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ARTICLE

Activity-Guided Fractionation to Characterize a Coffee Beverage that Effectively Down-Regulates Mechanisms of Gastric Acid Secretion as Compared to Regular Coffee

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In some individuals, the consumption of coffee beverages is related to symptoms of gastric irritation. Hot water steam-treatment of raw coffee beans is hypothesized to reduce the contents of stomach irritating compounds, and products to which this technology is applied are launched as stomach-friendly coffee. However, data on the effect of steam-treated coffee on gastric acid secretion are conflicting and it has not been proven yet as to which coffee components act as pro- or antisecretory stimulants. The work presented here aimed at the characterization of a coffee beverage that effectively down-regulates mechanisms of proton secretion in human gastric cells (HGT-1). At first, a regular coffee beverage was fractionated by using solvents of different polarity: water, ethylacetate, dichloromethane, and pentane. Functional assays on the proton secretory activity (PSA) of these solvent fractions revealed the least pronounced effect for the water fraction, for which quantitative analyses demonstrated the highest distribution of chlorogenic acid (95%), [β]N-alkanoyl-5-hydroxy-tryptamides (55%), and N-methylpyridinium (N-MP, >99%) among all fractions. Following experiments demonstrated that HGT-1 cells treated with regular coffee fortified with N-MP at a concentration of about 20 mg/mL N-MP showed a significantly decreased PSA as compared to cells which were exposed to coffee beverages containing higher (32−34 mg/L) or lower (5 mg/L) N-MP concentrations. Results from cellular pathway analyses of transcription (ATF-1 and Akt1) and signaling (cAMP and EGFr) factors and kinases (ERK1/2), and experiments on the gene expression of pro (histamine-HRH2 and acetylcholine-CHRM3)- and anti (somatostatin-SSTR1)-secretory receptors and H^+,K^+-ATPase verified this antisecretory activity of N-MP in coffee beverages.

KEYWORDS: Gastric acid secretion; intracellular proton index; HGT-1 cells; coffee; *N*-methylpyridinium

INTRODUCTION

Coffee is one of the most popular beverages and is highly appreciated for its stimulating effect on the central nervous system. Some consumers experience symptoms of gastric irritation or heartburn after coffee consumption, which might be caused by an increased acid secretion in the human stomach (*1, 2*). Aiming at the production of stomach-friendly coffees, manufacturers apply a hot water-steam treatment to raw coffee beans (*3, 4*) to reduce the contents of putative stomach irritating compounds, such as [β]N-alkanoyl-5-hydroxytryptamides and caffeine (*5*). However, mechanistic data on the gastric acid secretory activity of coffee beverage components is scarce.

In a former study, we presented a novel approach to determine the stomach acid secretory activity of coffee beverages (*6*). In that work, human gastric parietal cells treated with reconstituted freeze-dried coffee beverages, prepared from commercial samples labeled as stomach-friendly or decaffeinated, showed a lower stomach acid secretory activity than those treated with beverages prepared from regular coffee. However, we were not able to assign these differences to any of the coffee brew constituents. Quantitative data of putative stomach irritating compounds varied substantially, most likely due to the different processing technologies applied. The work presented here aimed at the characterization of coffee beverages prepared from steam-treated coffee under standardized conditions with respect to their effects on mechanisms of stomach acid secretory activity in human gastric parietal cells.

On the cellular level, stomach acid secretion is mediated by cell surface receptors and subsequent signal transduction pathway activation, resulting in the expression of associated genes and

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functional proteins, such as H^+,K^+-ATPase (*7*). Among the cell surface receptors involved in gastric acid secretion, the histamine H2 receptor with its most potent ligand, histamine, plays a pivotal role in this mechanism (*7*). Next to histamine, acetylcholine can stimulate the secretory activity of parietal cells by binding to the acetylcholine receptor M3 (*7*). Another potent pro-secretory transmitter is gastrin, acting by binding to the cholecystokinin β-receptor (*7*). The only antisecretory cell surface receptor known so far is the somatostatin receptor for which somatostatin is the most potent ligand (*7*–*9*). For any of these cell surface receptors, binding of a ligand results in subsequent intracellular signaling which is transmitted by cyclic AMP, phosphoinositide-3, and Ca^{2+} (*7*, *10*, *11*).

