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Effect of Coffee Combining Green Coffee Bean Constituents with Typical Roasting Products on the Nrf2/ARE Pathway in Vitro and in Vivo

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ABSTRACT: This study investigated Nrf2-activating properties of a coffee blend combining raw coffee bean constituents with 5-*O*-caffeoylquinic acid (CGA) as a lead component with typical roasting products such as *N*-methylpyridinium (NMP). In cell culture (HT29) the respective coffee extract (CN-CE) increased nuclear Nrf2 translocation and enhanced the transcription of ARE-dependent genes as exemplified for NAD(P)H:quinone oxidoreductase and glutathione-*S*-transferase (GST)A1, reflected in the protein level by an increase in GST enzyme activity. In a pilot human intervention study (29 healthy volunteers), daily consumption of 750 mL of CN-coffee for 4 weeks increased Nrf2 transcription in peripheral blood lymphocytes on average. However, the transcriptional response pattern of Nrf2/ARE-dependent genes showed substantial interindividual variations. The presence of SNPs in the Nrf2-promoter, reported recently, as well as the detection of GSTT1*0 (null) genotypes in the study collective strengthens the hypothesis that coffee acts as a modulator of Nrf2-dependent gene response in humans, but genetic polymorphisms play an important role in the individual response pattern.

KEYWORDS: *human intervention study, Nrf2, phase II enzymes, chlorogenic acid, N-methylpyridinium*

INTRODUCTION

One prominent way to protect cells and tissues from carcinogens and carcinogenic metabolites is the activation of phase II detoxifying and antioxidative enzymes such as glutathione *S*-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1), γ-glutamylcysteine ligase (γGCL), or heme oxygenase 1 (HO1).[1−3] The induction of these enzymes is mediated by the antioxidant or electrophile response element (ARE/EpRE), located in the promoter region of many of these phase II genes.[4−6] The transcription factor NF-E2 p45 subunit-related factor 2 (Nrf2) plays a key role in ARE-mediated gene expression. Under physiological conditions Nrf2 is retained in the cytoplasm, bound to the actin-anchored protein Keap1. Upon activation by oxidative or electrophilic stimuli Nrf2 is released and translocates into the nucleus, activating the transcription of important ARE-responsive genes.[7] Food constituents of different structure classes, including the coffee constituents kahweol and cafestol, have been found to interact with the Nrf2/ARE-pathway, increasing the nuclear Nrf2 protein level and modulating ARE-mediated gene expression.[8−15] Furthermore, *N*-methylpyridinium (NMP), a degradation product formed from trigonelline during roasting of coffee beans, was found to increase phase II enzyme activities in rats.[16] Recently, we identified NMP as well as chlorogenic acid (CGA), a major polyphenol in raw coffee beans, as potent activators of the Nrf2/ARE pathway, activating nuclear Nrf2-translocation as well as gene expression of different phase II enzymes in the colon carcinoma cell line HT29.[17] In a subsequent human intervention study we observed an increase of transcripts of phase II genes in peripheral blood lymphocytes (PBL) after 4 weeks of daily consumption of either a coffee rich in CGA or one rich in NMP.[18] Due to the Nrf2/ARE-activating effects of both CGA-rich coffee and NMP-rich coffee, we now addressed the question whether a combination of both characteristics, increased amounts of green coffee bean constituents as well as typical heavy roast products, enhances the Nrf2/ARE-activating properties of a coffee beverage in vitro and in vivo. To this end, an Arabica-based coffee blend was produced with a considerable content in chlorogenic acids

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(580.1 mg/L) combined with an increased NMP concentration (71.4 mg/L).[19] The coffee extract (CE) of this hybrid CGA/NMP-rich (CN) coffee contains amounts of NMP comparable to those recently reported in the NMP-rich coffee,[18] but a significantly higher concentration of CGA (Table 1). Because

Table 1. Concentrations of CGA and NMP in CGA-CO, NMP-CO, and CN-CO[a]

coffee constituent	CGA-CO (mg/L)	NMP-CO (mg/L)	CN-CO (mg/L)
CGA	1606.5	185.11	580.1
NMP	5.3	73.7	71.7

[a]Formulation: 600 mL of water for 29.5 g of ground coffee; see Materials and Methods. CGA, data given as the sum of the dominating derivatives 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, and 5-*O*-caffeoylquinic acid; NMP, *N*-methylpyridinium; CGA/NMP rich; CO, coffee brew.

degradation of CGA and formation of NMP show an inverted progression in relation to the roasting time/temperature during the process of roasting, a mixture of two different coffees was necessary to maintain the desired characteristics in the resulting CN coffee brew.[19] This hybrid coffee was used to test the hypothesis whether a combination of raw coffee bean constituents with heavy roast characteristics enhances the Nrf2/ARE-activating potency of coffee. The study was performed in a two-step approach: (1) With respect to the underlying mechanism, Nrf2/ARE activating properties of a respective coffee extract CN-CE were examined in HT29 colon carcinoma cells, a cell culture model that is well characterized in terms of cell signaling and known to be sensitive to the activation of Nrf2/ARE-dependent gene expression.[17] (2) The relevance of these findings for the in vivo situation was studied in a human pilot intervention study, investigating the effect of 4 weeks of daily consumption of the hybrid coffee on the transcription of Nrf2-dependent genes in human peripheral blood lymphocytes (PBLs) of the participants.

MATERIALS AND METHODS

Preparation of Coffee Extracts. The ground roast coffee powder (48 g) was placed in a standard coffee filter (size 4, Melitta, Germany), and the water tank of the drip filter machine (TCM, Germany) was filled with distilled water (900 mL). After brewing, the coffee beverage (820 mL) was aliquoted into glass dishes, frozen (−20 °C), and freeze-dried (2.1 mbar, 48 h, 25 °C). The fluffy material obtained was homogenized with a spatula and kept in brown glass screw-cap bottles until analysis/biological testing. For all assays freshly prepared solutions of the test compounds were used. All constituents and mixtures were dissolved in DMSO with a final concentration in the different test systems of maximum 1% except GST-enzyme activity, which was performed using solutions in 0.1% DMSO.

Preparation of Coffee Brew. The coffee used in this study consisted of a special roasted and blended Arabica coffee rich in both green and roasted beans constituents, especially in chlorogenic acid and NMP. Prior to the intervention, the ground coffee was portioned into vacuum-packed aliquots of 29.5 g/package each. The coffee brew was freshly prepared by paper filtration in a conventional coffee machine immediately before each individual consumed 250 mL (three times a day), using 600 mL of water for 29.5 g of ground coffee.

Cell Culture. HT29 cells (human colon adenocarcinoma, ATCC 299) were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). Cell culture media and supplements were purchased from GIBCO Invitrogen (Karlsruhe, Germany). The HT29 cells were cultured in Dulbecco's modified Eagle medium (DMEM with 4500 mg/L glucose and 4.0 mM L-glutamine without sodium pyruvate) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. The cells were kept in a humidified incubator at 37 °C, 5% CO_2. Cells were tested routinely and found to be negative for mycoplasm contamination. To suppress the accumulation of hydrogen peroxide in the cell culture medium, 100 U/mL catalase was added in all assays.

