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FOOD & FUNCTION

Coffees rich in chlorogenic acid or *N*-methylpyridinium induce chemopreventive phase II-enzymes via the Nrf2/ARE pathway in vitro and in vivo

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Recently, the coffee constituents 5-*O*-caffeoylquinic acid (CGA) and *N*-methylpyridinium (NMP) were identified as inducers of the Nrf2/antioxidant-response element (ARE) detoxifying pathway under cell-culture condition. To study the impact of CGA and NMP on the Nrf2-activating properties of a complex coffee beverage, two different model coffees were generated by variation of the roasting conditions: a low-roast coffee rich in CGA and a heavy-roast low in CGA but containing high levels of NMP. Activation of the Nrf2/antioxidant-response element pathway was monitored in vitro and in vivo.

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To prevent from ROS, cells possess different defense mechanisms like phase II-detoxifying-enzymes [1, 2]. A deficiency in phase II-enzyme activity is associated, e.g., with a higher risk of colon cancer [3, 4]. The expression of many phase II-genes is regulated by the activation of antioxidant-response elements (AREs), found in the 5′-flanking region of respective gene promoters. Via binding of the transcription factor, Nrf2/ARE is activated [5]. In quiescent cells, Nrf2 is sequestered in the cytoplasm. Activation by ROS provokes the release of Nrf2 and its nuclear translocation, and thus activating ARE-dependent genes [2, 6]. Nrf2-regulated genes include glutathione *S*-transferases (GST), γ-glutamate cysteine ligase (γGCL), NAD(P)H:quinone oxidoreductase 1 (NQO1), and heme oxygenase 1 (HO1) [7].

Recently, we demonstrated that 5-*O*-caffeoylquinic acid (CGA) and *N*-methylpyridinium (NMP) represent potent activators of Nrf2-nuclear translocation and subsequent ARE-dependent gene transcription in human colon carcinoma cells (HT29) [8].

To get a more profound estimation of the potential of CGA and NMP in the complex matrix coffee, we now examined two coffees, derived from the same batch of green coffee beans (Arabica Brazil): a light roast (CGA-CO; 260°C, 2 min in a technical scale fluidized bed coffee roaster) comprising a CGA concentration (819.2 mg/L) above the average and a dark roast (NMP-CO; 260°C, 5 min) extremely high in NMP (73.7 mg/L; Supporting Information Table I) in comparison to standard coffees [8–10].

Nrf2-translocation was investigated (incubation, 3 h) in HT29 cells by Western blot (for details, see Supporting Information). CGA-CE (extract of CGA-CO, for preparation,

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Abbreviations: ARE, antioxidant-response element; **CGA,** 5-*O*-caffeoylquinic acid; **GCL,** γ-glutamate cysteine ligase; **GST,** glutathione *S*-transferase; **HO1,** heme oxygenase 1; **NMP,** *N*-methylpyridinium; **NQO1,** NAD(P)H:quinone oxidoreductase 1

see Supporting Information) did not induce nuclear Nrf2-translocation, instead a reduction of nuclear Nrf2-protein at 100 μg/mL was observed (Fig. 1A). An aliquot of 10 μg/mL CGA-CE contains 16.5 μM CGA, a concentration at which the isolated constituent activated nuclear Nrf2-translocation [8]. Thus, CGA-dependent Nrf2-activation seems to be overlapped by compensating effects of other coffee constituents, such as trigonelline and catechol, which were already identified to diminish Nrf2-protein in HT29 cells in concentrations occurring in CGA-CE [8].

Furthermore, increased intracellular ROS levels after incubation with CGA-CE in concentration from 10^{-1} to 100 μg/mL (189.2 ± 21.8%) were apparent (Fig. 1B). High ROS levels have been already reported to induce the Nrf2/ARE pathway [11]. Cell cytotoxicity, inhibiting regular cellular response to oxidative stress, might decrease Nrf2-nuclear translocation, thereby explaining the here detected effects. Yet, no growth inhibition was observed in HT29 cells by CGA-CE up to ≥100 μg/mL (SRB-Assay, for detailed information on methods, see Supporting Information).

In HT29 cells, NMP-CE diminished the nuclear Nrf2 protein level in low concentrations (62.4 ± 15.4; 10^{-4} μg/mL), whereas in higher concentration ranges, Nrf2-translocation was clearly induced (Fig. 1A). Since total Nrf2-protein level was increased as well (data not shown), Nrf2 de novo synthesis might be set on. An increased de novo synthesis of Nrf2 was already discussed in different cell lines [5, 12]. To investigate whether the potent induction of Nrf2-translocation is caused by increased intracellular ROS levels, DCF assay was performed. NMP-CE did not modulate the intracellular redox status in HT29 cells (Fig. 1B). Growth inhibitory properties were also of no relevance up to concentrations of ≥100 μg/mL (data not shown). In 10 μg/mL NMP-CE, high nanomolar NMP concentrations are reached, known to induce nuclear Nrf2-translocation as a single compound [8].

