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Dominant negative FTase (DNFTα) inhibits ERK5, MEF2C and CREB activation in adipogenesis

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Abstract

We recently demonstrated that dominant negative FTase/GGTase I α-subunit-inhibited (DNFTα-inhibited) insulin-stimulated adipocytes differentiation. DNFTα interferes with Ras prenylation whereby ERK1/2, CREB and the differentiation cascade are downregulated. To further investigate prenylation in adipogenesis, we examined DNFTα's ability to inhibit activation of ERK5, MEF2C and CREB. DNFTα-inhibited insulin-stimulated expression, activation and nuclear translocation of ERK5. Inhibition was associated with decreased activation of MEF2C and CREB by 80 and 78%, respectively. PD98059 did not block activation of ERK5 and MEF2C, but inhibited CREB phosphorylation by 90%. ERK5 siRNA-inhibited MEF2C activation, whereas it reduced CREB phosphorylation only 50%. Pre-adipocytes expressing DNFTα or treated with PD98059 were unable to differentiate to mature adipocytes, whereas pre-adipocytes transfected with ERK5 siRNA showed moderate inhibition of insulin-induced adipogenesis. Taken together, these data suggest that prenylation plays a critical role in insulin-stimulated adipogenesis, and that the ERK5 plays an important, but less crucial role in adipogenesis as compared to ERK1/2.

Keywords: Farnesyltransferase; ERK5; CREB; Insulin; Adipogenesis; Differentiation

1. Introduction

Insulin plays an important role in adipocyte physiology, inducing both proliferation and differentiation and appears to be a major factor in the differentiation of pre-adipocytes into mature adipocytes. Adipocyte differentiation begins with clonal expansion, a proliferative event that includes DNA synthesis, increased expression of c-fos and c-jun and increased cell number, followed by increases in the intermediates of the differentiation cascade, such as the CCAAT/enhancer-binding proteins (C/EBP), peroxisome proliferator-activated receptor (PPAR) and cyclic AMP response element-binding protein (CREB) (Morrison and Farmer, 2000; Ntambi and Young-Cheul, 2000). Insulin-stimulated increases in adipocyte proliferation appear to occur via the Ras-mitogen-activated protein kinase (Ras-MAPK) pathways. Although the most characterized insulin-stimulated Ras-MAPK pathway is the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway, crosstalk between this pathway and the most recently discovered Ras-ERK5 pathway has been established (English et al., 1999; Kamakura et al., 1999; Kato et al., 1998). The ERK5 pathway has also been shown to be associated with proliferation and more importantly, differentiation (Cavanaugh et al., 2001; Dinev et al., 2001). Additionally, both the Ras-ERK1/2 and Ras-ERK5 pathways activate CREB (Watson et al., 2001), and thus, appear to be in concert with each other, mediating growth factor-stimulated proliferation and differentiation.

The interaction between the insulin-signaling pathway and the differentiation cascade pathway seems to be inextricable and important in the conversion of pre-adipocytes to fully mature adipocytes. However, the mechanism of linking the insulin-signaling pathway with the adipocyte-differentiation cascade remains unclear. Klemm et al. (2001a) have shown that CREB plays a pivotal role in adipocyte differentiation and that expression of a dominant negative CREB blocks insulin-stimulated adipocyte differentiation. Additionally, Klemm et al. (2001a) have shown that constitutively active CREB can induce adipogenesis in the absence of insulin, and therefore, CREB may be the "link" between the insulin-signaling pathway and the differentiation cascade.

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Several studies have convincingly shown that insulin stimulates the phosphorylation and activation of CREB (Klemm et al., 2001a; Reusch et al., 2000). Additionally, we have observed that insulin-stimulated phosphorylation of CREB and subsequent pre-adipocyte differentiation were blocked by the pharmacological inhibition of prenylation of p21Ras and RhoA, suggesting that prenylation of these small GTPases plays an important role in activation of insulin-stimulated pre-adipocyte growth and differentiation (Klemm et al., 2001a; Reusch et al., 2000).

Prenylation of p21Ras and RhoA is mediated by the protein prenyltransferase enzymes, farnesyltransferase (FTase) and geranylgeranyltransferase-1 (GGTase-1), respectively. FTase and GGTase-1 are heterodimers that share the same α-subunit, whereas the β-subunit confers substrate specificity (Seabra et al., 1991). The activities of FTase and GGTase-1 are stimulated by insulin, but not epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1) or platelet-derived growth factor (PDGF) (Goalstone et al., 1998). Inhibitors of FTase and GGTase-1 block insulin-stimulated prenylation of Ras and Rho proteins, thereby inhibiting insulin- and other growth factor-induced GTP-binding of Ras and RhoA, and subsequent activation of their respective downstream effectors (Leitner et al., 1997).

Recently, we designed a mutated form of the FTase/GGTase-1 α-subunit, FTase α-subunit [(S60A)(S62A)(Y63F)] (DNFTα) (Solomon and Goalstone, 2001). When over-expressed in mammalian cells, the mutated α-subunit acts as a dominant negative form for both FTase and GGTase-1 activity. DNFTα cannot be phosphorylated, and therefore, is unable to be activated. As a result, Ras and RhoA cannot be: (1) prenylated; (2) bind to their respective membranes; and (3) activated by growth factor-stimulated GTP-loading. We have also shown that expression of DNFTα blocks insulin-stimulated differentiation of pre-adipocytes and inhibits insulin-stimulated phosphorylation of CREB (Solomon et al., 2003). Furthermore, we demonstrated that expression of constitutively active CREB rescued adipocyte-differentiation process from the inhibitory influence of DNFTα (Klemm et al., 2001a).