Measurement of Intracellular ROS, Dichlorofluorescein (DCF) Assay. Forty thousand HT29 cells/well were seeded into 96-well plates and allowed to grow for 48 h. Cells were incubated with 50 μM dichlorodihydrofluorescein diacetate for 30 min. After a washing with phosphate-buffered saline, cells were incubated with the respective compound dissolved in DMSO for 3 h adopted in serum-free colorless DMEM (to a final DMSO concentration of 1%). The incubation was performed in the presence of catalase (100 U/mL) to prevent the formation of hydrogen peroxide in the cell culture medium. The fluorescence was measured at λ_{ex} = 485 nm and λ_{em} = 525 nm.

GST Enzyme Activity. HT29 cells (1×10^6) were seeded into Petri dishes (⌀ 10 cm) and allowed to grow for 48 h. Subsequently, cells were treated for 24, 48, or 72 h with the different compounds or the solvent control DMSO (0.1% v/v). The incubation was performed in the presence of catalase (100 U/mL) to prevent hydrogen peroxide formation in the cell culture medium. Cells were collected, and the viability was determined by trypan blue exclusion. Thereafter, aliquots corresponding to 1×10^6 cells were centrifuged (500*g*, 4 °C) for 10 min. The pellet was resuspended in 1 mL of PBS, and after a final centrifugation step for 10 min (500*g*, 4 °C), the pellet was stored at −80 °C until use. For substrate conversion the pellet was solved in 500 μL of potassium hydrogen phosphate buffer (100 nM K_2HPO_4, adjusted to pH 7.4 with 100 mM KH_2PO_4), and centrifuged (500*g*, 4 °C) for 10 min, and the supernatant (cytosolic protein) was collected. Eighty microliters cytosolic protein was added to 700 μL of reaction mixture (containing 660 μL of potassium hydrogen phosphate buffer and 40 μL of glutathione (20 mM)) and incubated (300 rpm, 37 °C) for 5 min. The reaction was initiated by adding 20 μL of 1-chloro-2,4-dinitrobenzene (40 nM), and the formation of thioether after 5 min of incubation was measured at 340 nm in a thermostated compartment at 25 °C. Total enzymatic activity of GST was expressed as micromoles per minute per milligram of protein and plotted as test over control (percent).

Western Blot Analysis. HT29 cells (4.5×10^6) were seeded per Petri dish and allowed to grow for 48 h. Cells were incubated for 3 h with the different compounds at respective concentrations in the presence of 100 U/mL catalase, thus preventing hydrogen peroxide accumulation. Thereafter, the cells were rinsed with ice-cold PBS, abraded on ice in 0.7 mL of PBS, and centrifuged for 5 min (800*g*, 4 °C). The pellet was resolved in 200 μL of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 μM DTT, and 1 μM protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) freshly added to buffer A). After 15 min of incubation, 17 μL of Nonidet-P40 (10%) was added, and the lysate was vortexed for 15 s. Thereafter, the lysate was repeatedly centrifuged for 10 min (800*g*, 4 °C) to separate the nuclear from the cytosolic fraction. The supernatant (cytosolic fraction) was stored on ice. The pellet was resuspended in 65 μL of buffer B (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 μM DTT, and 1 μM protease inhibitor cocktail (Roche Diagnostics) were freshly added to buffer B) and incubated on ice for 15 min, being vortexed for 10−15 s every 2 min. After a final centrifugation step for 10 min (20000*g*, 4 °C), the supernatant (nuclear extract) as well as the cytosolic extract were separated by SDS-PAGE (10% polyacrylamide gel). The proteins were transferred onto a nitrocellulose membrane, and Western blot was performed using rabbit polyclonal antibody against human Nrf2 (H-300, Santa Cruz, Heidelberg, Germany) and an anti-rabbit IgG peroxidase conjugate (Santa Cruz, Heidelberg, Germany) as secondary antibody. α-Tubulin was used as a loading control. The respective chemoluminescent signals (Lumi-GLO, Cell Signaling Technology) were analyzed for quantification using the LAS 3000 system with the AIDA Image Analyzer 3.52 software (Raytest, Straubenhardt,

Table 2. Effect of Coffee Consumption (BC3) in Relation to Wash-out Period (BC2) on Gene Transcription in PBL of the Participants Determined by Q-PCR[a]

proband	Nrf2	GPx	GSTM5	NQO1	γ-GCL	GSTT1	GSR	SOD1	CAT	HO1
1	**1.7**	1.1	0.5	0.9	**1.7**	0.8	1.4	1.2	1.0	**1.6**
3	**2.1**	0.9	0.6	0.8	0.7	nt	0.9	0.5	0.8	1.1
4	1.1	0.7	1.4	0.7	1.1	1.1	0.9	0.6	0.9	0.8
5	**2.2**	1.1	**2.1**	**1.7**	1.4	**1.5**	1.4	**2.2**	1.0	1.0
6	**1.6**	0.9	0.8	**1.7**	1.4	1.2	1.3	0.9	1.0	0.7
7	**1.9**	1.1	1.2	0.9	1.0	nt	0.9	0.4	0.8	0.3
8	0.7	0.5	0.4	1.1	0.8	0.9	0.7	0.3	0.8	0.3
9	0.8	0.9	1.0	1.2	1.3	1.2	1.3	0.4	1.0	0.4
10	**2.4**	1.4	1.1	0.8	1.1	1.0	1.2	1.0	1.2	0.9
11	0.6	0.7	1.2	1.1	1.1	n.t.	0.7	0.3	0.6	0.2
12	1.2	1.3	0.9	0.9	0.9	0.8	1.3	1.2	1.2	1.0
14	**2.4**	**2.2**	1.1	0.8	0.7	0.8	0.6	**2.5**	1.4	0.9
15	0.5	1.0	0.5	1.0	1.1	0.6	0.9	0.3	0.7	0.2
16	**1.9**	**1.5**	**1.5**	0.8	0.9	1.0	**1.9**	1.0	1.2	0.6
17	1.2	**1.8**	**2.4**	1.4	**1.5**	**1.5**	1.4	**1.8**	1.4	**1.7**
18	1.3	0.8	1.0	1.1	1.4	1.0	1.2	0.6	0.8	0.7
19	**2.2**	**2.1**	0.6	1.0	0.8	nt	1.1	1.0	**1.6**	1.2
20	0.7	0.8	1.2	1.2	1.2	1.0	0.9	**1.8**	1.3	0.7
21	0.8	**2.0**	0.9	1.3	0.6	0.8	0.6	1.3	1.4	0.8
22	0.8	**1.9**	1.2	**2.1**	**2.0**	1.1	1.3	0.5	**1.9**	0.2
23	**1.8**	0.9	0.6	1.2	0.9	0.8	0.9	0.4	0.6	0.3
26	**2.2**	**1.5**	1.3	0.6	0.6	0.8	1.2	0.7	0.7	**1.5**
29	**1.8**	**1.6**	0.2	0.8	0.8	0.8	0.7	1.0	1.3	0.6
30	1.2	1.1	0.6	1.1	1.1	0.9	1.4	0.4	1.2	1.0
31	1.2	0.5	n.t.	0.9	1.2	0.9	1.2	1.2	0.8	1.0
32	1.4	1.1	0.9	**1.5**	1.2	1.3	1.1	1.1	0.9	1.0
33	0.9	0.8	nt	1.3	**1.5**	nt	1.1	1.0	0.9	0.7
34	1.0	0.9	0.9	1.1	1.1	nt	0.9	0.7	0.7	0.6
35	0.7	0.6	0.6	1.2	1.0	nt	1.0	0.7	0.5	0.5

[a]Probands 13 and 25 study not completed; probands 2, 24, 27, 28, RNA material not evaluable; nt, no transcript.

Germany). Arbitrary light units were plotted as test over control (percent).

