RESEARCH ARTICLE

Multi-parametric approach to identify coffee components that regulate mechanisms of gastric acid secretion

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Scope: Chlorogenic acid (CA), caffeine (CAFF), pyrogallol (PYR), catechol (CAT), $^{\beta}$*N*-alkanoyl-hydroxytryptamides (C5HT) and *N*-methylpyridinium (N-MP) were evaluated for their influence on mechanisms of gastric acid secretion as single compounds and in biomimetic mixtures. **Methods and results**: Compounds were tested in coffee representative concentrations. Human gastric cancer cells (HGT-1) were used to study the proton secretory activity by Ussing chamber experiments and FACS analysis. For activation of EGFr, Akt1, ERK1/2, ATF-2 and cAMP levels, we performed pathway screening assays. Time-dependent expression of related genes were determined by real-time PCR. Part of the data was used for neural network modeling to identify the most relevant compounds. N-MP increased the expression of the anti-secretory somatostatin receptor by 114%, whereas C5HT decreased its expression by 52%. N-MP down-regulated the pro-secretory CHRM3 receptor by 36% and the H^+,K^+-ATPase by 36%. CAFF stimulated the secretory activity in the functional assays, whereas N-MP and CA decreased proton secretion. After applying a pathway analysis, we were able to discriminate between CAFF, CA, CAT, C5HT, PYR and histamine-activating EGFr signaling and N-MP-associated ERK1/2 signaling. **Conclusion**: By applying a multi-parametric approach, N-MP was shown to effectively down-regulate mechanisms of gastric acid secretion in human parietal gastric cells.

Received: July 6, 2011
Revised: September 7, 2011
Accepted: September 15, 2011

Keywords:
Coffee / Gastric acid secretion / H^+,K^+-ATPase / Neural network / SSTR2-receptor

1 Introduction

Habitual coffee consumption sometimes causes heartburn or stomach irritation [1–5], which, in some cases, is associated with increased gastric acid secretion. The stimulating effects of individual coffee constituents such as caffeine (CAFF) or chlorogenic acid (CA) as well as $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides (C5HT) on gastric acid secretion have already been demonstrated in animal and human studies [6–8].

To account for synergistic or anti-synergistic effects, we applied a multi-parametric approach, including multiple cellular regulatory pathways of gastric acid secretion, on the quantitatively most relevant coffee components. Our

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Abbreviations: ATP4A, H^+,K^+-ATPase α-subunit; **CA,** chlorogenic acid; **CAFF,** caffeine; **CAT,** catechol; **CHRM3,** acetylcholine receptor M3; **C5HT,** $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides; **EGFr,** epidermal growth factor receptor; **HIS,** histamine; **HRH2,** histamine receptor H2; **IPX,** intracellular proton index; **N-MP,** *N*-methylpyridinium; **OMP,** omeprazole; **PYR,** pyrogallol; **SOST,** somatostatin; **SSTR2,** somatostain receptor 2; **TRI,** trigonelline

previous results have shown that one coffee component, *N*-methylpyridinium (N-MP), down-regulated proton secretion in human parietal gastric tumor cells (HGT-1) [9], when applied in combination with a lyophilized regular coffee beverage. In contrast, we also found that other coffee components such as CAFF as well as C5HT stimulated proton secretion in HGT-1 cells when applied in coffee representative concentrations [10, 11].

The purpose of this study was to elucidate the impact of multi-compound combinations of the six quantitatively most relevant compounds discussed to be involved in stomach irritation on cellular pathways regulating stomach acid secretion, and to identify the most potent compound in this coffee-biomimetic matrix. Using HGT-1 cells as a well-established parietal cell line for studying mechanisms of stomach acid secretion [12, 13], we tested the effects of N-MP, CAFF, C5HT, pyrogallol (PYR), catechol (CAT), trigonelline (TRI) and CA on proton secretion, signal transduction, transcription factors or expression of genes relevant to gastric acid secretion. Synergistic effects were tested in combination experiments using N-MP, CAFF, C5HT, PYR, CAT and CA as for these compounds, the evidence from previous studies strongly pointed to regulatory effects on mechanisms of stomach acid secretion.

2 Material and methods

2.1 Cell culture

HGT-1 cells (Dr. C. Laboisse, Nantes, France) were cultured at 37°C and with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) with 20% fetal calf serum (FCS), 5% glutamine and 5% penicillin/streptomycin. Cells were synchronized for 24 h prior to experiments with FCS-depleted DMEM.

2.2 Single compound and combinatory experiments thereof

A total number of about 150 000 cells were treated with CAFF (3 mM), CA (3 mM), CAT (52 μM), PYR (32 μM), TRI (497 μM) (all Sigma-Aldrich, Munich, Germany), C5HT (total concentration 282 nM, mixture comprising the predominating derivatives in biomimetic ratios on molar basis: stearoyl-5HT: 2 nM, arachinoyl-5HT: 75 nM, behenoyl-5HT: 180 nM and lignoceroyl-5HT: 25 nM), or N-MP (145 μM) (synthesized as described previously [9, 10]). As reference compounds, omeprazole (OMP, 1 mM), somatostatin (SOST, 0.5 mM), histamine (HIS, 1 mM) and acetylcholine (ACH, 1 mM) (all Sigma-Aldrich) were tested. All compounds were either directly dissolved in phosphate-buffered saline (PBS) or in Ringer's solution, except for $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides which were first dissolved in tetrahydrofuran (1 mg/mL THF) and then added in a volume of 2 μL to 10 mL of buffer in order to reach the final concentration. For the combinatorial experiments, five coffee constituents (CA, CAFF, CAT, C5HT, PYR) were first paired individually with N-MP. Three-component mixes were then obtained by combining N-MP, CA and each of the remaining four compounds. Four-component mixes were obtained by combining N-MP, CA, CAFF and each of the three remaining compounds. Five-component mixes were obtained by combining N-MP, CA, CAFF, CAT and either C5HT or PYR. In addition, all of these cocktails were also applied without N-MP.

