African Journal of Biotechnology Vol. 10(5), pp. cc-733, 31 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.927
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

The gene expressions of DNA methylation/demethylation enzymes and cytochrome c oxidase subunit 4 in skeletal muscle of thyroidectomized rats

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Accepted 8 December, 2010

A decrease in mRNA levels for cytochrome c oxidase (COX) subunits was observed in skeletal muscle of hypothyroid rats. However, the precise expression mechanisms of the related genes in hypothyroid state still remain unclear. This study investigated gene expressions of DNA methyltransferases (*Dnmts*), DNA demethylases and *Cox4* in skeletal muscle of thyroidectomized rats. The findings showed that, the mRNA levels of genes encoding *Dnmts* and DNA demethylases were up-regulated while the mRNA levels of *Cox4* down-regulated. These results imply that, DNA methylation might down-regulate *Cox4* expression and induce a compensatory mechanism for avoiding an excessive incorporation of 5-^{m}C into DNA.

Key words: Gene expression, *Dnmts, Mbd4, Gadd45α, Cox4*.

INTRODUCTION

Hypothyroidism is known to be associated with muscular weakness, which is associated with low oxygen utilization and adenosine triphosphate (ATP) synthesis (Guerrieri et al., 1998). The cytochrome c oxidase (COX), the terminal enzyme in the respiratory chain, catalyzes the translocation of protons across the mitochondrial membrane. The translocated protons drive the synthesis of ATP (Kornblatt, 1980). It was reported that, the mRNA levels of COX subunits were generally decreased in skeletal muscle of hypothyroid rats (Wiesner et al., 1992). However, the precise expression regulation mechanisms of the related genes in hypothyroid state still remain unclear. Earlier studies, such as by Wong et al. (1989), reported that hyermethylation of Spot14 was consistent with its markedly reduced expression level in hepatic DNA derived from hypothyroid rats (Wong et al., 1989). These results lead to the speculation that, DNA methylation might be a potential regulation mechanism for transcription represssion of cyclooxygenase in skeletal muscle from hypothyroid animals.

The DNA methylation is carried out by DNA methyltransferases (*Dnmts*) which catalyze the covalent addition of a methyl group from a donor S-adenosyl methionine to the 5 position of cytosine, predominantly within the CpG islands (Robertson, 2001). The *Cox4* gene, encoding one subunit of COX, is located on chromosome19q12 with a CpG dense region (Virbasius and Scarpulla, 1990). CpG islands which exist in the promoter of *Cox4* gene provide possible DNA methylation sites.

Up till now, there are at least three *Dnmts: Dnmt1*, *Dnmt3a*, and *Dnmt3b* (Chen and Li, 2006). Among these, *Dnmt1* preferentially methylates hemi-methylated DNA

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Abbreviations: COX, Cytochrome c oxidase; *Dnmts*, DNA methyltransferases; **RT-PCR,** Real-time polymerase chain reaction amplification.

(Pradhan et al., 1999); *Dnmt3a* and *Dnmt3b* are responsible for *de novo* methylation during embryogenesis (Okano et al., 1999). Recent evidence suggests that, *Dnmt3a* and *Dnmt3b* also cooperate with *Dnmt1* for the maintenance function, which helps to transmit methylated DNA patterns from parental to daughter cells (Jones and Liang, 2009). *Dnmt3a* expression is ubiquitous and can be readily detected in most adult tissues, whereas *Dnmt3b* is expressed at very low levels in most tissues except the testis, the thyroid gland and bone marrow (Xie et al., 1999). The DNA methylation is reversible and DNA demethylation enzymes are responsible for active demethylation (Szyf, 2007), which is generally with induction of gene expression (El Kharroubi et al., 2001; Tinker and Brown, 1998). Although the biochemical properties of the enzymes responsible for active demethylation are still controversial, one proposal has been put forward that, a coupled mechanism of 5-^{m}C demethylation in Zebrafish, whereby activation induced deaminase (AID) deaminates 5-^{m}C, followed by thymine base excision by methyl-CpG binding domain 4(*Mbd4*), promoted by the growth arrest and DNA damage-inducible protein α(*Gadd45α*) (Rai et al., 2008). *Mbd4* and *Gadd45α* play a key role in DNA demethylation. *Mbd4 k*nockdown impairs parathyroid-hormone-induced DNA demethylation and subsequent transcriptional derepression (Kim et al., 2009). *Gadd45α* over expression elicits global genome demethylation in zebrafish embryos (Rai et al., 2008). Knockdown of *Gadd45α* leads to hypermethylation and gene inactivation (Barreto et al., 2007). However, it is not well clear whether *Cox4* is regulated by DNA methylation, such as little information of expression patterns of *Dnmts, Mbd4* and *Gadd45α* in hypothyroidism. Here, we hypothesize that, DNA methylation exists in hypothyroidism and regulates the mRNA levels of *Cox4* in skeletal muscle. Therefore, we test the mRNA levels of *Dnmt1, Dnmt3a, Mbd4, Gadd45α* and *Cox4* in hypothyroid rats.

