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Roasted coffee induction of aldo-keto reductase 1C3 expression in LNCaP human prostate cancer cells is associated with Nrf2 activation

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Abstract. A moderate intake of coffee is associated with a reduced risk of developing certain types of chronic diseases, including many types of cancer. However, the results of epidemiological studies conducted to explore the association between coffee consumption and prostate cancer are inconsistent. Prostate cancer is dependent on androgens for development and growth; therefore the current study analyzed the effect of coffee on the expression of enzymes involved in androgen metabolism in human prostate cancer LNCaP cells. It was observed that a small amount of coffee strongly increased the expression of aldo-keto reductase 1C3 (AKR1C3), which plays a pivotal role in the synthesis of testosterone and dihydrotestosterone. Treatment with 2.5% (v/v) coffee for 24 h resulted in 5-fold increase in *AKR1C3* gene expression. The active components in coffee were extracted using *n*-butanol and it was demonstrated that the major constituents including caffeine, caffeic acid, chlorogenic acid and trigonelline had no effect on *AKR1C3* expression even at high concentrations (100 μM). Roasting of coffee beans however enhanced the induction of *AKR1C3* gene expression. Activation of nuclear factor-erythroid-2-related factor 2 was observed prior to *AKR1C3* induction produced by coffee and this activation was enhanced by roasting the coffee beans. The results of the present study suggest that consumption of roasted coffee may activate androgenic *AKR1C3* expression mediated by Nrf2 in human prostate cancer cells and therefore may increase the risk of prostate cancer.

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Introduction

In many western countries, prostate cancer (PC) is the second leading cause of cancer-associated mortality among males (1). Androgens induce PC growth and development, therefore the standard treatment for PC is androgen deprivation therapy, which is aimed at decreasing the levels of testosterone and dihydrotestosterone in serum (2). The androgen receptor also induces epithelial cell growth in the prostate (3). Suppressing androgen receptor activity or blocking androgen synthesis may arrest the tumor growth for a limited period (2,4).

Intraprostatic androgens may be synthesized *de novo* from cholesterol or other molecular precursors, including dehydroepiandrosterone (DHEA; Fig. 1). DHEA is primarily produced from DHEA sulfate by steroid sulfatase (STS). A critical step in androgen synthesis is the conversion of androsenedione to testosterone, which is catalyzed by 17β-hydroxysteroid dehydrogenases (HSD17B) types 3 and 5, the latter of which is also known as aldo-keto reductase (AKR) 1C3 (5). Testosterone may subsequently be converted to dihydrotestosterone, the most potent androgen, by 5a-reductase (SRD5A) type 1 or 2. AKR1C3 is the primary enzyme involved in testosterone formation in prostate cells (6). Several studies have indicated that AKR1C3 is overexpressed in PC cells, suggesting that AKR1C3 expression increases as PC progresses (7-9).

Coffee is commonly consumed as a beverage and moderate coffee intake may reduce the risk of certain chronic diseases (10). A number of epidemiological studies have been performed to explore the association between PC and coffee consumption, however, the results obtained have been inconsistent (11,12). A meta-analysis of epidemiological studies demonstrated that observations in case-control studies were different to those of cohort studies (13). The results of a recent meta-analysis of prospective cohort studies conducted by Cao *et al* (14) suggested that coffee consumption may decrease the risk of PC. To investigate the effect of coffee consumption on PC progression, the expression levels of enzymes involved in androgen metabolism were analyzed in coffee-treated human prostate cancer LNCaP cells.