2.3 Gene expression experiments

After 5, 10, 15, 20, 25 and 30 min of exposure, cells were harvested for RNA extraction and gene expression analysis. For the isolation of RNA, the RNeasy Midi Kit (Qiagen, Hilden, Germany) was used. The RNA was quantified by measuring the absorption at 260 nm. The pureness was determined by measuring the 260/280 ratio. The following cDNA synthesis was performed with the High Capacity cDNA synthesis Kit (Applied Biosystems, Forster City, CA, USA). qPCR assays were performed by using the Brilliant SYBR Green Master Mix (Stratagene, Amsterdam, The Netherlands) on a MxPro3000 cycler (Stratagene). The primer sequences for real-time PCR are stated elsewhere [9]. The results from the time course gene expression experiments are only given for those genes that were significantly regulated by the compounds tested.

2.4 Signal transduction assay

The phosphorylation status of receptor tyrosine kinases and MAPK kinases was detected after 10 min of incubation by the following ELISAs: EGFr-ELISA Kit, ERK1/2 ELISA Kit (both Calbiochem/Merck, Nottingham, UK), Akt1 pathscan ELISA and ATF-2 pathscan ELISA (both Cell Signaling/New England Biolabs, Frankfurt a. M., Germany). Absorption was read out at 450 nm on an MRX plate reader (Dynex, Berlin, Germany).

2.5 Neural network data analysis

The neural networks for the three receptors histamine receptor H2 (HRH2), acetylcholine receptor M3 (CHRM3) and somatostatin receptor 2 (SSTR2) were individually trained on the set of coffee recombinate data. The neural network software, NeuralWorks Predict (NeuralWare, Pittsburgh, PA, USA), was used with default settings with the exception of the use of cascaded variable selection and forced treatment of the input as continuously variable numerical data. The relevance of individual coffee

components on gene expression in the neural networks is characterized by the frequency in which the particular component appears in the final population of the genetic algorithm that selects network variables. A maximum frequency of 100 for an input variable (coffee component) indicates that this variable is highly important for the neural network and is always chosen.

2.6 Quantitative determination of cyclic AMP (cAMP)

cAMP was quantified in cell supernatants, using a competitive cAMP ELISA kit (R&D Systems, Minneapolis, MN, USA) as described in manufacturer's protocol. The cells were lysed after 0.5 min of treatment with the test compounds.

2.7 Proton secretory activity

The measurement of proton secretion was performed on a fluorescence plate reader (Varisoscan Flash, Thermo, Munich, Germany) and adapted from the previously applied flow cytometry assay [11]. A total of 120 000 HGT-1 cells were sown into a 96-well plate and grown close to confluence for 24 h. Cells were washed two times with PBS before the pH-sensitive fluorescence dye SNARF-AM (Life Technologies, Darmstadt, Germany) was loaded in a concentration of 3 μM in DMEM at 37°C for 30 min. After another washing step ($1000 \times g$, 5 min), the cells were incubated with the compound of interest for 60 min. The fluorescence dye was excited at 488 nm and detected at 580 and 640 nm. For CA, which showed a rapid oxidation in the test assay, fluorescence was measured at 10 min of exposure. This time point was selected based on the results from previous experiments in which a peak secretory activity of HGT-1 cells after treatment with lyophilized coffee beverages was demonstrated after 10 min of exposure [11]. The intracellular pH was calculated referring to a calibration curve with 2 μM nigericin (PAA, Coelbe, Germany) treated HGT-1 cells in a K^+ clamp buffer consisting of 20 mM NaCl, 110 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 18 mM D-Glucose and 20 mM HEPES that was set to different pH calibration points (6.4–8.2) by titration with NaOH. Then, the intracellular proton index (IPX) was calculated by $\log_2$ transformation of the intracellular proton concentration ratio between treated cells and control cells. The higher the IPX, the more protons remain in the cell, indicating a lower secretory activity. A more negative IPX indicates a higher secretory activity [11].

2.8 Ussing chamber experiments

To test how single coffee components affect the ion transport from the basolateral to the apical side of a HGT-1 monolayer, we performed Ussing chamber experiments. HGT-1 cells were cultivated on polyester membrane SNAP-well permeable supports with 0.4 μm pores (Sigma-Aldrich, Munich, Germany). The surface of the cultivated area was about 1.13 cm^2. Cells were grown until confluence, confirmed by microscopic analysis and resistance measurement with an Endohm chamber (WPI, Berlin, Germany). SNAP-well inserts were mounted into Ussing chambers (Easy Mount Chambers, Harvard Apparatus, March-Hugstetten, Germany), bathed with Ringer's solution (Sigma-Aldrich, Munich, Germany) at 37°C, and were gently bubbled with air under pH control. All tested substances were dissolved in Ringer's solution. The transepithelial potential difference was measured by a pair of Ag/AgCl electrodes, connected to a voltage clamp apparatus (KMSCI, Aachen, Germany). The current and voltage electrodes were placed on each side of the monolayer, were inserted into an electrode tip and connected to the Ringer's solution by a 3M KCl-filled agar-bridge. Ion transport was measured as current in the short-circuit mode and displayed as μA/cm^2 (pulse duration, 0.2 s; pulse frequency, 1 Hz; pulse amplitude, 25 ms). All compounds were added to the apical compartment.

2.9 Statistical analysis

Statistical analysis was performed with Excel 2003 or SigmaStat (Systat Software GmbH, Erkrath, Germany). Data sets generated by qPCR and ELISA were transformed by logarithmic conversion to reach normal distribution and are indexed as $\log_2$ ratio in the figures. Single comparisons between treated and control cells were done by applying the two-tailed Student's *t*-test for equal variances unless otherwise stated. For time course analysis of gene expression, we performed a one-way ANOVA with Holm–Sidak post-hoc analysis for parametric data sets and the Kruskal–Wallis test with Dunn's post-hoc analysis for non-parametric data sets. Numbers of replicates for each experiment are stated in Section 3. In each diagram, the mean value of n independent biological experiments ± standard error (SE) is shown. Ussing chamber experiments were performed as single experiments and the originally recorded values are shown in the according diagrams. For gene regulation experiments, only those results are included that show significant changes of expression over time.

