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Phoenixin-14 stimulates differentiation of 3T3-L1 preadipocytes via cAMP/Epac-dependent mechanism

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ABSTRACT

Phoenixin-14 (PNX) is a newly discovered peptide produced by proteolytic cleavage of the small integral membrane protein 20 (Smim20). Previous studies showed that PNX is involved in controlling reproduction, pain, anxiety and memory. Furthermore, in humans, PNX positively correlates with BMI suggesting a potential role of PNX in controlling fat accumulation in obesity. Since the influence of PNX on adipose tissue formation has not been so far demonstrated, we investigated the effects of PNX on proliferation and differentiation of preadipocytes using 3T3-L1 and rat primary preadipocytes. We detected *Smim20* and *Gpr173* mRNA in 3T3-L1 preadipocytes as well as in rat primary preadipocytes. Furthermore, we found that PNX peptide is produced and secreted from 3T3-L1 and rat primary adipocytes. PNX increased 3T3-L1 preadipocytes proliferation and viability. PNX stimulated the expression of adipogenic genes (*Pparγ*, *C/ebpβ* and *Fabp4*) in 3T3-L1 adipocytes. 3T3-L1 preadipocytes differentiated in the presence of PNX had increased lipid content. Stimulation of cell proliferation and differentiation by PNX was also confirmed in rat preadipocytes. PNX failed to induce AKT phosphorylation, however, PNX increased cAMP levels in 3T3-L1 cells. Suppression of Epac signalling attenuated PNX-induced *Pparγ* expression without affecting cell proliferation. Our data show that PNX stimulates differentiation of 3T3-L1 and rat primary preadipocytes into mature adipocytes via cAMP/Epac-dependent pathway. In conclusion our data shows that phoenixin promotes white adipogenesis, thereby may be involved in controlling body mass regulation.

1. Introduction

Phoenixin (PNX) is a recently discovered peptide produced mainly in hypothalamus by proteolytic cleavage of a small integral membrane protein 20 (Smim20) [1]. The most important endogenous active isoforms of PNX are amided peptides, composed of 14 and 20 amino acids [2]. Previous data provided evidence that biological effects of PNX are mediated via GPR173 receptor activation [3,4]. Initially, PNX was characterized as a reproductive peptide which regulates pituitary gonadotropin secretion as well as GnRH expression in rats [1]. Further studies showed that PNX is involved in regulation of anxiety, itching sensation, pain, as well as memory [2,5–7]. There is strong evidence indicating that PNX is expressed outside of the central nervous system, such as in the gastrointestinal tract, heart, kidney and pancreatic islets [1,8]. Furthermore, PNX peptide was detected in the humans and rats circulation [9–11]. Interestingly, in humans PNX serum levels positively correlated with BMI [9,10]. These results collectively suggest that PNX may contribute to the regulation of energy homeostasis, thereby

Abbreviations: AKT, RAC-alpha serine/threonine-protein kinase; BMI, body mass index; BrdU, 5-bromo-2′-deoxyuridine; cAMP, cyclic adenosine monophosphate; C/ebpβ, CCAAT/enhancer-binding protein beta; Epac, exchange protein directly activated by cAMP; Fabp4, fatty acid binding protein 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPR173, G Protein-Coupled Receptor 173; IBMX, 3-isobutyl-1-methylxanthin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PNX, phoenixin; ORO, oil red O; Pparγ, peroxisome proliferator-activated receptor gamma; Smim20, small integral membrane protein 20; T3, triiodothyronine

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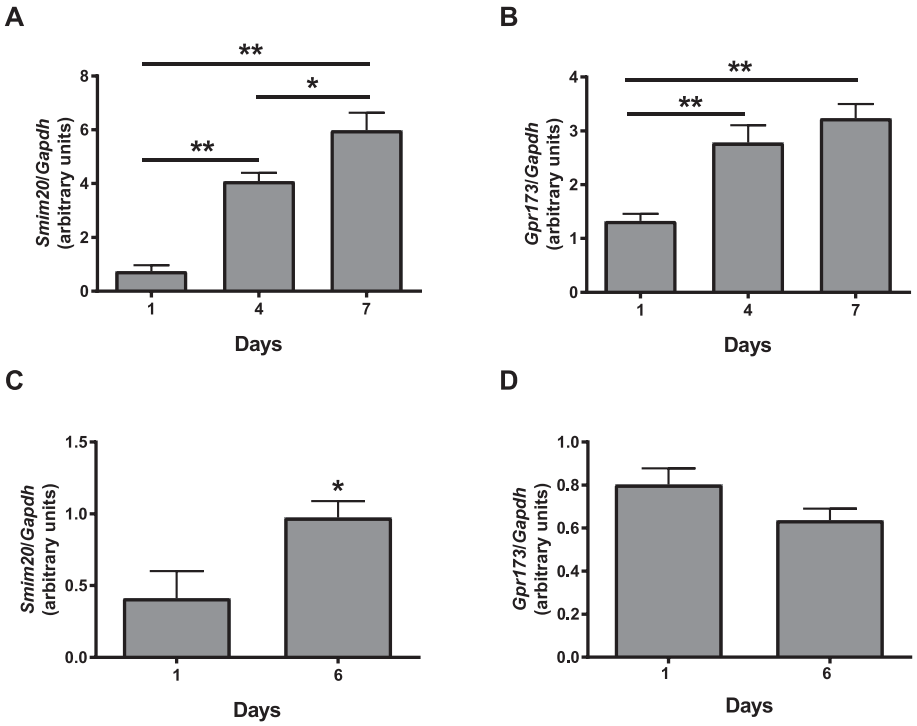

