, pH 7.5, 8.3 mM MgCl2, 0.1 mM EDTA, and 0.1 uM [^{3}H] cAMP (35000 cpm) as substrate. After dephosphorylation of 5' AMP to adenosine with crotalus atrox venom (Sigma, St Louis, MO), the product was separated from substrate using ion exchange chromatography (QAE Sephadex A25, pharmacia) and quantified by scintillation counting. PDE3 activity is that portion of total activity inhibited by 1.0 uM cilostamide (Calbiochem), a specific PDE3 inhibitor(IC50, ~ 17-80 nM) [2-4]. Vehicle (DMSO), added in equal quantities to samples without inhibitor, did not alter PDE activities, usually expressed as pmol cAMP hydrolyzed/mg/min (unless otherwise indicated).

siRNA Knockdown of PDE3B

Adipocytes, in 10-cm dishes, were lysed, using 1 ml of QIAzol lysis reagent (Qiagen, Chatsworth, CA). The lysates were collected, transferred into a microtube, and pipetted up and down until the samples were uniformly homogeneous. After incubatiion of samples (room temperature, 5 min), 300 µl of chloroform was added to each sample and the tubes were shaken vigorously. After 5 min, the solution was centrifuged (20,000 g, 4ºC, 15 min). The upper aqueous phase was transferred to a new tube and one volume of 70% ethanol was added. The solution immediately was applied to RNeasy Mini Spin Column (RNeasy mini kit), and total RNA was purified with on-column DNase digestion (RNase-Free DNase Set, Qiagen), according to the manual for RNeasy mini kit. Total RNA (100 ng) in triplicate was subjected to Real-time quantitative RT-PCR, using the HT7900 Sequence Detection System (Applied Biosystems) and QuantiTect SYBR green RT-PCR kit (Qiagen), according to manufacturer's protocols. PDE3B was normalized by cyclophilin A, which served as internal control for every sample. The sequences of primers for PDE3B are 5' CCAATTCCTGGCTTACCTCA 3' and 5'

CTGAGGTGCATTTGTAGCCA 3' (AF547435), spanning exon 11-12. The sequences of primers for cyclophilin A are 5' AGCATACAGGTCCTGGCATC 3' and 5' TTCACCTTCCCAAAGACCAC 3'.

Purification of FLAG-tagged recombinant MPDE3B protein in Sf21 cells

Sf21 cells (suspended in 3 ml buffer B containing 1% NP-40/ flask) were homogenized in a 40 ml dounce homogenizer (25 strokes), sonicated [2 x 20 pulses, output 2, 40 % Cycle, Sonifier cell disruptor 350 (Branson Sonic Power Co., Danbury, CT, USA)], and centrifuged (100,000 g, 1h, 4 ^{0}C, SW 41 Ti rotor) to obtain solubilized recombinant proteins. Recombinant MPDE3B (3 ml, containing 8-10 mg protein, 1500-2000 pmoles cAMP hydrolyzed/min/mg) was purified by directly passing the solubilized recombinant MPDE3B (4-5 times) through an anti-FLAG-M2-agarose affinity column [purified murine IgG1 monoclonal antibody covalently attached to agarose by hydrazide linkage (Sigma)]. Columns (1.0 ml) were washed with 20 ml buffer B (without sucrose) containing 1% NP-40. Recombinant MPDE3B bound to beads was eluted with 1 ml FLAG peptide (100 and 500 ug/ml), by sequentially passing the solutions at least 3 times through the affinity column. After SDS-PAGE, purity of protein and its concentrations were estimated by staining with Simply Blue Safe (Invitrogen) stain using BSA as standard.

Purification of anti-PDE3B antibodies

Affi-gel 10 (1 ml), a derivatized N-hydroxysuccinimide ester-crosslinked agarose gel bead support (Bio-rad), was coupled to 5 mg of peptides, PDE3B-CT (aa 1076 -1095) or PDE3B-NT (aa 2 - 16), by incubating (overnight,4 ^{0}C) in 5 ml 50 mM Hepes buffer, pH 7.5. Peptide-coupled beads were placed in a Poly-Prep chromatography column (cat# 731-1550, Bio-rad) and washed sequentially with 10 ml of 10 mM Tris, pH 7.5, 100 mM glycine, pH 2.5, 10 mM Tris, pH 8.8, and 100 mM triethylamine, pH 11.5, and then finally with 10 mM Tris, pH 7.5, until the effluent pH was 7.5. After passing 5 ml rabbit anti-serum, diluted (1:10) in 10 mM