3 Results

3.1 N-MP down-regulates the expression of H^+, K^+-ATPase α-subunit (ATP4A) and increases the anti-secretory SSTR2 receptor

Expression of ATP4A was solely regulated over time by CA, CAFF, PYR and N-MP ($n = 5$–6, Fig. 1A). Compared with non-treated control cells (= 0), CA and CAFF increased the

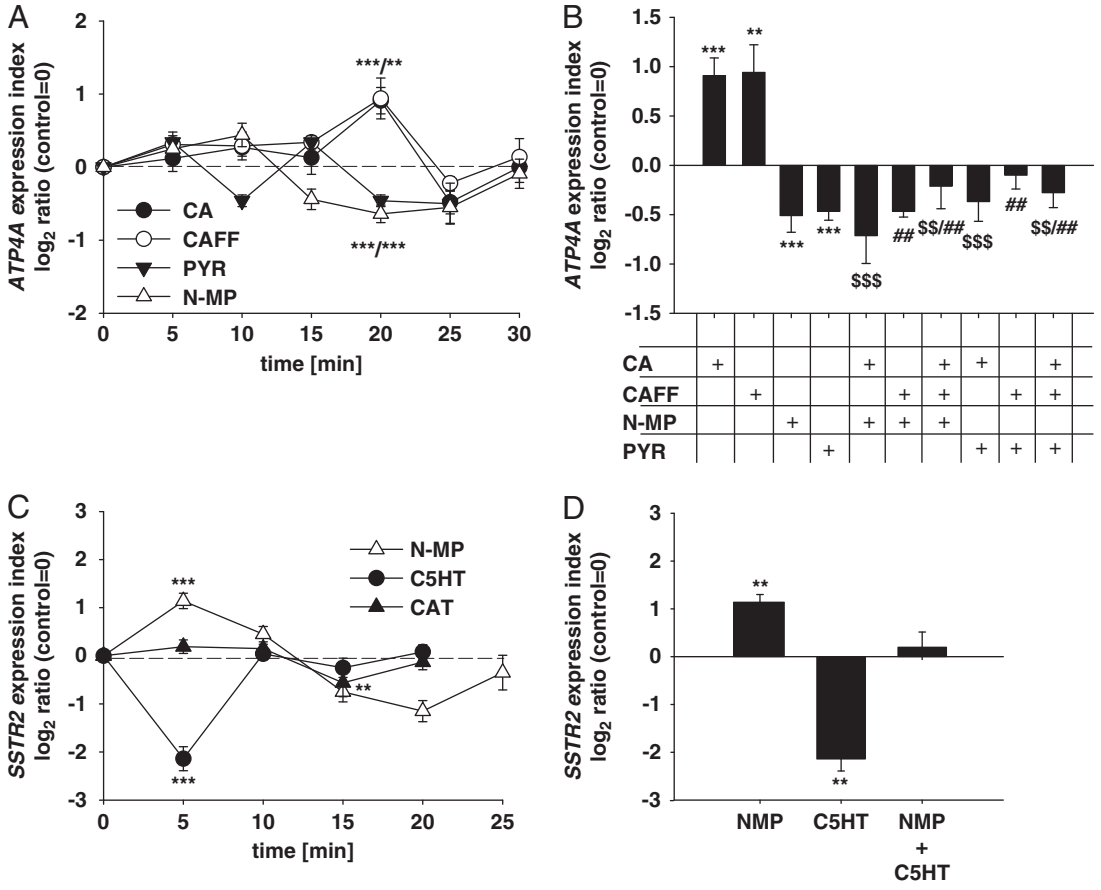

Figure 1. Effect of individual coffee compounds and combinations thereof on gene expression of ATP4A and SSTR2. (A) Expression of ATP4A (H^+,K^+-ATPase α subunit). (B) Combination effect of either CA or CAFF, or both in combination with N-MP on the expression of ATP4A. (C) Expression of SSTR2 after treatment with C5HT, CAT or N-MP. (D) Combination effect of N-MP and C5HT after five minutes on SSTR2 expression. ($^{**}p \leq 0.01$, $^{***}p \leq 0.001$: versus control; $^{\$\$}p \leq 0.01$, $^{\$\$\$}p \leq 0.001$: versus CA; $^{\#\#}p \leq 0.01$: versus CAFF, $n = 5$–9)

expression of ATP4A almost twofold to 0.91 ± 0.18 and 0.94 ± 0.28 ($n = 6$) after 20 min of exposure. In contrast, treatment of HGT-1 cells with N-MP and PYR led to a decrease in expression after 20 min to -0.64 ± 0.12 and -0.46 ± 0.08 ($n = 5$–6).

Compensating effects after 20 min of both PYR and N-MP were observed towards the up-regulating effect demonstrated for CA and CAFF (Fig. 1B) (N-MP/CA: -0.71 ± 0.28, N-MP/CAFF: -0.47 ± 0.09, N-MP/CA/CAFF: -0.21 ± 0.23 and PYR/CA: -0.37 ± 0.20, PYR/CAFF: -0.10 ± 0.14, PYR/CA/CAFF: -0.28 ± 0.15, $n = 4$–6).

The anti-secretory SSTR2 was regulated over time by N-MP, C5HT and CAT ($n = 4$–9, Fig. 1C). CAT decreased the expression of SSTR2 to -0.56 ± 0.29 after 15 min ($n = 6$). C5HT induced a strong decrease in SSTR2 receptor expression to -2.14 ± 0.25 after five minutes of exposure ($n = 4$). In contrast, N-MP enhanced the expression of the anti-secretory SSTR2 by more than twofold to 1.14 ± 0.16 after five minutes of treatment ($n = 5$) and compensated for the effect of C5HT (Fig. 1D).

3.2 Pro-secretory receptors HRH2 and CHRM3 are differentially regulated by compound combinations

CAT (-0.55 ± 0.24) and C5HT (-1.36 ± 0.22) (both $n = 5$) down-regulated the expression of HRH2 after five minutes of exposure (Fig. 2A). CA up-regulated the HRH2 receptor expression (0.55 ± 0.22, $n = 6$) after 20 min. The combination of C5HT with CA resulted in an up-regulation of the H2 receptor after five minutes (0.47 ± 0.04, $n = 4$–6, Fig. 2B) compared with control cells and treatment with C5HTs alone.

An up-regulation of the pro-secretory CHRM3 receptor expression after five minutes of incubation was demonstrated for CAFF (0.53 ± 0.12), PYR (1.14 ± 0.14) and N-MP (0.53 ± 0.25) ($n = 4$–6, Fig. 2C). However, counter-regulation was shown for N-MP (-0.76 ± 0.26, $n = 6$) after 20 min and for PYR (-0.70 ± 0.21, $n = 6$) after 15 min. In addition, there was no indication of a synergistic or compensating effect.