Isolation of Human Peripheral Blood Lymphocytes. Five milliliters of freshly collected human blood anticoagulated with sodium heparin was layered on 5 mL of Histopaque 1077 (Roche Diagnostics). After centrifugation for 25 min (400*g*, without break, 25 °C), the lymphocytes were collected from the layer between the plasma and Histopaque 1077 phases and were transferred into 6 mL of RPMI 1640 medium (Invitrogen Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Thereafter, the cell suspension was centrifuged for 10 min (250*g*), and the pellet was resolved in 4 mL of 10% FCS medium and repeatedly centrifuged. Cells were transferred into 1 mL of RNAlater stabilization reagent (QIAGEN).

RNA Extraction and Real-Time (Q)-PCR. Total RNA was extracted according to the manufacturer's handbook with the RNeasyMini Kit (QIAGEN). Two micrograms of RNA was reverse-transcribed at 37 °C for 60 min, using oligo-dT primers and the Quantitec Reverse Transcription Kit (QIAGEN), and cDNA obtained from the RT reaction (corresponding to 2 μg of total RNA) was subjected to RT-PCR using QuantiTect SYBR Green PCR (QIAGEN). A control without reverse transcriptase was included in each dilution series. The primer assays used were for GSTA1, Hs_GSTA1_2_SG, QT01671530; GSR, HS_GSR_1_SG, QT00038325; GPX, HS_GPX1_1_SG, QT00203392; SOD1, HS_SOD1_2_SG, QT017671551; CAT, HS_CAT_1_SG, QT00079674; GSTT, Hs_GSTT1_2_SG, QT01751638; γ-GCL, Hs_GCLC_1_SG, QT00037310; NQO1, Hs_NQO1_1_SG, QT00050281; HO1, Hs_HMOX1_1_SG, QT00092645; Nrf2, Hs_NFE2L2_1_SG, QT00027384; and β-actin, Hs_ACTB_1_SG, QT00095431 (QIAGEN). Primer concentrations used were according to the manufacturer's guidelines in the QuantiTect SYBR Green PCR Handbook 11/2005 (QIAGEN). Q-PCR reaction parameters were as follows: incubation at 50 °C for 2 min, incubation at 95 °C for 15 min, and thereafter 40 cycles of denaturation at 94 °C for 15 s, annealing at 50−60 °C for 30 s, and extension at 72 °C for 30 s. Each sample was determined in duplicate. A −RT control was included for all assays. Fold changes in expression of the target gene relative to the internal control gene (β-actin) were analyzed using Bio-Opticon software, and the Ct data were imported into Microsoft Excel 03. Data of all assays were analyzed by the $2-^{\Delta\Delta CT}$ method. This relative quantification compares the Q-PCR signal of the target transcript to the endogenous control gene β-actin. The $2-^{\Delta\Delta CT}$method is a convenient way to analyze relative changes in gene expression from real-time quantitative PCR experiments. The mean fold change in expression of the target gene was calculated using ΔΔCT = (CT-target − CT-actin). For the ΔΔCT calculation to be valid, the amplification efficiencies of the target and reference must be approximately equal as controlled for all investigated genes.

GSTT1 Genotyping. From the pilot coffee intervention trial 18 male participants consented to be involved in saliva donation and genotyping studies. All volunteers were informed of the objectives of the study, and consent was received for their participation. Each participant was asked to use the OG-500 Oragene·DNA sample collection kit for saliva donation. Genomic DNA was then purified as follows: of a total of 2 mL of Oragene/saliva sample, 1 mL was transferred into a 1.5 mL microcentrifuge tube; 40 μL of Oragene Purifier was added and mixed gently by inversion. After 10 min of incubation on ice, the sample was centrifuged for 3 min at 15000*g* (room temperature). Thereafter, the clear supernatant was transferred into a 15 mL centrifuge tube. One milliliter of 95% ethanol was added to the supernatant and gently mixed by inverting it 10 times. After a

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further incubation step (10 min, RT), the sample was centrifuged for 2 min at 15000*g* (room temperature). After the supernatant was discarded, 500 μL of 70% ethanol was layered carefully onto the pellet and incubated for 1 min at room temperature. Subsequently, the ethanol was carefully removed without disrupting the pellet. After the DNA pellet was completely dried, the pellet was dissolved in 500 μL of distilled water. To fully resuspend the DNA, a further incubation step for 20 min at 50 °C was added. After ethanol precipitation, the final concentration of DNA was quantified using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA).

To amplify the polymorphic region of the GSTT1 gene, 40 ng of gDNA was amplified with 4 μL of GoTag Flexi buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.19 μM of each GSTT1 primer (see Table 4), and 0.125 μM of each β-actin primer, and Taq polymerase was added to a total reaction volume of 20 μL (Table 2). Amplification was performed on a VeritiTM 96-well Thermal Cycler (Applied Biosystems) with an initial denaturation of 94 °C for 3 min, followed by 39 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1.25 min, followed by a final extension of 72 °C for 5 min. Following amplification, PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide for 30 min at 80 V. The positive GSTT1 genotype was observed as a 480 bp GSTT1 fragment, with β-actin migrating to a 289 bp fragment.[20] The GSTT1*0 (null) genotype produced only a band at 289 bp for β-actin amplification. If β-actin was not present, sample genotyping and analysis was repeated. The multiplex PCR method used for GSTT1 genotype determination does not distinguish between homozygote and heterozygote GSTT1 positive genotypes. However, it does conclusively identify the homozygous null genotype.

RESULTS

Modulation of Nrf2 Translocation in Vitro. To investigate the impact on Nrf2 signaling, HT29 cells were treated for 3 h with CN-CE, and nuclear translocation of Nrf2 was determined by Western blot analysis. Because Nrf2 nuclear translocation might be initiated by the onset of intracellular oxidative stress, the accumulation of intracellular ROS was determined in the DCF assay, demonstrating that under these experimental conditions intracellular redox status was not modified significantly (tested range: 10^{-5}–100 μg/mL; data not shown). Nevertheless, CN-CE activated Nrf2 translocation in the concentration range from 10^{-1} μg/mL (127 ± 12%) to 100 μg/mL (183 ± 45%) (Figure 1), whereas the cytosolic Nrf2 protein level was not significantly modulated (data not shown). In comparison, NMP-CE increased Nrf2 nuclear levels in concentrations of 10–100 μg/mL, as reported previously,[17] and CGA-CE even failed to induce Nrf2 translocation at any test concentration. Moreover, nuclear Nrf2 levels were slightly decreased by CGA-CE and also by NMP-CE at concentrations <1 μg/mL (Figure 1).[17] In comparison to CGA-CE and NMP-CE the hybrid coffee extract CN-CE displayed the most potent Nrf2-activating properties of the tested CEs.

Induction of Phase II Gene Transcription in Vitro. To determine whether the detected potent Nrf2-translocation is passed downstream, the influence of CN-CE on the transcription of ARE-dependent phase-II-genes was determined, exemplified for HO1, NQO1, γGCL, and GSTA1 by Q-PCR. No significant modulation of HO1 gene transcription was observed in HT29 cells after 24 h of incubation with CN-CE (Figure 2A). In contrast, NMP-CE significantly induced HO1 gene transcription at 100 μg/mL, whereas CGA-CE slightly decreased HO1 transcription levels at 10 μg/mL. Of note, CN-CE significantly enhanced the transcription level of NQO1 at 100 μg/mL (1.7 ± 0.2; Figure 2B). In contrast, CGA-CE and NMP-CE failed to induce NQO1 gene transcription in HT29 cells after 24 h of incubation. Furthermore, in a tight concentration window (10^{-3} μg/mL; Figure 2C) a significant induction of GSTA1 gene transcription by CN-CE (up to 2.5 ± 0.2) was observed, whereas CGA-CE and NMP-CE were not effective.

