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Coffee constituents as modulators of Nrf2 nuclear translocation and ARE (EpRE)-dependent gene expression

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Abstract

Oxidative cellular stress initiates Nrf2 translocation into the nucleus, thus inducing antioxidant response element (ARE)-mediated expression of Phase II enzymes involved in detoxification and antioxidant defence. We investigated whether coffee extracts (CEs) of different proveniences and selected constituents have an impact on the Nrf2/ARE pathway in human colon carcinoma cells (HT29). Assessed as increased nuclear Nrf2 protein, Nrf2 nuclear translocation was modulated by different CEs as observed by Western blot analysis. In addition to the known Nrf2 activator 5-*O*-caffeoylquinic acid (CGA), pyridinium derivatives like the *N*-methylpyridinium ion (NMP) were identified as potent activators of Nrf2 nuclear translocation and ARE-dependent gene expression of selected antioxidative Phase II enzymes in HT29. Thereby, the substitution pattern at the pyridinium core structure determined the impact on Nrf2-signalling. In contrast, trigonelline was found to interfere with Nrf2 activation, effectively suppressing the NMP-mediated induction of Nrf2/ARE-dependent gene expression. In conclusion, several coffee constituents, partly already present in the raw material as well as those generated during the roasting process, contribute to the Nrf2-translocating properties of consumer-relevant coffee. A fine tuning in the degradation/formation of activating and deactivating constituents of the Nrf2/ARE pathway during the roasting process appears to be critical for the chemopreventive properties of the final coffee product.

Keywords: Anti-oxidative; Chemopreventive; *N*-Methylpyridinium; Chlorogenic acid; Trigonelline

1. Introduction

Diet and lifestyle are known to play a major role in the development of many life-threatening diseases such as cardiovascular syndromes and cancer. Of special interest are foods and foodstuffs, supporting the body's defences against such diseases. Coffee as one of the most widely consumed beverages in the world has been associated with a reduced risk to develop Parkinson's disease or type 2 diabetes mellitus. A variety of publications propose caffeine as well as various diterpenes as the most potent biological active constituents of coffee [1–5]. Its antioxidative activity is ascribed mostly to phenolic constituents and/or Maillard reaction products which are formed during the roasting process [6,7]. A meta-analysis, comprising five cohort and 12 case-control studies, revealed a potential protective role of coffee in colon carcinogenesis [8], but the molecular mechanisms involved in these effects have not been fully elucidated yet.

One pivotal mechanism to guard against cancer, neurodegenerative diseases, aging and atherosclerosis is the protection of cells against reactive oxygen species (ROS)-induced damage [9,10]. In this regard, cells have developed defence mechanisms, such as the induction of the expression of Phase II detoxifying enzymes [11,12]. Phase II enzymes play a critical role in converting reactive electrophiles or xenobiotics into less toxic products. The balance between the Phase I potentially carcinogen-activating and the Phase II detoxifying enzymes seems to be crucial for the risk to develop cancer. A deficiency in Phase II enzyme activity is associated with an increased risk of colon cancer [13,14]. The expression of many Phase II detoxifying genes is regulated by the activation of antioxidant or electrophile response elements (ARE/EpRE) which are located in the 5′-flanking region of their respective promoters. ARE, a *cis*-active element, is activated *via* binding of the transcription factor Nrf2 [nuclear factor-erythroid 2 p45 subunit (NF-E2)-related factor 2], a member of the cap'n'collar family of the basic region leucine zipper proteins [15]. Increased nuclear levels of Nrf2 provoke gene

Abbreviations: AB, Arabica Brazil; AC, Arabica Columbia; ARE/EpRE, antioxidant or electrophile response element; CE, coffee extract; CGA, 5-*O*-caffeoylquinic acid; DMP, dimethylpyridinium; NMP, *N*-methylpyridinium ion; Nrf2, NF-E2 p45 subunit-related factor 2; RI, Robusta India; T/C, test over control.

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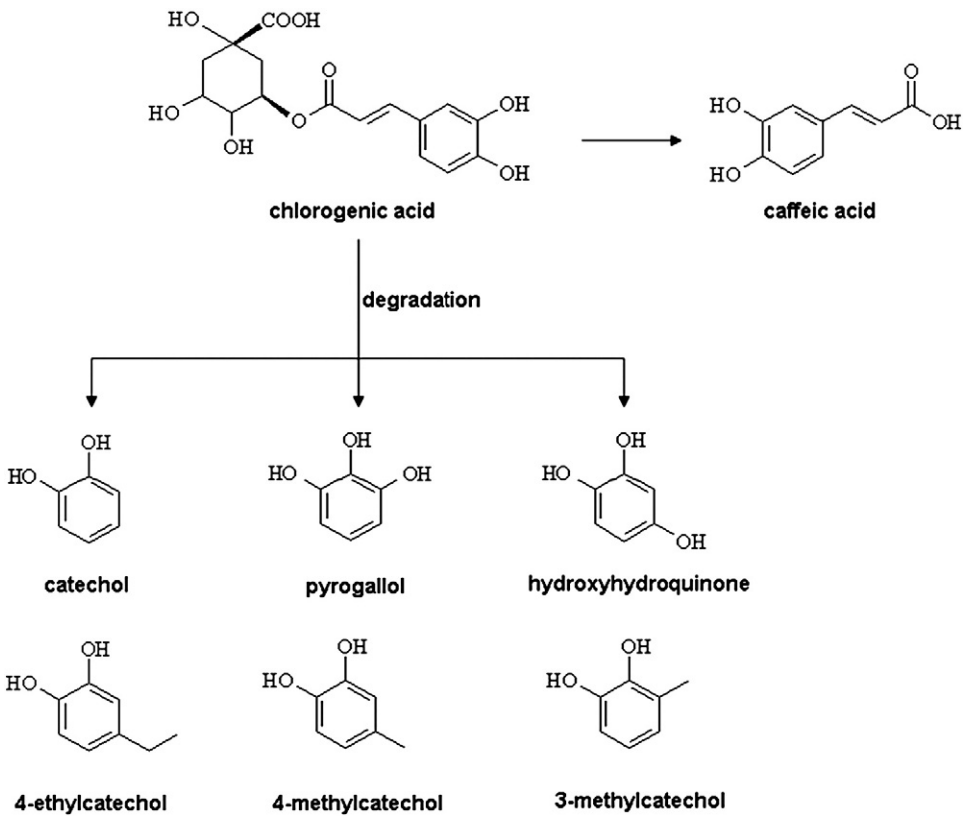

Fig. 1. Chlorogenic acid degradation during coffee roasting.

transcription of Phase II detoxifying enzymes [16]. In quiescent cells, Nrf2 is sequestered in the cytoplasm associated with Keap1, an E3 ubiquitin ligase substrate adaptor [17]. Activation by ROS or upstream protein kinases induces the translocation of Nrf2 into the nucleus, its binding to ARE and the onset of the transcription of Phase II enzymes such as glutathione *S*-transferases (GST), UDP-glucuronyltransferases (UGT), γ-glutamate cysteine ligases (γGCL), NAD(P)H-quinone oxidoreductase 1 (NQO1) or heme oxygenase 1 (HO1) [16–21].

Curcumin, sulforaphane, caffeic acid phenethyl ester, sequiterpene lactones, as well as the diterpenes kahweol and cafestol have been reported to activate the Nrf2/ARE pathway [5,19,22]. Furthermore, the activation of Phase I and Phase II enzyme activity in mice liver

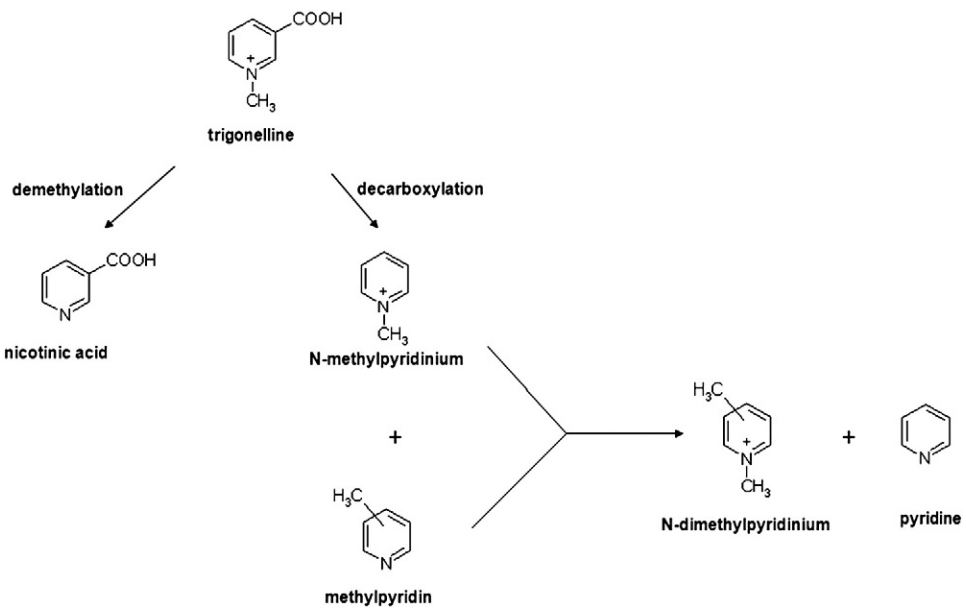

Fig. 2. Trigonelline degradation during coffee roasting.