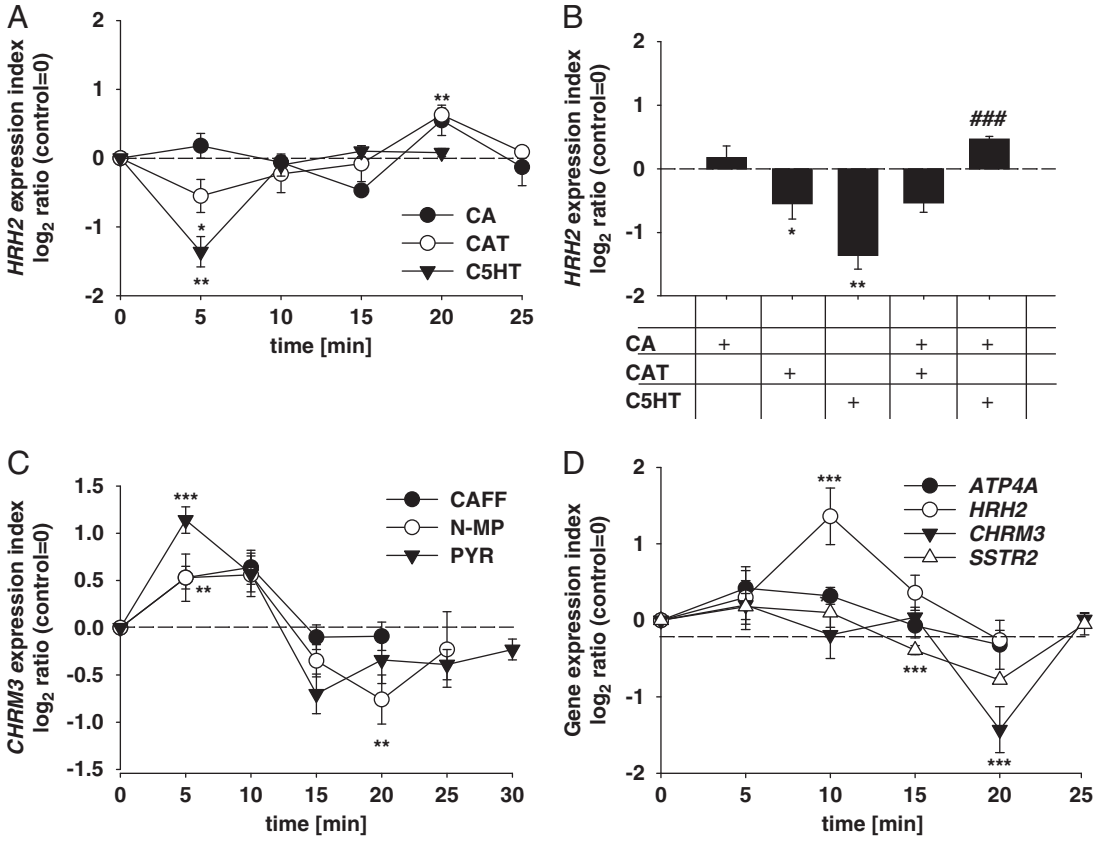

Figure 2. Effect of single compounds and combined compounds on the gene expression of the HRH2 and the CHRM3. (A) Regulation of HRH2 after treatment with C5HT, CAT or CA. (B) Combination effect of CA and C5HT on the expression of HRH2. (C) Expression of CHRM3 after treatment with PYR, CAFF or N-MP. (D) Expression of ATP4A, HRH2, CHRM3 and SSTR2 after incubation with a combination of CAFF, CA, PYR, CAT, C5HT and N-MP. ($^{**}p \leq 0.01$, $^{***}p \leq 0.001$: versus control, $^{\#\#\#}p \leq 0.001$: versus C5HT, $n = 5$–9)

3.3 Combination experiments reveal synergistic effects of coffee compounds on gene expression

Our findings confirmed the compensatory effects of N-MP and PYR on the expression of ATP4A, which remained unchanged after a 20-min treatment with all compounds (Fig. 2D, compared with Fig. 1B).

SSTR2 was shown to be up-regulated by N-MP and to be adversely regulated by C5HT (compared with Fig. 1C and D). However, treatment with all compounds confirmed counteracting effects of C5HT/N-MP ($n = 6$, Fig. 2D).

The mRNA levels of HRH2 after treatment with all compounds appeared to be different from experiments with two combined compounds. Since treatment with C5HTs and CA increased the mRNA levels of HRH2 after five minutes (Fig. 2A and B), after 10 min, the HRH2 mRNA levels increased to 1.36 ± 0.37 due to the treatment with all tested compounds combined ($n = 6$, Fig. 2D).

For CHRM3, the treatment with all compounds down-regulated the expression after 20 min to -1.43 ± 0.30 ($n = 6$, Fig. 2D) to a stronger extent compared with the effect of N-MP or PYR alone (compared with Fig. 2C).

3.4 Identification of combinatory effects on receptor gene expression by neural network analysis

Various combinations of the tested compounds were used for the evaluation of effects due to combination of single compounds with a neural network (Table 1, Fig. 3). N-MP, CAFF and CA were identified as the most dominant compounds in a multi-compound solution. N-MP was considered to be an important factor in 94% (R-value = 0.94) of all calculated scenarios for the expression of the HRH2. The compound with highest relevance for this receptor was CAFF with 100%. PYR and CA had an R-value of 0.71 and 0.79, whereas C5HT and CAT had a lower R-value of 0.50 and 0.29, respectively. In total, the calculated scenarios correlated with experimental data for the expression of the HRH2 with R-value = 0.94. The experimental results for the expression of the CHRM3 also had a correlation of R-value = 0.94 with the calculated model. Here, CA had the highest R-value with 1. N-MP was used in 91% (R-value = 0.91) of all calculated scenarios as an important factor in combination with other components. CAFF had an R-value of 0.82. CAT (0.59), C5HT (0.68) and PYR (0.23) were of