As a consequence of enhanced nuclear Nrf2-translocation, increased transcription of Nrf2/ARE-dependent genes is to be expected. Changes in the transcription of selected ARE-dependent genes in HT29-cells were determined by RT-PCR (for detailed information on methods, see Supporting Information). Modulations by CGA-CE after 3 h incubation were inconsistent with a decreased γGCL-transcript level in low concentrations ($\leq 10^{-1}$ μg/mL) and an increase in GSTT1-transcription, an important member of GST superfamily [11] at 100 μg/mL (2.4 ± 0.3). The data presented indicate that though no increase in nuclear Nrf2 protein was detected Nrf2-dependent gene transcription was set on, suggesting additional activation mechanisms. This effect was even more visible after long time incubation (24 h) of HT29 cells with CGA-CE, significantly enhancing the γGCL-transcription, especially in low extract concentrations (10^{-3} μg/mL; 2.8 ± 0.7). However, 10 μg/mL CGA-CE significantly diminished γGCL- and HO1-transcription levels (0.3 ± 0.2 and 0.5 ± 0.1), possibly resulting from the significant decrease of nuclear Nrf2-protein after 3-h incubation. It is worth noting that HO1-transcription was increased by 100 μg/mL CGA-CE (1.7 ± 0.3, Fig. 2B).

Thus, depending on the incubation time and concentration range, a different transcription pattern with rather up or downregulated gene transcription was observed. However, Cavin et al. reported from a marked induction of mRNA expression of HO1, NQO1, and γGCL in rat liver and primary hepatocytes treated with 800 μg/mL of standard coffee for 24 h [13]. The different outcome might be explained by differences between the standard coffee [12] and the designed extract, used here. However, the potent effect of CGA as a single compound on ARE-dependent

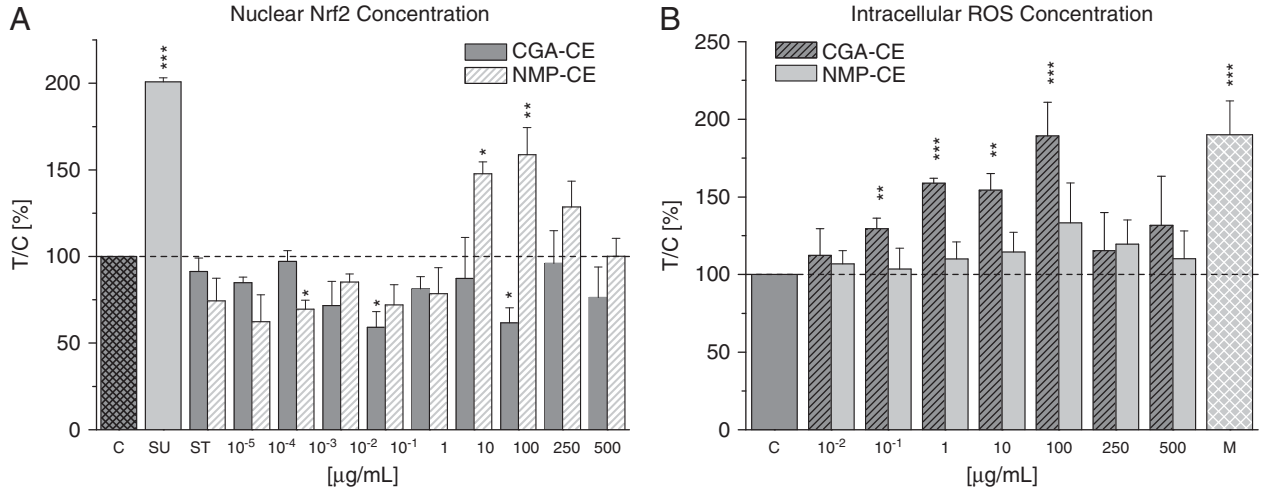

Figure 1. (A) Western blot of nuclear Nrf2-protein level in HT29 cells with CGA-CE and NMP-CE; positive control: L-sulforaphane (30 μM, respective Western blot, see Supporting Information Fig. I). (B) DCF assay of CGA-CE and NMP-CE; positive control: Menadion (20 μM); data are mean ± SD of three independent experiments; significances are calculated using Student's *t*-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$) (for detailed information on methods, see Supporting Information).