MATERIALS AND METHODS

Animals and treatment

Male Sprague-dawle rats (weight 180 to 220 g) were divided in two groups. Group 1 was surgically thyroidectomized as described previously by Katyare (Katyare and Rajan, 2005). The sham-operated control animals received a similar treatment without removal of their thyroid glands. All animals were fed with *ad libitum*. The rats were kept in 12 h light: 12 h darkness cycles in a temperature controlled room. 30 days after the surgery, the rats were anesthetized by intraperitoneal injection of Pentobarbital Sodium (30 mg/kg) and killed by decapitation. Then, quadriceps muscles were excised and immediately put into liquid nitrogen and kept at -80°C

Quantification of mRNA expression

Frozen tissues were homogenized in Trizol reagent solution (TransGen, China), and then the total RNA was extracted according to the manufacture's instructions. The purity of the RNA was quantified and assessed by Protein Nucleic Acid Analyzer (Beckman, Germany). An amount of 1 μg total RNA, mixed with reverse transcription primers, was incubated at 65°C for 5 min, then, mixed with the reaction buffer and dNTPs and revert Aid M-MuLV reverse transcriptase (Fermentas, Lithuania) for 1 h at 42°C. Real-time polymerase chain reaction amplification (PCR) assay, involved LineGene (Bioer, China) technology associated rapid thermocycling with on-line fluorescence detection of the PCR products. PCR reactions were performed in a volume of 20 μL containing oligonucleotide primers (0.2 μM of each), cDNA and SYBR premix Ex Taq (Takara, China). Amplification occurred in a two-step procedure: denaturation at 95°C for 30 s and 40 cycles with denaturation at 95°C for 5 s, 60°C for 30 s. The forward and reverse primer sequences were shown in Table 1. Specificity of primers was validated through the verification of RT-PCR product specificity. Quantification data were analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

The independent sample *t*-test was used for significant test between two groups by SPSS15.0.

RESULTS AND DISCUSSION

In our study, the mRNA expressions *Dnmt1* mRNA and *Dnmt3a* were increased ~2.5 and ~4.4-fold, while the *Cox4* mRNA expression was reduced (74%) in hypothyroid rats relative to the level of control animals, respectively (Figure 1). As for *Mbd4* and *Dnmt3a* genes, their expressions were elevated ~2.2 fold and ~2.3-fold, respectively (Figure 2). Previously, *Dnmt1* and *Dnmt3a* are responsible for maintenance and *de novo* CpG methylation and the expressions of both genes are higher in the rat hepatoma, in which the promoter of tumor suppressor genes are methylated, when compared with the liver (Hsieh, 1999; Pradhan et al., 1999). In 1998, Nan et al also reported that, CpG methylation of the promoter region inactivates gene expression. According to latest publication, our result of significant elevation of *Dnmt1* and *Dnmt3a* is consistent with the result in rathippocampus (Sui and Li, 2010). Meanwhile, the significant low *Cox4* mRNA level is also consistent with previous report (Wiesner et al., 1992).

Some genes, such as Spot4, reeling gene and brain-derived neurotrophic factor (BDNF) gene, are reported to be methylated in hypothyroidism (Sui and Li, 2010; Wong et al., 1989). Considering DNA methylation usually inactivating gene expression, the results of up-regulation of *Dnmt1* and *Dnmt3a* mRNA while down-regulation of *Cox4* mRNA indicated that, DNA methylation might regulate the transcription of *Cox4.*

To our best knowledge, the publication related to expression profiles of DNA demethylases in hypothyroid animals is fairly lacking, including the genes involved in DNA demethylation such as *Mbd4* and *Gadd45α* (Rai et al., 2008). Previously, recent publications report that, *Mbd4* knockdown impairs parathyroid-hormone-induced DNA demethylation and subsequent transcriptional derepression and that Gadd45α overexpression activates

Table 1. Real-time PCR, primers.

Gene	Genbank accession number	Forward primer (5′–3′) reverse primer (5′–3′)	PCR product size (bp)	Annealing temperature (℃)
β-actin	NM_031144	TGGGTATGGAATCCTGTG GTGTTGGCATAGAGGTCTTT	91	60
Dnmt1	NM_053354	ACCTACCACGCCGAC AT AGGTCCTCTCCGTACTCCA	104	60
Dnmt3a	NM_001003958	CAGCAAAGTGAGGACCATTA AACACCCTTTCCATTTCAG	123	60
Cox4	NM_017202	GCAGCAGTGGCAGAATGT ATCAGGCAAGGGGTAGTCA	158	60
Mbd4	XM_001059437	CTGGGTGGAGAAAAGAGA GAGGGAATCACAACAAATG	120	60
Gadd45α	XM_575660	ATTCGTGCTTTCTGTTGC GCTCTTGTCGTTCTCCAGTA	96	60

The *β-actin* cDNA was used as a housekeeping gene for the relative quantification of cDNA of *Cox4, Dnmt1, Dnmt3a, Mbd4* and *Gadd45α*.

