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Is Reduced Adiponectin mRNA Associated with Alterations in Transcription Factor ATF3 in Adipose Tissue of Nutritionally Programmed Microswine Offspring?

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Abstract

Poor prenatal and accelerated postnatal growth is associated with elevated cardiometabolic risk in adulthood, but underlying mechanisms are unknown. We previously showed that microswine offspring whose mothers ate a low protein diet (low protein offspring, LPO) exhibited lower adipose tissue (AT) mRNA levels for adiponectin, a cardiometabolically protective hormone, vs. normal protein offspring (NPO) controls. LPO also exhibit hyperreactivity to norepinephrine in vasculature. Therefore, we hypothesized that increased sensitivity to β adrenergic receptors (βAR) in AT causes low adiponectin mRNA level in LPO, and that this is mediated by increases in both CREB, an effector of βAR signaling, and CREB's downstream transcriptional repressor of adiponectin, ATF3. Nuclear proteins and total mRNA were isolated from AT of juvenile LPO and NPO; dot blot and real-time PCR were used to analyze ATF3 and CREB nuclear protein and mRNA, respectively. ATF3 and CREB mRNA levels were reduced in LPO vs. NPO in subcutaneous (SC)-AT, but not in intra-abdominal (ABD)-AT. There was no difference in phosphorylated CREB level based on maternal diet, post-weaning diet, or sex. In ABD-AT, ATF3 mRNA was reduced by post-weaning caloric restriction (CR) in NPO; LPO-CR had higher ATF3 mRNA than NPO-CR. CR also reduced ATF3 in NPO, but not in LPO, in SC-AT. ATF3 protein in ABD-AT was 2.4 times higher in male LPO than in male NPO, though this difference was not statistically significant. Because patterns of ATF3 and CREB do not match those predicted, the βAR system is probably not responsible for reductions in adiponectin mRNA levels.

Keywords: Adiponectin, Fetal Programming, Transcription Factors

1. Introduction

The perception of adipose tissue (AT) being solely an energy storage site has been extended to include an endocrine-like function that plays a pivotal role in regulation of energy homeostasis and metabolism. Apart from secreting free fatty acids, AT also produces and releases numerous substances (adipocytokines) that have autocrine, paracrine, and endocrine functions, as well as anti- and pro-inflammatory effects. Adipocytokines include leptin, tumor necrosis factor α, adipsin, resistin, and adiponectin; they function in regulation of energy intake and expenditure, and regulation of glucose and lipid metabolism[5].

Adiponectin is the gene product of the adipose most abundant transcript 1 gene (APM1). It consists of 247 amino acids, with 4 domains: a signal sequence (removed in the mature protein), a globular domain, a non-conserved variable region, and a collagen-like domain[10]. It is induced during adipocyte differentiation. Paradoxically, in obese subjects with more adipose tissue, adiponectin concentration is lower compared to lean subjects[5]. Adiponectin is a beneficial hormone with multiple effects. It enhances insulin sensitivity by increasing fatty acid oxidation and decreasing hepatic glucose production. It decreases lipid accumulation and increases glucose uptake in muscles. It decreases

cardiomyopathy in the heart, and also decreases glucose production in the liver. In the vasculature, it increases vasodilation, and decreases atherosclerosis and thrombosis[5].

The concept of Developmental Origins of Health and Disease (DOHaD) describes how poor fetal and accelerated childhood growth "program" individuals for elevated cardiometabolic risks, including cardiovascular disease, obesity, type 2 diabetes, osteoporosis, chronic obstructive lung disease, some mental illnesses, and some forms of cancer[6]. Environmental exposure including diet, exercise, parental lifestyle, smoking, and exposure to chemicals and toxins have been shown to modulate these risks.