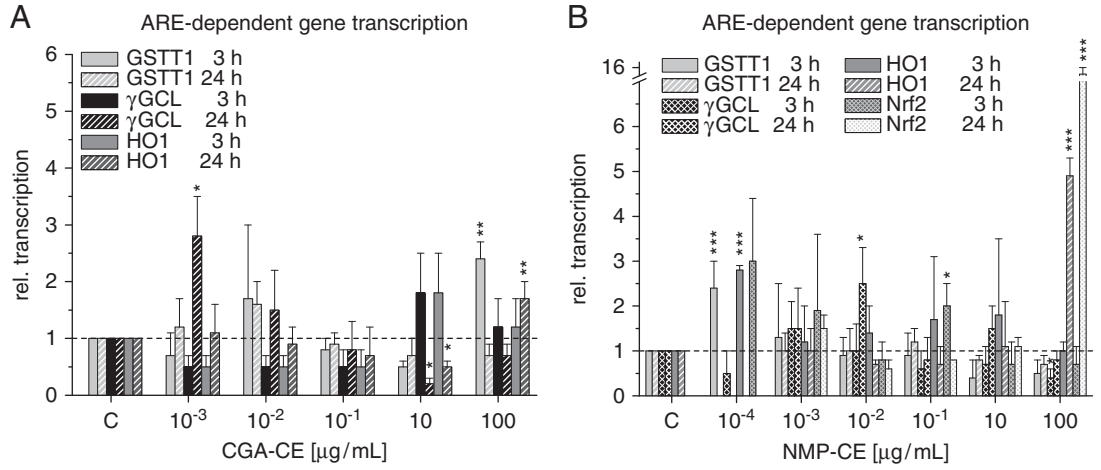

Figure 2. Increase of GSTT1-, γGCL-, HO1-, NQO1- and Nrf2-transcription in HT29 cells after incubation with (A) CGA-CE and (B) NMP-CE performed by RT-PCR. Mean ± SD of four independent experiments (duplicates); normalized to β-actin expression and presented as relative transcription of control = 1; significances calculated using Student's *t*-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$) (for detailed information on methods, see Supporting Information).

transcription in HT29 cells [8] was not retrieved by the use of respective CGA concentrations in complex coffee.

Modulation of ARE-dependent gene transcription by NMP-CE (3 h) was already apparent in low concentrations (10^{-4} μg/mL), with GSTT1-, HO1-, and Nrf2-transcript levels being elevated. Nrf2-transcription was also increased by 10^{-1} μg/mL NMP-CE (2.0 ± 0.5). On the contrary, at 100 μg/mL NMP-CE a decrease of GSTT1- and γGCL-transcription was observed. After 24-h incubation, a completely different pattern was apparent, potently increasing γGCL-transcription in the concentrations of 10^{-2} μg/mL (2.5 ± 0.8). At 100 μg/mL, the HO1- (4.9 ± 0.4) and Nrf2-transcription (13.6 ± 2.4) were enhanced. On the contrary, GSTT1-transcription was not modulated by NMP-CE in concentrations ranging from 10^{-3} to 100 μg/mL (Fig. 2B).

NMP has been previously reported to increase GSTT1- and Nrf2-ranscription after 3-h incubation of HT29 cells [8]. Hence, a contribution of NMP to the Nrf2/ARE-activating properties of the respective coffee extract is suggested. However, the effects on Nrf2/ARE-dependent gene expression appear to depend on the concentration range, the incubation time, and the respective genes.

It is worth noting that Nrf2-transcription was elevated at high NMP-CE concentrations (24 h). Recently, a dose-dependent increase in Nrf2-luciferase reporter activity in AREc32-cells by coffee was reported [14], supporting the present results.

Taken together, both coffee extracts modulated the transcription of Nrf2/ARE-dependent genes in HT29-cells, albeit with a different activity pattern with respect to time, concentration, and potential target genes.

In preparation of a respective human trial, microarray analysis was used to assess whether additional ARE-dependent genes are likely to be affected. The array (for detailed information on methods, see Supporting Information) profiles more than 260 genes related to cell stress and cell toxicity. In the context of this publication, however, only selected genes will be discussed. Pooled human PBLs were incubated (3 h) ex vivo with the coffee extracts, confirming an induction of GSTT1-gene expression by CGA-CE (+2.2), but not by NMP-CE. Furthermore, HO1- and NQO1-gene expression was elevated by NMP-CE (+2.6 and +3.7, Supporting Information Table II).

A pilot intervention trial (Ethic votum 1737/07, TU Munich) was performed, where 27 (average, 26 ± 1 years; BMI, 23.2 ± 0.5) healthy nonsmoking moderate coffee drinker (~400 mL coffee/day) had to follow 2 wk of washout (abstinence of coffee and polyphenol-poor nutrition). Subsequently, 4-wk coffee intervention was conducted with consumption of 500 mL/day of the respective coffee without sugar or milk. At the beginning of each period, blood was withdrawn to isolate PBLs to determine the effects on gene transcription (Fig. 3A).