Tris, pH 7.5, through the affinity column (2-3 times), and washing (20 ml,10 mM Tris, pH 7.5), antibodies were eluted using 100 mM glycine, pH 2.5, and adjusted to pH 7.5 using 1 M Tris pH 8.0. Affinity purified anti-PDE3B-NT and anti-PDE3B-CT antibodies were used for immunofluorescence microscopy. For Western blotting and immunoprecipitation experiments, anti-PDE3B-NT, -RD and -CT antibodies were also purified using Immunopure® IgG protein G purification kits (Pierce).

Results:

To monitor the transfection efficiency of siRNA in adipocytes, cells were transfected with RNA-induced silencing complex (RISC)-free siGLO®-Red, a fluorescent labeled, non-targeting siRNA with impaired ability for RISC formation. Almost all cells (>90%) were positive to siGlo®-Red fluorescence 24 h post-transfection, indicating that adipocytes were efficiently transfected (Fig. 1A). Selective depletion of PDE3B using siRNA was performed. Control (non-targeting siRNA) or PDE3B-siRNA were introduced to the cultures of differentiating adipocytes on day 10. At 56 h post-transfection, cultures were serum-starved for 16 h and then incubated with or without insulin (Figure 1). PDE3B enzymatic activity and protein expression (Fig. 1B) were severely blunted in the PDE3B siRNA-transfected cultures compared with untreated or non-targeting control transfected cultures. In contrast, protein levels of β-actin, a cytoskeleton protein, were not significantly changed. siRNA-mediated specific knock-down of PDE3B mRNA was validated using cyclophilin A as internal control for each sample for quantitative RT-PCR (Fig. 1C).

Overexpression of 14-3-3 in adipocytes reduced insulin-induced activation of PDE3B by almost 20% (Fig. 2).

After incubation of 3T3-L1 adipocytes with insulin, PDE3B and PKB were co-immunoprecipitated from solubilized microsomal membranes with anti-PKB antibodies (Fig. 3, panel 1,2). Incubation of 3T3-L1 adipocytes with wortmannin, which inhibits PI3-K, blocked phosphorylation of PKB (Fig. 3, panel 5) and activation of PDE3B (Fig. 3, lower panel). Wortmannin inhibited not only

phosphorylation of PKB (pPKB) (panel 5) and activation of membrane-associated PDE3B (bottom), but also the interaction and co-immunoprecipitation of PDE3B and PKB/pPKB (panel 1). Another PI3-K inhibitor, LY294002, and the tyrosine kinase inhibitor, genistein [5]**,** which blocked insulin receptor-mediated tyrosine phosphorylation of IRS-1 (panel 6), also inhibited insulin-induced activation of PKB (panel 5) and PDE3B (bottom) and their co-immunoprecipitation (panel 1)**.**