In previous studies, pregnant microswine sows were randomized to a normal (NP) or low protein (LP) diet. Offspring of these sows were randomized to an Ad Lib (AL) or Calorie Restriction (CR) post-weaning diet, in order to study the effects of maternal and offspring diet on the level of adiponectin and other growth factors. Results have shown that adiponectin mRNA level in adipose tissue (AT) was significantly lower in the low protein offspring (LPO) compared to the normal protein offspring (NPO), regardless of sex (male/female), tissue depot (ABD/SC), maternal diet (NP/LP)[3], or post-weaning diet (AL/CR; unpublished observation). In this study, we continued with the same animal model to identify the mechanism leading to lower adiponectin mRNA in the LPO compared to NPO in previously observed. Classically, alterations in mRNA level are driven by transcriptional rate. We hypothesized that reduced adiponectin mRNA level was caused by an increase in transcriptional repression of the adiponectin gene, possibly by two transcription factors proposed to be related to the regulation of adiponectin via the βAR signaling pathway.

The first transcription factor is Activating Transcription Factor 3 (ATF3), which is a member of ATF/CREB family. In contrast to the implication of its name, ATF3 is a repressor of adiponectin, especially when induced by a variety of stress signals[1,8]. The cAMP Response Element Binding Protein (CREB) is the classical mediator of the βAR-induced gene expression, and is induced via cAMP and activation of protein kinase A (PKA) by phosphorylation on Serine 133[4,9]. CREB triggers the expression of the transcriptional repressor ATF3, which leads to a down-regulation of adiponectin **(Fig 1**).

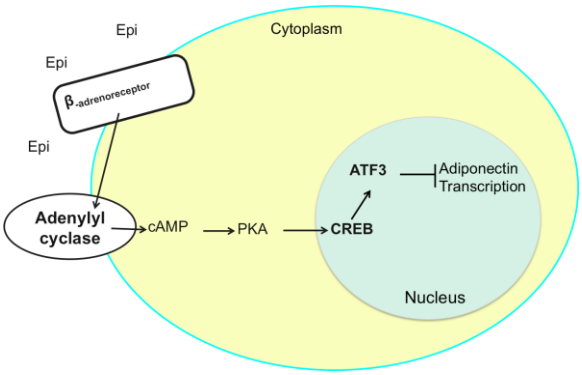

Figure 1: The β-adrenergic signaling pathway, mediated via cAMP and PKA that may influence adiponectin transcription via CREB and ATF3**.**

Epinephrine (Epi) binds the β-adrenoreceptor; $G\alpha_s$ then activates adenylyl cyclase. Cyclic AMP (cAMP) is produced and activates protein kinase A (PKA). PKA phosphorylates CREB, which in turn activates ATF3, which then inhibits adiponectin transcription.

We hypothesized that reduced adiponectin mRNA level in LPO observed in previous studies was due to a repression by transcription factor ATF3 as a result of excessive βAR signaling. We predicted that ATF3 was up- regulated in LPO compared to NPO controls. We also predicted that CREB was hyperactivated by PKA via the βAR signaling pathway, leading to a down-regulation of adiponectin.

2. Methodology

2.1. Animal Model And Experimental Design

The results in this study were obtained from the same animals described in previous studies[2,3]. General methods as well as those specific to this study are reported here. For details, refer to previous papers[2,3]. Pregnant Yucatan

microswine sows were randomized to either a control diet (NP) (14% protein/75% carbohydrate), or an isocaloric low-protein (LP) diet (1% protein, 88% carbohydrate) applied during the last fourth of gestation until 2 weeks postnatally. At 2 weeks, sows were returned to a standard diet. Offspring were weaned at 4 weeks, and randomized to either a standard ad libitum (AL) diet (20% protein, 4% fat), or a calorie restricted (CR) diet. The CR diet was the same content as the AL diet, but provided in reduced amount designed to prevent accelerated growth previously observed in LPO offspring fed an AL diet [2]. Thus, the CR diet restricted growth in LPO to keep each animal's body weight reduced throughout the study by the same percentage reduction for that animal at 2 weeks of age compared with sex-matched NPO-AL controls.