Table 1. Data for the calculation of a neural network for receptor expression

	Single compounds included: yes (100)/no (0)						Receptors		
	N-MP	CA	CAFF	CAT	C5HT	PYR	*HRH2*	*CHRM3*	*SSTR2*
Control	0	0	0	0	0	0	100%	100%	100%
N-MP	100	0	0	0	0	0	137%	138%	129%
R-value							**0.94**	**0.91**	**0.96**
CA	0	100	0	0	0	0	103%	156%	96%
R-value							0.79	**1.00**	**1.00**
CAFF	0	0	100	0	0	0	138%	190%	103%
R-value							**1.00**	0.82	**1.00**
CAT	0	0	0	100	0	0	118%	139%	120%
R-value							0.29	0.59	**0.96**
C5HT	0	0	0	0	100	0	117%	119%	98%
R-value							0.50	0.68	0.25
PYR	0	0	0	0	0	100	107%	151%	90%
R-value							0.71	0.23	0.83
Mix	100	100	100	100	100	100	263%	99%	111%
N-MP/CA	100	100	0	0	0	0	107%	129%	113%
N-MP/CAFF	100	0	100	0	0	0	186%	223%	134%
N-MP/CAT	100	0	0	100	0	0	181%	235%	134%
N-MP/C5HT	100	0	0	0	100	0	135%	142%	93%
N-MP/PYR	100	0	0	0	0	100	165%	155%	80%
N-MP/CA/CAFF	100	100	100	0	0	0	107%	148%	115%
N-MP/CAT/CA	100	100	0	100	0	0	147%	241%	164%
N-MP/C5HT/CA	100	100	0	0	100	0	154%	175%	97%
N-MP/PYR/CA	100	100	0	0	0	100	157%	148%	92%
N-MP/CA/CAFF/CAT	100	100	100	100	0	0	101%	156%	125%
N-MP/C5HT/CA/CAFF	100	100	100	0	100	0	137%	166%	84%
N-MP/PYR/CA/CAFF	100	100	100	0	0	100	142%	149%	105%
N-MP/CA/CAFF/CAT/C5HT	100	100	100	100	100	0	90%	133%	121%
N-MP/PYR/CA/CAFF/CAT	100	100	100	100	0	100	179%	162%	89%
CA/CAFF	0	100	100	0	0	0	62%	69%	36%
CA/CAT	0	100	0	100	0	0	72%	79%	35%
CA/C5HT	0	100	0	0	100	0	54%	59%	29%
CA/PYR	0	100	0	0	0	100	66%	78%	31%
CA/CAFF/CAT	0	100	100	100	0	0	74%	90%	59%
CA/CAFF/C5HT	0	100	100	0	100	0	91%	90%	51%
CA/CAFF/PYR	0	100	100	0	0	100	57%	73%	111%
CA/CAFF/CAT/C5HT	0	100	100	100	100	0	79%	84%	223%
CA/CAFF/CAT/PYR	0	100	100	100	0	0	89%	106%	266%
CA/CAFF/CAT/PYR/C5HT	0	100	100	100	100	0	86%	101%	193%

Relevance (*R*-value, expressed as percent relevance in the model, if significant in bold numbers) of individual coffee components in the neural networks for gene expression of the HRH2, CHRM3 and SSTR2 receptors are given ($n = 3$).

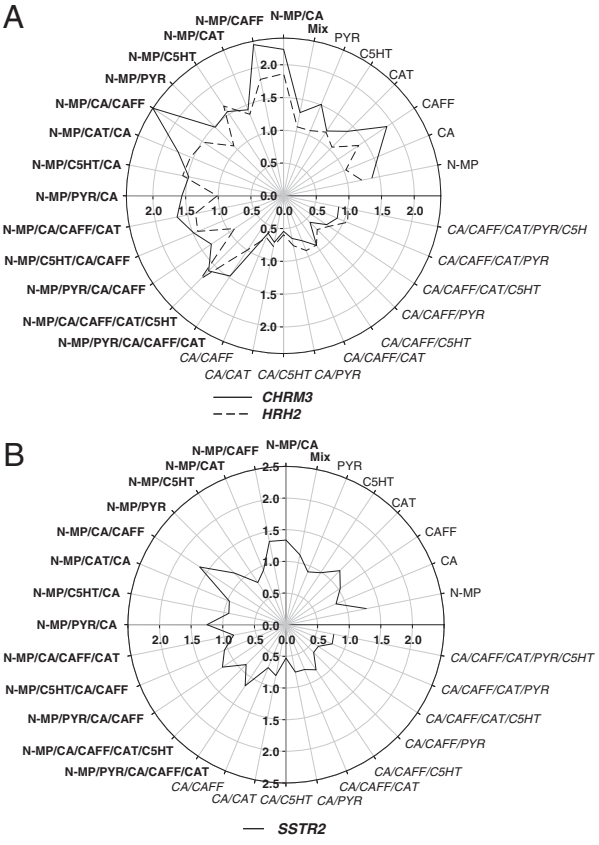

Figure 3. Combinatory effects of tested compounds on gene expression of gastric acid secretion related cell surface receptors used for the calculation of a neural network. (A) The relative expression values of the pro-secretory receptors HRH2 and CHRM3 used for the calculation of a neural network are visualized in a polar plot after 10 min of incubation. (B) Relative expression values of the anti-secretory SSTR2 receptor.

minor importance. The neural network for the anti-secretory SSTR2 receptor correlated with an acceptable correlation of $R = 80\%$ with the experimental data. Apart from the C5HT (0.25), all compounds showed an *R*-value above 0.83 (PYR). The highest weight factor was calculated for CA and CAFF with 1. Both N-MP and CAT showed an *R*-value of 0.96.

3.5 cAMP concentrations are elevated in gastric parietal cells after treatment with all selected compounds, except CA

HRH2 signaling in parietal cells is strongly related to elevated cAMP levels due to activation of adenylate cyclase [14]. Treatment with histamine increased the cAMP concentration in HGT-1 cells to 71 ± 19 fmol/mL ($n = 3$; Fig. 4A) compared with control cells (23 ± 3, $n = 3$). Similar to histamine, also CAFF (56 ± 18 fmol/mL), PYR (70 ± 24 fmol/mL), CAT (74 ± 31 fmol/mL), C5HT (40 ± 1 fmol/mL) and N-MP (56 ± 13 fmol/mL) increased cAMP levels in HGT-1 cells ($n = 3$). In contrast, treatment with CA (12 ± 2 fmol/mL) decreased the cAMP concentration ($n = 3$). The full combination ($n = 4$) of all compounds also increased the cAMP concentration to 46 ± 7.1 fmol/mL compared with controls.