First, mRNA of four randomly chosen participants was analyzed by microarray analysis to identify responsive genes. GSTT1- and NQO1-expression levels were elevated by CGA-CO in comparison to washout (11.1 ± 4.7 and 4.1 ± 1.6), whereas HO1- and NQO1-gene expression was increased by NMP-CO (6.4 ± 2.0 and 2.8 ± 1.2, Supporting Information Table II). Taken together, the microarray results indicated a potential of both coffees to influence gene expression in vivo.

Subsequently, the PBLs, which had been collected during the trial, were investigated by RT-PCR. A slightly increased NQO1-transcription was apparent after CGA-CO consumption in the average of all 27 participants, whereas the following washout caused a potent decrease in NQO1-transcripts which was compensated after NMP-CO consumption, elevating NQO1-transcript levels to baseline (Fig. 3B). A Nrf2-dependent increase of NQO1- and

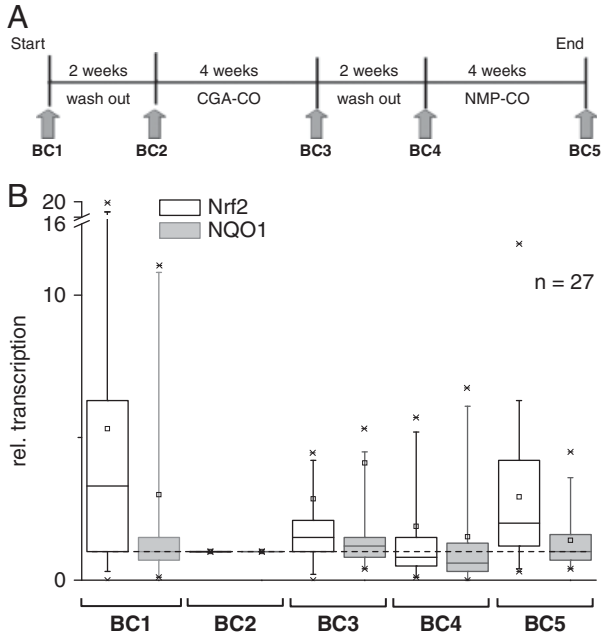

Figure 3. (A) Study design; blood samples of 27 participants were collected at each blood collection (BC). (B) Modulation of NQO1 and Nrf2-transcripts in human PBLs during the course of the trial. The data (BOX diagrams) are normalized to β-actin expression and represent relative transcription levels of individual at the five different BC points. The data (performed in duplicates) are represented as relative transcription of the BC2 = 1 (for detailed information on methods, see Supporting Information).

γGCL-transcription has been already reported in mice, fed with a coffee-containing diet [12].

Only in 17 participants GSTT1-transcription could be detected, probably due to the others carrying a GSTT1*0 genotype, resulting in gene deletion [15]. In the average of these participants though, GSTT1-gene transcripts were slightly downregulated after both CGA-CO and NMP-CO consumption. Furthermore, only consumption of NMP-CO, not CGA-CO, increased HO1-transcript levels (~1.5-fold; data not shown).

Nrf2 was modulated by the polyphenol-poor diet prior to coffee intervention, reducing Nrf2-transcripts in the PBLs. Both CGA-CO and NMP-CO consumption partly compensated this effect by increasing Nrf2-transcript levels in the mean in comparison to the washout (Fig. 3B). A closer look on the modulations of Nrf2-gene transcription revealed differences between the influences of the COs. NMP-CO seems to possess higher Nrf2-activating properties, resulting in >0.5 elevated transcription levels in 59% ($n = 16$) of the participants (41%, $n = 11$, after CGA-CO intervention). However, 22% of the participants ($n = 6$) displayed a very weak Nrf2-transcription during the complete trial (Supporting Information Fig. II). Different authors already reported about polymorphisms in the Nrf2-gene affecting its transcriptional activity, with 20% of the Caucasian population carrying such less sensitive Nrf2 genotypes [16], thereby leading to a likely explanation why 22% of the participants did not show any modulations during the course of the study. Recent studies also indicate a weaker luciferase activity of different UGT-isoforms after treatment with coffee in cells carrying these SNPs [17]. However, the mean of the weak-responding individuals displayed modulated Nrf2-transcription after NMP-CO consumption, suggesting a potential of this CO as an Nrf2-activator even in generally weak responding individuals.

Together, our data point out that CGA-CE and NMP-CE modulate ARE-dependent transcription. Yet, the NMP-CE represented the more potent extract in vitro and in vivo, tempting to speculate an important role of the isolated constituent NMP.

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