

Coffee Induces 11 β -Hydroxysteroid Dehydrogenase Type 2 Activity in Human Placental Choriocarcinoma Cells, JEG-3

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Coffee is the beverage consumed during pregnancy that is most commonly associated with adversely affecting the developing fetoplacental unit. Since placental 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD-2) is an important determinant for maintenance of fetal glucocorticoid levels during pregnancy, we investigated the effect of coffee on 11 β -HSD-2 expression in the human placental choriocarcinoma cell line, JEG-3. Addition of coffee to the culture medium of JEG-3 cells increased 11 β -HSD-2 gene expression after 24 hr in a dose-dependent manner. Cellular 11 β -HSD-2 activity, which was calculated by the amount of cortisol that was converted to cortisone in the culture medium, also increased in the coffee-treated JEG-3 cells. None of the major constituents of coffee, such as caffeine, caffeic acid or chlorogenic acid, showed any ability to induce 11 β -HSD-2 gene expression. The 11 β -HSD-2-inducing activity in coffee was extractable with ethyl acetate. Increasing the degree of roasting improved the ability of the coffee extract to induce 11 β -HSD-2 gene expression indicating that the active constituent(s) is produced during the roasting process.

Key words — coffee, 11 β -hydroxysteroid dehydrogenase type 2, JEG-3, placenta, trophoblast

INTRODUCTION

Although glucocorticoids are essential for normal fetal organ growth and maturation,¹⁾ excess glucocorticoid exposure *in utero* reduces fetal growth and may predispose to hypertension and diabetes mellitus in adult life.^{2–4)} Throughout pregnancy, the majority of the glucocorticoids in the fetal circulation originate from the mother via the placenta. Thus, precise control of the transplacental transfer of maternal glucocorticoids to the fetus is critical for normal fetal development.

Fetal glucocorticoid levels are lower than maternal levels.⁵⁾ This difference in levels is ensured by placental 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD-2), which catalyses the rapid metabolism of cortisol and corticosterone to inert 11-keto steroids, cortisone and 11-dehydrocorticosterone.⁶⁾ Therefore, precise regulation of 11 β -HSD-2 activity in placenta is also important for normal fetal growth and development.

Coffee is the beverage consumed during pregnancy that is most commonly associated with adversely affecting the developing fetoplacental unit. Maternal coffee intake has been reported to be associated with a reduction in birth weight.^{7–10)} However, the underlying mechanisms of the adverse effect of coffee on fetal growth have not yet been elucidated. We hypothesized that coffee may affect fetal glucocorticoid levels during pregnancy thereby leading to growth retardation. Because of the importance of placental 11 β -HSD-2 for maintenance of fetal glucocorticoid levels during pregnancy,¹⁾ we tested the effects of coffee on 11 β -HSD-2 expression and activity in the human placental choriocarcinoma cell line, JEG-3.

MATERIALS AND METHODS

Materials — [¹⁴C]-hydrocortisone was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Reagents for the polymerase chain reaction (PCR) were obtained from Applied Biosystems, Inc. (Warrington, U.K.). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The human placental choriocarcinoma cell line JEG-3 was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Columbian Arabica coffee beans were

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purchased from Nakaya Coffee (Tokyo, Japan).

Preparation of Coffee Extract — Blended coffee powder was obtained from Starbucks Coffee Japan (Tokyo, Japan). Coffee extracts were prepared using a common method, in which 8 g of powder was extracted with 140 ml hot water (95°C). The extract was then filtered, divided into small aliquots and stored at -80°C until use. Undiluted extract, with a dry weight of 8.4 mg/ml, was assigned a concentration of 100% (v/v). The filtrate was sequentially extracted with an equal volume of the solvents hexane, ethyl acetate, and *n*-butanol. Organic and aqueous phases were then concentrated by evaporation and dissolved in 16 µl dimethyl sulfoxide (DMSO). The effects of each extract on JEG-3 cells were monitored using 1 µl aliquots of these fractions.

Roasting of Coffee Beans — Colombian Arabica coffee beans were roasted in a roaster at 220°C for 5, 10, 15, 20 and 25 min. At each roasting time, coffee beans were pulverized, and 8 g of the sample was extracted with 140 ml of boiling water.

Cell Culture and Treatments: JEG-3 cells were cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, sodium pyruvate, and penicillin/streptomycin. Cells were maintained in a 10-cm dish (Corning, Lowell, MA, U.S.A.) at 37°C in a 5% CO₂ humidified incubator. The medium was changed every 3–4 days. To study the effects of coffee extracts on 11β-HSD-2 activity, cells were passaged onto 12-well plates and cultured to confluence. Cells in triplicate wells were exposed to a given dose of coffee extracts for 24 hr or as stated otherwise. Controls (also in triplicate) were treated similarly but without the addition of coffee extracts.