Materials and methods

Materials. Caffeine, chlorogenic acid, caffeic acid and trigonelline were purchased from Sigma-Aldrich (St Louis, MO, USA). Androgen-sensitive human prostate adenocarcinoma LNCaP cells were obtained from the RIKEN BioResource Center (Tsukuba, Japan). Antibodies for β-actin, nuclear factor-erythroid-2-related factor 2 (Nrf2), AKR1C3 and lamin B were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Preparation of coffee extract. Blended coffee powder (Arabica beans; *Coffea arabica*) was obtained from Starbucks Coffee Japan (Tokyo, Japan). Coffee extracts were prepared by a common method, in which 8 g of powder was extracted with 140 ml hot water (95˚C) through a coffee paper filter. The extract was filtered, divided into small aliquots and stored at -80˚C. The undiluted extract, with a dry weight of 8.4 mg/ml, was assigned a concentration of 100% (v/v).

Roasting of coffee beans and solvent extraction. Colombian Arabica coffee beans (*Coffea arabica*; Nakaya Coffee, Tokyo, Japan) were roasted in a MR-101 coffee bean roaster (Dainichi Co., Ltd., Niigata, Japan) at 220˚C for 5, 10 and 20 min. Coffee beans were pulverized at each roasting time point and 8 g of each sample was extracted with 140 ml boiling water.

Each 150 ml aliquot of coffee extract was sequentially extracted with 30 ml *n*-hexane, ethyl acetate and *n*-butanol. The residual solution was labeled as the water fraction. Prior to the experiments, the solvents were evaporated and the residual material was dissolved in a small amount of dimethyl sulfoxide (DMSO; 1/50 of the initial solvent volume). The dissolved materials were subsequently diluted with DMSO and added to the medium at 0.1% (v/v) DMSO.

Cell culture. LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham MA, USA), 2 mM glutamine, 10 U/ml penicillin and 10 U/ml streptomycin, and maintained in an incubator, at 37˚C with a humidified atmosphere containing 5% CO_2. The medium was changed every 4-5 days. The cells were seeded at a concentration of 5×10^5 cells/ml in 6-well plates coated with poly-D-lysine (Iwaki Co., Ltd., Tokyo, Japan) and cultured for 2 days. The cells were further cultivated for up to 24 h with varying concentrations of coffee.

Cytotoxicity assay. Cytotoxic effects on cell metabolism were assayed using WST-1, a tetrazolium family compound. Cells (1×10^4) were seeded in 96-well plates and grown for 2 days at 37˚C. Following incubation for 24 h with varying concentrations of coffee, cells were washed with phosphate-buffered saline and subjected to the WST assay according to the manufacturer's protocol (Nacalai Tesque, Inc., Kyoto, Japan). Following incubation for 2 h with the WST-1 reagent, the absorbance of each sample was measured at 450 nm.

Gene expression analysis. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to analyze levels of gene expression. Total RNA was isolated from the cultured cells using the guanidinium thiocyanate-phenol-chloroform extraction method. First-strand cDNA was synthesized from 1 µg of total RNA using 1 unit of ReverTra® Ace reverse transcriptase (Toyobo Life Science, Inc., Osaka, Japan) with random primers, according to the manufacturer's protocol. The primers used for cDNA amplification were based on the published sequences and were as follows: *AKR1C3*, forward, 5'-TAAAGCCAGGTGAGGAAC TTTCA-3' and reverse, 5'-GGATGACATTCTACCTGGTTG CA-3'; *STS*, forward, 5'-TCAAGGCCGAACATCATCCT-3' and reverse 5'-TCCGTAGGACACTTCTCCGA-3'; sulfotransferase 2B1 (*SULT2B1*), forward, 5'-CCCGCCGAGCCC CAGATCC-3' and reverse, 5'-ATGAGGCGGGGGCTGTAC TGGTC-3'; *HSD17B2*, forward, 5'-AGTTGCTTCCATCCA ACCTG-3' and reverse, 5'-GGAAATTCCGCTGTGCTA AG-3'; *SRD5A1*, forward 5'-CCACCCCTAGCACGTGGAT-3' and reverse, 5'-TTAGTGATGGACTCAAAGGCAAAG-3'; *SRD5A2*, forward, 5'-TCATTAGCAGCCTTCCCTTG-3' and reverse, 5'-AATCGAATACAGGGCACAGC-3'; HSD3B1, forward, 5'-TCATCCGCCTCTTGGTGAAG-3' and reverse, 5'-CGCAATAAACGTCATTGAAAGG-3'; heme oxygenase 1 (*HO-1*), forward, 5'-CAGGCAATGGCCTAAACTTC-3' and reverse, 5'-GAAACTCAGGGCTTTTGGAG-3'; *Nrf2*, forward, 5'-CGGTATGCAACAGGACATTG-3' and reverse, 5'-TTGGCTTCTGGACTTGGAAC-3'. Real-time qPCR was performed in an ABI 7300 Real-Time PCR System using a SYBR® Green PCR Core Reagent kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Samples were denatured at 94˚C for 10 min and cDNA products were amplified with 40 cycles of denaturation at 94˚C for 30 sec, followed by annealing and extension at 60˚C for 60 sec. The expression of each target gene relative to the reference gene (18S rRNA) was quantified using the ΔCq method.