2.2. Tissue Collection

NPO-AL (n=16; 7 males, 9 females), LPO-AL (n=16; 9 males, 7 females), NPO-CR (n=11; 6 males, 5 females), and LPO-CR (n=7; 4 males, 3 females) offspring were sacrificed at 14-22 weeks of age. Subcutaneous adipose tissue (SC-AT) from the ventral abdomen, and intra-abdominal adipose tissue (ABD-AT) from the lateral interior abdominal wall were quickly harvested from animals under isoflurane anesthesia, snap frozen in liquid nitrogen, and stored at -80ºC until use.

2.3. Nuclear Extract Preparation From Frozen Adipose Tissue

Frozen AT was minced, then homogenized using a Dounce homogenizer in ENE1 buffer from the Episeeker Nuclear Extraction Kit (Abcam, Cambridge, MA). Extracts were centrifuged at 11,752 x g for 10 min at 4ºC. The supernatant was saved as the cytoplasmic fraction. The pellets were re-incubated in ENE2 buffer with DTT. Re-suspended pellets were then sonicated (3x10s), followed by another centrifugation step at 16,000 x g for 10 min at 4ºC. The supernatant was transferred to new tubes and stored at -80ºC as the nuclear fraction until analysis via dot blot.

2.4. Real-Time Polymerase Chain Reaction (PCR)

Snap-frozen tissue was homogenized in TRIzol (Invitrogen Corporation, Carlsbad, CA), and total ribonucleic acid (RNA) was extracted according to manufacturer instructions. RNA was treated with DNase I (Invitrogen), and reversed transcribed using random hexamer primers (Eurofins MWG Operon, Huntsville, AL) and SuperScript II reverse transcriptase (Invitrogen).

The SYBR Green method was used to analyze cDNA in triplicate. Gene-specific primers to ATF3 (Fwd: 5'-gtggagacaggagcaaaatg-3'; Rev: 5'-GGATGGCGAACCTCAGCTC-3'), CREB (Fwd: 5'-gagtgccaaggattgaagaggag-3'; Rev: 5'-AGTGGCAGCTGCATTGGTC-3') and 18s ribosomal ribonucleic acid (rRNA) (Fwd: 5'-cagcagccgcggtaattc-3'; Rev: 5'-ACGAGCTTTTTAACTGCAGCAA-3') were designed using Primer Express software (Life Technologies, Grand Island, NY). All primers were synthesized by Operon Biotechnologies. Amplification reactions were run on an ABI Prism 7500 (Life Technologies) at 50ºC for 2 min, 95ºC for 10 min, then 40 cycles of 90ºC for 15 s and 60ºC for 60 s. Relative gene expression was calculated by the standard curve method using serial dilutions of a pool of a large subset of samples, and normalized to the endogenous control gene (18s rRNA).

2.5. Dot Blot

AT nuclear extracts (4µg) were blotted directly onto nitrocellulose membranes and allowed to dry. The proteins were detected using specific antibodies for either phospho-CREB (1:500) (Cell Signaling Technology, Danvers, MA) or ATF3 (1:500) (Abcam, Cambridge, MA), and horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:1000) (Cell Signaling Technology), and were visualized by enhanced chemiluminescence using film. Spots were analyzed by densitometry using NIH Image J software (NIH, Baltimore, MD).

2.6. Statistical Analysis

For dot blot, each animal's raw densitometry values were converted to % of male NPO-AL mean. These were analyzed by ANOVA using maternal diet, post-weaning diet, and sex as factors. For real-time PCR, data are shown as a ratio of gene-of-interest to endogenous control (18s rRNA). For analysis, ratios were Ln transformed, then analyzed by

ANOVA using maternal diet, post-weaning diet, sex, and AT depot as factors. Data are presented as mean ± S.E.M. For all statistical tests, a P-value < 0.05 was considered significant.