Analysis of 11β-HSD-2 Gene Expression — Total RNA was isolated from the cultured cells using guanidinium thiocyanate-phenol-chloroform extraction. First strand cDNA was synthesized from 10 µg of total RNA using 1 unit of M-MLV reverse transcriptase and oligo (dT) primers, according to the manufacturer's protocol. PCR was performed with *Taq* DNA polymerase using this cDNA as a template. The primers used for the amplification of 11β-HSD-2 cDNA were designed based on the published sequence as follows (5' to 3'): AGCTGCATGGAGGTGAATTCT and CTTTCCCACTGACCCACGTT. Quantitative real-time PCR was performed in an ABI 7300 thermal cycler using a SYBR green PCR core reagent kit (Applied Biosystems Inc.). Samples were denatured at 94°C for 10

min, and cDNA products were amplified using 40 cycles of denaturation at 94°C for 30 s followed by annealing and extension at 60°C for 60 s. The initial amounts of mRNA were calculated according to the cycle threshold method.¹¹⁾ The mRNA levels were normalized to 18S RNA levels, which were quantified using real-time PCR.

Assay of 11β-HSD-2 Activity — The level of 11β-HSD-2 activity in intact cells was determined by measuring the rate of cortisol to cortisone conversion as described previously.¹²⁾ Briefly, the cells were treated with coffee (0–5%) for 24 hr and the cells were then incubated for 4 hr at 37°C in serum free medium containing [¹⁴C]cortisol (0.1 µCi) and 10 nM unlabeled cortisol. Preliminary studies indicated that the rate of the reaction was linear from 1 to 12 hr at cell density and this substrate concentration. At the end of the incubation, the medium was collected and steroids were extracted with ethyl acetate. The extracts were dried, and the residues were resuspended. A fraction of this suspension was spotted and separated on silica-gel 60 F₂₄₅ plates (Merck, Darmstadt, Germany) using chloroform : methanol (9 : 1, v : v) as the mobile phase. The radioactivity on the TLC plate was visualized using an FLA7000 device (Fuji Film, Tokyo, Japan). A non-radioactive standard mixture was included on the plate and visualized under UV light. The rate of cortisol to cortisone conversion was calculated based on the density of the cortisol and cortisone spots. The blank values were subtracted, and the results are expressed as the percentage of the initial cortisol that was converted to cortisone. The migration of standard steroids was used to identify the radioactive steroids on the same plate.

Statistical Analysis — Data were statistically analyzed using Dunnett's multiple comparison tests. Results were considered significant when *p* < 0.05.

RESULTS

Coffee Extract Induces 11β-HSD-2 Expression in JEG-3 Cells

In order to elucidate the effects of coffee extract on the expression and the activity of 11β-HSD-2 in human placenta we used a human placental choriocarcinoma cell line, JEG-3. As observed in the human placenta,¹³⁾ expression of the 11β-HSD-2 gene, but not the 11β-HSD-1 gene, was observed in JEG-3 cells by PCR analysis (Fig. 1A). To test the effect of coffee on expression of the 11β-HSD-2 gene,