Preparation of whole cell lysates and nuclear extracts. Prior to nuclear extraction, cells were lysed with buffer A [10 mM Hepes-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA (pH 8.0) and 0.1% Nonidet™ P-40 (NP-40); supplemented with protease inhibitor cocktail (Nacalai Tesque, Inc.)], and centrifuged at 2,300 x *g* for 1 min. Nuclear fraction pellets were lysed with NP-40 lysis buffer (50 mM Tris-HCl at pH 7.4, 10% glycerol, 50 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 20 mM NaF and 0.2 mM Na_3VO_4; supplemented with protease inhibitor cocktail). Whole cell lysates were prepared using NP-40 lysis buffer.

Western blot analysis. Protein samples (15 µg) were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and subsequently electrotransferred to a polyvinylidene difluoride membrane (EMD Millipore; Billerica, MA, USA). The membrane was incubated overnight at 4˚C in blocking buffer consisting of Tris buffered saline (20 mM Tris-HCl at pH 7.6 and 137 mM NaCl) and 1% skimmed milk. Following one wash with 0.1% Tween-20 in Tris buffered saline (TTBS), the membrane was incubated for 1 h at 37˚C with the antibodies for AKR1C3 (sc-23795), Nrf2 (sc-722), lamin B (sc-1615) or β-actin (sc-6217; all from Santa Cruz Biotechnology Inc., Dallas, TX, USA) diluted in Can Get Signal® Immunoreaction Enhancer Solution (1:1,000 dilution; Toyobo Life Science, Inc.). Following 3 additional washes with TTBS, the membrane was incubated for 1 h at

37˚C with anti-rabbit IgG linked to horseradish peroxidase (GE Healthcare Life Sciences; Chalfont, UK). The membrane was subsequently washed five times with TTBS and immunoreactive proteins were visualized using the SuperSignal™ West Pico Chemiluminescent Substrate (Pierce Protein Biology Products; Thermo Scientific), detected with a VersaDoc™ 5000 system and quantified with Quantity One Software version 4.6.3 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. The data were analyzed using Dunnett's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Coffee induced AKR1C3 gene expression in LNCaP cells. To elucidate the effect of coffee on androgen metabolism in human prostate cancer, the effects of coffee on the expression of enzymes involved in androgen synthesis at the mRNA and protein levels in human prostate cancer LNCaP were investigated. No cytotoxicity was observed following exposure to coffee concentrations as high as 5% (v/v) (data not shown), therefore, LNCaP cells were treated with coffee concentrations ≤2.5% for 24 h. Coffee increased *AKR1C3* gene expression in a dose-dependent manner (Fig. 2A), whereas it reduced *HSD17B2* expression (Fig. 2C). Coffee did not significantly alter the expression of other steroid metabolizing enzymes (*HSD3B1, SRD5A1*, and *SRD5A2*) (Figs. 2B, D and E), prostate specific antigen (Fig. 2F), or *SULT2B1* and *STS*, which encode enzymes involved in DHEA sulfoconjugation (data not shown). Fig. 3A demonstrates the time-dependent induction of AKR1C3 protein expression by 2.5% coffee. After 8 h, AKR1C3 protein abundance reached its maximum level. Western blot analysis indicated that the coffee-mediated increase in AKR1C3 protein levels occurred in accordance with changes in its transcript abundance (Fig. 3B).