3. Data

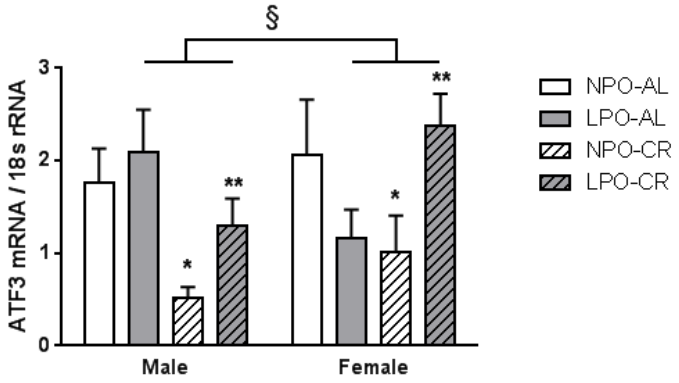

Figure 2. ATF3 mRNA level in ABD-AT of NPM-AL (n=7), LPM-AL (n=9), NPM-CR (n=6), LPM-CR (n=4), NPF-AL (n=9), LPF-AL (n=7), NPF-CR (n=5), and LPF-CR (n=3) microswine offspring, shown as a ratio of ATF3 to endogenous control. *P=0.02 compared to NPO-AL; **P=0.002 compared to NPO-CR; §P=0.04 interaction between sex and postweaning diet (LPO only).

ATF3 mRNA level in ABD-AT was decreased significantly in NPO-CR (NPM-CR: 0.52 ± 0.30; NPF-CR: 1.01 ± 0.88) compared to the NPO controls (NPM-AL: 3.23 ± 3.96; NPF-AL: 2.07 ± 1.78; P=0.02; **Fig 2).** In the LPO groups, there was a decrease in ABD-AT ATF3 mRNA in response to CR for males (LPM-AL: 2.60 ± 1.95; LPM-CR: 1.29 ± 0.59; **Fig 2**); however the females showed an increase in ATF3 mRNA (LPF-AL: 1.16 ± 0.75; LPF-CR: 2.38 ± 0.59; P=0.04 for interaction) under similar conditions, although neither observation alone was significant. LPO-CR also showed a significant increase in ABD-AT ATF3 mRNA compared to NPO-CR in both males (LPM-CR: 1.29 ± 0.59; NPM-CR: 0.52 ± 0.30) and females (LPF-CR: 2.38 ± 0.59; NPF-CR: 1.01 ± 0.88; P=0.002 for combined sexes; **Fig 2)**.

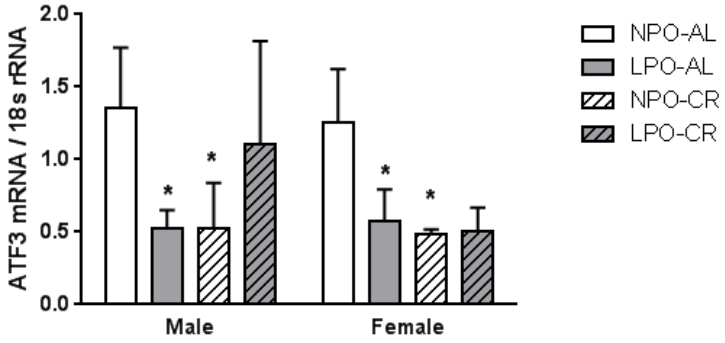

Figure 3. ATF3 mRNA level in SC-AT of NPO-AL (n=16; 7 males, 9 females), LPO-AL (n=16; 9 males, 7 females), NPO-CR (n=11; 6 males, 5 females), and LPO-CR (n=7; 4 males, 3 females) microswine offspring, shown as a ratio of ATF3 to endogenous control. *P<0.05 compared to NPO-AL.