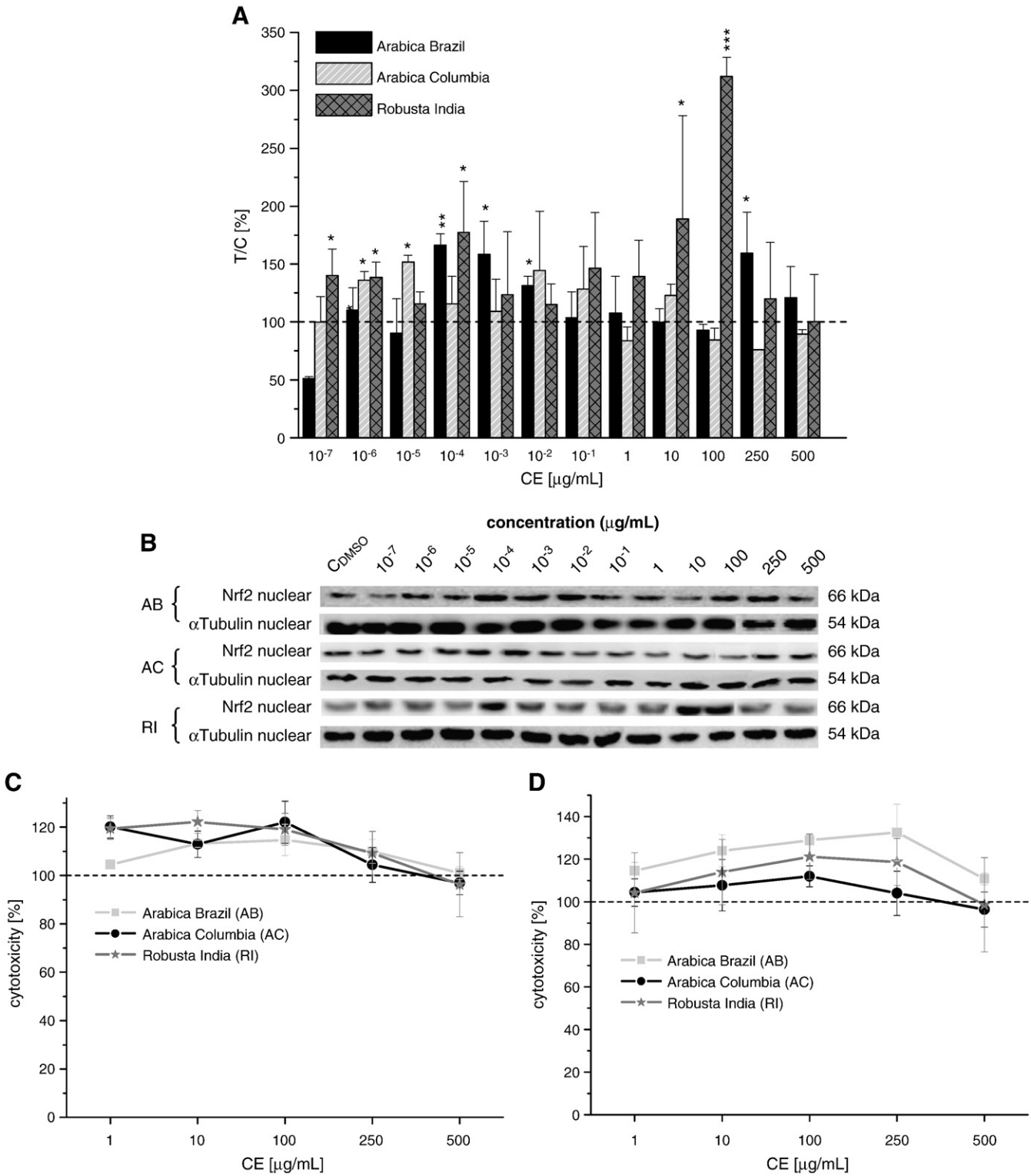

Fig. 3. Impact of AB, AC and RI on nuclear Nrf2 protein in HT29 cells after 3 h of treatment. α-Tubulin was included in the test as a loading control. (A) The data are presented as test over control [T/C (%)] with the control being cells treated with 1% DMSO. The data are the mean±S.D. of at least three independent experiments with similar outcome. The significances indicated refer to the comparison of the respective concentration with the solvent control and are calculated using Student's *t* test (*P<.05; **P<.01; ***P<.001). (B) Figure of a representative Western blot experiment. Inhibition of cell growth in HT29 cells by the CEs after 24 h (C) and 48 h (D) determined in the sulforhodamine B assay. Growth inhibition was calculated as percent survival of treated cells over control cells (treated with the solvent 1% DMSO)×100 (T/C %). The values given are the mean±S.D. of at least three independent experiments, each performed in quadruplicate. C_{DMSO}: Solvent control; AB: Arabica Brazil CE; AC: Arabica Columbia CE; RI: Robusta India CE.

after feeding diets containing 3% or 6% coffee for 5 days has been described, proposing the Nrf2/ARE pathway as a potential mechanism [5,23]. Therefore, in the present study, we investigated the induction of Nrf2 translocation into the nucleus by different coffee extracts (CE) in human colon carcinoma cells (HT29). We addressed the question whether known Nrf2 activators such as chlorogenic acid [24,25] or yet

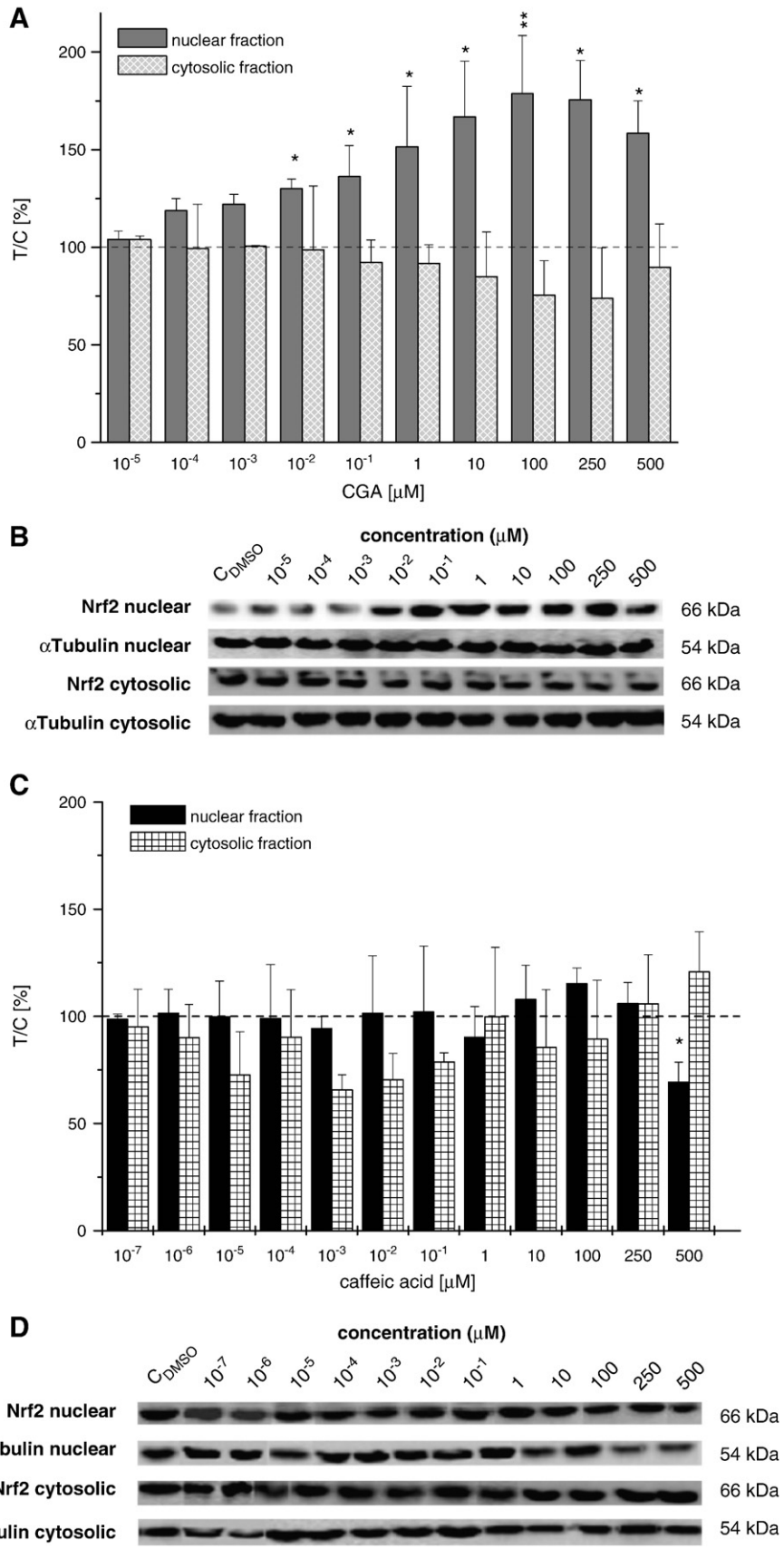

Fig. 4. Western blot analysis of the nuclear and cytosolic Nrf2 protein in HT29 cells after 3 h of treatment with 5-*O*-CGA (A+B), caffeic acid (C+D) or its degradation products catechol (E+F) and hydroxyhydroquinone (G+H). The data are presented as test over control [T/C (%)] with the control being cells treated with 1% DMSO. The data are the mean±S.D. of at least three independent experiments with similar outcome, with Panels B, D, F and H showing the respective Western blots. The significances indicated refer to the comparison of the respective concentration with the solvent control and are calculated using Student's *t* test (*$P<.05$; **$P<.01$). C_{DMSO}: Solvent control.