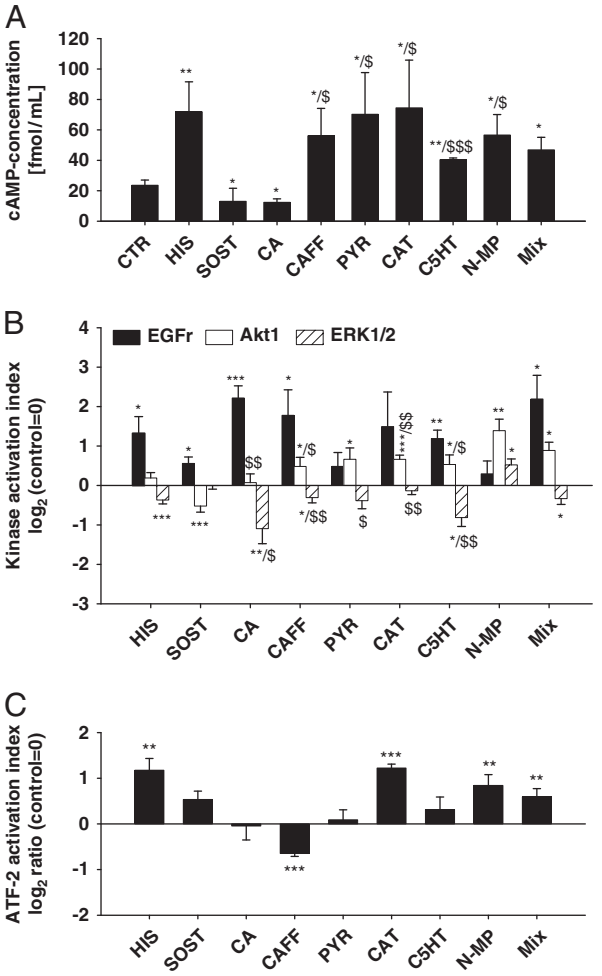

Figure 4. Influence of single compounds and mixes on cyclic AMP concentrations, activation of kinases and the transcription factor ATF-2. (A) Intracellular cyclic AMP concentration after treatment with HIS, CA, CAFF, CAT, PYR, C5HT and N-MP. (B) Activation of epidermal growth factor receptor (EGFr), Akt1 and ERK1/2 after treatment with HIS, CA, CAFF, CAT, PYR, C5HT and N-MP. (C) Phosphorylation of the cyclic AMP responsive element binding protein, known as transcription factor ATF-2, after treatment with HIS, CA, CAFF, CAT, PYR, C5HT and N-MP. ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$: versus control; in A: $^{\$}p \leq 0.05$, $^{\$\$\$}p \leq 0.001$: versus CA, in B: $^{\$}p \leq 0.05$, $^{\$\$}p \leq 0.01$: versus N-MP; $n = 3$–7)

3.6 Activation of signal transduction proteins by combination of all compounds depends on cluster effects of single compounds

EGF receptor tyrosine kinase phosphorylation increased after addition of histamine (1.32 ± 0.42), CA (2.22 ± 0.31),

CAFF (1.78±0.65) or C5HT (1.19±0.21) ($n = 3$–7; Fig. 4B), whereas N-MP did not change its activation status compared with control cells. Akt1, a downstream signaling protein of gastric secretion, was only slightly activated by CAFF (0.48±0.23), PYR (0.66±0.29), CAT (0.66±0.11) and C5HT (0.53±0.24), but more strongly by N-MP (1.39±0.29, $n = 7$). Furthermore, we examined whether extracellular regulated kinases 1/2 (ERK1/2) show a different phosphorylation status after treatment with histamine or single compounds. Only N-MP (0.52±0.15) led to activation of ERK1/2 ($n = 3$). In contrast, treatment with histamine (−0.37±0.10), CA (−1.09±0.38), C5HT (−0.81±0.23) and CAFF (−0.31±0.13) even resulted in a decreased activation of ERK1/2 in comparison to control cells ($n = 7$). The treatment with all compounds, however, activated EGFr (2.19±0.60) and Akt1 (0.88±0.21), but not ERK1/2 (−0.33±0.15). This result indicated a predominant effect of the other tested compounds over N-MP in ERK1/2 signaling.

3.7 cAMP responsive transcription factor ATF-2 is activated by all selected coffee constituents except CAFF

Since the genes ATP4A and SSTR exhibit cAMP responsive elements [15, 16], we investigated the role of coffee constituents on activation of ATF-2. Histamine (1.18±0.26), CAT (1.22±0.09) and N-MP (0.84±0.24) activated ATF-2. Solely CAFF (0.65±0.06) caused a phosphorylation status that was lower than that in control cells ($n = 7$; Fig. 4C). Treatment with all compounds led to an increased activation of ATF-2 compared with controls ($n = 4$).

3.8 Proton secretion by human gastric parietal cells is differentially affected by single coffee constituents as compared with biomimetic mixtures thereof

The intracellular proton index (IPX) [11] was used to analyze proton secretion after treatment of biomimetic compound mixtures of which the concentrations of individual compounds corresponded to those of a regular coffee beverage. Trigonelline was included as a coffee compound into the functional assays. Histamine caused a strong secretory stimulation by decreasing the IPX to −1.12±0.08 ($n = 11$; Fig. 5A). The effect of CA was anti-secretory with an IPX of 0.29±0.12 ($n = 4$; Fig. 5B). Combination of all compounds lacking CA also indicated an anti-secretory effect of CA in the omission experiment (−0.52±0.18) compared with a treatment with all compounds ($n = 7$; Fig. 5C). In contrast, CAFF stimulated proton secretion in the most pronounced way, to −2.01±0.16 ($n = 12$; Fig. 5A). N-MP decreased proton secretion ($n = 11$; Fig. 5A) and, in the omission experiment, treatment with all compounds