Characterization of active coffee components. To determine which constituents were responsible for the coffee-mediated induction of AKR1C3 gene expression, the inducible activity of the major constituents of coffee, including caffeic acid, caffeine, trigonelline and chlorogenic acid were measured. None of these components significantly altered the expression of AKR1C3 in the treated LNCaP cells, even though the concentration of each compound (100 mM) was similar to that in 10-50% coffee (Fig. 4A) (15).

The activity of green coffee bean extracts that had undergone varying degrees of roasting prior to brewing were assayed to examine the possibility that active components are formed during the roasting process. Extracts from coffee beans roasted for 0-20 min were used for gene expression assays using RT-qPCR. It was observed that increasing the degree of roasting resulted in a greater induction of *AKR1C3* gene expression (Fig. 4B).

To further analyze the active constituents in coffee, brewed coffee was sequentially extracted using one-third volume of the solvents *n*-hexane, ethyl acetate and *n*-butanol and the effect each extract had on gene expression was measured. Expression of *AKR1C3* was primarily induced by the *n*-butanol extract (Fig. 4C), which itself increased HO-1 expression (Fig. 4D).

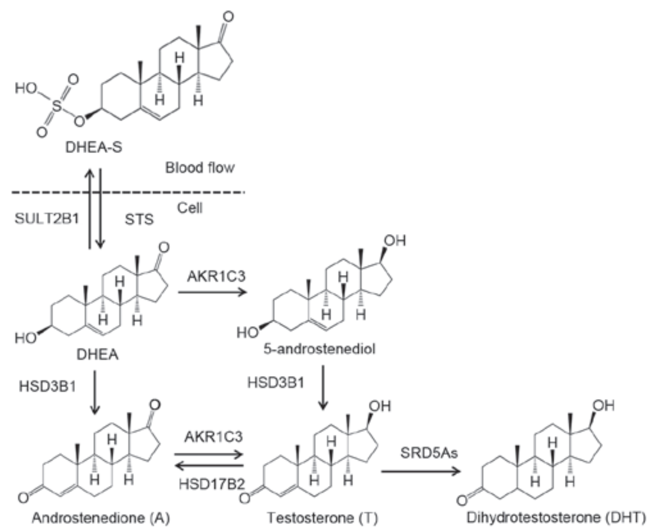

Figure 1. Schematic representation of androgenic metabolic pathway in prostate cells. DHEA-S, dehydroepiandrosterone sulfate; SULT2B1, sulfotransferase 2B1; STS, steroid sulfatase; AKR1C3, aldo-keto reductase 1C3; DHEA, dehydroepiandrosterone; SRD5A, 5a-reductase; HSD3B1, 17β-hydroxysteroid dehydrogenase B type 1.

Induction of AKR1C3 gene expression by coffee is associated with Nrf2. Previous studies have demonstrated that constituents of coffee are able to activate Nrf2 nuclear translocation in human cells (16,17). Therefore, levels of Nrf2 were measured in coffee-treated LNCaP cells. Immunoblot analyses indicated that coffee treatment increased both cytosolic and nuclear Nrf2 content in 2.5% coffee-treated LNCaP cells (Fig. 5A and B). The appearance of Nrf2 protein in the nuclei occurred prior to the induction of *AKR1C3* gene expression (Figs. 3 and 5). As presented in Fig. 5C, coffee-mediated activation of Nrf2 was dependent on the roasting process. Nrf2 activation was produced by the extract of coffee beans roasted for 5 min and the potency increased as the roasting time was increased to 20 min. No change was observed in *Nrf2* transcripts in the coffee-treated LNCaP cells (data not shown). Furthermore, Nrf2 activation was greatest in cells treated with the *n*-butanol coffee extract, in comparison with cells treated with the other extracts (Fig. 5D).