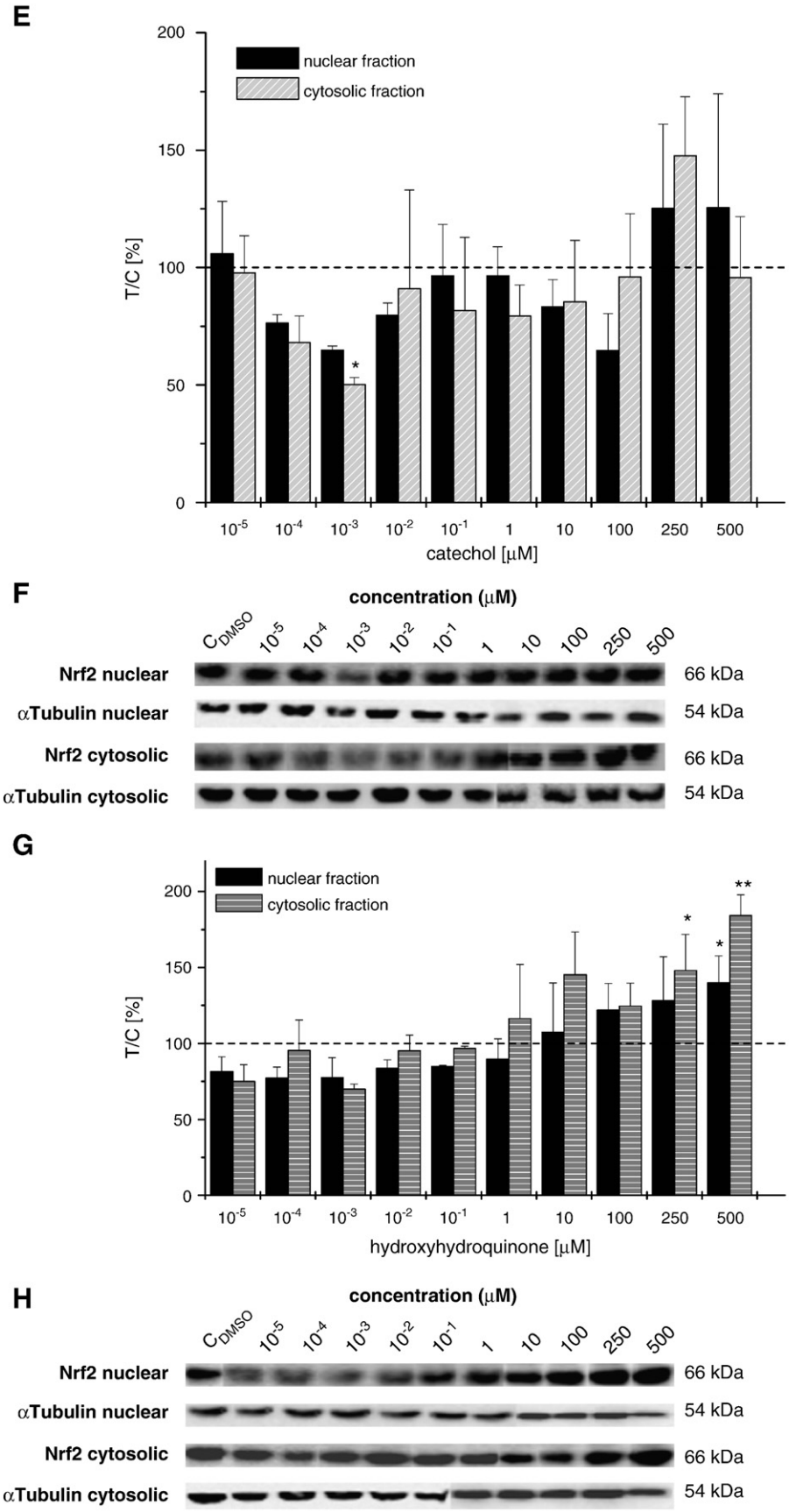

Fig. 4 (*continued*).

unknown Nrf2 activators in the CEs are crucial for the activation of the Nrf2/ARE pathway. The impact of various constituents of both the phenolic fraction of coffee with CGA as a parent substance (Fig. 1) [26–29] and a cationic fraction of coffee including trigonelline (*N*-methylnicotinic acid) on the nuclear translocation of Nrf2 was investigated. Besides caffeine, trigonelline represents one of the major alkaloids in raw coffee beans, leading to the formation of *N*-methylpyridinium iodide (NMP) and related picolinium analogues (Fig. 2) upon roasting [30,31]. To underline the potential health benefit of coffee consumption in terms of chemoprevention, we investigated whether the modulation of Nrf2 nuclear translocation by coffee constituents is reflected subsequently by enhanced transcription of ARE-dependent Phase II genes. As a suitable cell culture model, the colon carcinoma cell line HT29 was selected, originating from the gastrointestinal tract, well characterized with respect to cell signalling and known to be sensitive to the activation of Nrf2/ARE-dependent gene expression.

2. Materials and methods

2.1. Chemicals

The chemicals 5-*O*-caffeoylquinic acid (CGA), caffeic acid, trigonelline hydrochloride, hydroxyhydroquinone (1,2,4-trihydroxybenzene), catechol, pyridine, 2-methylpyridine, 3-methylpyridine, 4-methylpyridine, methyl iodide, [$^2H_3$]-methyl iodide, ammonium formate, methanol and acetonitrile were purchased from Sigma Aldrich (Steinheim, Germany). Solvents were HPLC grade; water was Millipore quality.

Coffee beverages were prepared from samples of Arabica Brazil (AB), Arabica Columbia (AC) and Robusta India (RI) using a common coffee drip filter machine (TCM, Germany) with 48 g of roast coffee powder and 900 ml of bidistilled water. An aliquot (500 ml) of the freshly prepared coffee beverage was frozen with liquid nitrogen and lyophilized (0.77 mbar, 48 h) to yield the CEs of AB, AC and RI.

For all assays, freshly prepared solutions of the compounds were used. All constituents and extracts were dissolved in DMSO and adopted in serum-free media to a final DMSO concentration of 1%.

2.2. Synthesis of N-methylated pyridininium derivatives

N-Methylpyridinium iodide and 1,2-, 1,3- and 1,4-dimethylpyridinium iodides (DMPs) were synthesized and purified following the protocol reported earlier [30,31]. Briefly, a solution of the individual pyridine derivative (5 mmol) and methyl iodide (8 mmol) in dry acetonitrile (3 ml) was refluxed for 3 h, then cooled in an ice bath to crystallize the iodide salt of the target compound. After filtration, the title compound was recrystallized twice from acetonitrile and then kept under vacuum. [$^2H_3$]-*N*-Methyl-4-methylpyridinium iodide was prepared using the same protocol with 4-methylpyridine as the starting material.

The spectroscopic data of NMP (81% yield) were consistent with those described in the literature [30].

1,2-Dimethylpyridiniumiodid (1,2-DMP, 86.3% yield): MS(ESI$^+$): *m/z* (%) 108 (100, [M$^+$]); MS/MS(ESI$^+$): *m/z* (%) 93 (100, [M-CH_3]$^+$); ^{1}H NMR (d_6-DMSO, 400 MHz, DQF-COSY): δ/ppm 2.80 (s, 3H, H-C(8)), 4.26 (s, 3H, H-C(7)), 7.94 (t, 1H, *J*=6.69 Hz, H-C(5)), 8.06 (d, 1H, *J*=7.83 Hz, H-C(3)), 8.47 (t, 1H, *J*=7.83 Hz, H-C(4)), 9.01 (d, 1H, *J*=6.19 Hz, H-C(6)); ^{13}C NMR (d_6-DMSO, HMQC, HMBC): δ/ppm 20.83 (CH_3, C8), 46.38 (CH_3, C7), 125.62 (CH, C5), 129.95 (CH, C3), 145.55 (CH, C4), 146.62 (CH, C6), 156.36 (C, C2).

1,3-Dimethylpyridiniumiodid (1,3-DMP, 93.1% yield): MS(ESI$^+$): *m/z* (%) 108 (100, [M$^+$]); MS/MS(ESI$^+$): *m/z* (%) 93 (100, [M-CH_3]$^+$). ^{1}H NMR (d_6-DMSO, 400 MHz, DQF-COSY): δ/ppm 2.47 (s, 3H, H-C(8)), 4.30 (s, 3H, H-C(7)), 8.03 (t, 1H, *J*=6.98 Hz, H-C (5)), 8.41 (d, 1H, *J*=7.94 Hz, H-C(4)), 8.81 (d, 1H, *J*=5.88 Hz, H-C(6)), 8.92 (s, 1H, H-C (2)); ^{13}C NMR (d_6-DMSO, HMQC, HMBC): δ/ppm 17.95 (CH_3, C8), 4.32 (CH3, C7), 127.27 (CH, C5), 138.59 (C, C3), 143.26 (CH, C6), 145.37 (CH, C2), 145.89 (CH, C4).

1,4-Dimethylpyridiniumiodid (1,4-DMP, 92.6% yield): MS(ESI$^+$): *m/z* (%) 108 (100, [M$^+$]); MS/MS(ESI$^+$): *m/z* (%) 93 (100, [M-CH_3]$^+$); ^{1}H NMR (d_6-DMSO, 400 MHz, DQF-COSY): δ/ppm 2.58 (s, 3H, H-C(8)), 4.28 (s, 3H, H-C(7)), 7.96 (d, 2H, *J*=6.62 Hz, H-C (3,5)), 8.84 (d, 2H, *J*=6.62 Hz, H-C(2,6)); ^{13}C NMR (d_6-DMSO, HMQC, HMBC): 21.92 (CH_3, C8), 47.48 (CH_3, C7), 128.32 (CH, C3,5), 144.66 (CH, C2,6).

[$^2H_3$]-N-Methyl-4-methylpyridiniumiodid ([$^2H_3$]-1,4-DMP, 72% yield): MS(ESI$^+$) : *m/z* (%) 111 (100, [M]$^+$). MS/MS(ESI$^+$): *m/z* (%) 93 (100, [M-C[$^2H_3$]]]$^+$. ^{1}H NMR (d_6-DMSO, 400 MHz, DQF-COSY): δ/ppm 2.58 (s, 3H, H-C(8)), 7.96 (d, 2H, *J*=6.62 Hz, H-C(3,5)), 8.84 (d, 2H, *J*=6.62 Hz, H-C(2,6)). ^{13}C NMR (d_6-DMSO, HMQC, HMBC): 21.92 (CH3, C8), 47.48 (CD3, C7), 128.32 (CH, C3,5), 144.66 (CH, C2,6).

