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Roles of 14-3-3β and γ in regulation of the glucocorticoid receptor transcriptional activation and hepatic gluconeogenesis

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ABSTRACT

The glucocorticoid receptor (GR) is a ligand-dependent transcription factor that mediates the effects of glucocorticoids, and plays a crucial role in cell growth, development, inflammation, and gluconeogenesis. The 14-3-3 proteins bind to target proteins via phosphorylation, and influence many cellular events by altering their subcellular localization or by acting as chaperones. However, the mechanisms by which 14-3-3 proteins regulate GR transactivation and their involvement in gluconeogenesis remain uncharacterized. We found that 14-3-3β and γ increased GR transcriptional activity and the promoter activities of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase in the presence of glucocorticoids. Inhibition of the endogenous 14-3-3β and γ decreased dexamethasone- and cAMP-stimulated PEPCK expression. Further, both 14-3-3β and γ increased glucose production in response to glucocorticoids. Our findings suggest that 14-3-3β and γ function as positive regulators of GR transactivation and glucocorticoid-mediated hepatic gluconeogenesis.

1. Introduction

The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily and is a ligand-dependent transcription factor. The GR mediates the effects of glucocorticoids in diverse cellular processes, including homeostasis, growth, development, and inflammation [1–4]. The GR contains three major structural domains [5]. The AF1 transactivation domain in the N-terminus interacts with components of the transcriptional machinery, and the DNA-binding domain in the center associates with specific glucocorticoid response elements (GREs) within the target genes [6,7]. The C-terminal ligand-binding domain contains overlapping functional domains, which are responsible for ligand binding, nuclear translocation, dimerization, transactivation, and binding with the 90 kDa heat-shock protein (HSP90) [8]. The unliganded GR in the cytoplasm forms a complex with HSP chaperones, including HSP90 and HSP70; however, once activated by ligand binding, the GR translocates to the nucleus and functions as a transcription factor [9].

The 14-3-3 proteins are a family of highly conserved acidic proteins, expressed in wide range of organisms and tissues [10,11]. The 14-3-3 proteins bind to target proteins through phosphorylation [12]. In humans, seven 14-3-3 isoforms have been identified [13]. The mammalian 14-3-3 isoforms, β, γ, ε, ζ, η, θ, and σ, are encoded by seven individual genes [10]. These 29–31 kDa acidic proteins bind to phosphorylated serine/threonine motifs on target proteins [13,14]. Possible modes of action of 14-3-3 on target proteins include directed conformational changes, modification of nuclear/cytoplasmic localization, protection of the phosphorylated state, masking a phosphorylated region of a target protein, and scaffolding [14]. The 14-3-3 proteins play crucial roles in diverse processes such as cell cycle regulation, DNA repair, apoptosis, cell differentiation, signal transduction, and cell adhesion [11].

It has been reported that 14-3-3η serves as a positive regulator of the GR and activates GR transactivation in response to glucocorticoids [15]. Under a fasting state, secretion of the pancreatic hormone glucagon and adrenal glucocorticoids is increased, and these hormones induce hepatic glucose production [16]. This process regulates hepatic gluconeogenesis, which involves the transcriptional activation of key metabolic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [17]. The gluconeogenesis process is regulated by the transcriptional levels of the gluconeogenic genes. In this study, we found that 14-3-3β and γ regulate the transcriptional activity of the GR in a ligand-dependent manner and are both involved in hepatic gluconeogenesis.

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2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), antibiotic-free Opti-MEM, fetal bovine serum (FBS), and Lipofectamine 2000 reagent were purchased from Invitrogen (Carlsbad, CA, USA). Anti-GR and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Genefectin reagent was purchased from Genetrone Biotech Co. (Seoul, South Korea). The luciferase assay system was purchased from Promega (Madison, WI, USA). Dexamethasone (Dex) and cAMP were obtained from Sigma (St. Louis, MO, USA).

2.2. Cell culture

HEK 293A and HepG2 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 incubator.

2.3. Plasmids and transient transfection

The plasmids of rat PEPCK-Luc (-2371/+70), human G6Pase-Luc (-1227/+57) were kind gifts from Hueng-Sik Choi (Chonnam National University, South Korea). Rat PEPCK promoter was amplified by PCR and was cloned into the pGEM-T easy vector (Promega). HepG2 cells were plated at a density of 3×10^5 cells/well in a 12-well plate. After 18–24 h, cells were cotransfected with 0.5 μg of the reporter gene plasmid and the experimental plasmid using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h of transfection, cells were grown in the same medium supplemented with 0.5% FBS for 24 h. Serum-starved cells were used for further assays.