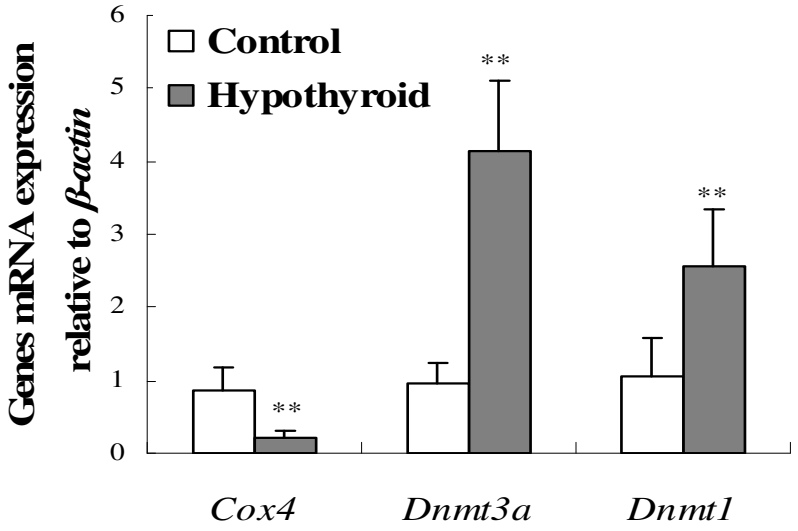

Figure 1. Effects of hypothyroidism on expressions of *Cox4, Dnmt1* and *Dnmt3a* in the skeletal muscle of rats. Results are presented as mean ± S.D (n = 5-8). **P < 0.01 was the result of significant analysis between hypothyroid group and control group.

methylation-silenced reporter plasmids and promotes global DNA demethylation (Balada et al., 2007; Kim et al., 2009). In this study, it is surprising that the pattern of change in *Mbd4* and *Gadd45α* mRNA levels was similar to that for *Dnmts* in the skeletal muscle of hypothyroid rats. The significant elevation of *Mbd4* and *Gadd45α* mRNA levels in our test suggested that, DNA demethylation might also exist in hypothyroid animals as well as DNA methylation. However, we cannot exactly conclude the reason for the co-existence between DNA methylation and DNA demethylation in hypothyroidism. Referring to Balada et al. (2007), the possible explanation is the existence of a compensatory mechanism for avoiding an excessive incorporation of 5-^{m}C into DNA.

In brief, we have studied the gene expressions of *Dnmt1, Dnmt3a, Cox4, Mbd4* and *Gadd45α* in mRNA level in thyroidectomized rats. Our results suggested that, the transcription of *Cox4* might be regulated by DNA methylation in the skeletal muscle of hypothyroid rats and DNA demethylation might exist in hypothyroidism.However, the exact sites of methylation in *Cox4* and the interaction of DNA methylation/demethylation enzymes in

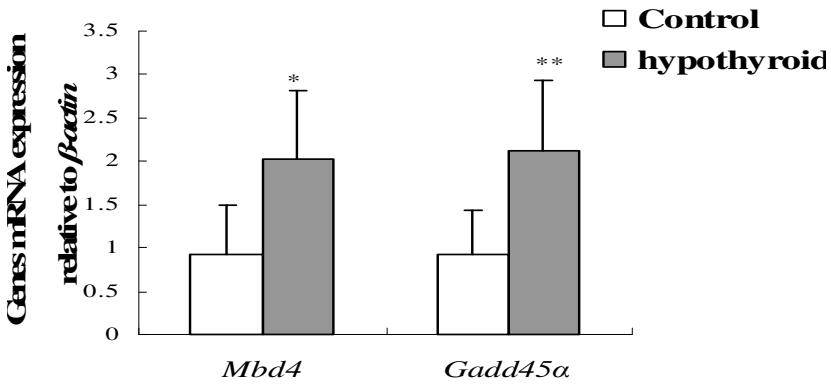

Figure 2. Effects of hypothyroidism on expressions of *Mbd4* and *Gadd45α* in the skeletal muscle of rats. Results are presented as mean ± S.D (n = 5-8). *P < 0.05 and **P < 0.01 were the results of significant analyses between hypothyroid group and control group.

hypothyroidism need further studies demonstration.

ACKNOWLEDGEMENTS

This study was supported by grant from the Major State Basic Research Development Program of China (973 Program) (2006CB504702) and by the science and technology plan projects of Jiangxi Provincial Education Department(GJJ11548). We would like to thank the staff of the Laboratory of traditional Chinese medicine germplasm for their help. We thank also Kaifeng Chen for the care of animals. Finally, we thank Dr. Qinglong Shu for suggestions of the revising manuscript.

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