Discussion

The present study demonstrates that coffee-induced AKR1C3 expression is associated with the activation of cytoprotective transcription factor Nrf2. Nrf2 activation was triggered by constituent(s) in coffee produced by the roasting process (Fig. 5C) and not by the major constituents of coffee beans (Fig. 4A).

AKR1C3 is the primary enzyme responsible for catalyzing testosterone formation in prostate cells (6). It is crucial for the synthesis of testosterone and dihydrotestosterone, which are among the most potent stimuli for growth, proliferation and metastasis in PC cells (7). A recent study indicated that AKR1C3 overexpression may be developed as a biomarker

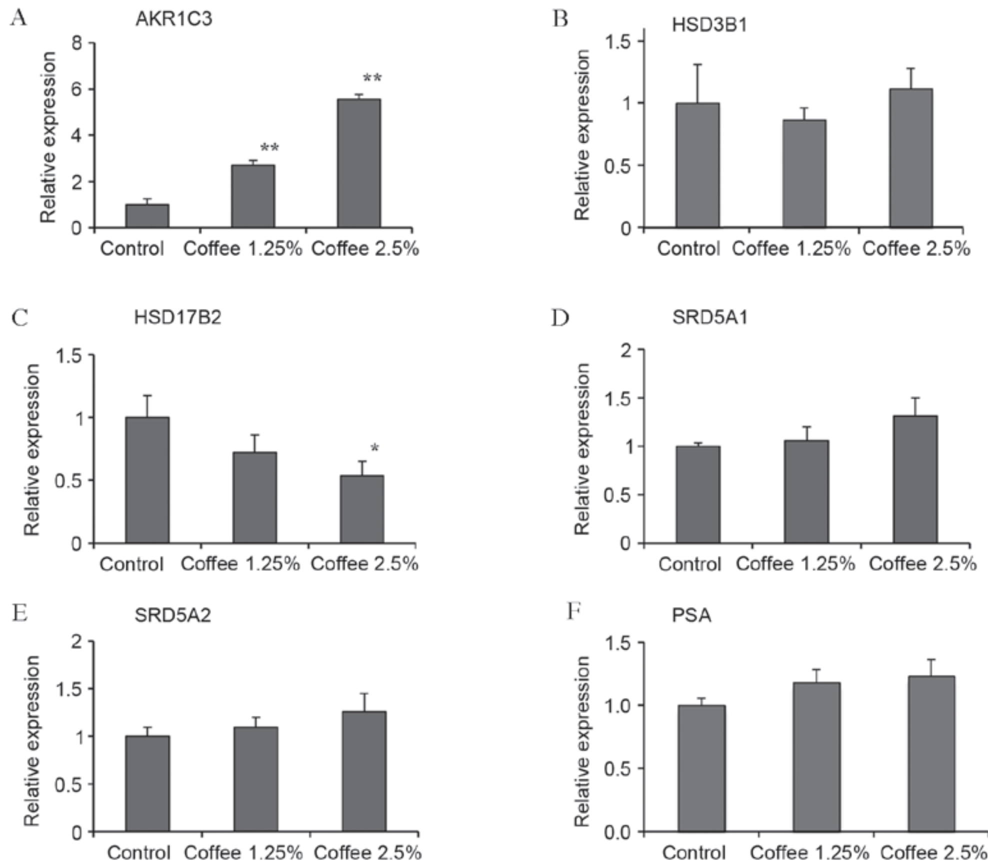

Figure 2. Effect of coffee on the expression of genes involved in androgenic metabolism. Total RNA was isolated from LNCaP cells treated with coffee for 24 h at the indicated concentrations. (A-F) The extracted RNA was used to generate cDNA, which was used for analysis via reverse transcription-quantitative polymerase chain reaction with specific primers for each gene. *P<0.05 and **P<0.01 vs. control.