Another receptor reported to be involved in the regulation of gastric acid secretion is the receptor for the epidermal growth factor (EGFr) (*12*, *13*). This receptor contains intracellular domains that are phosphorylated at single tyrosine residues following receptor activation. Subsequent signaling of EGFr activates different MAPK kinases such as Akt1 and ERK1/2 (*14*–*17*). It has also been reported that the EGFr can be activated by histamine and that downstream intracellular EGFr signaling is activated by intracellular Ca^{2+} concentrations (*7*). Overall, activation of pro-secretory receptors may lead to (a) activation of signal transduction pathways through MAP kinases, (b) gene and protein expression of the histamine H2 receptor, the acetylcholine receptor M3, and the cholecystokinin β-receptor, and H^+,K^+-ATPase as well as (c) restructuring of the actin-cytoskeleton and incorporation of the proton secreting H^+,K^+-ATPase into the cell membrane (*9*, *18*). Since H^+,K^+-ATPase exhibits a binding site for the transcription factor ATF-2 (*19*, *20*), an increase in ATF-2 levels is also considered as a pro-secretory signal.

In a previous study, we reported that the expression of genes encoding for pro-secretory receptors and for the H^+,K^+-ATPase gene is increased in human parietal stomach cells after treatment with caffeine and chlorogenic acid in coffee beverage representative concentrations (*21*). Here, we investigated reconstituted freeze-dried coffee beverages and quantitatively dominating components thereof for their effects on cellular mechanisms of stomach acid secretion. In order to characterize a coffee brew with minimum impact on stomach acid secretion, a solvent fractionation with quantitation of putative stomach irritating compounds in each fraction was performed. Selection of compounds to be quantitated was based on their hypothetical stomach irritating effects, such as $^\beta N$-alkanoyl-5-hydroxytryptamides (*22*), or based on their losses during the steam-treatment applied to produce stomach-friendly coffee such as chlorogenic acid, pyrogallol, and catechols (*3*). Furthermore, we also quantitated *N*-methylpyridinium, which is formed during roasting and has been reported to have chemopreventive activities in vivo and in vitro (*23*).

The solvent fractions were tested for their secretory activity in human parietal gastric cells (HGT-1) which are known to express all important transporters and receptors for gastric acid secretion (*24*–*26*). When the quantitative data on caffeine, chlorogenic acid, pyrogallol, catechol, *N*-methylpyridinium, and $^\beta N$-alkanoyl-5-hydroxytryptamide were related to the functional data on secretory activity, a coffee beverage with the lowest pro-secretory activity could be characterized. These results were finally proven in experiments in which coffee beverages prepared from standardized coffee samples were studied for their effects on cellular mechanisms of stomach acid secretion.

MATERIAL AND METHODS

Sample Preparation. For solvent fractionation, 54 g of homogeneous ground roast coffee powder (Arabica Brazil, not treated, coffee A) were weighed into a paper filter commonly used for the home preparation of coffee beverage (Melitta Gold, Nr. 4, Aldi, Germany). Portions of freshly boiled tap water ($T \sim 90$–$95\,°C$, approximately 100 mL each) were poured over the powder, and the hot filtrate was collected in a volumetric flask of 1000 mL. Exactly 500 mL of the coffee solution was transferred to crystallizing dishes, frozen ($-20\,°C$) immediately, and finally dried by lyophilization (48 h, 0.77 mbar, 25 °C) yielding 6.35 g of fluffy brown lyophilizate. The second part of the coffee beverage (500 mL) was transferred to a separation funnel, and the aqueous phase was sequentially extracted with pentane (4×500 mL), dichloromethane (4×500 mL), and ethyl acetate (4×500 mL), with the aqueous phase as the residue. All fractions obtained were reduced in a vacuum, taken up in water, frozen ($-20\,°C$), and freeze-dried (48 h, 0.77 mbar, 25 °C). The yields were determined by gravimetry (water fraction, 5.5 g (87%); dichloromethane fraction, 0.42 g (6.6%); ethyl acetate fraction, 0.39 g (6.1%); pentane fraction, 0.04 g (0.3%)). Solvent fractions were then used for functional assays on the secretory activity of human gastric parietal cells in concentrations according to their respective yields.