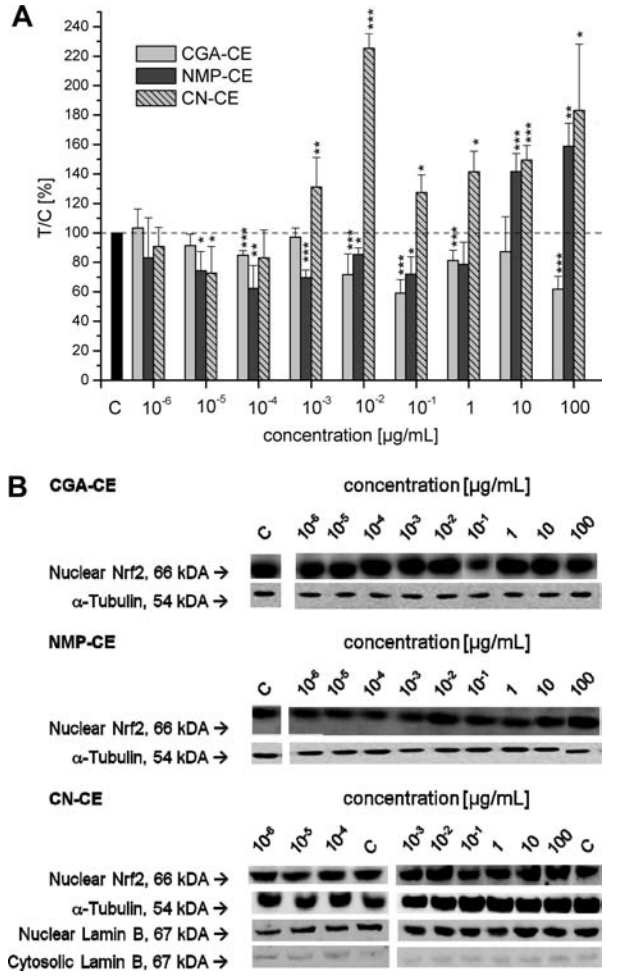

Figure 1. (A) Western blot of Nrf2 nuclear translocation in HT29 cells after 3 h of treatment with CN-CE in comparison to CGA-CE and NMP-CE.[17] (B) Representative Western blot of Nrf2 protein and the respective controls. α-Tubulin was included in the test as a loading control and Lamin B as control for a division of nuclear and cytosolic fraction. The data are presented as test over control (T/C) in percent with the control being cells treated with 1% DMSO. The data are the mean ± SD of at least three independent experiments with similar outcomes (n = 2 for CGA-CE and NMP-CE at 10^{-6} μg/mL). The significances indicated refer to the comparison of the respective concentration with the solvent control and are calculated using Student's t test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Nrf2, erythroid 2p45 (NF-E2)-related factor 2; C, solvent control.

Activation of the Nrf2/ARE pathway might also be linked to an elevation of Nrf2 de novo synthesis.[21] Thus, changes in Nrf2 transcription levels after incubation of HT29 cells were assessed. However, no onset of Nrf2 gene transcription was detected over the tested concentration range, comparable to the results reported earlier for CGA-CE.[17] From the three coffee extracts only NMP-CE potently enhanced Nrf2 gene transcription in HT29, but only in the highest concentration of 100 μg/mL (Figure 2D[17]). Thus, on the transcription level CN-CE appeared not to combine the effects of CGA-CE and NMP-CE in HT29 cells but to exhibit a distinct pattern of gene modulation. These results raised the question whether the

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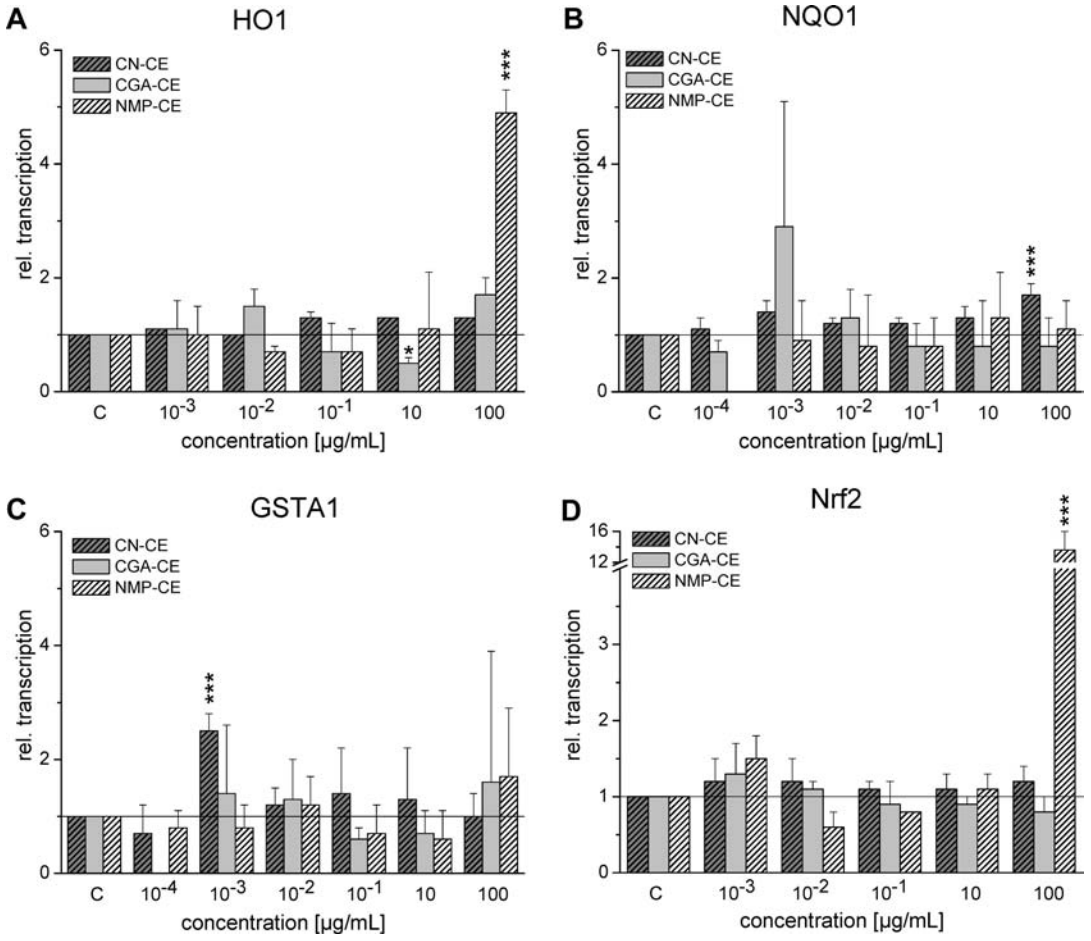

Figure 2. Modulation of gene transcription of (A) HO1, (B) NQO1, (C) GSTA1, and (D) Nrf2 in HT29 cells after 24 h of incubation of CN-CE in comparison to CGA-CE and NMP-CE.[17] The data are the mean ± SD of a minimum of three independent experiments performed in duplicates. Data are normalized by β-actin expression and presented as relative transcription of the solvent control (1% DMSO) = 1. The significances indicated are calculated compared to the solvent control using Student's t test (*, $p < 0.05$; ***, $p < 0.001$). GST, glutathione *S*-transferase; HO1, heme oxygenase 1; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, erythroid 2p45 (NF-E2)-related factor 2.

effects on gene transcription were reflected subsequently on the protein level, exemplified for GST enzyme activity.