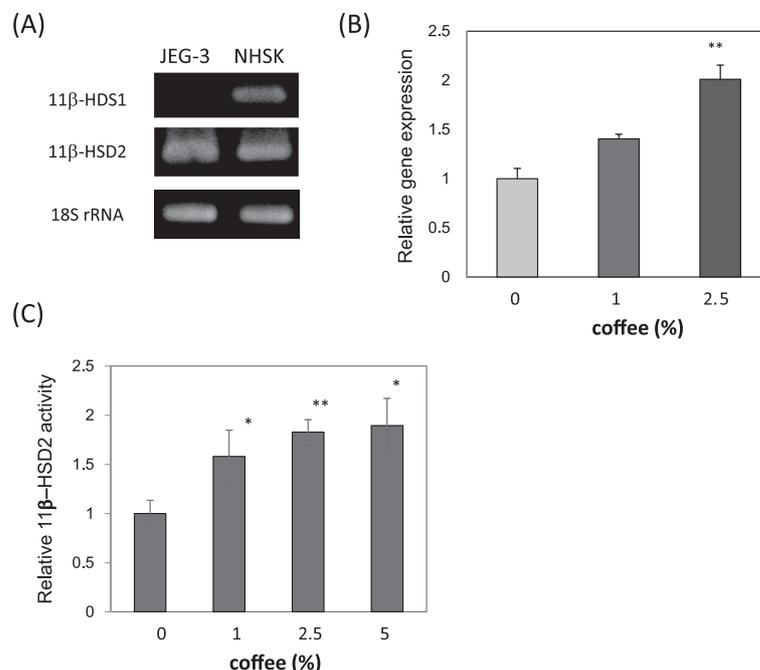


Fig. 1. Induction of 11β-HSD-2 Expression in JEG-3 Cells Treated with Coffee

(A) The mRNA expression of the two types of the 11β-HSD gene was assayed in normal human skin keratinocytes (NHSK) and JEG-3 cells using reverse transcription-PCR. The 18S rRNA was used as a control. (B) JEG-3 cells were incubated with coffee (0, 1 or 2.5%) for 24 hr and expression of the 11β-HSD-2 gene was quantified using real-time PCR analysis. (C) The activity of cellular 11β-HSD-2 in the JEG-3 cells treated with coffee for 24 hr was calculated by calculation of the amount of cortisol that was converted to cortisone in the culture medium. The 11β-HSD-2 activity is expressed relative to that of the non-treated control. The bars represent means and standard deviations of three measurements. * $p < 0.05$, ** $p < 0.01$.

JEG-3 cells were incubated with coffee (0–2.5%, v/v) for 24 hr and the amount of 11β-HSD-2 mRNA was measured using real-time PCR. As shown in Fig. 1B, coffee increased the mRNA level of 11β-HSD-2 in a coffee dose-dependent manner. Significant induction was observed after 6 hr of incubation (data not shown). No induction of 11β-HSD-1 gene expression was detected (data not shown). Cellular 11β-HSD-2 activity in the coffee-treated JEG-3 cell, which was calculated by the amount of cortisol that was converted to cortisone in the culture medium, was also elevated in accordance with increasing coffee concentration (Fig. 1C).

Chemical Properties of the Active Coffee Compounds

The major compounds in coffee extract, such as caffeine, chlorogenic acid, and caffeic acid exhibit specific physiological activities. To determine the constituents of the coffee extract that induce 11β-HSD-2 mRNA expression, we tested the effect of major coffee constituents on expression of the 11β-HSD-2 gene. We did not detect any significant effect of these compounds on gene expression (Fig. 2A).

To characterize the chemical properties of the

active coffee components, coffee was sequentially extracted with an equal volume of the solvents, hexane, ethyl acetate *n*-butanol or chloroform. Each extract was assayed for its ability to induce 11β-HSD-2 gene expression. As shown in Fig. 2B, the 11β-HSD-2 gene activity was recovered in the ethyl acetate extract.

To further characterize the active coffee constituents, we next tested the activity of extracts of beans that had been roasted for different time periods, as well as that of green beans. Increasing the degree of roasting enhanced the ability of the extract to induce 11β-HSD-2 gene expression (Fig. 3).

DISCUSSION

In this study, we demonstrated that coffee stimulates the expression of 11β-HSD-2 in JEG-3 via induction of gene expression. We showed that the active compound is not a major constituent of coffee, that it is produced during the roasting process, and that it exhibits slightly hydrophobic characteristics. These data suggest that chronic consumption of coffee by a pregnant woman could potentially result in sufficient levels of this compound in the body

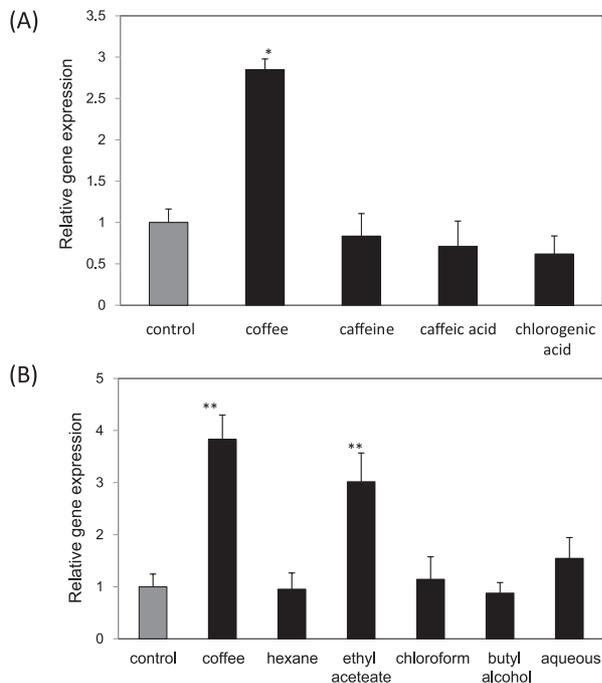


Fig. 2. Properties of the Active Constituents in Coffee Extracts

(A) The effect of major coffee constituents (caffeine, caffeic acid and chlorogenic acid; 100 μ M) on 11 β -HSD-2 gene expression in JEG-3 cells was measured using real-time PCR. Control, no addition; coffee, 2.5% coffee. (B) Coffee was sequentially extracted with an equal volume of the solvents hexane, ethyl acetate, chloroform and *n*-butanol. Organic and aqueous phases were then concentrated and dissolved in DMSO. The effect of each extract on 11 β -HSD-2 gene expression in JEG-3 cells was monitored using real-time PCR and 1 μ l aliquots of these fractions which are calculated to be equivalent to 2.5% coffee at the final concentrations. The bars represent means and standard deviations of three measurements. * p < 0.05, ** p < 0.01.