Studies on the cellular mechanisms of gastric acid regulation were performed with two c. Arabica Brazil (coffee A) and two c. Robusta Vietnam (coffee R) samples. One of each coffee varieties was a regular coffee, neither steam-treated nor decaffeinated, and the other matching sample was subjected to steam-treatment (coffee AT and coffee RT), on the basis of the manufacturer's practice to remove putative stomach irritating compounds which followed Darboven's patent (*3*). All coffee samples were representative for commercial coffees and were provided by a local coffee manufacturer. Briefly, raw coffee beans were subjected to hot water-steam extraction at a pressure of 19 to 22 psi for 10–30 min. Afterward, the remaining humidity is evaporated by heating the beans up to 130–150 °C. Finally, the evaporated water steam is released from the apparatus, whereas the original water content of raw coffee beans is restored in a vacuum atmosphere. From each of these four coffees (A, AT, R, and RT), beverages were prepared following a standard recipe which is typically used for household preparations.

Cell Culture. Human parietal carcinoma cells (HGT-1) were provided by Dr. C. Laboisse (Laboratory of Pathological Anatomy, Nantes France) and cultured at 37 °C and 5% CO_2. Dulbecco's modified Eagle medium (DMEM, PAA, Coelbe, Germany) with glucose (4%, PAA, Coelbe, Germany) was used as culture medium and supplemented with 20% fetal calf serum (PAA, Coelbe, Germany), 2% L-glutamine (PAA, Coelbe, Germany), 2% penicillin streptomycin (PAA, Coelbe, Germany), and 2% 4-(2-hydroxyethyl)-1-piperazineethanesulphonicacid (HEPES)-buffer (PAA, Coelbe, Germany). For each of the experiments, cells were seeded at a density of 500,000 cells in a T75 flask, and cell growth was controlled by transepithelial electrical resistance. Cells were cultivated with FCS for a period of four to five days since at this time, they showed a confluence of 90–100%. Synchronization of the cells was performed for 24 h in DMEM media without FCS. According to the literature, HGT-1 cells are well differentiated for experiments at the confluent state (*24*). Cells were treated with 2.5 mg/mL coffee lyophilizate reconstituted in phosphate buffered saline (*6*). Cell viability was tested to be stable during incubation with lyophilized coffee brews by Trypan Blue staining (PAA, Coelbe, Germany).

RNA Isolation and cDNA Synthesis. A total of 100,000 cells were seeded in six-well plates. After sample treatment, cells were harvested for the analysis of total RNA which was isolated using the Rneasy Midi Kit (Qiagen, Hilden, Germany). DNase I digest was performed on-column with RNase free DNase Kit (Qiagen, Hilden, Germany). Prior to qPCR, total RNA contents were quantitated photometrically at 260 nm. The cDNA was synthesized using the cDNA High Capacity Synthesis Kit (Applied Biosystems, Munich, Germany), as described in the manufacturer's manual.

Gene Expression Assays. Gene expression analyses were performed after sample treatment of the cells for 5, 10, 15, or 20 min. Primers for the H^+,K^+-ATPase α-subunit (*ATP4A*), the histamine H2 receptor (*HRH2*), the somatostatin receptor (*SSTR2*), and the acetylcholine receptor M3 (*CHRM3*) were designed with Beacon Designer 7.0 (PremierBiosoft, Palo Alto, CA) and validated by standard and melting curve analysis as stated elsewhere (*21*). The correct sequence of PCR products was verified by sequencing (Medigenomics, Martinsried, Germany, data not shown). Realtime-PCR assays were performed on a Mx3000p cycler (Stratagene, Amsterdam, Netherlands) using Brilliant SYBR Green Kit (Stratagene,

Amsterdam, Netherlands). Cycling conditions were as follows: 10 min/95 °C (activation), 30 s/95 °C (denaturation), 30 s/60 °C (annealing with fluorescence measurement), 30 s/72 °C (elongation).