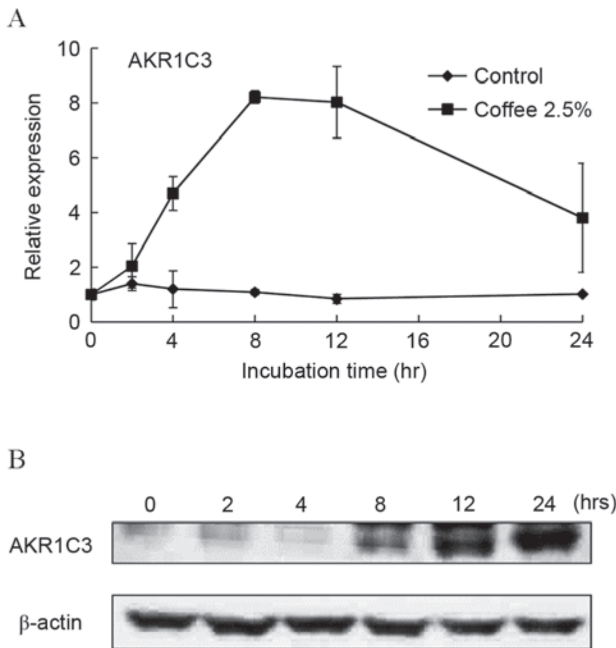

Figure 3. Changes in (A) *AKR1C3* gene expression and (B) AKR1C3 protein abundance in the coffee-treated LNCaP Cells. LNCaP cells were treated with 2.5% coffee for up to 24 h. At the indicated times, total RNA and protein were extracted. *AKR1C3* mRNA expression was analyzed by reverse transcription-quantitative polymerase chain reaction and cytosolic AKR1C3 protein abundance was assessed via immunoblot analyses. Control cells were not treated with coffee. β-actin was used as a cytosolic marker. AKR1C3, aldo-keto reductase 1C3.

for PC progression (9). However, the findings of the current study suggest that chronic consumption of coffee may enhance AKR1C3 expression in prostate cells, leading to false diagnoses when AKR1C3 is used as a PC progression biomarker. Further investigations, including animal and human studies, are required to clarify this issue.

It has been reported that Nrf2-dependent pathways regulate several members of the AKR superfamily (18,19). Nrf2 is a transcriptional activator of cytoprotective genes that activates transcription in response to reactive oxygen species (ROS) and electrophiles. In the current study, it was observed that roasting coffee beans increased the Nrf2 activation elicited by coffee bean extracts (Fig. 5C). Previous studies have reported that coffee components, including diterpene kahweol, activate Nrf2 (16,17,20). However, brewed coffee contains limited amounts of diterpenes, as paper coffee filters absorb coffee diterpenes (21). Furthermore, the preliminary data collected in the current study indicated that kahweol produced only a weak induction of *AKR1C3* gene expression in LNCaP cells (unpublished data). Coffee is rich in Maillard reaction products (MRPs) (22) and Sauer *et al* (17) have reported that MRPs activate Nrf2 in human cells. Boettler *et al* (16) have indicated that degradation derivatives of chlorogenic acid and trigonelline may activate Nrf2 nuclear translocation in human colon cancer cells. Furthermore, Vicente *et al* (23) have demonstrated that the administration of brewed coffee for 28 days activated Nrf2 expression in the rat liver. Taken together, these results indicate