Figure Legend

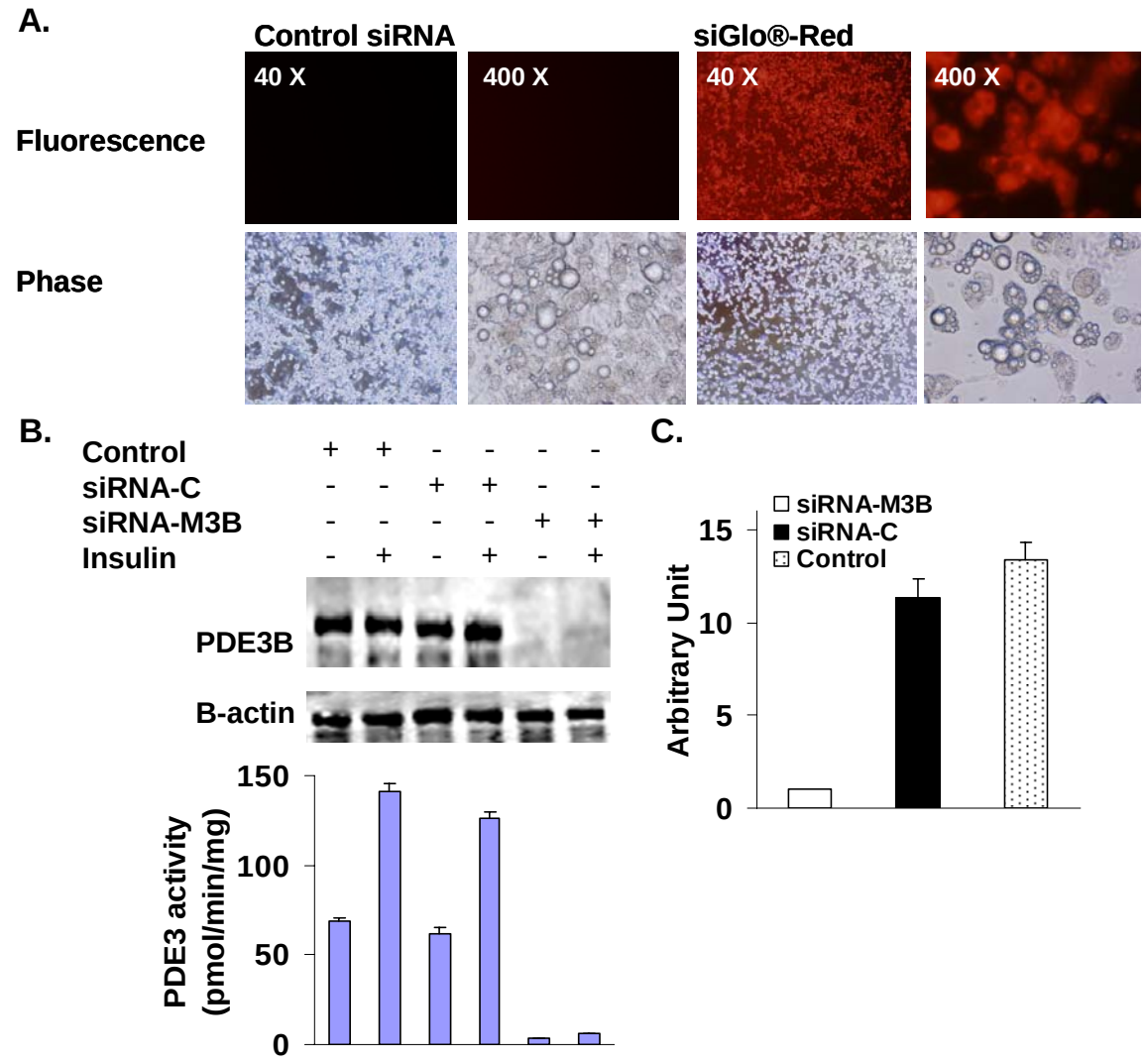
Supplemental Figure 1. siRNA-mediated knock-down of PDE3B in 3T3-L1 adipocytes.

A. Transfection efficiency of differentiated adipocytes was examined by transfecting the cells with a fluorescent-tagged RNA duplex siGlo®-Red (Cat# D-001630-02), RISC-independent siRNA obtained from Dharmacon, which is chemically modified to localize to the nucleus. Fluorescence of siGlo®-Red and control siRNA was monitored on Olympus IX51 microscope, using texas-red filter (upper panel) and with phase contrast (lower panel). **B.** Adipocytes (Control) were transfected with 100 nM non-targeting (siRNA-C) or PDE3B siRNA (siRNA-M3B). 72 h after transfection, knock-down specificity was analyzed by immunoblotting, PDE3 activity assay, and RT-PCR. After incubation of adipocytes for 10 min without or with 100 nM insulin, solubilized membrane proteins, from either untreated cells (*Control*) or cells transfected with non-targeting control siRNA (*siRNA-C*) or PDE3B siRNA (*siRNA-M3B*), were subjected to SDS-PAGE (30 ug protein/lane) and immunoblotted with antibodies targeting PDE3B and β-actin; PDE3 activity was analysed as described in methods. Results are representative of two experiments. **C.** Adipocytes were also lysed in QIAzol lysis reagent, and total RNA prepared using RNeasy mini kit (Qiagen). RNA (100 ng), in triplicate, was subjected to Real-time quantitative RT-PCR as described in methods. Values represent mean +/- ½ the range, n=2 experiments.