Quantitative Determination of Cyclic AMP. A total of 50,000 cells were seeded in 24-well plates and treated with the respective sample for 0.5 min. For the determination of cyclic AMP in cell supernatants, we applied the competitive cyclic AMP ELISA parameter kit (R&D Systems, Minneapolis, MN) as described in the manufacturer's protocol. Supernatants were isolated after 1 min of incubation.

Signal Transduction Assay. Phosphorylation status of receptor tyrosine kinases and MAPK kinases was detected by enzyme linked immuno sorbent assays. Total protein was isolated from HGT-1 cells after incubation with the tested coffees for 10 min as recommended by the manufacturer's protocol. Total protein content was quantified photometrically using Bradford's reagent (Bio-Rad, Munich). The following ELISAs were used for the determination of the phosphorylation status: 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 a MRX plate reader (Dynex, Berlin, Germany) or Varioscan Flash plate reader (Thermo Electron Cooperation, Walthman, MI) with additional reading of the reference wavelength at 620 nm if indicated by the manufacturer's protocol.

Quantitative Analyses of Coffee Compounds. Chlorogenic acids and caffeine were quantified by HPLC-DAD as reported (*6*). *N*-Methylpyridinium (*6*), pyrogallol, catechol (*27*), and *N*-alkanoyl-5-hydroxytryptamides (*28*) were measured by HPLC-SIDA-MS/MS according to the methods given in the references in parentheses.

Secretory Activity. A volume of 2 mL cell suspension, corresponding to 2,000,000 cells, was used for each biological independent experiment and was treated for 10 min with 2.5 mg of a freeze-dried coffee beverage per mL of PBS, histamine (1 mmol in PBS), or different solvent fractions at 37 °C. The secretory acitivity was measured by the determination of the intracellular proton index (IPX) (*6*). The fluorescence dye Carboxy-SNARF-AM (Invitrogen, Karlsruhe, Germany) was used for the measurement of the intracellular pH by flow cytometry. The method was validated as described elsewhere, and histamine (1 mmol/L) was used as a well-known reference compound that stimulates stomach acid secretion (*6*). Briefly, HGT-1 cells were loaded with 3 μM dye for 30 min on ice. The intracellular pH was calculated referring to a calibration curve with 2 μM nigericin (PAA, Coelbe, Germany) treated HGT-1 cells in K+ clamp buffer consisting of 20 mM NaCl, 110 mM KCl, 1 mM CaCl2, 1 mM MgSO$_4$, 18 mM D-glucose, and 20 mM HEPES that was set to different pH calibration points (6.8−8.2) 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, whereas lower IPX values indicate a higher proton secretion.

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 displayed as indexed log$_2$ ratios in the figures. Single comparisons between treated and control cells were done with the two-tailed Student's *t*-test for equal variances. For time course analysis of gene expression, we performed the one-way ANOVA with Holm-Sidak posthoc analysis for parametric data sets and the Kruskal−Wallis test with Dunn's posthoc analysis for nonparametric data sets. Numbers of replicates for each experiment are stated in the results section, but were at least *n* = 3. In each diagram, the error bars represent the standard error (SE).

RESULTS

Secretory Activity of Solvent Fractions Prepared from Regular Coffee. Very recently, we introduced a novel approach to evaluate the gastric acid secretory activity of coffee beverages in human gastric cancer cells (HGT-1). In this assay, cells treated with reconstituted freeze-dried coffee beverages prepared from decaffeinated and/or steam-treated coffee showed a reduced secretory activity compared to that of cells treated with samples prepared from regular coffee (*6*). However, in this first approach,