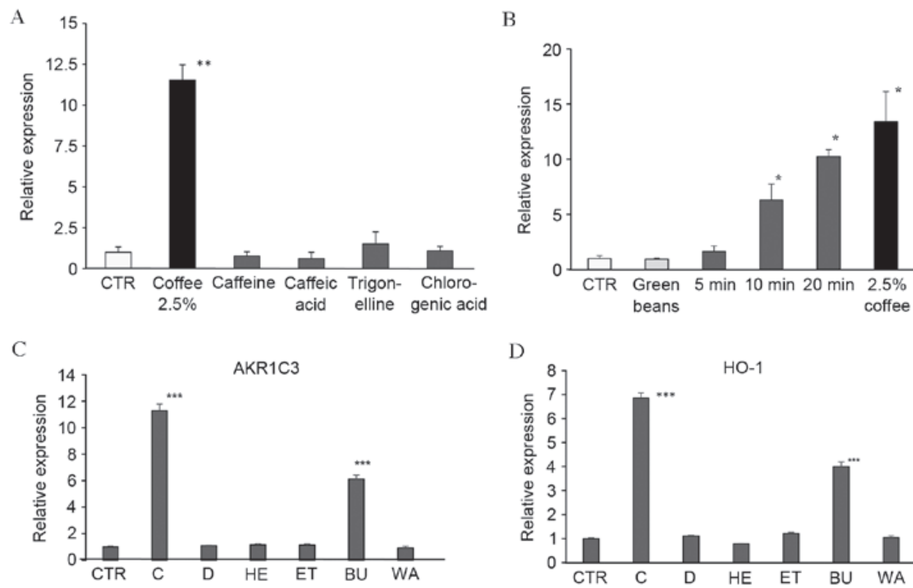

Figure 4. Characterization of the active component(s) in coffee responsible for the induction of *AKR1C3* gene expression. (A) Effects of major coffee constituents on *AKR1C3* gene expression in LNCap cells. Cells were incubated with 100 μM of each compound for 24 h, and *AKR1C3* gene expression was subsequently measured using RT-qPCR. (B) Arabica coffee beans were roasted at 220˚C for 5, 10 or 20 min and the beans were pulverized and extracted with boiling water as previously (Materials and Methods section). The cells were incubated for 24 h in media with each extract added at a concentration equivalent to 2.5% (v/v) coffee. *AKR1C3* mRNA was measured using RT-qPCR. The data are expressed relative to CTR. (C) Coffee was sequentially extracted with one-third volume of *n*-hexane, ethyl acetate and *n*-butanol. The organic and aqueous phases were concentrated by evaporation and dissolved in DMSO, and their effects on *AKR1C3* gene expression in LNCaP cells were measured using RT-qPCR. Data are expressed relative to CRTs. (D) The effects of solvent-extracted coffee fractions on HO-1 gene expression were measured, n=3. *P<0.05, **P<0.01, ***P<0.001. CTR, non-roasted control values; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; AKR1C3, aldo-keto reductase 1C3; C, 2.5% coffee; D, 0.1% (v/v) dimethyl sulfoxide; HE, *n*-hexane; ET, ethyl acetate; BU, *n*-butanol; WA, water phase; HO-1, heme oxygenase-1.