2.3. Quantitative analysis of N-methylpyridinium derivatives

An aliquot (1 ml) of a freshly prepared coffee beverage was spiked with 0.1 ml of an aqueous solution of [$^2H_3$]-*N*-methylpyridinium iodide (1 μmol/ml) and [$^2H_3$]-*N*-methyl-4-methylpyridinium iodide (10 nmol/ml) as the internal standards. After equilibration for 15 min and solid-phase cartridge clean-up, the sample was analysed by means of HPLC-MS/MS as described recently [26,30,31]. For HPLC-MS/MS analysis, an Agilent 1200 Series HPLC-system, consisting of a pump, a degasser and an autosampler (Agilent, Waldbronn, Germany), was connected to a 3200 API triple quadrupole system (Applied Biosystems/MDS Sciex, Darmstadt, Germany) with an electrospray ionization (ESI) device running in positive ionization mode. Chromatography was performed on a GROM-Sil80 SCX column (50 mm×2 mm, 5 μm; Grom, Herrenberg, Germany) at a flow rate of 350 μl/min with isocratic elution (10 min) using aqueous methanol (50%) which contained ammonium acetate as modifier (50 mmol/L). For mass spectrometry, nitrogen was used as the nebulizer gas and curtain gas and set at 65 and 25 psi, respectively. Heater gas was set at 60 psi; the temperature was 350°C. Detection was performed in multiple reaction monitoring mode, recording the transitions from the parent ion [M]$^+$ to the fragments after collision-induced dissociation. For tuning, aqueous solutions of the compounds were individually infused using a syringe pump at a flow rate of 10 μl/min and the parameters for the mass transitions were optimized using the "quantitative optimization" tool provided by the software (Analyst 1.4.1, Sciex, Toronto, Canada). *N*-Methylpyridinium (NMP): *m/z* 94→*m/z* 79, *m/z* 78, *m/z* 52; [$^2H_3$]-*N*-methylpyridinium ([$^2H_3$]-NMP): *m/z* 97→*m/z* 79, *m/z* 78, *m/z* 52; *N*-methyl-2-methylpyridinium, *N*-methyl-3-methylpyridinium, and *N*-methyl-4-methylpyridinium: *m/z* 108→*m/z* 93, *m/z* 92; [$^2H_3$]-*N*-Methyl-4-methylpyridinium: *m/z* 111→*m/z* 93, *m/z* 92. The declustering activity was 50, the cell exit activity was 15 and the collision energy was 33. The dwell time for each mass transition was 75 ms. The quadrupoles operated at unit mass resolution; ion spray voltage was 5500 V. Calibration of the internal standard was performed by analysing mixtures of analyte and internal standard in molar ratios from 1:10 to 10:1. A regression line was calculated from a graph prepared from area ratios (analyte/internal standard) vs. concentration ratios (analyte/internal standard). The equation for NMP/[$^2H_3$]-NMP was $y=1.1108x+0.002$ ($R^2=0.9992$); the equation for 1,4-dimethylpyridinium/[$^2H_3$]-*N*-methyl-4-methylpyridinium was $0.8306x+0.025$ ($R^2=0.9999$).

2.4. Quantitation of chlorogenic acids

A sample of the freshly prepared coffee beverage was diluted with water (1+9 or 1+1, v+v), and an aliquot (10 μl) was injected into the HPLC-DAD system monitoring the effluent at 324 nm. The HPLC consisted of a Merck/Hitachi pump (L-7100), a degasser (Merck L-7612) and a detector (Merck/Hitachi L-7450). Samples were injected using an autosampler (Merck/Hitachi L-7200). Chromatography was performed on a Luna phenyl-hexyl column (4.6×250 mm, 5 μm, Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.8 ml/min using a solution of aqueous 0.25 mM ammonium formate (pH 3.5) as solvent A and methanol as solvent B. For chromatography, solvent B was increased from 22% to 25% within 25 min, then to 100% within 5 min, followed by isocratic elution for 5 min. Quantitation of the sum of 3-, 4- and 5-*O*-caffeoyl quinic acids was performed using a five-point external calibration (range 1–200 mg/L) with the commercially available 5-*O*-caffeoyl quinic acid.

2.5. 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 Invitrogen Life Technologies (Karlsruhe, Germany). The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg/L glucose, 4.0 mM L-glutamine, without sodium pyruvate, supplemented with 10% FCS and 1% penicillin/streptomycin in a humidified atmosphere at 37°C and 5% CO_2. Cells were tested routinely and found to be negative for mycoplasm contamination.

2.6. Western blot analysis

4.5×10^6 HT29 cells were seeded per Petri dish and allowed to grow for 48 h. Cells were incubated for 3 h with the different compounds at their 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 PBS and centrifuged for 3 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]. Subsequently 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 3 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 every 2 min for 10–15 s. After a final centrifugation step for 5 min (15,000×*g*, 4°C), the supernatant (nuclear extract) as well as the cytosolic extract was 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 (Santa Cruz, Heidelberg, Germany) and an antirabbit

IgG peroxidase conjugate (Santa Cruz) as secondary antibody. Alpha-tubulin was used as a loading control. The respective chemoluminescent signals (Lumi-GLO, Cell Signaling Technology) were analysed for quantification using the LAS 3000 system with the AIDA Image Analyzer 3.52 software (Raytest, Straubenhardt, Germany). Arbitrary light units were plotted as test over control (%).

2.7. Sulforhodamine B assay

Cytotoxic effects on HT29 cells were determined by the sulforhodamine B assay (SRB assay) according to a modified method of Skehan [32]. Briefly, cells were incubated with the respective compounds for 24 h in serum-containing medium (10%

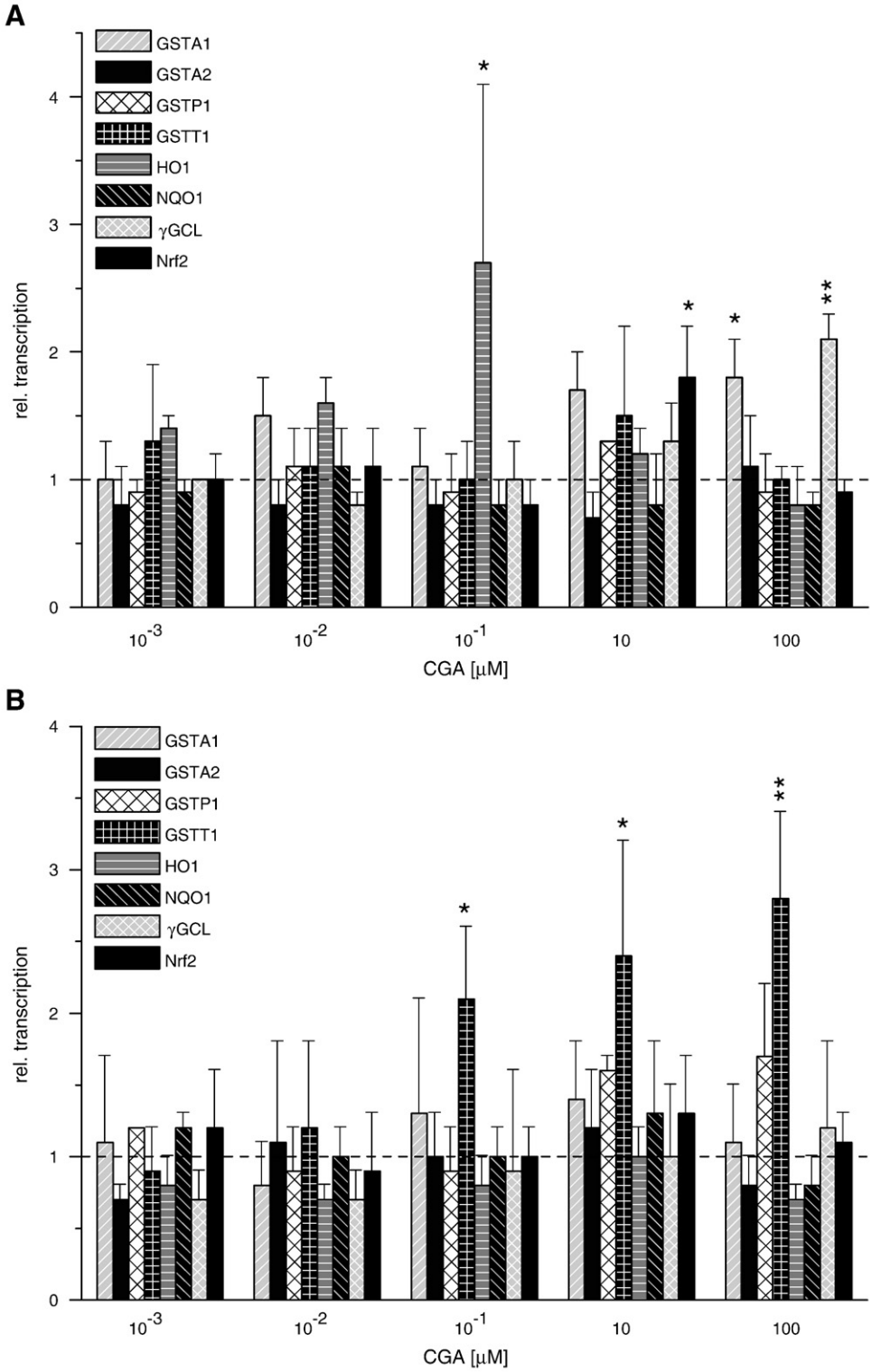

Fig. 5. Gene transcription analysis of ARE-dependent genes GSTA1, GSTA2, GSTP1 and GSTT1 as well as γGCL, HO1, NQO1 and Nrf2 in HT29 cells after incubation with CGA for (A) 3 h and (B) 24 h. The data are the mean±S.D. of four independent experiments performed in duplicates. Data are normalized by β-actin 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 (*$P<.05$; **$P<.01$). GST: Glutathione *S*-transferase; γGCL: γ-glutamate cysteine ligase; HO1: heme oxygenase 1; NQO1: NAD(P)H-quinone oxidoreductase 1; Nrf2: NF-E2 p45 subunit-related factor 2.

