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The hexosamine biosynthetic pathway induces gene promoter activity of acetyl-CoA carboxylase beta

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ABSTRACT

The cardiac isoform of acetyl-CoA carboxylase (ACCβ) produces malonyl-CoA, a potent inhibitor of mitochondrial fatty acid (FA) uptake. Higher ACCβ activity decreases FA utilization, potentially leading to intracellular myocardial lipid accumulation and insulin resistance (IR). Since increased hexosamine biosynthetic pathway (HBP) flux is linked to IR onset, we hypothesized that HBP activation leads to the induction of ACCβ gene promoter activity. Rat H9c2 cardio-myoblasts were transiently transfected with a 1317 bp human ACCβ promoter-luciferase construct (pPIIβ−1317) ± an expression construct encoding the HBP rate-limiting step, i.e., glutamine:fructose 6-phosphate amidotransferase (GFAT) ± various HBP modulators. The administration of L-glutamine (HBP substrate) dose-dependently increased, while HBP inhibitors attenuated pPIIβ−1317 activity. Co-transfections with dominant-negative GFAT constructs diminished pPIIβ−1317 activity. To explore underlying transcriptional mechanisms, we co-transfected with upstream stimulatory factor (USF) expression constructs and found that USF2 induced pPIIβ−1317 activity vs. controls. Moreover, co-transfection of a GFAT expression construct + USF reporter-promoter construct (with consensus USF binding elements) led to induction of pPIIβ−1317 activity vs. controls. We next performed transfections with GFAT ± full length ACCβ and seven truncated promoter-luciferase constructs, respectively. Here GFAT-mediated ACCβ promoter induction was blunted when co-transfected with the pPIIβ−38/+65 deletion construct indicating that USF2 binds to the proximal ACCβ promoter region (near start codon). Our study demonstrates that HBP activation induces ACCβ gene promoter activity in H9c2 cells via USF2. We propose that such ACCβ induction may elicit serious downstream effects, e.g. the inhibition of FA β-oxidation and the onset of IR.

1. Introduction

The incidence and prevalence of insulin resistance/type 2 diabetes is rapidly increasing and constitutes a major concern for both developed and developing countries [9,15]. These alarming projections therefore necessitate the delineation of underlying, molecular mechanisms that drive the onset of such pathophysiologic conditions, with the eventual aim to develop improved therapeutic interventions and diagnostic tools.

There are several biochemical and molecular pathways that contribute to the onset of insulin resistance and type 2 diabetes. However, our focus is on the muscle- and cardiac-enriched isoform of acetyl-CoA carboxylase (ACCβ), encoding a 280-kDa enzyme that plays a key role in controlling mitochondrial fatty acid oxidation (FAO) [1,22]. ACCβ catalyzes the ATP-dependent carboxylation of cytosolic acetyl-CoA to form malonyl-CoA, a potent inhibitor of the mitochondrial FA transfer enzyme carnitine palmitoyltransferase 1 (CPT1) [13]. As the molecular control of the ACCβ gene promoter is not well understood, we began to investigate its regulation and found that it can be activated in a glucose-responsive manner (depending on cell-type) [12]. Since four E-box consensus sequences (CAANTG) were previously identified on the human ACCβ gene promoter [11], upstream stimulatory factors (USFs) emerged as ideal candidate transcription factors regulating its activation. This rationale is based on USFs ability to bind consensus E-box elements located within the promoter regions of several metabolic enzyme-encoding genes [23,24]. Our earlier study data found that upstream stimulatory factor 1 (USF1) forms part of the transcriptional machinery orchestrating ACCβ promoter activation in neonatal cardiomyocytes and CV-1 fibroblasts [12]. We proposed that such an induction would increase malonyl-CoA levels, thereby inhibiting mitochondrial FAO and eventually contributing to the build-up of intracellular long-chain FA levels. In support,

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others have suggested that lower FAO rates found in some type 2 diabetic individuals may be attributed to increased ACCβ gene expression and the subsequent accumulation of malonyl-CoA [4].

For the present study, we further investigated ACCβ gene promoter regulation and evaluated whether its control is linked to hyperglycemia-activated metabolic pathways implicated in the onset of insulin resistance, i.e., the hexosamine biosynthetic pathway (HBP). The HBP usually acts as a fuel sensor that repartitions substrates into suitable storage depots within the body [18]. Here fructose-6-phosphate enters the HBP, forming glucosamine-6-phosphate in a reaction catalyzed by glutamine:fructose-6-phosphate amidotransferase (GFAT), the HBP rate-limiting enzyme [2]. Glucosamine-6-phosphate is ultimately converted to UDP-N-acetylglucosamine (UDP-GlcNAc), the HBP end product. UDP-GlcNAc is a substrate for *O*-linked β-*N*-acetylglucosaminyl transferase transferase (OGT) that catalyzes the *O*-linked transfer of GlcNAc moieties to specific serine/threonine residues of target proteins.

However, chronically activated HBP is maladaptive contributing to pathophysiologic phenotypes, e.g., insulin resistance, mitochondrial impairment, inflammation and cell death [3,6,8,16,17]. Furthermore, *O*-GlcNAc modification is implicated in the modulation of transcriptional mechanisms, thus also contributing to the regulation of gene expression [5]. In light of this, we here hypothesized that HBP activation results in the transactivation of the ACCβ gene promoter in cardio-myoblasts.