In SC-AT, ATF3 mRNA was reduced in LPM-AL (0.53 ± 0.35) compared to NPM-AL (1.36 ± 1.01; P<0.05; **Fig 3**). The same pattern was observed in SC-AT of females: LPF-AL (0.58 ± 0.57) had lower ATF3 mRNA level compared to NPF-AL (1.26 ± 1.09; P<0.05; **Fig 3**). There was also a decrease in ATF3 mRNA in response to CR in both NPM-CR (0.53 ± 0.83) and NPF-CR (1.91 ± 3.19) compared to their NPO-AL control (NPM-AL: 1.36 ± 1.01; NPF-AL: 1.26 ± 1.09; P<0.05; **Fig 3)**. There was a non-significant increase in the LPM-CR (1.10 ± 1.43) compared to LPM-AL

(0.53 ± 0.35; P=NS; **Fig 3)**. However, LPF-CR (0.51 ± 0.28) showed a blunted reduction in ATF3 mRNA compared to LPF-AL (0.58 ± 0.57; P=NS; **Fig 3)**.

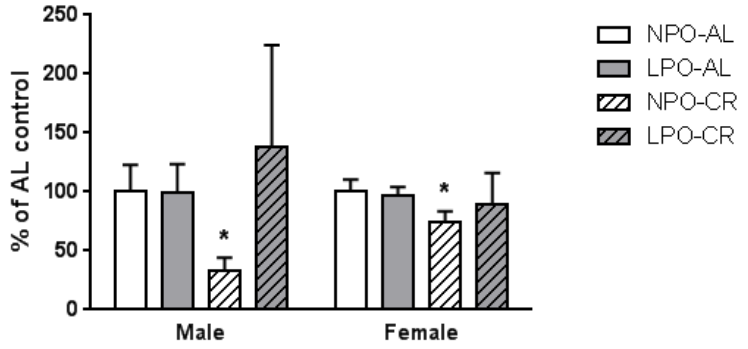

Figure 4. ATF3 protein level in ABD-AT of NPM-AL (n=7), LPM-AL (n=9), NPM-CR (n=6), LPM-CR (n=4), NPF-AL (n=9), LPF-AL (n=7), NPF-CR (n=5), and LPF-CR (n=3) microswine offspring, shown as % of AL control. *P<0.05 compared to NPO-AL.

ATF3 protein in ABD-AT was decreased in the NPM-CR compared to NPM-AL (NPM-CR: 32.9 ± 29.5%; NPM-AL: 100 ± 56.4%; **Fig 4**), as well as between NPF-CR (73.8 ± 21.6%) and the NPF-AL control (100 ± 30.6%; P<0.05 for combined sexes; **Fig 4**). No maternal diet effect was observed for ATF3 protein level in ABD-AT.

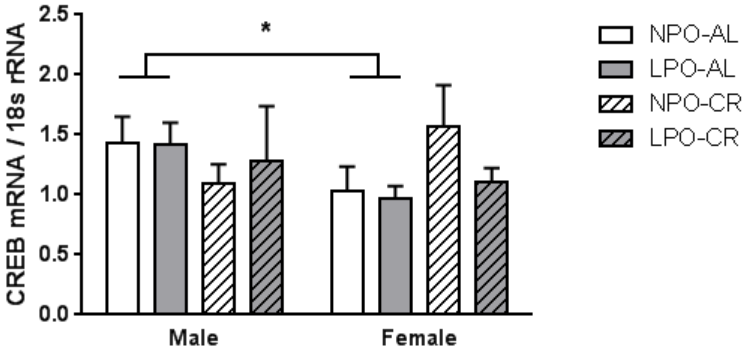

Figure 5. CREB mRNA level in ABD-AT of NPO-AL (n=16; 7 males, 9 females), LPO-AL (n=16; 9 males, 7 females), NPO-CR (n=11; 6 males, 5 females), and LPO-CR (n=7; 4 males, 3 females) microswine offspring, shown as a ratio of CREB to endogenous control. *P<0.05 female AL compared to male AL.