to affect the level of 11 β -HSD-2 activity in the placenta. Since human placental 11 β -HSD-2 is thought to play a crucial role in controlling glucocorticoid access to the fetus,¹⁾ coffee-induced alteration in 11 β -HSD-2 activity may affect the glucocorticoid exposure of the fetus *in utero*. This alteration might be related to the epidemiological studies that show that maternal coffee intake reduces fetal growth and predisposes to hypertension and diabetes mellitus in adult life,²⁻⁴⁾ although the effect of coffee on 11 β -HSD-2 activity reduced glucocorticoid exposure to fetus. Over exposure of glucocorticoids on the fetus during late gestation may lead to fetal growth retardation, on the other hand, proper concentrations of glucocorticoids at the early stage of gestation is thought to be necessary to stimulate differentiation of a wide range of tissues.¹⁴⁾ Perturbation of glucocorticoid concentrations by coffee constituents at the early stage of gestation might affect not only tissues essential for neonatal survival but also those in-

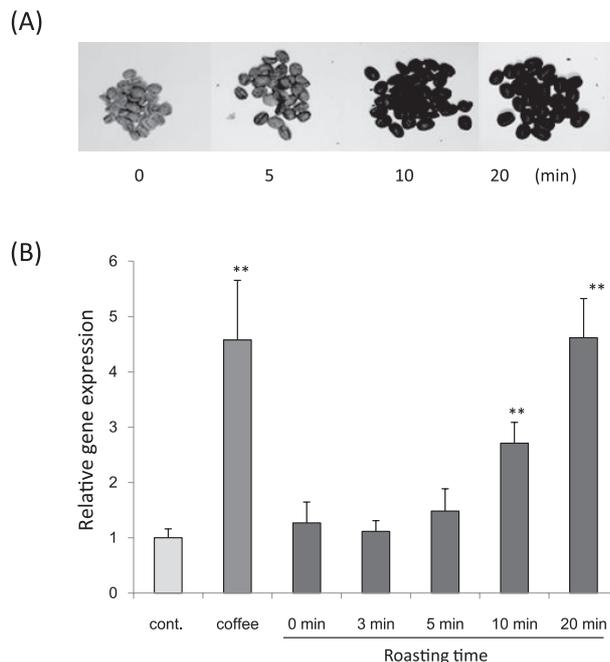


Fig. 3. Effect of the Degree of Roasting on Induction of the 11 β -HSD-2 Gene by Coffee Extracts

Extracts were made from green beans (0 min) and from beans that were roasted for different time periods as shown in (A). (B) JEG-3 cells were incubated with the indicated coffee extract (2.5%) for 24 hr. The level of 11 β -HSD-2 gene expression was measured using real-time PCR analysis. "Coffee" indicates 2.5% coffee extracts similar to those used for the experiments in Figs. 1 and 2. The bars represent means and standard deviations of three measurements. * p < 0.05, ** p < 0.01.

involved in more long-term adaptation to extrauterine life.

We demonstrated that the active coffee compound is produced during the roasting process. Several bioactive compounds have been shown to be produced during roasting.¹⁵⁾ There have been several studies regarding the regulation of 11 β -HSD-2 gene expression.¹⁶⁻¹⁹⁾ This regulatory pathway involves the protein kinase A pathway, but not the protein kinase C pathway,¹⁶⁾ the cAMP-mediated cascade¹⁷⁾ or the glucocorticoid receptor.¹⁸⁾ Recently, Sharma *et al.* reported the involvement of p38 MAPK in the regulation of 11 β -HSD-2 gene expression in human trophoblast cells.¹⁹⁾ Regarding the effects of coffee, there is accumulating evidence to suggest that coffee constituents act as modulators of nuclear transcription factors such as nuclear factor- κ B (NF- κ B),²⁰⁾ NF-E2-related factor 2 (Nrf2)²¹⁾ and aryl hydrocarbon receptor (AhR).²²⁾ Further investigation is necessary in order to understand the mechanism that underlies coffee-mediated induction of the 11 β -HSD-2 gene in JEG-3 cells.

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