Induction of GST Enzyme Activity. To elucidate whether the detected increase of GSTA1 gene transcription after incubation of CN-CE is also reflected in the level of protein activity, modulation of GST activity was determined after 24, 48, and 72 h of incubation of HT29 cells. CN-CE (10 μg/mL) significantly increased GST activity (112.2 ± 2.6%) after 48 h of incubation (Figure 3). To investigate a possible contribution of the coffee constituents CGA and NMP to this effect, both compounds were included in the assay and tested in an extract-relevant concentration. For comparison, the modulation of GST activity after incubation with NMP-CE and CGA-CE was determined. CGA (10 μM) potently enhanced GST activity after both 24 h (119.6 ± 4.3%) and 48 h (118.7 ± 3.1%). In a comparable way, 10 μg/mL CGA-CE induced GST activity after 24 h (112.6 ± 3.1%) and 48 h (105.9 ± 1.7%). Of note, neither NMP-CE (10 μg/mL) nor NMP (10 μM) significantly affected GST enzyme activity in the tested time and concentration range (Figure 3).

Induction of Phase II Gene Transcription by Coffee Consumption in Vivo. In a pilot human intervention study (approved by the local ethics committee of Rheinland-Pfalz, no. 837.207.08 (6204)) the effect of the hybrid coffee CN-CO on the activation of Nrf2/ARE-dependent gene expression was investigated, monitoring the transcription pattern in PBL in 29 healthy volunteers (male, nonsmoking, ages 20−44 years; for details of the study design see ref 19). Participants consumed a total amount of 750 mL of coffee per day, divided into three servings, for 4 weeks (Figure 4[19]). Before and after the coffee intervention, 4 weeks of wash-out period were entailed when no coffee was consumed and the participants had to adhere to a polyphenol-poor diet. From respective blood samples (Figure 4) total RNA was isolated from PBLs before and after each stage of the study, and gene transcript levels of selected genes were analyzed by Q-PCR. At the start of the intervention study (blood collection 1 = BC1) a broad range in the Nrf2 transcription levels was observed within the test collective, which was clearly decreased after the first wash-out period (BC2; Figure 5A). After 4 weeks of daily consumption of the test coffee CN-CO (BC3), the average Nrf2 gene transcription level of the study participants was significantly increased (1.4 ± 0.6) in comparison to the transcript status at the end of the wash-out phase (BC2). However, using a "response" threshold level of at least 1.5-fold for changes in relative transcription, a separation into "responder" (12/29) and "non-responder" (17/29) became apparent (Figure 5A; Table 2). After the second wash-out (BC4), the transcription level of Nrf2 was on average down-regulated even below the level of the first wash-out phase (compare BC2/BC4, Figure 5A). A prescreening approach was

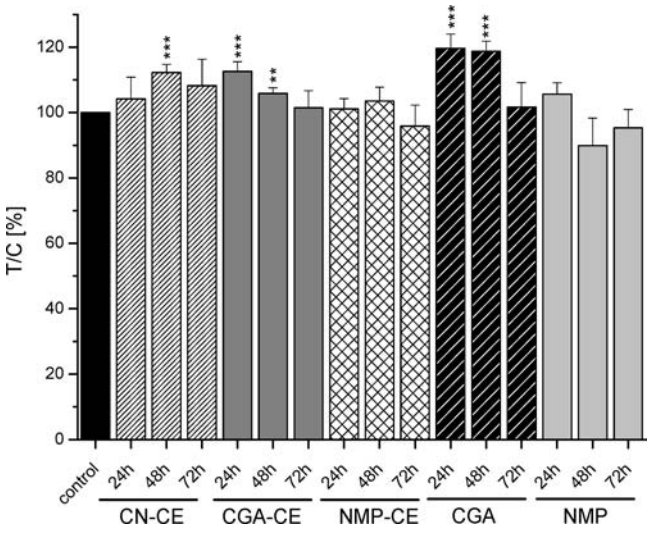

Figure 3. Effects of CN-CE, CGA-CE, NMP-CE, NMP, and CGA on GST enzyme activity in HT29 cells relative to the solvent control (=100%). HT29 cells were exposed to 10 μg/mL CN-CE, 10 μg/mL CGA-CE, 10 μg/mL NMP-CE, 10 μM NMP, or CGA for 24, 48, and 72 h. The data are the mean ± SD of at least three independent experiments. The significances indicated refer to the comparison of the respective concentration with the solvent control and are calculated using Student's t test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

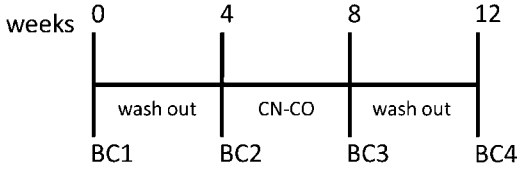

Figure 4. Study design of 3 month pilot intervention study (for details see ref 19).

used to identify potentially responsive phase II genes. Changes in the transcription pattern BC2/BC3 were determined in the blood samples of six randomly chosen participants of the intervention trial by a microarray comprising 260 genes (OligoGEArray Human Toxicology and Drug Resistance Microarray, SABiosciences). On the basis of these results (data not shown) together with the data on coffee-responsive phase II genes reported previously,[17] GSTM5, HO1, GSTT, NQO1, CAT, SOD1, GPX, γ-GCL, and GSR were selected for analysis by Q-PCR. However, a heterogeneous gene response pattern was observed in the study collective, which did not show a significant increase of transcription of Nrf2/ARE-dependent genes in the average of the participants after 4 weeks CN-CO consumption (Figure 5B–J; Table 2), with a low number of responders versus nonresponders: GSTM5 (3/24), HO1 (3/26), GSTT1 (2/20), NQO1 (4/25), CAT (2/27), SOD1 (4/25), GPX (8/21), γ-GCL (4/25), and GSR (1/28). Of note, a significant decrease in the transcript levels in the average of the participants was observed for HO1 and SOD1.

GSTT1 Genotyping. As no transcripts for the GSTT1 gene were obtained for seven participants in the study, a genotyping assay was performed to examine polymorphisms of the GSTT1 gene. GSTT1 has a known null genotype that results in gene deletion. Of 18 genotyped samples (due to limited availability of test material and/or declared consent for genotypic analyses), 5 individuals did not show a GSTT1 gene band, only a 289 bp β-actin control band, characteristic for the GSTT1*0 (null) genotype, with 13 participants demonstrating a positive GSTT1 genotype (Figure 6). Q-PCR results confirmed that the samples possessing a GSTT1*0 genotype coincided with the individuals lacking GSTT1 gene transcripts.

Correlation of Q-PCR Analysis with Biochemical Parameters. The impact of CN-CO consumption on the transcription of Nrf2-dependent genes was compared with biochemical parameters in the blood samples of these participants, reported previously,[19] such as the status of oxidative DNA damage after coffee consumption in the white blood cells (measured as fpg-sensitive sites in the comet assay), the change in oxidative DNA damage (difference between wash-out = BC2 and end of coffee consumption = BC3) and the modulation of plasma glutathione (GSH, Table 3, for details see ref 19). In total, only the relative transcription level of Nrf2 and the decrease of oxidative DNA damage observed after coffee consumption indicated some, but, if at all, comparably weak, association (R = 0.50, Table 3). The strongest correlation was observed for the transcription levels of GPx and CAT, which is, however, difficult to interpret. All other correlation coefficients above 0.50 might simply reflect the coincidence of lacking transcriptional modulation.