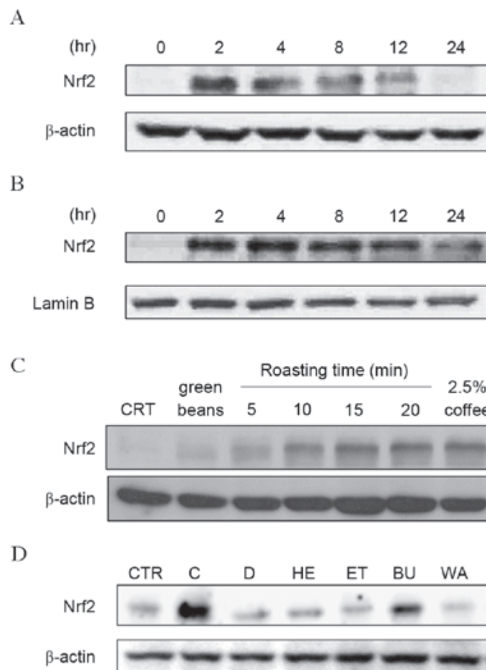

Figure 5. Nrf2 activation in the coffee-treated LNCaP cells. LNCaP cells were treated with 2.5% coffee for up to 24 h. (A) Whole cell lysates or (B) nuclear extracts (15 μg each) were subjected to immunoblot analyses for Nrf2. (C) LNCaP cells were treated with 2.5% coffee extracts prepared from beans roasted for varying periods of time (0-20 min) at 220˚C. Cytosolic Nrf2 was detected via immunoblot analysis and β-actin was used as a loading control. (D) Nrf2 activation by the coffee extracts obtained with each solvent was assessed with immunoblot analysis. β-actin was used as a loading control. CTR, non-roasted control values; C, 2.5% coffee; D, 0.1% (v/v) dimethyl sulfoxide; Nrf2, nuclear factor-erythroid-2-related factor 2; HE, *n*-hexane; ET, ethyl acetate; BU, *n*-butanol; WA, water phase.

that there are many candidate compounds in roasted coffee that may be responsible for the activation of Nrf2 in LNCaP cells. Further investigation is necessary to identify the active components responsible for coffee-mediated Nrf2 activation in LNCaP cells.

It remains unclear whether the active component(s) in coffee are absorbed and transferred to the prostate gland. It has been demonstrated that >50% of the major components in coffee, including caffeine, caffeic acid, trigonelline and chlorogenic acid, are rapidly absorbed in the stomach and small intestine and distributed to tissue around the body (10). The active component(s) in coffee was extractable with *n*-butanol (Fig. 4), therefore, it may be absorbed by the gastrointestinal tract. Further studies are required to clarify this point.

Increased nuclear accumulation of Nrf2 in coffee-treated LNCaP cells was not caused by increased transcription of *Nrf2*, as Nrf2 transcript levels were unchanged by exposure to coffee (unpublished data). Two Nrf2 activation mechanisms have been proposed to explain the inhibition of Keap1-dependent Nrf2 degradation and subsequent translocation into the nucleus (17). i) Electrophiles and ROS may modify specific cysteine thiols of Keap1, thus stabilizing cytoplasmic Nrf2. ii) Phosphorylation of serine and threonine residues of Nrf2 via protein kinases may result in the release of Nrf2 from Keap1. Sauer *et al* have suggested that the active constituents of roasted coffee extract generate H_2O_2, an ROS, which triggers the translocation of Nrf2 in human cells (17). It was observed that the induction of *HO* and *AKR1C3* transcript expression was produced by *n*-butanol (Fig. 4C and D), therefore, ROS may be a trigger for Nrf2 activation. Further

studies are required to elucidate the mechanism of Nrf2 activation in coffee-treated LNCaP cells.

Epidemiological studies exploring the association between coffee consumption and PC have been conducted; however, the results remain inconsistent. Lu *et al* (12) have suggested that high (e.g., ≥4 or 5 cups/day) coffee consumption is associated with a lower risk of overall PC as well as fatal and high-grade PC. However, the results of the present study suggest that chronic coffee consumption induces AKR1C3 expression in prostate cells when its active constituents are absorbed from the intestines. Furthermore, it was observed that coffee reduced HSD17B2 gene expression in LNCaP cells (Fig. 2C), however, the mechanism underlying the reduction remains unknown. The results of the current study suggest that daily coffee consumption may increase intraprostatic androgen levels and thus stimulate the growth, proliferation, and metastasis of PC cells (24). However, the effect of coffee on androgen metabolic enzymes does not explain the possible association between higher coffee consumption and reduced PC risk. Further studies are required to investigate the association between AKR1C3 expression and coffee consumption in patients with PC.

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