2. Materials and methods

2.1. Cell culture

H9c2 rat cardiac-derived myoblasts (ECACC No. 88092904) were maintained at 37 °C (5% CO_2 and 95% humidity) in 5.5 M glucose DMEM (Sigma–Aldrich, St. Louis MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad CA) and 4 mM L-glutamine (Invitrogen, Carlsbad CA). Cells were not allowed to grow to a confluency greater than 70–80% and were cultured for a maximum of 8 passages before growing new cells. We routinely employed passages #9–15 for transfection experiments.

2.2. Transfection studies

Myoblasts were cultured as described above and plated at 35,000 cells per well on 12-well culture plates for 24 h prior to transfection. The next day, cells were transiently transfected for 48 h using FuGene 6 reagent (Roche, Mannheim, Germany) according to the supplier's instructions. Here we employed 0.25 μg of the full-length 1317 bp human ACCβ promoter-luciferase reporter construct (pPIIβ−1317) as previously described [12] ±various co-transfection combinations: (a) 0.25 μg of a human pcDNA3-GFAT expression vector; (b) 0.25 μg pcDNA3-GFAT577 dominant negative expression vector; (c) 0.25 μg pcDNA3-GFAT667 dominant negative expression vector; (d) 0.25 μg of the pUC-SRα-USF1 expression construct; (e) 0.25 μg of the psv-USF2 expression construct; and (f) 0.25 μg of the TransLucent USF Reporter Vector (USF-L) that contains promoter recognition sites for both USF1 and USF2 (Panomics, Redwood City CA). The pPIIβ−1317 construct was generously provided by Dr. Kyung-Sup Kim (Yonsei University College of Medicine, Seoul, Korea) [11]. The GFAT constructs were donated to us by Dr. Cora Weigert (University of Tubingen, Germany) [26], the pUC-SRα-USF1 expression construct provided by Dr. Tetsuya Kamataki (Hokkaido University, Japan) [21], and the psv-USF2 expression construct received from Dr. Michele Sawadogo (University of Texas TX) [14].

In additional experiments, promoter deletion constructs pPIIβ−1090/+65, pPIIβ−800/+65, pPIIβ−569/+65, pPIIβ−349/+65, pPIIβ−93/+65, pPIIβ−38/+65 and pPIIβ−18/+65 were transfected ± pcDNA3-GFAT as earlier described. Here we employed 0.25 μg of each deletion construct, respectively, for co-transfection experiments. The serial deletion human ACCβ promoter-reporter luciferase constructs have been previously described [12] and were kindly provided by Dr. Kyung-Sup Kim (Yonsei University College of Medicine, Seoul, Korea). The total amount of DNA transfected for each experiment was 0.75 μg and DNA concentrations were equalized with pGL3-Basic (vector only) (Promega, Madison WI) to normalize results according to cell number and transfection efficiency.

Twenty-four hours after transfection, media was changed and various drugs added (in separate experiments) to further test our hypothesis. We employed the following agents: (a) 0.1 mM, 1 mM and 2 mM alloxan, respectively, to inhibit OGT; (b) 40 μM azaserine to inhibit GFAT; and (c) 40 μM 6-diazo-5-oxo-L-norleucine (DON) as a GFAT inhibitor. We performed several experiments in our laboratory to test different doses for DON and azaserine (data not shown). These studies allowed us to determine inhibitor concentrations that actually resulted in the predicted effects, i.e., to inhibit GFAT. Thus we eventually employed similar concentrations as also found by others, i.e., 40 μM DON [9] and 40 μM azaserine [7]. For the alloxan doses, we based the concentrations on what was previously reported [10]. We also evaluated the effects of varying L-glutamine (HBP substrate) concentrations (0, 4 and 8 mM) on ACCβ ± GFAT transfections. It is important to note that the media used in culturing cells utilizes 4 mM L-glutamine for standard growing conditions, and that cells that were cultured at 0 mM L-glutamine grew slower than those with higher L-glutamine concentrations. We therefore had to ensure that the L-glutamine concentration of our growing media was maintained at the same level for all experiments to ensure that results were reproducible. Transfections were performed in triplicate for each experiment and repeated to generate the necessary numbers for statistical analysis. At the end of the overall transfection period cells were lysed, protein extracted and expression of luciferase measured using a luminometer as before [12].

2.3. HBP assessment by flow cytometry

We evaluated HBP activation by employing a method previously described by us [20]. Cells were transfected with the GFAT overexpression ± the GFAT dominant negative (GFAT/577) constructs. The total amount of DNA transfected for each experiment was 8 μg. Following 48 h of transfection, cells were harvested, trypsinized, fixed and permeabilized with methanol at 20 °C for 10 min. Thereafter the cells were washed with ice cold PBS and blocked with 5% donkey serum in PBS for one hour at 4 °C. Cells were then incubated overnight at 4 °C in primary anti-*O*-GlcNAc antibody (RL-2 Santa Cruz Biotechnology, Dallas TX) made up in 5% donkey serum in PBS. Following this, the H9c2 cells were washed with ice-cold PBS and incubated for 30 min with fluorescent anti-mouse antibody at 4 °C. *O*-GlcNAc levels were analyzed using a flow cytometer (Becton–Dickinson, Franklin Lakes, NJ) and quantified by determining the mean of fluorescence for each treatment. We typically analyzed 10,000 cells per experiment and completed an $n = 4$ for these experiments.

2.4. Mitochondrial FA utilization

We employed a FAO flow cytometry kit (Abcam, Cambridge MA) that can measure the levels of several FA utilization enzymes. Since we added oleic acid as fuel substrate in these experiments, our analysis focused on acyl-CoA dehydrogenase very long-chain (ACADVL) since it metabolizes both long-chain and very long-chain FAs. Briefly, H9c2 myoblasts were cultured and transfected as

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