CREB mRNA level was significantly lower in the female AL compared to the male AL (NPM-AL: 1.43 ± 0.59; NPF-AL: 1.03 ± 0.61; LPM-AL: 1.42 ± 0.54; LPF-AL: 0.96 ± 0.26; P<0.05; **Fig 5**). We did not observe any significant changes in CREB mRNA level in SC-AT **(Fig 6)**. There was no maternal diet or caloric diet effect in CREB mRNA level that would explain our adiponectin data in previous studies.

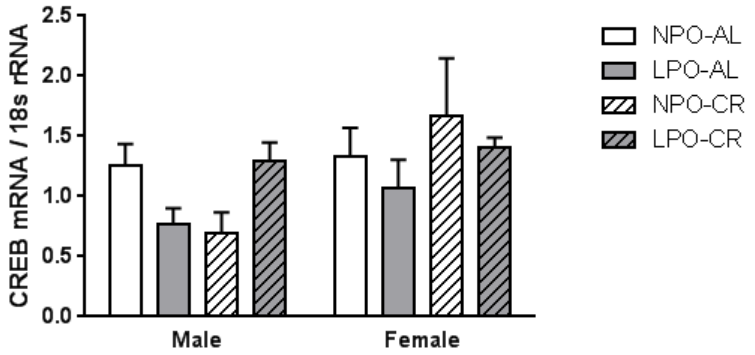

Figure 6. CREB mRNA level in SC-AT of NPO-AL (n=16; 7 males, 9 females), LPO-AL (n=16; 9 males, 7 females), NPO-CR (n=11; 6 males, 5 females), and LPO-CR (n=7; 4 males, 3 females) microswine offspring, shown as a ratio of CREB to endogenous control.

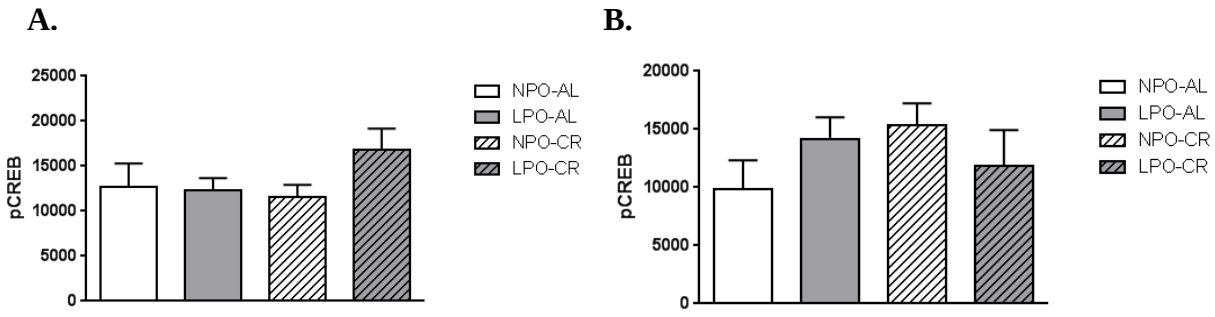

Figure 7. Level of phosphorylated CREB in ABD-AT of **A.** male (n=26: NPO-AL, 6; LPO-AL, 9; NPO-CR, 6; LPO-CR, 3) and **B.** female (n=24: NPO-AL, 9; LPO-AL, 7; NPO-CR, 5; LPO-CR, 3) microswine offspring.

No significant difference was observed for phospho-CREB level in ABD-AT based on maternal diet or postnatal diet **(Fig 7)**. Comparison of males to females was not possible because sexes were analyzed in separate assays.