In the group of individuals with enhanced Nrf2 transcription levels (Table 3, responders) an association was observed between the level of oxidative DNA damage after coffee consumption and the transcription of GSR, NQO1, and GSTM5, respectively, which was not seen for the non-responders. Within this group, enhanced transcription of Nrf2 appeared to be associated to some extent with GPx transcription (R = 0.57). Of note, if a threshold was used to classify the participants with respect to the decrease in oxidative DNA damage by coffee consumption of at least 2.5% tail intensity, the resulting groups of responders and nonresponders were overlapping by 66% with the groups classified by Nrf2 transcription levels.

■ DISCUSSION

Recently we demonstrated that coffees rich in either raw bean constituents, with CGA as a lead compound, or typical roasting products such as NMP enhanced Nrf2/-ARE-dependent gene transcription both in vitro and in vivo.[4] On the basis of these findings, an Arabica coffee blend, rich in both CGA and NMP, was produced now to determine the Nrf2-activating properties of a coffee beverage combining both characteristics. In vitro this hybrid coffee extract CN-CE indeed showed a clearly enhanced potency to increase nuclear Nrf2 translocation (Figure 1), indicating effective activation of Nrf2 signaling. The amounts of NMP in CN-CE (71.7 mg/L) and in the NMP-rich coffee extract NMP-CE (73.3 mg/L) [17] were comparable and also in the range where the isolated constituent possesses strong Nrf2 activating properties.[17] Thus NMP, a known activator of Nrf2 translocation, might indeed contribute to the effects of both coffee extracts.

Furthermore, we addressed the question of whether the translocation of Nrf2 into the nucleus is reflected downstream by the induction of Nrf2/ARE-dependent gene expression. The in vitro data indicate that the presence of green coffee bean constituents suppresses the onset of HO1 transcription, whereas a high amount of dark roasted coffee compounds seems to be crucial for HO1 expression. Of note, the induction of NQO1 gene transcription by CN-CE appeared in the same concentration range where maximal Nrf2 nuclear translocation was observed, indicative for downstream transfer of Nrf2

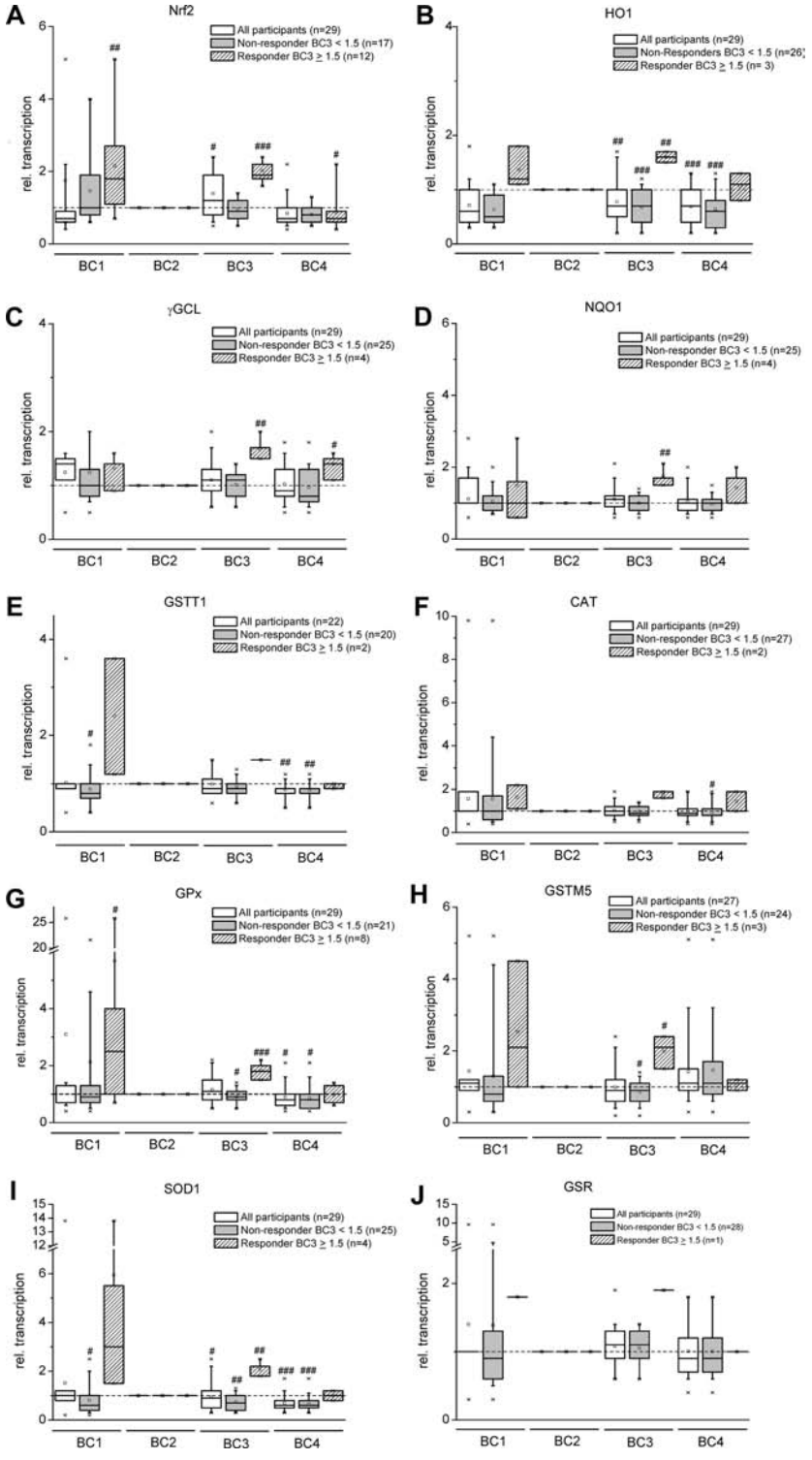

Figure 5. Modulation of Nrf2, GSTM5, HO1, GSTT1, NQO1, CAT, SOD1, GPX, γ-GCL (catalytic subunit), and GSR transcripts in human PBLs after a 4 week intervention study with consumption of 750 mL of CN-CO, respectively, per day (for study design see ref 19). The data presented are the average of 29 (exceptions: GSTT1, 22, and GSTM5, 27) independent experiments performed in duplicates. The data are normalized by β-actin expression and represented as relative transcription of individual levels of the participants before the study (BC1), after a 4 week wash-out period with a polyphenol-poor diet and no coffee consumption (BC2), after 4 weeks of daily 750 mL CN-CO consumption (BC3), and after a second wash-out period of 4 weeks equal to the first (BC4). The data are represented as relative transcription of the BC2 = 1. Statistical analysis was carried out using underlying ΔCt values. The Shapiro−Wilk test was applied for checking on normal distribution of variables or logarithmically transformed variables. In the case of normally distributed data significant differences between BC1, BC3, and BC4 compared to BC2 were calculated using Student's *t* test. Otherwise, the Wilcoxon rank sum test was used (#, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$). BC, blood collection; CO, freshly brewed coffee; Nrf2, NF-E2 p45 subunit-related factor 2; GST, glutathione *S*-transferase; NQO1, NAD(P)H:quinone oxidoreductase; CAT, catalase; SOD1, superoxide dismutase 1; GPX, glutathione peroxidase 1 γ-GCL, γ-glutamylcysteine synthase; HO1, heme oxygenase 1; GSR, glutathione reductase.

