Chemico-Biological Interactions 195 (2012) 68–75

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Inheritable stimulatory effects of caffeine on steroidogenic acute regulatory protein expression and cortisol production in human adrenocortical cells

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ARTICLE INFO

Article history:
Received 25 August 2011
Received in revised form 29 October 2011
Accepted 3 November 2011
Available online 12 November 2011

Keywords:
Caffeine
Steroidogenesis
Steroidogenic acute regulatory protein
Cortisol
DNA methylation

ABSTRACT

Caffeine is the most widely consumed psychoactive substance in the world. It can elevate the level of glucocorticoid which is involved in metabolism regulation, stress response, and immune function. However, the specific mechanism has yet to be elucidated. Glucocorticoid is steroid hormone synthesized in adrenal cortex and the key rate-limiting step in its biosynthesis is mediated by steroidogenic acute regulatory protein (StAR). This study was designed to investigate the direct effects and inheritable epigenetic mechanisms of caffeine on cortisol production and StAR expression in human adrenocortical cells. The human adrenocortical cell line NCI-H295A was cultured with 0.4–40 μM caffeine. There was a significant increase of the cortisol production in cells. In both acutely and chronically caffeine-treated cell groups, mRNA and protein expressions of StAR were stimulated in a dose-dependent manner. DNA methylation detection via bisulfite-sequencing PCR (BSP) uncovered a single site CpG demethylation at nt −682 within the StAR promoter region. Then we investigated how long the increased StAR expression and the single CpG demethylation could last. The caffeine was withdrawn after 48 h of treatment and then the cells were continually subcultured for up to 5 and 10 passages, respectively. The results showed that the StAR expression at post-caffeine passage 10 still increased, as compared with that in the control. The caffeine-induced demethylation at nt −682 in StAR promoter underwent a similar time course as StAR expression does. The present study reveals the direct effect and possible inheritable epigenetic mechanism of caffeine on steroidogenesis in human adrenocortical cells and has implications for our understanding of the consumption of caffeine.

1. Introduction

Caffeine is a xanthine alkaloid widely consumed in the form of coffee, tea, soft beverage, food and some analgesic drugs. For example 52% of all persons in the US over 10 years of age consume coffee [1]. Since caffeine is the most widely consumed psychoactive substance, it is important that it be investigated. While caffeine induces several well-documented positive effects such as increased alertness and cognition, caffeine can induce several potentially negative consequences. It has been shown that people who intake caffeine-contained food or drink are highly susceptible to metabolic syndrome [2–4]. Metabolic syndrome is a common complex trait comprising a set of risk factors for cardiovascular disease and type 2 diabetes mellitus. Abdominal obesity, ectopic lipogenesis, low-grade chronic inflammation, and resultant insulin resistance are the main processes thought to be responsible for the clinical manifestations of the metabolic syndrome. These processes are closely related with high glucocorticoid levels [5,6]. It is noteworthy that caffeine elevates glucocorticoid levels in both animals and humans. Acute caffeine increases plasma cortisol levels when injected into healthy subjects [7,8] and elevates plasma corticosterone levels in rats [9]. But the specific mechanism of caffeine induced glucocorticoid release has yet to be elucidated.

The glucocorticoids cortisol (humans and primates) and corticosterone (rodents) are steroid hormones synthesized from cholesterol in the zona fasciculata of the adrenal cortex. In humans, cortisol is the principal glucocorticoid, involved in metabolism regulation, stress response, and immune function [10]. Steroidogenic

Abbreviations: BSP, bisulfite-sequencing PCR; DNMT3b, DNA methyltransferase 3b; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LCAH, lipoid congenital adrenal hyperplasia; MDBP, methyl-DNA binding proteins; MeCPs, methyl-CpG binding proteins; MSP, methylation-specific PCR; SDS–PAGE, sodium dodecyl sulfate–polyacryamide gels; SF-1, steroidogenic factor-1; SIT, selenium/insulin/transferrin; SREBPs, sterol regulatory element-binding proteins; StAR, steroidogenic acute regulatory protein.

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doi:10.1016/j.cbi.2011.11.001

acute regulatory protein (StAR) mediates the translocation of cholesterol from the outer to the inner mitochondrial membrane, which is the initial and rate-limiting step in adrenocortical steroid biosynthesis [11–13]. The crucial role of StAR in the regulation of steroidogenesis has been observed in patients suffering from lipoid congenital adrenal hyperplasia (LCAH), an autosomal recessive disorder in which both adrenal and gonadal steroid biosyntheses are severely impaired [13,14]. Recent advances in tissue-specific and hormone-induced expression of the StAR provide insights into a number of human endocrinological health issues including developmental and reproductive abnormalities [15–18]. Our previous studies and other lab's research data have reported that several chemicals and cytokines, such as nicotine, polychlorinated biphenyls, apigenin, interleukin-1 and adiponectin, affect StAR expression and the subsequent steroidogenesis in adrenal [16,19–22]. However, whether caffeine could alter StAR expression in adrenal to elevate glucocorticoid levels is unknown, not to mention the underlying mechanisms.

Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence [23]. In the mammalian genome, DNA methylation is one of the most commonly occurring epigenetic events resulting in the covalent addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring [24]. The patterns of DNA methylation are heritable marks that ensure accurate transmission of the chromatin states and gene expression profiles over many cell generations. Aberrant DNA methylation occurs in human adrenocortical tumorigenesis that is often accompanied by abnormal hormone production [25]. Kwon et al. have reported that caffeine treatment can affect the DNA methylation status and *in vitro* development of porcine nuclear transfer embryos in relation to nuclear reprogramming [26]. Our recent study showed that caffeine can affect several genes involved in the epigenetic modifications in primary human fetal adrenal cortex cells by using gene array assays (Supplemental Data). For example, caffeine decreased the expression of DNA methyltransferase 3b (DNMT3b), which indicated that caffeine could change the DNA methylation patterns of genes. In this study, we investigated the direct effects of caffeine on adrenal steroidogenesis and StAR expression in human adrenocortical cells using the adrenal NCI-H295A cell line, an *in vitro* model of adrenal steroidogenesis. We showed that caffeine stimulated StAR mRNA/protein expression and cortisol production. Furthermore, we identified a caffeine-sensitive CpG site of demethylation in StAR promoter and suggest a possible inheritable epigenetic mechanism of caffeine toxicity *in vitro*.

2. Materials and methods

2.1. Chemicals

Caffeine and selenium/insulin/transferrin (SIT) were obtained from Sigma–Aldrich Co. (St Louis, Mo). RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B and Ex Taq™ DNA Polymerase were purchased from Invitrogen Co. (Carlsbad, Calif). Rabbit polyclonal anti-StAR, were purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology Co. (Beverly, MA). All primers were synthesized by Integrated DNA Technologies (Coralville, Iowa). All other chemicals and reagents were of analytical grade.

2.2. Cell culture and treatment

Human adrenocortical cell line (NCI-H295A) was kindly provided as gift by Dr. W.L. Miller (University of California, San Francisco, California, USA). Cells were cultured in a 5% CO_2 humidified incubator at 37 °C. Standard medium for NCI-H295A cells is RPMI-1640 supplemented with 2% FBS, 0.1% SIT and penicillin/streptomycin [27]. The medium was changed every other day and the cells were split at a ratio of 1:3 with trypsin after reaching confluence. After NCI-H295A became subconfluent (at 70–80% confluence), the cells were cultured with non-serum medium overnight before the start of all experiments. Then cells were treated with 0.4, 4, and 40 μM caffeine for indicated days. In the experiment of 10 days treatment, the cell culture was started with very low density because of the long time culture and the avoidance of cell subculture. Then the medium were changed every three days with fresh medium containing different concentrations of caffeine. In some experiments, caffeine treatment was withdrawn 48 h later and then the cells were continually subcultured for up to 5 and 10 passages, respectively.

2.3. MTT assay

NCI-H295A cells were cultured in 96-well plates and the media contain 0.4, 4, and 40 μM caffeine. 48 h later, the viability of cells were detected using MTT assay kit (Cayman Chemical Co., Ann Arbor, Michigan) following the manufacturer's instruction. Absorption intensity was measured using enzyme-linked immunosorbent assay reader (TECAN, Australia) at 490 nm.

2.4. Cortisol concentration

The cortisol released into culture medium was determined by commercial ELISA assay kit (DRG Instruments GmbH, Marburg, Germany). Basically, NCI-H295A cells were treated with various concentrations of caffeine for 48 h, and the culture medium was then collected for the analysis of cotisol levels according to the manufacturers' protocol.

2.5. RNA extraction and RT-PCR

The cells were seeded into 60 mm culture dishes in DMEM with caffeine for 48 h or 10 d. Total RNA was extracted from the cultured cells using RNeasy Mini kit (Qiagen, Hiden, Germany) following the manufacturer's protocol. The concentration and purity of RNA were determined using Nanodrop2000 spectrophotometer (Thermo scientific, USA) and adjusted to 1 μg/μl. Single-strand cDNA was prepared by the SuperScript™ II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, Calif) from 2 μg of total RNA and was stored at −20 °C until use. The sequences of specific primers for StAR are as follows: forward: 5′-TGAGCAGAAGGGTGTCATCAGG-3′; reverse: 5′-CGCAGGTGGTTGGCAA AATC-3′. PCR conditions were 30 s at 94 °C, 30 s at 59 °C, 25 s at 68 °C for 25 cycles. The expression of a housekeeping gene, GAPDH, was served as internal controls.

2.6. Genomic DNA extraction and sodium bisulfite modification

NCI-H295A cells on 60 mm culture dishes were treated by different concentrations of caffeine for 48 h. Genomic DNA samples were prepared using DNeasy Blood & Tissue kit (Qiagen, Hiden, Germany) and then subjected to bisulfite modification using EZ DNA methylation-direct kit (Zymo research corporation, Orange, CA) according to the manufacturer's instruction. The basic principle of bisulfite modification of DNA is that in the bisulfite reaction, all unmethylated cytosines are deaminated and sulfonated, converting them to thymines, while methylated cytosines (5-methylcytosines) remain unaltered [28]. Modified DNA was used immediately or stored at −80 °C for future use within six months.