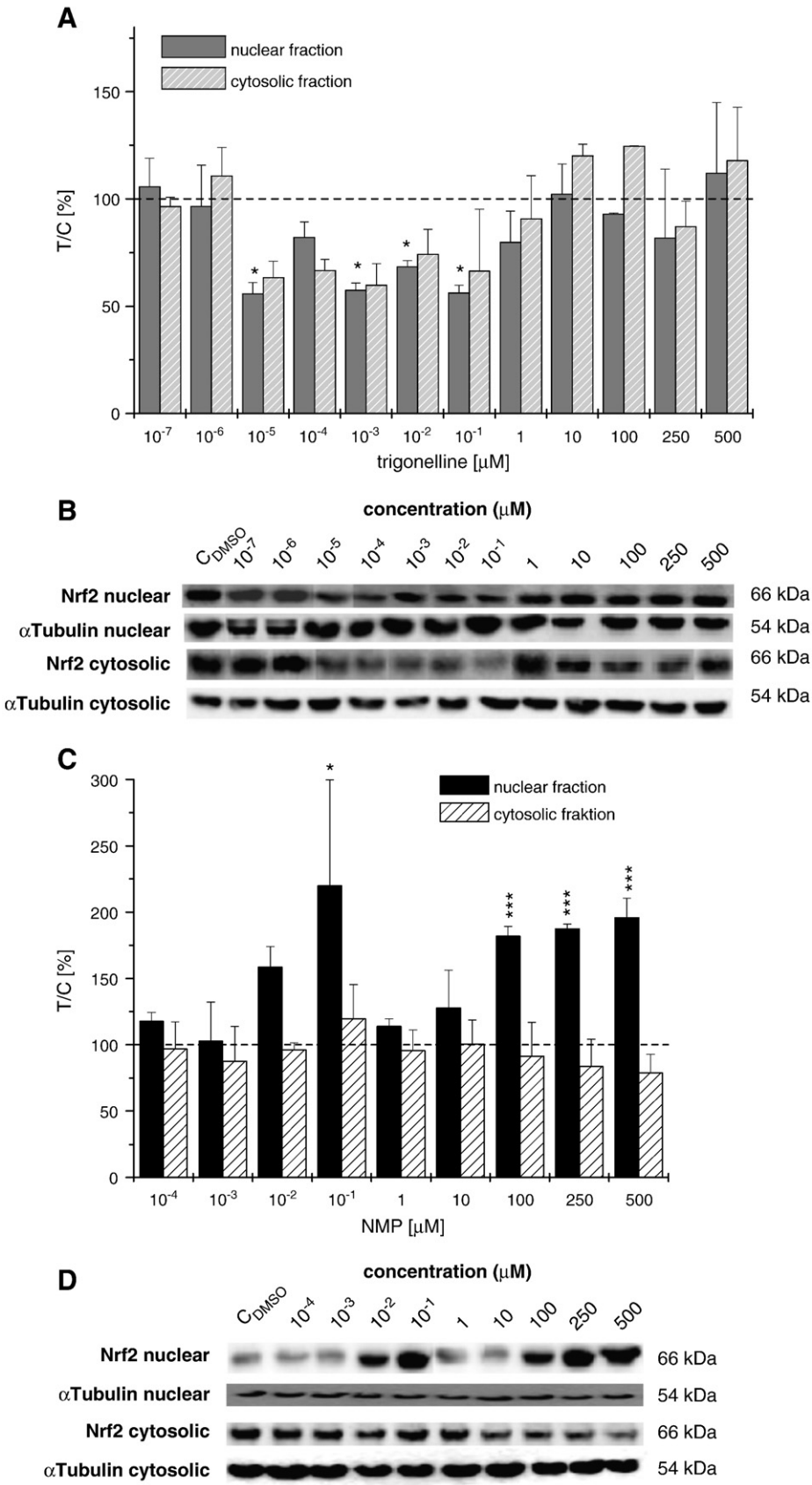

Fig. 6. Western blot analysis of the nuclear and cytosolic Nrf2 protein level in HT29 cells after 3 h of treatment with trigonelline (A and B) and its degradation products NMP (C and D), 1,3 DMP (E and F), 1,2 DMP (G and H) and 1,4 DMP (I and J). The data are presented as test over control [T/C (%)] with the control being cells treated with 1% DMSO. The data are the mean±S.D. of at least three independent experiments with similar outcome, with Panels B, D, F and G showing the respective Western blots. The significances indicated refer to the comparison of the respective concentration with the solvent control and are calculated using Student's *t* test (*P<.05; **P<.01; ***P<.001). C_{DMSO}: Solvent control.

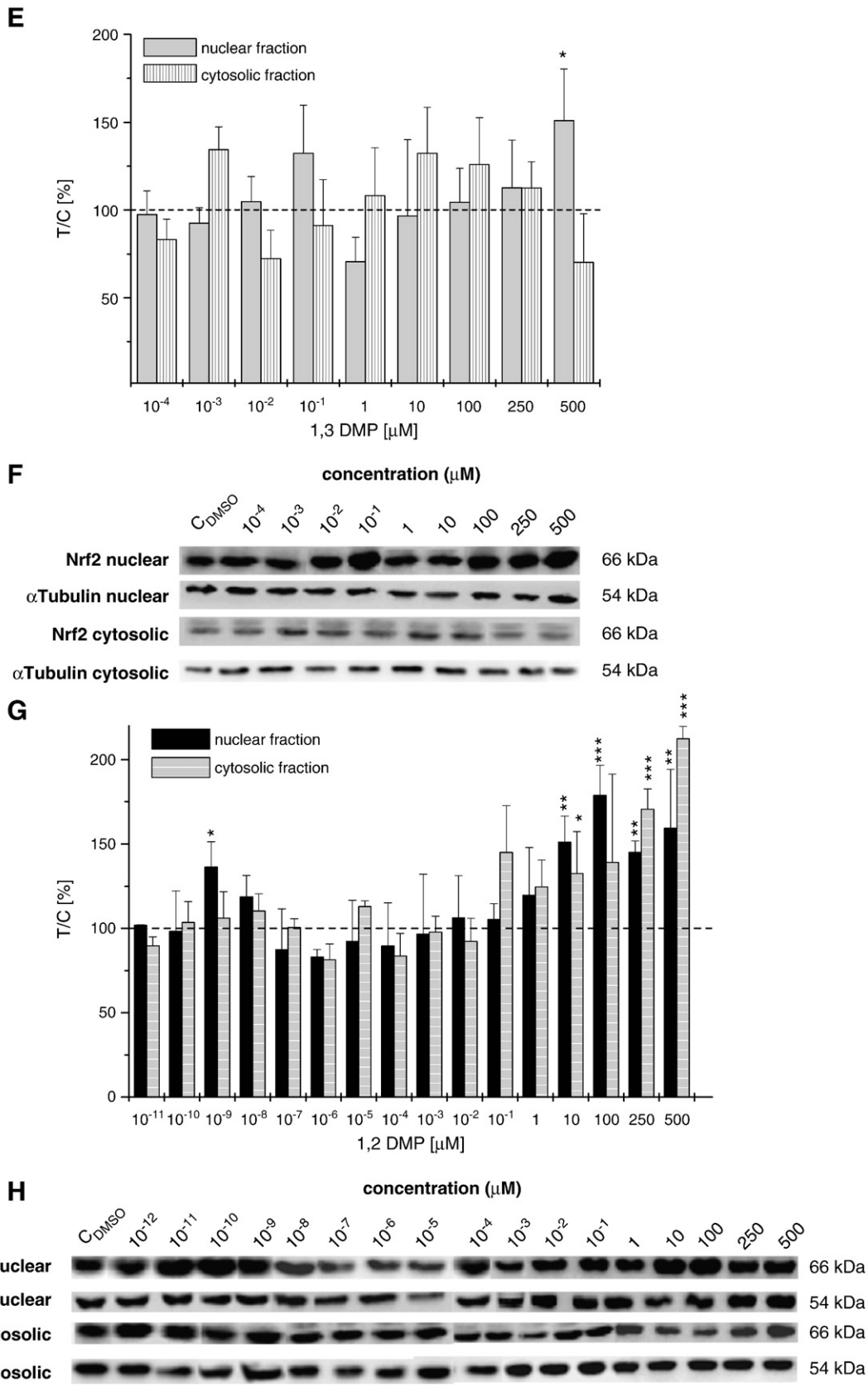

Fig. 6 (*continued*).

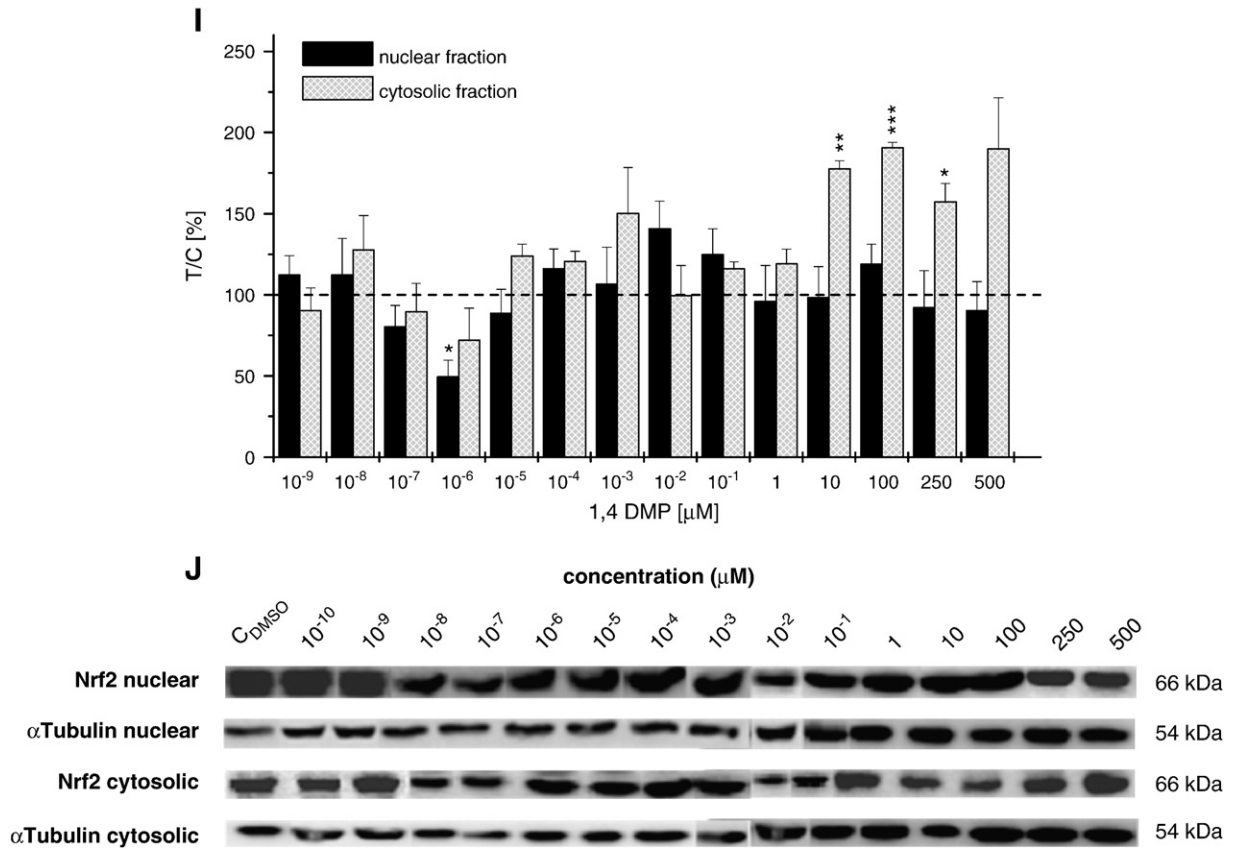

Fig. 6 (*continued*).

FCS) in the presence of 100 U/ml catalase. Cytotoxicity was determined as percent survival, determined by the number of treated over control cells (% T/C).