Supplemental Figure 2. Effect of 14-3-3 overexpression on activation of PDE3B in 3T3-L1 adipocytes. Differentiated adipocytes were infected with Ad-hemagglutinin-tagged (HA)-14-3-3 adenoviruses (Vector Biolabs) (6 plates) or Adβgal (supplied by Dr. B Baum, NINDR, NIH) (6 plates), and incubated for 10 min without or with 100 nM insulin. Solubilized total membranes (centrifugation of 1,000 g supernatant at 175,000 g) were prepared, and PDE3B, 14-3-3 and HA-14-3-3 were detected after SDS-PAGE (30 ug protein/lane) and Western blotting, with antibodies targeting PDE3B, HA, and 14-3-3, as indicated. PDE3 activity (expressed as pmol/min/mg) in solubilized total membranes was assayed in duplicate; values represent mean +/- SEM, n=3 dishes. Results are representative of two experiments.

Supplemental Figure 3. Co-immunoprecipitation of PDE3B and PKB from solubilized membranes of insulin-treated adipocytes: inhibition by wortmannin, LY294002 and genistein. Solubilized total membranes (3 mg protein), were prepared from adipocytes incubated for 10 min without or with 100 nM insulin, or with insulin after incubation with 100 nM wortmannin (30 min), or 50 uM LY294002 (30 min) or 300 uM genistein **[5]** (15 min). Fractions (1 mg protein) were cleared and immunoprecipitated with anti-PKB antibody (20 ul), and subjected to SDS-PAGE and Western immnoblotting with the indicated antibodies, including anti-IRS-1 [pY^{612}]. INPUT proteins: 30 ug of solubilized membrane proteins. Effects of wortmannin, LY or genistein on insulin-stimulated PDE3 activity (expressed as pmol/min/mg) in solubilized total membranes (10 ug) prepared from adipocytes, assayed as described in methods; values represents mean +/- ½ range. The results are representative of two experiments.

Supplemental Figure 1



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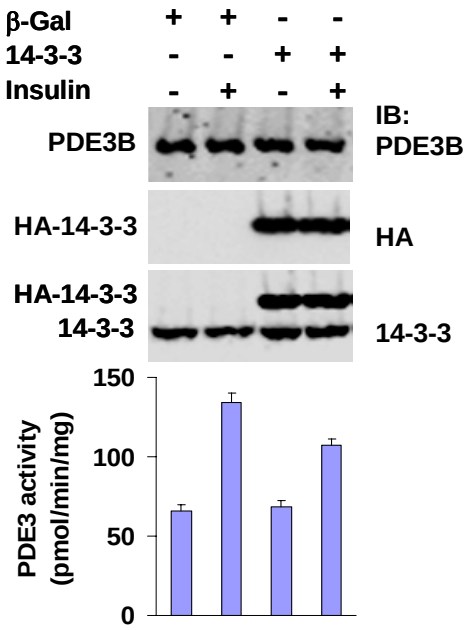
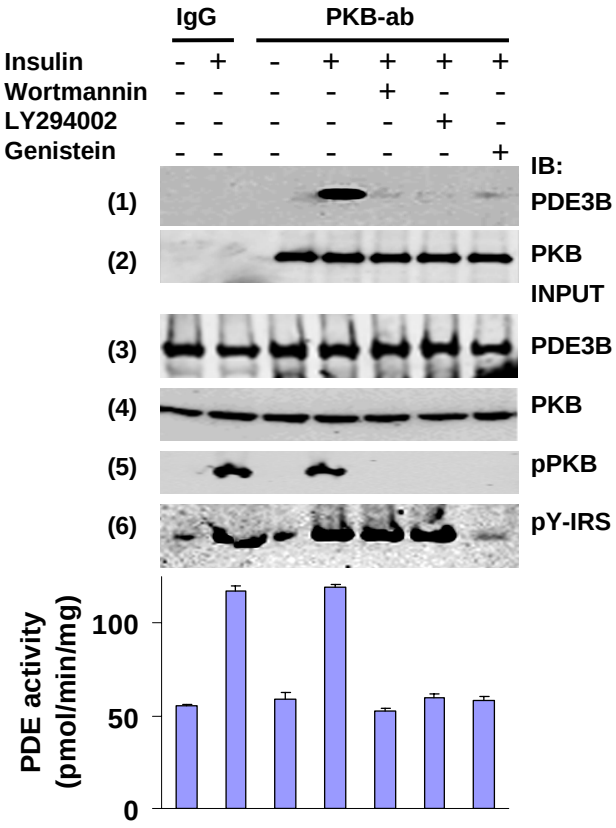


Supplemental Figure 3



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