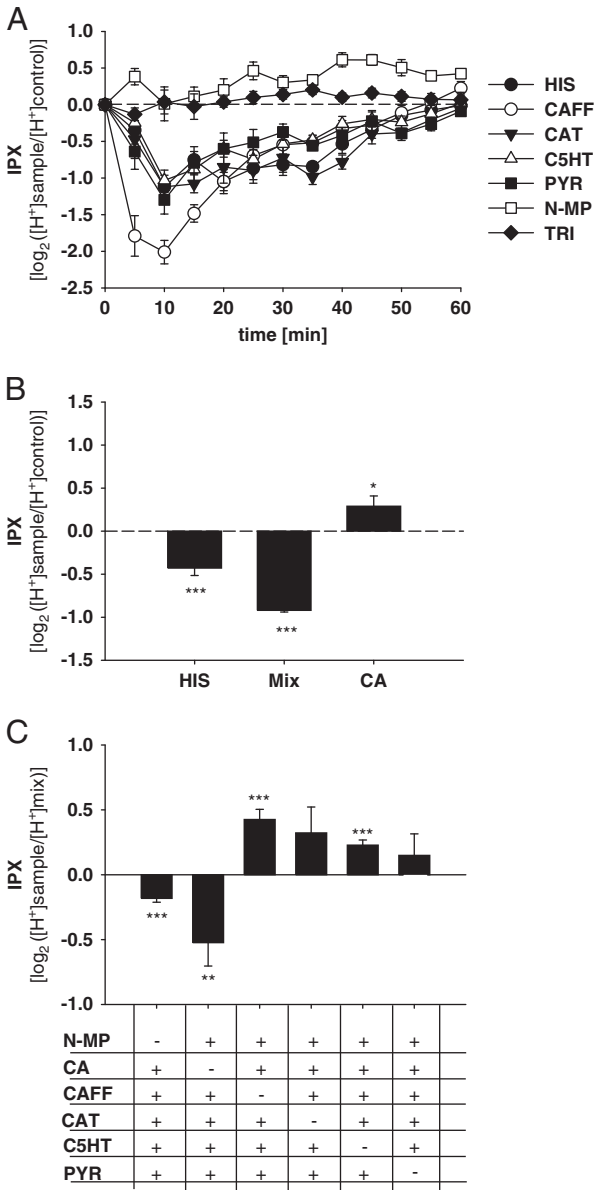

Figure 5. Measurement of proton activity by the intracellular proton index (IPX). (A) Treatment of HGT-1 cells with HIS, CAFF, PYR, CAT or C5HT and measurement of the IPX. ($p \leq 0.01$–0.001; $n = 10$–12). (B) IPX after treatment of HGT-1 cells with CA. (C) N-MP, CA, CAFF, CAT, C5HT or PYR were singly omitted from the full recombination of all tested compounds (mix) to elucidate synergistic effects of each compound. ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ versus control and versus mix respectively, $n = 3$–9)

except N-MP caused an IPX of −0.18±0.03 (Fig. 5C). Interestingly, treatment of HGT-1 cells with TRI, the precursor of N-MP in raw coffee beans, also showed an anti-secretory effect with a decreased proton secretion of 0.20±0.06 ($n = 11$, Fig. 5A). The results from the treatment with CAT indicated a stimulatory effect on HGT-1 cells with an IPX of −1.12±0.16 ($n = 11$; Fig. 5A). Combination of all

compounds lacking CAT resulted in a decreased proton secretion (0.32 ± 0.20, $n = 5$, Fig. 5C). In contrast, incubation with C5HT led to increased proton secretion (-1.03 ± 0.14) after 10 min ($n = 12$, Fig. 5A). Treatment with all compounds lacking C5HT caused a decreased proton secretion (0.23 ± 0.04) compared with the combination of all tested compounds ($n = 5$; Fig. 5C). Finally, treatment with PYR stimulated proton secretion with an IPX of -1.30 ± 0.19 compared with control cells ($n = 11$; Fig. 5A).

3.9 Total ion flux confirms the pro-secretory effect of CAFF, C5HTs and CAT, whereas N-MP, TRI and PYR have anti-secretory effects

The total ion flux ($\mu A/cm^2$) after application of histamine was elevated to 44 $\mu A/cm^2$ (Fig. 6A) and was inhibited by subsequent treatment with OMP, a specific and irreversible inhibitor of the H^+,K^+-ATPase, and then returned to resting levels (2 $\mu A/cm^2$, Fig. 6A). In a similar way, the current increased to 70 $\mu A/cm^2$ after apical application of C5HT indicating an increased total ion flux (Fig. 6A). CAFF also increased the total ion flux to 16 $\mu A/cm^2$ (Fig. 6A). The total ion flux after treatment with CAT was hardly changed compared with histamine (Fig. 6A). In contrast, addition of CA, PYR or TRI decreased the total ion flux to −13.5, −24 and −25 $\mu A/cm^2$, respectively, whereas the application of N-MP did not impair the total ion flux to −7 $\mu A/cm^2$ (Fig. 6B).

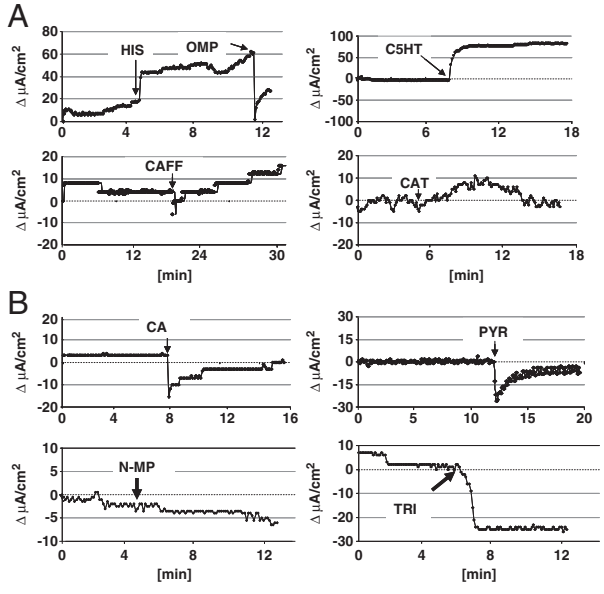

Figure 6. Measurement of total ion flux in the Ussing chamber. (A) From left to the right, apical treatment with HIS and subsequent apical admission of omeprazol (OMP), a specific inhibitor of the H^+,K^+-ATPase. Apical addition of C5HT. Apical application of CAFF. Apical addition of CAT. (B) From left to the right, apical admission of CA. Apical admission of PYR. Apical application of N-MP. Apical addition of TRI.