4. Discussion

Adiponectin is a cardiometabollically beneficial hormone with multiple effects. Previous studies have found a lower adiponectin mRNA level in microswine offspring exposed to maternal perinatal protein restriction (LPO) compared to NPO, and the trend was consistent regardless of sex, AT depot, maternal diet[3], and postweaning diet (unpublished observations). In order to explain the reduction in adiponectin mRNA level in LPO compared to NPO, we assessed ATF3 and CREB mRNA level in ABD and SC-AT, as well as ATF3 and phospho-CREB protein level in ABD-AT. CREB, and its downstream transcription factor ATF3, are mediated via the βAR signaling pathway. Binding of epinephrine to the adrenergic receptor triggers the production of cAMP, which in turn activates CREB and ATF3 transcription, leading to a down regulation of adiponectin **(Fig 1)**.

We found no significant maternal diet effect that would explain our reduced adiponectin mRNA pattern previously observed[3]. There was a significant decrease in ATF3 mRNA in response to CR in the NPO-CR in both ABD and SC-AT compared to the NPO-AL control (**Fig 2 & 3**). However, the result was in the opposite direction of what we predicted. In order to have a decrease in adiponectin level in LPO, there must be a higher ATF3 and/or CREB mRNA level compared to the NPO-AL control, since ATF3 and CREB negatively regulate adiponectin transcription via the βAR signaling pathway[9]. There was a significant decrease in ATF3 protein level between the NPO-AL and NPO-CR; however, there was no change in ATF3 between the LPO-AL and LPO-CR **(Fig 4)**. Our ATF3 protein data do not explain the adiponectin pattern previously observed; however, the lack of response to CR suggests a vulnerability or inappropriate response to stress conditions in the LPO.

All cells exhibit alterations in gene expression in response to extracellular stress signals, such as elevated temperature, lack of nutrients, and exposure to toxins. By in situ hybridization, pervious studies have demonstrated that the steady-state mRNA level of ATF3 increased greatly upon exposure to a variety of stresses[11]. The induction of ATF3 is a common cellular response to many stress signals; it is neither tissue- nor stress-specific. Considering the LPM-CR and LPF-CR, they showed changes in ATF3 level in opposite directions in response to CR compared to their corresponding LP-AL control (**Fig 2, 3 & 4**), indicating a potential response mechanism that differs between sexes in stressful situations.

Although there were no significant changes in CREB mRNA level, those results were not surprising because CREB activation is typically regulated through phosphorylation by PKA; therefore, we also measured phospho-CREB level and found no significant difference. CREB translation and activation seem to have been maintained regardless of being nutritionally programmed (**Fig 5, 6 & 7**). This indicated that CREB was not a likely candidate for transcriptional modification of adiponectin mRNA level in LPO.

Wolfgang et al. have found an unexpected ATF3-binding site in its own promoter, which was efficient for autorepression by ATF3. The binding site differed from the consensus ATF/CRE-binding site, and it was immediately downstream from the TATA box[11]. Those findings suggested that ATF3 could repress its own promoter, which played an important role in the prevention of sustained gene expression. The results of this study being opposite from our expectation could be due to ATF3 autorepressing its own promoter, especially in times of stress when the gene was continuously induced. Clearly, this is only a speculation at this point, and much work is required to test this supposition.

For future studies, we need to probe additional transcription factors, such as peroxisome proliferator- activated receptor γ (PPARγ) and C/EBPα, to explain the observation of reduced adiponectin in LPO-AT. PPARγ regulates the expression of a large number of genes involved in adipocite differentiation, lipid, carbohydrate metabolism, and adipokine synthesis[1,2]. C/EBPα has been found to regulate the expression of many immunoglobulins, cytokines, chemokines, and growth factors. Recently, it has been found that co-expression of PPARγ and C/EBPα synergistically activates the expression of many key metabolic adipocyte genes[7]. We need to consider non-transcriptional modification of mRNA level, such as mRNA instability, or miRNA, which regulates gene splicing, and also test ATF3 activation by nutritionally responsive pathways.

5. Acknowledgements

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