Here, we show for the first time a novel mechanism by which the insulin-signaling pathway is linked to the adipocyte-differentitaion cascade pathway; that insulin-stimulated activation of CREB is also mediated by the phosphorylation, activation and nuclear translocation of ERK5. We demonstrate that expression of DNFTα inhibits phosphorylation and nuclear translocation of ERK5 as well as activation of MEF2C and CREB. Taken together, these results indicate that insulin-stimulated activation of ERK1/2 and ERK5, leading to the phosphorylation and activation of CREB, may be the mechanism by which insulin enhances clonal expansion and differentiation of pre-adipocytes to mature adipocytes.

2. Materials and methods

2.1. Materials

All general laboratory reagents were from Sigma (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM) (Cat. #11885-084), fetal bovine serum (Cat. #16000-036) and phosphate buffered saline (PBS) 1× were from Gibco/Invitrogen (Carlsbad, CA). Fungizone (250 μg/mL) was from Gemini Bio-Products (Calabasas, CA). Gene microarrays were from SuperArray Bioscience Corp. (Frederick, MD) (Cat. #MM-017-N4 and MM-030-N4). RNA-STAT 60 was from Tel-Test Inc. (Friendswood, TX). Transfection reagents, Lipofectamine 2000, Oligofectamine™, Plus Reagent and Opti-MEM were from Invitrogen (Carlsbad, CA). Mammalian expression vector pCMV/FLAG/Tag2A was from Stratagene (La Jolla, CA). 3T3-L1 pre-adipocytes were from ATCC (Manassas, VA, Cat. #CL173). Biotin-labeled 16-UTP was from Roche Applied Science (Indianapolis, IN). SDS-polyacrylamide gel (SDS-PAGE) and Western blot equipment and supplies were from Bio-Rad (Hercules, CA). Anti-phospho-IRβ, anti-IGF-1Rβ, anti-phospho-tyrosine, anti-CREB, anti-PPARγ and anti-C/EBPα antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MEF2C, ERK1/2, phospho-ERK1/2, ERK5 and phospho-ERK5 antibodies were from Cell Signaling (Beverly, MA). Anti-phospho-threonine and anti-FLAG antibodies were from Sigma (St. Louis, MO), and anti-phospho-CREB antibodies were from Upstate (Lake Placid, NY). NE-PER nuclear and cytoplasmic extract kit was from Pierce (Rockford, IL).

2.2. Cell cultures

The 3T3-L1 pre-adipocytes were grown in DMEM growth medium (DGM) (5.5 mM glucose, 10% FBS, 50 μg/mL gentamicin, 1 mM glutamine, 110 mg/L sodium pyruvate, 1.25 μg/mL fungizone and pyrodoxine hydrochloride) at 37 °C and 5% CO_2 until approximately 100% confluent. Prior to treatment, growth medium was replaced with serum-free medium for 24 h. Subsequently, cells were pre-incubated for 1 h in the absence or presence of the MEK1/2 inhibitor, PD98059 (20 μM) followed by incubation without or with indicated concentrations of insulin and reagents for designated times to determine the effect of insulin on pre-adipocytes.

2.3. Pre-adipocytes stable cell lines

Transfection was performed as per manufacturer's protocol. Briefly, pre-adipocytes were grown in 6-well dishes to nearly 30% confluence in DGM. The growth medium was aspirated and cells were washed with 1× sterile PBS. The PBS was removed and replaced with a 500 μL of Opti-MEM. A 100 μL solution of a 6.7 μg/mL DNA, Lipofectamine 2000 and Plus Reagent was added to each well and cells were incubated at 37 °C 5% CO_2 for 4 h. The Opti-MEM/DNA solution was aspirated and replaced with DGM. Cells were incubated at 37 °C 5% CO_2 for 48 h and then incubated in DGM containing 250 μg/mL of geneticin (G418) for 14 days with repeated refreshing of DGM plus G418 every 2–3 days. Stable and viable transfected clones were isolated and pooled. Pre-adipocytes transfected with empty vector, pCMV/FLAG/Tag2A, were considered controls in designated experiments, whereas pre-adipocytes transfected with the expression vector, pCMV/FLAG/Tag2A/DNFTα (Solomon and Goalstone, 2001) were considered the "DNFTα-expressing" group and verified to have the DNFTα expression using an ant-FLAG antibody. Both cell lines were maintained in growth medium containing 250 μg/mL of geneticin to maintain the stable presence of the empty or expression vector plasmids.

2.4. RNA isolation and microarray hybridization

Gene expression analysis was performed as per the manufacturer's protocol. Briefly, cells were grown to nearly 100% confluence in DGM. DGM was replaced with serum-free medium for 24 h then incubated in this same medium in the absence or presence of indicated insulin and reagent concentrations for designated times. RNA was isolated, quantified and reverse transcribed to cDNA incorporating biotin-labeled 16-dUTP. Labeled cDNA was hybridized to oligonucleotide gene microarray membranes as per manufacturer's (SuperArray, Frederick, MD) protocols, incubated in CDP-Star reagent and exposed to X-ray film. Images were quantified by densitometry and signals from genes were normalized to housekeeping gene expression.

2.5. Western blot analysis

Wild type or cells stably transfected with either empty vector or DNFTα were grown to approximately 100% confluence in DGM. Subsequent to incubation in