4 Discussion

Consumption of coffee has been reported to be associated with heartburn or stomach irritation, which can both be induced by increased stomach acid secretion [1, 8]. The effect of coffee beverages on gastric irritation and the intragastric pH in humans was first studied by Ehrlich et al. [6]. After oral administration of 150 mL of a coffee beverage prepared from either a regular or a steam-treated coffee, the latter induced significantly less mucosal irritation in healthy volunteers than the regular coffee beverage. As a result, steam-treatment of coffee was hypothesized to significantly reduce stomach-irritating compounds in roasted coffee beans, and coffee manufacturers started to label steam-treated coffee as stomach-friendly. This technology was patented in 1997 [17] and was initially developed to remove CAFF and CA. Next to these compounds, C5HT are also discussed to have ulcerogenic effects [7]. However, for neither of these compounds is the stomach-irritating activity in humans proven, and even results on the stomach irritating potential of mild or steam-treated coffee versus regular coffee are conflicting [6, 9, 11]. Using human parietal gastric tumor cells (HGT-1) as a model system, we recently demonstrated a less pronounced proton secretory activity of reconstituted freeze-dried coffee beverages prepared from commercial samples labeled stomach-friendly or decaffeinated compared with lyophilizates from regular, neither steam-treated nor decaffeinated, coffees [11]. The results from the following studies revealed that the proton secretory activity of human stomach cells, induced by coffee beverages, is lowered by increasing concentrations of N-MP in the coffee [9]. However, the impact of other components such as CAFF, CA, or C5HT at a cellular level has not been extensively investigated, nor have interactions between individual coffee constituents in coffee representative biomimetic mixtures. The aim of the multi-parametric studies of this work was to elucidate the effect of single coffee constituents on different regulatory and functional parameters of gastric acid secretion. One of our major findings on the level of gastric acid-related gene regulation demonstrated that the effects of the tested compounds on the expression of specific gastric secretion related mRNAs depend on their synergistic effects. Therefore, we hypothesize that results from studies using individual coffee compounds are less likely to be representative for the effects of the complexity of components present in coffee beverages. For the ATP4A, we demonstrate that N-MP and PYR predominantly decreased mRNA levels, whereas CAFF or CA had opposite effects. In contrast, neither N-MP nor PYR affected mRNA levels of the pro-secretory HRH2 (data not shown), whereas treatment of the HGT-1 cells with CAT as well as C5HT down-regulated HRH2 gene expression. However, the combination of CAT and C5HT with CA did not show a down-regulating effect that could be of potential importance for the reduction of stomach acid secretion. N-MP did increase the mRNA levels of the anti-secretory

SSTR2, whereas C5HT led to a decreased expression of this receptor. N-MP also down-regulated the mRNA expression of the pro-secretory CHRM3. This finding is in accordance with the anti-secretory effect of N-MP after its addition to a lyophilized coffee beverage [9] and is also supported by an anti-ulcerogenic effect described in rats for 1-methylnicotinamide, a structure closely related to N-MP [18]. Thus, substituted pyridine structures may exhibit a stomach protective potential.

With regard to the signal transduction in parietal cells, we also demonstrated that coffee constituents change the activation level of signaling proteins in specific patterns. Phosphorylation of ERK1/2 is associated with a lower secretory activity of parietal cells, whereas activation of EGFr and Akt1 is known to be associated with a higher secretory activity [19–22]. N-MP was the only compound that activated ERK1/2, whereas all of the other compounds tested reduced the phosphorylation of ERK1/2. Treatment with combinations of all compounds led to an increased phosphorylation status of Akt1, EGFr and also ERK1/2. A similar result was achieved for cAMP, which is closely associated with stimulation of gastric secretion [14, 23]. With the exception of CA, which showed a lowering effect, the intracellular cAMP concentration was elevated after treatment with all of the other coffee constituents tested individually. Treatment of the parietal cells with a biomimetic mixture of coffee components also resulted in elevated cAMP levels compared with control cells, despite the presence of CA. For the activation of the transcription factor ATF-2, N-MP, CAT and C5HT increased its phosphorylation status, whereas CAFF did not. In previous studies, it has already been shown that lyophilized coffee beverages with higher contents of N-MP increase ERK1/2 activation compared with controls, whereas lyophilisates of coffee beverages with lower contents of N-MP decreased the activity below control levels [9].

On a functional level, a pro-secretory effect was observed for CAFF, CAT, PYR and C5HT, whereas N-MP, TRI and CA decreased proton secretion compared with non-treated control cells. Except for PYR, these effects were confirmed for each individual compound by analyzing the total ion flux. Experiments with biomimetic combinations as well as omission experiments revealed that N-MP and CA partially compensated the pro-secretory effect of other pro-secretory compounds. These results are in accordance with the previous findings of our group [7, 9, 11, 24], which also demonstrated that the pro-secretory activity of C5HT depends on the number of carbons in the C-chain of the corresponding fatty acid [10]. Furthermore, the effect of CAFF on gastric acid secretion has been often described as pro-secretory [3, 8, 25].

In this work, we have shown, for the first time that CA exhibits an anti-secretory effect as a single compound and obviously might compensate the pro-secretory effect of a biomimetic mix on a functional level.

In order to discriminate between individual coffee components, we applied a neural network model, which showed that although treatment with only N-MP decreased the mRNA expression of the CHRM3, the expression of this pro-secretory receptor was up-regulated when parietal cells were treated with different multi-compound solutions containing N-MP. In addition, the expression of the anti-secretory SSTR2 receptor was up-regulated when N-MP was added compared with the same mixture without N-MP, which was in accordance with the effect of N-MP as a single compound. The evaluation of expression data using a neural network led to the identification of CAFF, CA and N-MP as the most relevant compounds in the biomimetic mixtures.

In conclusion, these results give evidence for an anti-secretory effect of N-MP on the level of gene regulation, signal transduction and proton secretion as an isolated compound and in biomimetic mixtures. In addition, CA and PYR have been shown to be partially anti-secretory, whereas CAFF, C5HT and CAT were identified as pro-secretory coffee constituents. The results further demonstrate that the application of neural networks is a useful approach to identify key compounds in complex solutions that closely represent the real matrix.

This research project was supported by a grant from FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn, Germany) the AiF (Arbeitsgemeinschaft industrieller Forschung), the German Ministry of Economics (Project No. 14042 N) and the German Academic Exchange Service (DAAD).

The authors thank Dr. C. L. Laboisse (Inserm 94-04, Faculte de Medicine, Nantes, France) for kindly providing the HGT-1 cells, clone 6.

The authors have declared no conflict of interest.

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