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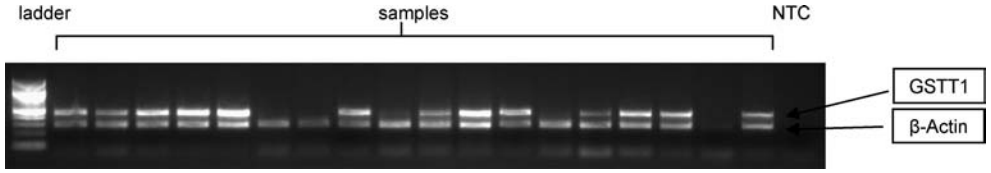

Figure 6. GSTT1 polymorphic PCR products resolved by agarose gel electrophoresis, showing the GSTT1 null (lanes 7, 8, 10, 14, and 18) and positive genotypes (lanes 2−6, 9, 11−13, 15−17, and 19). Ladder: 100 bp DNA ladder (New England BioLabs); NTC, no template control.

Table 3. Correlation Matrix of the Analyzed Biochemical End Points, Calculated for All Evaluable 29 Test Subjects as well as Separately for Responders (Nrf2 Transcription ≥ 1.5, 12 Subjects) and Nonresponders (Nrf2 Transcription < 1.5, 17 Subjects)[a]

	transcription										
	HO1	CAT	SOD1	GSR	γGCL	NQO1	GSTM5	GPx	Nrf2	rel GSH level	Δ ox DNA
All Subjects											
ox DNA level	−0.36	0.07	−0.18	0.13	0.18	0.31	0.18	−0.16	−0.09	−0.3	0.00
Δ ox DNA	0.37	0.14	0.08	0.08	−0.10	−0.34	0.10	0.21	**0.50**	−0.02	
rel GSH level	0.27	−0.02	0.09	0.11	−0.30	−0.23	−0.19	−0.04	0.00		
Nrf2	0.46	0.15	0.35	0.22	−0.29	−0.33	0.18	0.43			
GPx	0.32	**0.78**	0.44	0.03	−0.16	0.04	0.31				
GSTM5	0.23	0.24	0.38	0.34	0.16	0.21					
NQO1	−0.22	0.29	0.10	0.17	**0.64**						
γGCL	−0.01	0.20	0.03	0.24							
GSR	0.22	0.16	−0.01								
SOD1	**0.51**	0.43									
CAT	0.22										
Responder (Nrf2-Transcr ≥ 1.5)											
ox DNA level	−0.26	0.01	0.21	**0.53**	0.37	**0.58**	**0.50**	−0.37	0.04	−0.19	−0.12
Δ ox DNA	−0.13	0.23	−0.39	−0.10	−0.02	−0.19	−0.17	0.15	0.20	0.24	
rel GSH level	0.13	**0.65**	0.06	0.04	−0.35	−0.39	−0.36	0.49	0.28		
Nrf2	0.29	0.33	0.45	0.04	−0.45	−0.32	0.42	**0.57**			
GPx	0.20	**0.80**	0.48	−0.24	−0.48	−0.43	−0.01				
GSTM5	−0.01	−0.11	0.44	**0.52**	0.11	0.27					
NQO1	−0.22	−0.10	0.24	0.18	**0.59**						
γGCL	0.15	−0.07	0.19	−0.02							
GSR	−0.03	−0.09	−0.16								
SOD1	0.24	**0.51**									
CAT	0.13										
Nonresponder (Nrf2-Transcr < 1.5)											
ox DNA level	−0.43	0.10	−0.46	−0.17	0.08	0.16	0.02	−0.07	−0.29	−0.35	0.07
Δ ox DNA	**0.62**	0.07	0.35	0.20	0.02	−0.33	0.24	0.06	**0.53**	−0.05	
rel GSH level	0.42	−0.28	0.18	0.21	−0.37	−0.24	−0.10	−0.21	0.30		
Nrf2	**0.75**	0.12	0.33	**0.58**	0.14	−0.08	0.15	0.11			
GPx	0.30	**0.81**	0.34	0.22	0.22	0.61	**0.50**				
GSTM5	0.38	0.42	0.33	0.17	0.25	0.23					
NQO1	−0.11	**0.63**	0.09	0.24	**0.63**						
γGCL	−0.01	0.41	0.01	**0.61**							
GSR	0.47	0.37	0.14								
SOD1	**0.73**	0.38									
CAT	0.26										

[a]The correlation coefficient *R* was calculated for any combination between the induction of transcription of various genes associated with oxidative stress response (see Table 2) and the modulation of biochemical end points as reported previously in ref 19. (I) Status of oxidative DNA damage in blood cells after coffee consumption measured as fpg-sensitive sites in the comet assay, calculated as tail intensity (%); (II) change in the status of oxidized DNA in blood cells induced by coffee intervention[19] (difference in comet tail intensities between BC2 and BC3); (III) relative GSH level in the blood after coffee consumption (calculated as percentage of the GSH level before coffee intervention, BC2). Correlation coefficient values $R \geq 0.5$ shown in boldface.

signals. A significant decrease of NQO1 mRNA expression in $nrf2^{(-/-)}$ mice in comparison to wild type mice, indicating the regulation of NQO1 gene transcription by Nrf2, has already been reported.[22] Furthermore, $nrf2^{(-/-)}$ mice respond significantly more weakly in terms of NQO1 induction after tertiary butylhydroquinone (tBHQ) treatment in the liver and intestine than the respective wild type, also supporting this conclusion.[7] In previous studies we demonstrated increased NQO1 transcript levels in HT29 cells after 24 h of incubation with 10 μM NMP.[17] CN-CE concentrations inducing significant NQO1 gene transcript induction (100 μg/mL CN-CE) comprise 5 μM NMP, thus within the effective range of

Table 4. GSTT1 Primer Sequences

primer	sequence	ref
GSTT1 forward	5′-TTCCTTACTGGTCCTCACATCTC-3′	20
GSTT1 reverse	5′-TCACCGGATCATGGCCAGCA-3′	
β-actin forward	5′-CGGAACCGCTCATTGCC-3′	20
β-actin reverse	5′-ACCCACACTGTGCCCATCTA-3′	20

this compound. However, the NMP-rich coffee extract itself failed to induce NQO1 gene transcription after 24 h, indicating that green coffee constituents contribute substantially to the effects of CN-CE on NQO1 transcription. Yet, coffee high in only green coffee bean constituents such as CGA-CE displayed only a very weak potential to induce NQO1 gene transcription, supporting the hypothesis that a hybrid of both green and dark roast coffee constituents enhances the Nrf2/ARE-activating potential. Modulation of NQO1 has already been reported by other coffee constituents. Feng et al. determined an increased NQO1 activity in JB6 mouse epithelial cells after 18 h of treatment with 40 and 80 μM chlorogenic acid.[23] Furthermore, the coffee-specific diterpenes cafestol and kahweol have been reported to increase NQO1 mRNA,[14] hinting at the contribution of coffee constituents other than NMP to the observed effects.