2.8. RNA Extraction and real-time PCR

1.6×10^6 HT29 cells were seeded per Petri dish in DMEM supplemented with FCS (10%) and 1% penicillin/streptomycin to examine the influence of the test compounds on the transcription of different GST isoforms, HO1, NQO1, γGCL and Nrf2. All compounds were dissolved in DMSO and adopted in serum-free medium (3 h of incubation) or 10% FCS medium (24 h of incubation) to a final DMSO concentration of 1%. The incubation was performed in the presence of catalase (100 U/ml) to prevent hydrogen peroxide formation in the cell culture medium. After incubation, the culture medium was removed and the cell monolayer was rapidly rinsed with ice-cold PBS. Total RNA was extracted according to the manufacturer's handbook with the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Two micrograms of RNA was reverse transcribed at 37°C for 60 min using Oligo-dT primers and the Omniscript Reverse Transcription Kit (QIAGEN). cDNA obtained from the reverse transcriptase (RT) reaction (amount corresponding to 2 μg of total RNA) was subjected to PCR using QuantiTect SYBR Green PCR (QIAGEN). A control without RT (−RT) was included for each dilution series. The primer assays used were as follows: for γGCL: Hs_GCLC_1_SG QuantiTect Primer Assay (200), QT00037310; for NQO1: Hs_NQO1_1_SG QuantiTect Primer Assays (200), QT00050281; for HO1: Hs_HMOX1_1_SG QuantiTect Primer Assay (200), QT00092645; for Nrf2: Hs_NFE2L2_1_SG QuantiTect Primer Assay (200), QT00027384; and for β-actin: Hs_ACTB_1_SG QuantiTect Primer Assays (200), QT00095431 (QIAGEN). Primers were used in concentrations according to the manufacturer's guidelines in the *QuantiTect SYBR Green PCR Handbook* 11/2005 (QIAGEN). PCR reaction parameters were as follows: incubation at 95°C for 15 min and thereafter 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Each sample was determined in duplicate. The −RT control was included for all assays. The fold changes in expression of the target gene relative to the internal control gene (β-actin) were analysed using Opticon 2 software (Bio-Rad) and the CT data were imported into Microsoft Excel 03. Data of all assays were analysed by the $2^{-\Delta\Delta CT}$ method [14]. This relative quantification compares the PCR signal of the target transcript to the endogenous control gene β-actin. The $2^{-\Delta\Delta CT}$ method is a convenient way to analyse the 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 genes must be approximately equal.

3. Results

3.1. Impact of different CEs on Nrf2 nuclear translocation in HT29 cells

Exemplarily, three commercially available coffees of different representative proveniences (Arabica Brazil, Arabica Columbia and Robusta India) were used to prepare the respective CEs. The influence of the different CEs on the nuclear translocation of Nrf2 in human colon carcinoma cells was investigated by Western blot analysis. Recently, several reports indicated the formation of hydrogen peroxide in cell culture media, due to a reaction of polyphenols with yet unknown components of the cell culture media [33–35]. To avoid hydrogen peroxide accumulation and respective experimental artefacts, cell incubation experiments were performed in the presence of catalase (100 U/ml). HT29 cells were incubated for 3 h with extracts of AB, AC and RI. AC induced Nrf2 nuclear translocation at concentrations of 1 and 10 pg/ml by 136±4.4% and 152±6.0%, respectively (Fig. 3A and B). The intracellular localisation of Nrf2 was not significantly effected by higher concentrations of AC. In contrast, the extract of AB increased the nuclear Nrf2 level in HT29 cells significantly and most effectively at 100 pg/ml and 250 μg/ml (166±9.8% and 150±35.5%, respectively), displaying a U-shaped curve profile (Fig. 3A and B). The extract of RI presented the most effective CE with respect to Nrf2 nuclear translocation. Already at the lowest concentration of 100 fg/ml, RI increased nuclear Nrf2 levels up to 140±22.8%. The U-shaped curve was marked by two peak maxima at 100 pg/ml and 100 μg/ml with an increase in nuclear Nrf2 of

177±43.9% and 312±16.6%, respectively (Fig. 3A and B). Cytosolic Nrf2 protein level was not significantly modulated by any of the three extracts (data not shown). The potent induction of Nrf2 nuclear translocation at 250 μg/ml AB and 100 μg/ml RI was not accompanied by any cytotoxic effects of these CEs in HT29 cells as shown by the SRB assay (Fig. 3C and D). None of the tested CEs displayed cytotoxic properties up to 500 μg/ml after 24 or 48 h of incubation. In contrast, all three CEs showed weak growth stimulatory properties.

3.2. Modulation of Nrf2 nuclear translocation by CGA and its thermal degradation products

Roasting of coffee is associated with the degradation of CGA into the substructures caffeic acid and quinic acid and, among others, the subsequent formation of catechol and hydroxyhydroquinone (Fig. 1). The impact of CGA and its potential degradation products on Nrf2 nuclear translocation in HT29 was determined by Western blot. CGA was found to enhance Nrf2 nuclear levels at concentrations ≥10 nM up to a maximum of 178±29.9% at 100 μM (Fig. 4A and B), whereas the cytosolic Nrf2 levels were not significantly modulated by CGA. In contrast, caffeic acid had no impact on Nrf2 nuclear translocation up to 250 μM. Of note, 500 μM caffeic acid significantly diminished the nuclear Nrf2 level (Fig. 4C and D). The CGA degradation product catechol significantly reduced both the cytosolic and the nuclear proportion of the Nrf2 protein at a concentration of 1 nM (Fig. 4E and F). In higher concentrations, catechol did not show any effects on Nrf2 protein expression. Hydroxyhydroquinone elevated both the nuclear and the cytosolic Nrf2 protein levels significantly at 250 and 500 μM (Fig. 4G and H).

3.3. Induction of ARE-dependent gene expression by CGA

From the phenolic coffee constituents tested so far, CGA showed the highest Nrf2-translocating properties in HT29 cells. Therefore, we investigated by real-time PCR whether downstream in the signalling cascade the transcription of ARE-dependent genes was modulated. The level of transcripts of GSTA1 was significantly increased after 3 h of incubation with CGA ≥10 μM (1.7±0.3). Nrf2 transcripts were also elevated by 10 μM CGA (1.8±0.3) indicating an increased *de novo* synthesis of Nrf2. γGCL transcription was only affected at a concentration of 100 μM (2.1±0.2) (Fig. 5A). HO1 seemed to be the most sensitive gene being modulated after incubation with CGA. The HO1 transcript level was significantly altered at 0.1 μM CGA (2.7±1.4) (Fig. 5A). In contrast, GSTA2, GSTP1, GSTT1 and NQO1 as further ARE-dependent genes were not significantly affected in their transcription levels after 3 h of incubation with CGA. Prolonged incubation (24 h) resulted in a different transcription pattern. GSTT1 transcripts were significantly increased at CGA concentrations ≥0.1 μM (2.1±0.5), whereas none of the eight investigated ARE-dependent genes was significantly modulated after 24 h of incubation with CGA (Fig. 5B).

3.4. Modulation of Nrf2 nuclear translocation by trigonelline and its thermal degradation products

Upon coffee roasting, the alkaloid trigonelline is known to degrade to NMP and to various picolinium analogues such as 1,2- dimethylpyridinium (1,2-DMP), 1,3-dimethylpyridinium (1,3-DMP) and 1,4-dimethylpyridinium (1,4-DMP) (Fig. 2). Trigonelline did not provoke an increased Nrf2 nuclear translocation in HT29 cells up to 500 μM as shown by Western blot analysis. Of note, a significant drop in Nrf2 total protein was observed at concentrations ranging from 10 pM up to 100 nM trigonelline (Fig. 6A and B). In contrast, the degradation product NMP significantly increased Nrf2 nuclear translocation in a U-shaped curve which was marked by a potent induction at 100 nM NMP (219.9±79.9%) and in the concentration range of 100–500 μM NMP (182.0±7.4% to 195.9±14.9%, respectively). The cytosolic fraction of Nrf2 was not significantly modulated by NMP (Fig. 6C and D). The potent induction of Nrf2 translocation by NMP appeared not to be due to the onset of oxidative stress as detected in the DCF assay (data not shown).

1,3-DMP mediated a significant increase of nuclear Nrf2 protein only at the highest concentration of 500 μM (150.9±29.5%) (Fig. 6E and F). In contrast, 1,2-DMP already initiated a significant induction of Nrf2 nuclear translocation at a concentration of 1 fM up to 136.3±15.0%, following a U-shaped curve progression reaching a maximum of 178.9±17.8% at 100 μM. Of note, the cytosolic Nrf2 protein increased at concentrations ≥100 μM 1,2-DMP in parallel, indicating additional mechanisms affecting total cellular Nrf2 levels (Fig. 6G and H). In contrast to the other analogues, the 1,4-substituted dimethylpyridinium significantly decreased the nuclear Nrf2 protein content at a concentration of 1 pM by 49.4±10.3% but did not affect the Nrf2 nuclear protein level significantly at higher concentrations. At concentrations ≥10 μM, the cytosolic Nrf2 proportion was significantly increased to a maximum of 190.6±3.3% at 100 μM 1,4-DMP (Fig. 6I and J). Thus, from the *N*-methylpyridinium analogues, NMP displayed the most potent Nrf2 translocating properties, whereas trigonelline effectively suppressed Nrf2 translocation in submicromolar concentrations. These two extremes were selected for further studies addressing the question of whether the modulation of Nrf2 translocation is of impact for ARE-dependent gene transcription.