2.4. RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from cells with TRIzol (Invitrogen) according to the manufacturer's protocol. Approximately 2 μg of total RNA was used to prepare cDNA using the Superscript First Strand cDNA Synthesis Kit (Bioneer, Daejeon, South Korea). The experimental conditions were as follows: PEPCK, G6Pase, 14-3-3 β, γ, ε, ζ, η, θ and σ (96 °C for 40 s, 60 °C for 1 min, and 72 °C for 40 s for 30 cycles), GAPDH (96 °C for 40 s, 60 °C for 40 s, and 72 °C for 40 s for 35 cycles). Primer pairs of PEPCK, G6Pase, 14-3-3 β, γ, ε, ζ, η, θ, σ, and GAPDH are shown in Table 1. GAPDH was amplified as an internal control. The PCR products were electrophoresed on a 2% (w/v) agarose gel in 1 × Tris-acetate-EDTA buffer, and stained with ethidium bromide solution. All the PCR reactions were repeated at least three times. The intensity of each band amplified by RT-PCR was analyzed using UV image analyzer (Vilber Lourmat, Germany), and normalized to that of GAPDH mRNA in corresponding samples.

2.5. Luciferase reporter gene activity assay

The transfected cells were washed twice with ice-cold PBS and lysed in culture dishes with reporter lysis buffer. Luciferase activities were recorded in a Luminometer 20/20^n (Turner BioSystems, Sunnyvale, CA, USA) according to the manufacturer's instructions. Luciferase activity was normalized with β-galactosidase activity. For the β-galactosidase assay, pSV40-β galactosidase was cotransfected with the luciferase reporter gene. Cell extracts were assayed for β-galactosidase activity using the β-galactosidase enzyme assay system (Promega) and analyzed by a DU530 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). The ratio of luciferase to β-galactosidase activity was determined in triplicate samples. All data are presented as the mean ± S.E.M. of at least three independent experiments.

Table 1
Primer sequences used in this study.

Gene	Type	Primer sequence
PEPCK	Forward	AGCCTTTGGTCAACAACTGGC
	Reverse	CCTCTGAGCTCCACTCCTTAT
G6Pase	Forward	GGCTGTGCAGCTGAATGTCT
	Reverse	CTGGGCTTTCTCCAGAGTCC
14-3-3β	Forward	TAGTCGACCATGACAATGGATAAAAGT
	Reverse	GAGCGGCCGCTTAGTCTCTCCCTCCCC
14-3-3γ	Forward	TAGTCGACCATGGTGGACCGCGA
	Reverse	TTGCGGCCGCTTCATTGTTGCCTT
14-3-3ε	Forward	TAGTCGACCATGGATGATCGAGAGGAT
	Reverse	GAGCGGCCGCTCACTGATTTTCGTCTTC
14-3-3ζ	Forward	TAGTCGACCATGGATAAAAATGAGCTGG
	Reverse	TTGCGGCCGCTTCATTTTCCCCTCCTT
14-3-3η	Forward	TAGTCGACCATGGGGGACCGGGAGCAG
	Reverse	GAGCGGCCGCTCAGTTGCCTTCTCCTGCTT
14-3-3θ	Forward	TAGTCGACCATGGAGAAGACTGAGC
	Reverse	TTGCGGCCGCTTCGTTTTCAGC
14-3-3σ	Forward	TAGTCGACCATGGAGAGAGCCAGT
	Reverse	TTGCGGCCGCTCCGCTCTGGG
GAPDH	Forward	CCATCACCATCTTCCAGGAG
	Reverse	CCTGCTTCACCACCTTCTTG

2.6. GST pull-down assay

HEK 293A cell were seeded into 10 mm culture dish at a density of 5×10^6 cells per well. The GR, GST-14-3-3β and γ were transfected into HEK 293A cells using Genefectine reagent. For the GST pull-down assay, approximately 30 μl of glutathione Sepharose 4B beads were incubated with 2 mg of cell lysates for 18 h. The beads were then washed for five times with cold PBS by centrifugation at 3000 × g for 3 min and boiled at 100 °C for 10 min. The binding proteins were resolved by 10% SDS-polyacrylamide gel and analyzed by Western blotting using monoclonal antibodies against GR and GST.

2.7. Glucose production assay

Glucose production from HepG2 cells was measured according to the manufacturer's protocol, using a colorimetric glucose oxidase assay kit (Sigma). Briefly, after the indicated experimental time period, cells were washed three times with PBS. Then the cells were incubated for 6 h at 37 °C in glucose production buffer (glucose-free DMEM, pH 7.4, containing 20 mmol/L sodium lactate and 2 mmol/L sodium pyruvate without phenol red). Six hours later, the glucose level in the media was measured. The glucose production assays were performed in triplicate, and the intra-assay coefficient of variation was <5%.

2.8. Statistical analysis

Data are presented as mean ± S.E.M. Statistical evaluation was performed using GraphPad Prism Software 5 (GraphPad Software, San Diego, CA, USA). Two-tailed *t*-test *p* values of 0.05 or less were considered statistically significant.

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