Fig. 1. Expression of *Smim20* and *Gpr173* mRNA in 3T3-L1 cells and rat primary adipocytes during differentiation process. Expression of *Smim20* (A) and *Gpr173* (B) in 3T3-L1 cells differentiated for 1, 4 or 7 days. Expression of *Smim20* (C) and *Gpr173* (D) in rat preadipocytes differentiated for 1 or 6 days. Results are the mean ± SEM, (n = 6).

influencing body weight.

It is well known that the adipose tissue is one of the most pivotal organs involved in regulation of energy homeostasis and body composition. Adipocytes store energy and produce numerous hormones, and metabolites which modulate energy balance as well as glucose and lipid metabolism [12]. Increased number and size of adipocytes which originate from preadipocytes [13], and particularly, their dysfunction, are hallmarks of obesity [14].

In view at a suggested association between PNX systemic concentrations and BMI a question arises whether PNX may control adipose tissue formation and adipocyte's functions e.g. maturation processes of adipocytes. Thus, in the present study we characterized the effects of PNX on proliferation and differentiation preadipocytes into mature fat cells. For this purpose, we utilized 3T3-L1 preadipocytes as well as rat primary preadipocytes.

2. Materials and methods

2.1. Materials

3T3-L1 fibroblast (ATCC® CL-173™) cell line (a well-characterized cellular model suitable to study adipogenesis [15]) was from ATCC, LGC Standards, (Manassas, VA, USA). Phoenixin-14 amide was from Novazym (Poznań, Poland). Cell culture media and supplements were from Biowest (Nuaillé, France). Phospho-AKT (#9275L), AKT (#9272S) antibodies were from Cell Signalling Technology (Danvers, MA, USA). Phoenixin-14 amide antibody (H-079-01) was from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA). ESI09 and LY290042 were from Tocris Bioscience (Bristol, UK). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

2.2. Isolation of rat primary preadipocytes

Rat primary preadipocytes were obtained from male Wistar rats, weighing 80–100 g (5–6 weeks old). Cells were isolated and differentiated as previously described [16].

2.3. 3T3-L1 preadipocytes culture and differentiation

3T3-L1 cells were cultured in a standard growth medium (SGM), composed of Dulbecco's Modified Eagle Medium supplemented with 10% FCS and antibiotics (100 kU/l penicillin, 100 mg/l streptomycin). Cells were maintained at 37 °C in a humidified atmosphere (95% air, 5% CO_2). Differentiation of 3T3-L1 cells into adipocytes was induced 2–3 days post-confluence in SGM containing 1 μmol/l dexamethasone, 500 μmol/l IBMX and 1 μmol/l insulin in the presence or absence of 1, 10 or 100 nmol/l PNX. After 48 h medium was replaced by SGM containing 1 μmol/l insulin with or without PNX. Cells were incubated for additional 2 days. Thereafter, medium was replaced by SGM with or without PNX. Cell differentiation into mature adipocytes was confirmed by evaluation *Fabp4* expression (> 1000-fold increase in preadipocytes comparing to cells differentiated for 7 days).

2.4. Proliferation

3T3-L1 cells proliferation was assessed using Cell Proliferation BrdU Elisa Kit (Roche Diagnostics, Penzberg, Germany). Briefly, cells were seeded (5×10^3 cells/well) into 96-well plates. After 24 h, cells were cultured for 24 h in SGM without serum and then treated with 1, 10 or 100 nmol/l PNX for further 24 or 48 h. Thereafter, BrdU solution (10 μmol/l) was added and the assay was performed according to the manufacturer's protocol.

2.5. Cell viability

3T3-L1 preadipocytes were prepared in the same way as described in cell proliferation experiments. Cells were treated with 1, 10 or 100 nmol/l PNX for 48 h. Cell viability was assessed using MTT assay as previously described [17].

2.6. Cell death

Cell death was evaluated in 3T3-L1 preadipocytes treated with PNX (100 nmol/l) for 48 h using Cell Death Detection ELISA kit (Roche Diagnostics), as described [17].

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