3.5. Effects of trigonelline and NMP on ARE-dependent gene transcription

NMP significantly increased the transcripts of various GST isoforms already after 3 h of incubation (Fig. 7A). GSTA1 transcripts were significantly increased in a concentration-dependent manner from 10 to 100 μM NMP (2.3±1.3 to 4.3±1.5). The transcription of GSTT1 was increased in the range from 0.1 to 10 μM (3.2±0.6). In contrast, GSTA2 and GSTP1, as further genes of the GST isoenzyme family, were not modulated after 3 h of incubation with NMP (Fig. 7A). γGCL transcripts were already increased after 3 h of incubation with 0.1 μM NMP up to 3.7±1.2, whereas HO1 and NQO1 transcription was not modulated at this time point (Fig. 7A). To address the question of whether nuclear translocation of Nrf2 by NMP is fortified by an increased *de novo* synthesis of Nrf2, the level of Nrf2 transcripts was also determined. The incubation of HT29 cells with NMP for 3 h caused an increase in Nrf2 transcripts at 10–100 μM NMP (1.4±0.1 and 2.2±0.5, respectively) in comparison to the solvent control (Fig. 7A), indicative for the onset of Nrf2 *de novo* synthesis. To investigate whether the here shown effects could also be seen after prolonged incubation, HT29 cells were treated for 24 h with NMP. As a result, GSTA1 transcripts were also significantly increased from 10 to 100 μM NMP (1.9±0.3 to 2.3±0.8), yet not as potent as after 3 h of incubation. Furthermore, GSTP1 transcription level was increased from 10 (1.7±0.2) to 100 μM (2.0±0.6) NMP and GSTT1 was significantly increased at 10 μM NMP (4.2±0.4). At an NMP concentration of 100 μM, the GSTT1 transcript level was enhanced with 2.0±0.5. In contrast, the GSTA2 transcription level was not modulated after 24 h of incubation with NMP (Fig. 7B). Furthermore, 10 μM NMP significantly increased γGCL (2.1±0.2), HO1 (5.1±1.9) and NQO1 (3.2±1.0) transcription. To investigate whether a long-term modulation of Nrf2 gene transcription by NMP occurs, transcript levels after 24 h of incubation were determined. A strong increase in Nrf2 transcription was detected in HT29 cells at NMP concentrations from 0.1 μM to 10 μM (2.1±1.3 and 4.4±1.8, respectively), indicating an even more potent long-term induction of Nrf2 gene transcripts (Fig. 7B).

Furthermore, we addressed the question of whether the decrease of Nrf2 protein in the nucleus after incubation with trigonelline was reflected by the suppression of the transcription of Nrf2 or Nrf2-

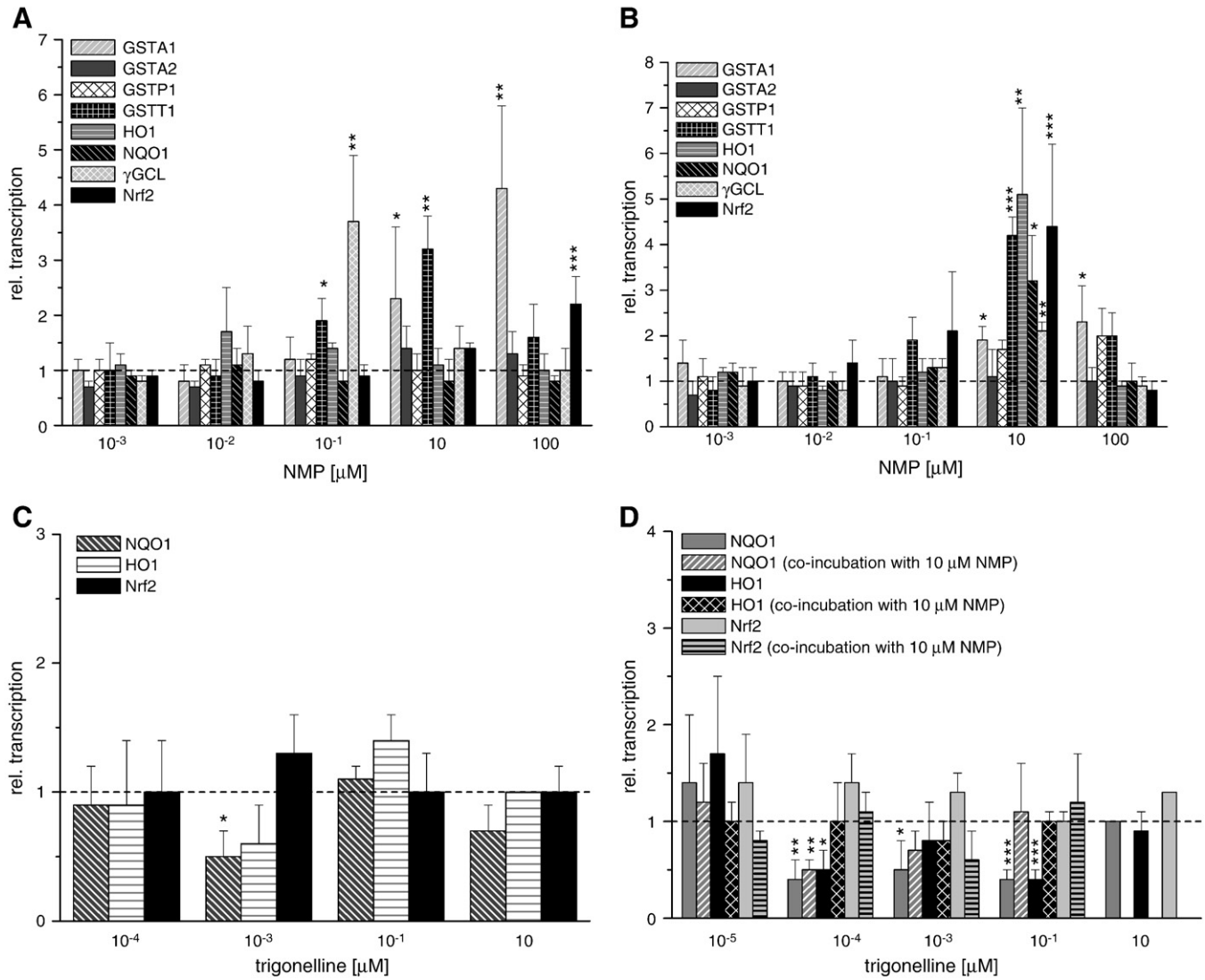

Fig. 7. Gene transcription analysis of ARE-dependent genes GSTA1, GSTA2, GSTP1 and GSTT1, as well as γGCL, HO1, NQO1 and Nrf2 in HT29 cells after incubation with NMP for 3 h (A) and 24 h (B), as well as 3 h of incubation with trigonelline (C) and 24 h of co-incubation with NMP and trigonelline (D). The data are the mean±S.D. of four 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 (*$P<.05$; **$P<.01$; ***$P<.001$). GST: Glutathione *S*-transferase; γGCL: γ-glutamate cysteine ligase; HO1: heme oxygenase 1; NQO1: NAD(P)H-quinone oxidoreductase 1; Nrf2: NF-E2 p45 subunit-related factor 2.

dependent genes like NQO1 or HO1. Incubation with trigonelline for 3 h did not affect Nrf2 gene transcription in comparison to the solvent control (Fig. 7C). In contrast, the transcription of NQO1 (0.5±0.2) and HO1 (0.6±0.3) decreased at a concentration of 0.001 μM trigonelline, reflecting the disadvantageous effects of trigonelline on Nrf2 translocation. Long-time incubation with trigonelline (24 h) did not cause a significant modulation of Nrf2 transcription (Fig. 7D), thus Nrf2 *de novo* synthesis seemed not to be affected.

We furthermore investigated the effect of a combination of the Nrf2 activator NMP and the Nrf2 suppressor trigonelline on the transcription of ARE-dependent genes. Of note, Nrf2 transcription was not significantly modified by co-incubation with trigonelline and NMP. However, over the whole concentration range the presence of trigonelline potently suppressed the stimulatory effects of NMP (10μM) on Nrf2 transcription. These effects were even more pronounced with respect to the transcription of HO1 and NQO1 (Fig. 7D).

By treatment of HT29 cells with trigonelline as a single compound, the level of HO1 transcripts was significantly decreased at trigonelline concentrations of 0.0001 μM (0.5±0.2) and 0.1 μM (0.4±0.1). Concomitantly, a significant suppression of NQO1 gene transcription was observed at ≥0.0001 μM (0.4±0.2) up to 0.1 μM (0.4±0.1). These effects were partially compensated by co-incubation with NMP (10 μM) (Fig. 7D). However, on the other hand, the presence of trigonelline substantially suppressed the effects of NMP on the transcription of the ARE-dependent genes HO1 and NQO1.

4. Discussion

4.1. Impact of CEs and selected constituents on the Nrf2/ARE pathway

The activation of the Nrf2/ARE pathway, an important mechanism to protect cells from ROS-mediated damages, is mediated *via* translocation of the transcription factor Nrf2 into the nucleus. Three different CEs (AB, AC and RI) were investigated with respect to their impact on the Nrf2/ARE pathway in human colon carcinoma cells. *Coffea arabica* and *Coffea canephora* var. *robusta* are the most consumed coffee varieties worldwide. All three selected CEs were

found to affect the translocation of Nrf2 to the nucleus, although differences in the potency and bioactive concentration ranges were observed (Fig. 3A and B). Further studies are needed to clarify the impact factors on Nrf2 translocation, with coffee variety, provenience and roasting conditions as potential contributors.

Cytotoxic properties of the three extracts were not detected in tumour cells after incubation of 24 and 48 h. Thus, a modulation of the Nrf2/ARE pathway due to cytotoxic effects might not be of relevance (Fig. 3C and D). The *Coffea robusta* extract RI was the most effective CE at increasing Nrf2 nuclear protein levels, probably providing a more efficient protection from ROS-mediated damages in HT29 cells. Daglia et al. [36,37] reported that *robusta* samples contained higher amounts of reducing constituents and were more effective regarding *ex vivo* protective activity than *arabica* samples. In contrast to our findings where the two *arabica* extracts differed regarding their potential to increase Nrf2 nuclear translocation, they observed only minor differences between *arabica* samples in protective activity and reducing constituents. In our study, the roasting degree, known as an important factor with respect to antioxidant activity [7], played no crucial role since all provided extracts were from medium roasted coffees. Growing conditions like climate, altitude and soil are also known to have a strong impact on concentrations and constituent pattern in the growing bean, even of the same variety. The different modulation of Nrf2 nuclear translocation by AC and AB might therefore be a result of a differing constituent pattern between the two extracts. The three CEs AB, AC and RI differed, *e.g.*, in the concentration of CGA and NMP (Table 1). AC contained higher amounts of CGA (743 mg/L) and less NMP (22 mg/L=0.23 mmol/L), a marker for trigonelline and its degradation products. RI exhibited an inverse pattern of these constituents (CGA: 640 mg/L and NMP: 30 mg/L=0.32 mmol/L) [38]. Therefore, we investigated the contribution of selected coffee constituents to the effective increase in nuclear Nrf2 protein levels.