Moreover, CN-CE significantly up-regulated the GSTA1 gene transcripts in HT29 cells after 24 h of incubation (Figure 2C). An induction of different GSTs by Nrf2 has already been demonstrated;[21,24] thus, a contribution of the Nrf2/ARE pathway on the GSTA1 activation seems to be likely. Increased GSTA1 gene transcription and activation of the ARE sequence in the GSTA1 promoter as well as elevated GST activity after incubation with the coffee constituent CGA have already been reported.[17,23] In vivo up-regulation of GST activity in rats has been described after feeding with the coffee constituents NMP, cafestol, and kahweol.[15,16] The profile of the significantly up-regulated genes in HT29 cells after treatment with CN-CE seems to differ from the profiles of NMP-rich and CGA-rich CE, reported earlier.[17] However, the overall potency to up-regulate phase II gene transcripts seems to be comparable.

Significantly increased GST activity was detected in HT29 cells after 48 h of incubation with CN-CE (Figure 3). However, the concentration was 10^3-fold higher than the one inducing GSTA1 gene transcription in these cells (Figure 2C). Increased GST activity in human intestinal Caco-2 cells after 72 h of incubation with a coffee extract has already been reported.[16] Furthermore, induction of GST activity by coffee, coffee beans, and coffee extract has been demonstrated in vivo,[14−16] further supporting our findings. Moreover, in the present study a significant increase in GST activity after incubation with CGA (10 μM) was observed. Elevation of GST enzymatic activity after 18 h of incubation with 20 and 40 μM CGA has been reported in mouse epidermal JB6 cells.[25] Furthermore, 10 μg/mL CGA-CE significantly induced GST enzyme activity after 24 and 48 h in HT29 cells. The 10 μg/mL CN-CE extract contains 0.5 μM CGA, thus a power of 10 lower concentration than the concentration at which the isolated constituent increased GST enzyme activity, hinting at a contribution of other coffee constituents to the effects of the extract. Neither NMP-CE nor the isolated coffee constituent NMP significantly affects GST activity, questioning a substantial contribution of NMP to the GST enzyme activating properties of coffee under the tested conditions.

On the basis of the positive outcome of an onset of the Nrf2/ARE pathway with the hybrid coffee extract in vitro, a pilot human intervention study was performed with the respective coffee beverage. To allow a direct comparison with the effects of coffee high in either green coffee bean constituents or characteristic roast products, determined in a precedent intervention trial,[17] a comparable study design with 4 weeks of coffee consumption was chosen. As expected from the results of ref 17, in the present study a significant activation of Nrf2 gene transcription could be observed in the mean of the participants. However, large variations in the individual response became apparent. Whereas 12 individuals reflected a significant response (≥1.5 in comparison to previous wash-out period), Nrf2 gene transcription did not change in 17 participants of the trial after coffee consumption, thus exhibiting a lower response rate than observed previously for CGA-CO or NMP-CO.[17] In contrast to NMP-CO, no significant induction of NQO1 transcription was observed in the average of the participants, but even a down-regulation of HO1 and SOD1 transcript levels was detected. Overall, a heterogeneous transcript pattern of the tested Nrf2-dependent genes within the test collective was monitored (Figure 5B−J) with 8 of 29 participants (27%) exhibiting an induction of three or more of the tested genes by a factor of ≥1.5 at BC3 (Table 2). In contrast, 11 of 29 participants (34%) failed to show any significant modulation after coffee consumption (BC3) in the transcription levels of the selected genes (transcript level <1 .5). Critical consideration of these results raised the question of why such a large variation in the induction of Nrf2 transcripts as well as phase II gene transcripts was observed within the test collective. One possible explanation might be the occurrence and functional effect of genetic polymorphisms. Several single-nucleotide polymorphisms (SNP) have already been identified in the Nrf2 gene. Of special relevance seems to be the −617C/A polymorphism and the −651G/A SNP, which are located in the promoter region of the gene. Both SNPs were found to reduce the transcriptional activity of Nrf2, resulting in decreased Nrf2-dependent gene transcription.[26−28] Determining the Nrf2 genotype in a subset of the present study collective displayed a contribution of the Nrf2 genotype to the changes in Nrf2 gene transcription after coffee consumption.[29] The analysis of the Nrf2 genotype was compared to the transcription pattern for GSTM5, HO1, GSTT, CAT, SOD1, GPX, γ-GCL, and GSR. From 4 of 27 individuals displaying an increase in NQO1 gene transcription, 3 (75%) have been found to carry the WT sequence at each of the determined Nrf2 SNPs. This frequency is much higher than expected, with a frequency of only 22% of all genotyped individuals possessing a WT at each of the three investigated SNPs, thus hinting at a contribution of the Nrf2 genotype to the response in NQO1 gene transcription.

However, genetic variations in the respective ARE-dependent genes could further contribute to the large variations within the response. Thus, an 8-fold lower HO1 promoter activity has already been reported if a SNP at position −413 in the promoter region of the HO1 gene is present.[30] Moreover, there is clear evidence that several SNP in the γGCL gene have a functional role in gene expression.[31] A genotyping assay for the detection of polymorphisms of the GSTT1 gene furthermore confirmed that the samples possessing a GSTT1*0 genotype coincided with the individuals lacking GSTT1 gene transcripts, emphasizing the necessity of polymorphism characterization of the test collective.

To address the question of whether the modulation of Nrf2/ARE-dependent gene transcription represents a suitable biomarker for chemopreventive properties in vivo, the impact of coffee consumption on the transcription was compared with respective data on biochemical parameters in the blood samples, reported previously,[19] including the status of oxidative DNA damage after coffee consumption in the white blood cells (measured as fpg-sensitive sites in the comet assay), the change in oxidative DNA damage (difference between wash-out and end of coffee consumption), and the modulation of plasma glutathione (GSH). However, only the increase in Nrf2 transcription and the reduction of oxidative DNA-damage resulted in a weak association (Table 3). Thus, dividing the participants into two groups, one showing a reduced oxidative DNA of >2.5 tail intensity (percent) and the other showing no protective effect after coffee consumption, classified as group of responders and nonresponders showed an overlap of 66% with the groups classified as Nrf2 responders (increase in relative transcription after coffee consumption $\geq$ 1.5) and Nrf2 nonresponders, respectively. Thus, the level of Nrf2 transcription seems to represent a promising molecular biomarker for the chemopreventive/antioxidative properties of foods in humans.

Taken together, the tested coffee blend CN-CE, which combines elevated levels of characteristic raw coffee bean constituents with typical dark-roast products, displays potential chemopreventive properties by elevating nuclear Nrf2 translocation and increased transcription levels of the Nrf2-dependent phase II enzymes GSTA1 and NQO1 as well as increased GST enzyme activity in vitro. In humans, 4 weeks of consumption of respective coffee brews significantly increased mean Nrf2 gene transcripts in PBLs of the test subjects. However, the response pattern of Nrf2-dependent genes demonstrated considerable individual variability. Nevertheless, the data indicate an association (R = 0.5) between Nrf2 transcription and a decrease in oxidative DNA damage in PBLs observed after coffee consumption, supporting the hypothesis that coffee-mediated effects on gene expression are of chemopreventive relevance.

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ABBREVIATIONS USED

CE, coffee extract; CO, coffee beverage; CGA, 5-*O*-caffeoylquinic acid; FCS, fetal calf serum; γGCL, γ-glutamylcysteine ligase; GST, glutathione *S*-transferase; HO1, heme oxygenase 1; HT29, human colon carcinoma cell line; NQO1, NAD(P)H:quinone oxidoreductase 1; NMP, *N*-methylpyridinium; PBLs, human peripheral blood lymphocytes.

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