CGA was identified as a potent inducer of Nrf2 nuclear translocation (Fig. 4A and B). Since CGA (10–250 μM) significantly reduced the intracellular ROS level after 3 h of incubation in HT29 cells, assessed by DCF assay (data not shown), it can be excluded that the observed activation of the Nrf2/ARE pathway is caused by an increase of ROS in the cells. The effect of CGA on the transcription of ARE-dependent genes in HT29 cells was time dependent. After 3 h of incubation, an increase in the transcription levels was observed for γGCL (100 μM), HO1 (0.1 μM) and GSTA1 (≥10 μM), whereas other GST isoforms such as GSTA2, GSTP1 and GSTT1 remained unaffected. Interestingly, Nrf2 transcription was elevated by 10 μM CGA, indicating an increased *de novo* synthesis (Fig. 5A). Long-time incubation (24 h) with CGA significantly induced GSTT1 transcription (≥0.1 μM), although none of the other selected ARE-dependent genes was affected after 24 h of incubation with CGA (Fig. 5B).

Coffee represents one of the major sources of CGA, based on a daily intake of 0.5–1 g CGA [39,40]. Several studies reported CGA as a phenolic food constituent with promising antioxidative properties [41]. Feng et al. [24] showed a CGA-mediated induction of Nrf2 nuclear translocation in murine epidermal cells in micromolar concentrations, supporting our results on the induction of Nrf2 nuclear translocation in HT29 cells. Together, the data emphasize that the isolated constituent, CGA, represents a potent activator of Nrf2 translocation in different cell lines. The coffee extract AC, though richest in CGA (Table 1), induced Nrf2 nuclear translocation in HT29 cells exclusively in picograms per milliliter concentrations (Fig. 3A and B) corresponding to picomolar concentrations of CGA. Yet, CGA as a single compound did not modulate the Nrf2/ARE pathway in HT29 cells in the respective concentration range. Thus, CGA appeared to be only of minor relevance for Nrf2 nuclear translocation in CEs. These results are supported by the data of Del Castillo et al. [7], indicating that *in vitro* antioxidant activity was lower in green coffee, known to be rich in CGA, in comparison to roasted coffee with lower CGA concentrations. The finding that the Nrf2-translocating potential of CGA alone was not reflected by a respective potential of CGA-rich CE implies that other coffee constituents contribute substantially to the modulation of the Nrf2/ARE pathway. Interestingly, the degradation of CGA to caffeic acid was associated with a complete loss of Nrf2-translocating potency (Fig. 4C and D), probably due to a limited cellular uptake because of the high polarity of the acid group. Catechol, another degradation product, significantly reduced both cytosolic and nuclear Nrf2 levels in the nanomolar concentration range. Higher catechol concentrations had no significant influence on Nrf2 protein levels (Fig. 4E and F). The concentration of catechol in the coffee beverages was in the range of 5 mg/L, representing a concentration of ~0.27 mg/g in the freeze–dried brew. Thus, 1 μg/ml CE corresponded to roughly 2.45 nM catechol. However, no influence on Nrf2 protein levels in low microgram per milliliter extract concentrations was observed, questioning a substantial contribution of catechol to the effect of the whole extract. Hydroxyhydroquinone increased both nuclear and cytosolic Nrf2 levels in a concentration-dependent manner (≥250 μM), indicating a potent onset of the Nrf2 pathway (Fig. 4G and H). Genotoxic effects, discussed for both catechol and hydroxyhydroquinone, might be due to hydrogen peroxide formation during autoxidation [42]. Hence, catechol and hydroxyhydroquinone are regarded critically in food. Hiramoto et al. [43] associated effects as the formation of single-strand breaks by coffee with hydroxyhydroquinone-mediated ROS production. Thus, the observed induction of Nrf2 nuclear translocation by hydroxyhydroquinone in our studies might be due to hydrogen peroxide formation [15]. With an average concentration of 5 mg/L hydroxyhydroquinone in CE, about 200 nM hydroxyhydroquinone is expected in a final CE concentration of 100 μg/ml. This concentration was far below the effective concentration affecting Nrf2 translocation, also excluding hydroxyhydroquinone as a major contributor to the Nrf2-translocating properties of CEs.

NMP was identified as a potent inducer of Nrf2 nuclear translocation, marked by a U-shaped curve progression with two peak maxima (100 nM and 100–500 μM; Fig. 6C and D). Subsequently, NMP significantly increased the transcription of several ARE-dependent genes. However, the pattern of transcriptional modulation was different from the effects of CGA. After 3 h of incubation, a significant increase was observed for GSTA1, GSTT1 and γGCL, whereas GSTA2, GSTP1, HO1 and NQO1 transcription levels were not affected (Fig. 7A). However, the nuclear translocation of Nrf2 by NMP seems to be fortified by an increased *de novo* synthesis of Nrf2 since 3 h of incubation of HT29 cells with NMP caused a very consistent increase in Nrf2 transcripts (10–100 μM; Fig. 7A). Furthermore, it can be excluded that the here seen activation of the Nrf2/ARE pathway is caused by an NMP-induced intracellular increase of ROS, since no alteration of the intracellular ROS level was detected after 3 h of incubation with NMP in HT29 cells (data not shown). In contrast to CGA, long-time incubation (24 h) with NMP enhanced the transcript levels of several GST isoforms (Fig. 7B). Moreover, 0.1 μM NMP significantly increased γGCL, HO1, NQO1 and Nrf2 transcription levels, demonstrating the potency of NMP for long-term induction of Nrf2 gene transcription (Fig. 7B).

Table 1
Content of NMP and CGA in different CEs

Coffee extract	CGA		NMP	
	mg/L	mM	mg/L	μM
AC	743	2.1	22	234
AB	693	1.9	27	287
RI	640	1.8	30	320

AC, Arabica Columbia, AB Arabica Brazil, RI Robusta India.

The *C. robusta* extract RI presented the highest NMP concentration with 30 mg/L (Table 1), equivalent to a concentration of 320 μM NMP in the brew, corresponding to 1.6 mg/g in the lyophilisate. The high nanomolar NMP concentration in 100 μg/ml RI and in 250 μg/ml AB hints at the possibility that the coffee constituent NMP might contribute substantially to the significant increase of nuclear Nrf2 protein level induced by the RI extract. Furthermore, both extracts showed a U-shaped curve progression with two peak maxima, also seen after NMP incubation. Somoza et al. [26] reported increased GST activity in rat liver after feeding NMP-enriched coffee for 15 days. They discussed the induction of the Nrf2/ARE pathway as a potential mechanism of action [26], which has now been demonstrated in the present study.

NMP and related picolinium analogues are formed during the roasting process out of the natural precursor trigonelline (Fig. 2). In contrast to NMP, trigonelline did not induce Nrf2 nuclear translocation and even decreased the amount of Nrf2 protein in the nucleus as well as the total Nrf2 protein level (Fig. 6A and B) over a broad concentration range. Real-time PCR demonstrated that the decrease of Nrf2 in the nucleus was indeed associated with suppressed transcription of ARE-dependent genes (Fig. 7C). This effect was partially suppressed by co-incubation with NMP (Fig. 7D). However, it has to be underlined that nanomolar concentrations of trigonelline were sufficient to compensate the effect of 10 μM NMP, indicating that the content of these two coffee constituents with controversial effects on Nrf2 signalling might be critical for the biological activity of the whole CE. Thus, despite antioxidative properties in cell-free models [44], trigonelline appears to suppress cellular defence mechanisms within the cellular context.

Trigonelline degrades during the roasting process to NMP and to further products such as 1,2-, 1,3- and 1,4-DMP [36] (Fig. 1). The average concentration of trigonelline in coffee is about 300 mg/L [38]. Thus, 1 μg/ml CE comprises about 100 nM trigonelline, a concentration which was found to significantly suppress Nrf2 signalling in HT29 cells. However, the CEs AC, AB and RI had no suppressive impact on Nrf2 nuclear translocation at 1 μg/mL probably due to the compensatory effects of other CE constituents.

The loss of the carboxyl group seems to be a structural requirement for the antioxidative properties of pyridinium structures. Thus, NMP, the decarboxylation product of trigonelline, was, as mentioned above, a potent inducer of Nrf2. To investigate whether the position of the methyl group within the arising degradation products (DMPs) is also of relevance, isolated DMPs were examined. Nuclear Nrf2 translocation was induced by 1,3-DMP only at the highest test concentration of 500 μM (Fig. 6E and F). In contrast, 1,2-DMP appeared to be more effective, increasing Nrf2 nuclear protein at a concentration of 1 fM and ≥10 μM (Fig. 6G and H). Concomitantly, cytosolic Nrf2 protein levels were increased as well, suggesting interference with protein degradation or modulation of Nrf2 *de novo* synthesis. Kobayashi et al. [18] discussed that oxidative stress induces an inhibition of ubiquitinylation and increases *de novo* synthesis of Nrf2 protein. However, to our knowledge, a compound-mediated induction of Nrf2 *de novo* synthesis has not been described so far. 1,4-DMP increased the level of cytosolic Nrf2 significantly (≥10 μM), without affecting nuclear Nrf2 levels (Fig. 6I and J). In contrast, 1,4-DMP decreased the overall Nrf2 protein in picomolar concentrations. In sum, a total concentration of about 1 mg/L for all the DMP isomers in the CEs is present in coffee [38]; thus about 25 aM DMPs would be expected in 100 pg/mL CE. 1,2-DMP, the most effective DMP, caused increased Nrf2 nuclear translocation only at about 100-fold higher concentrations. Thus, a substantial impact of DMPs on the observed Nrf2 nuclear translocations by AB, AC and RI seems to be unlikely.

In summary, our data show a potent induction of Nrf2 nuclear translocation by incubation with different CEs in HT29 cells. The coffee constituents CGA and NMP were identified as potent inducers of Nrf2 translocation. However, the resulting pattern of ARE-dependent transcription was different. The formation of CGA degradation products during the roasting process was associated with a loss of activity. In contrast, NMP, formed under roasting conditions, represented a potent long-term enhancer of Nrf2/ARE-dependent gene expression. The NMP precursor trigonelline was identified as a potent suppressor of Nrf2 signalling, effectively compensating the effects of NMP. Thus, a fine tuning in the degradation/formation of activating and deactivating constituents of the Nrf2/ARE pathway during the roasting process appears to be critical for the potential chemopreventive properties of the final coffee product.

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