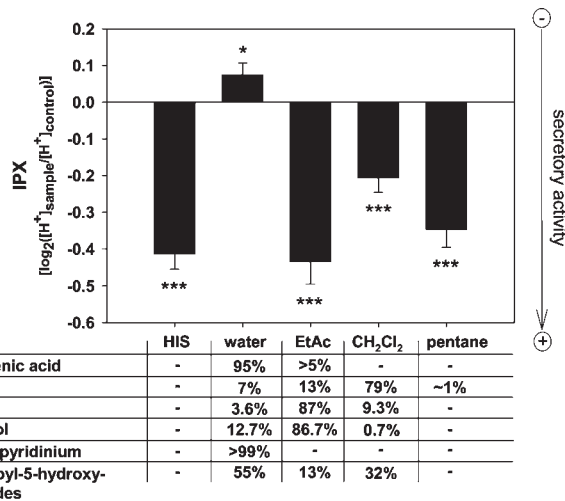|	HIS	water	EtAc	CH$_2$Cl$_2$	pentane
Chlorogenic acid	-	95%	>5%	-	-
Caffeine	-	7%	13%	79%	~1%
Catechol	-	3.6%	87%	9.3%	-
Pyrogallol	-	12.7%	86.7%	0.7%	-
N-methylpyridinium	-	>99%	-	-	-
βN-alkanoyl-5-hydroxy-tryptamides	-	55%	13%	32%	-

Figure 1. Intracellular proton index (IPX) of HGT-1 cells treated for 10 min with histamine (HIS, 1 mmol/L) or one of the solvent fractions prepared from a coffee beverage in concentrations according to their quantitative yields: water (H$_2$O, 2.14 mg/mL), ethyl acetate (EtAc, 0.16 mg/mL), dichloromethane (CH$_2$Cl$_2$, 0.18 mg/mL), and pentane (0.005 mg/mL). Distribution of quantitated compounds over the solvent fraction is given in the table below (statistics: two-tailed *t*-test vs control cells, *** = $p \leq 0.001$, *n* = 9).

we were not able to identify which of the coffee components affect mechanisms of proton secretion. In a following approach, we were able to identify different *N*-alkanoyl-5-hydroxytryptamides as potent stimulators of proton secretion in HGT-1 cells depending on their alkanoylic substitution (*28*). However, other coffee constituents may have an effect on stomach acid secretion as well.

Proton secretory activity can be analyzed by flow cytometry, using the pH-sensitive dye SNARF-AM that serves as a measure of the intracellular pH. Data are calculated as the intracellular pH index (IPX), on the basis of the hypothesis that the intracellular concentration of H$^+$ decreases due to proton secretion. The higher the IPX, the more protons remain in the cell, indicating a lower secretory activity, whereas lower IPX values indicate a higher proton secretion. IPX values are finally log$_2$ transformed for variance stabilization (*21*). In the work presented here, treatment of HGT-1 cells with the physiological stimulant histamine led to a stimulation of secretory activity as indicated by a significant decrease of the IPX (-0.41 ± 0.04; $p \leq 0.001$) compared to nontreated control cells (**Figure 1**). Cells treated with those fractions extracted from a commercial regular coffee blend with ethyl acetate, dichloromethane, and pentane reacted in a similar way, with IPX values of -0.43 ± 0.6, -0.20 ± 0.03, and -0.34 ± 0.04, respectively (two-tailed *t*-test, $p \leq 0.001$ vs nontreated control cells for each fraction; **Figure 1**). In contrast, cells exposed to the water extract demonstrated a significantly decreased secretory acitivity compared to that of control cells, as indicated by positive IPX values of $+0.07 \pm 0.03$ (two-tailed *t*-test, $p \leq 0.05$; **Figure 1**). Considering the distribution of the quantitated compounds among the solvent fractions (**Table 1**), the pentane fraction contained only one of them, caffeine, with about 1% of the total amount quantitated in all fractions.

Most of the compounds quantitated were distributed among the water, the ethyl acetate, and the dichloromethane fraction, with catechol and pyrogallol predominantly extracted with ethyl acetate and caffeine mainly ending up in the dichloromethane extract (**Table 1**). The water extract, which showed antisecretory activity in the functional assay, contained the majority of chlorogenic acid and, surprisingly, $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides.

Table 1. Yields of Fractions Obtained by Sequential Solvent Extraction of Coffee Beverage (Coffee A) and Concentrations of Putative Stomach Irritants in the Respective Fraction and the Reconstituted Solution Applied in the Cell Culture Experiments

	total	water	ethyl acetate	dichloromethane	pentane
yield (g)[a]	12.7	11.1	0.77	0.84	0.04
yield (%)[a]		87	6.1	6.6	0.3
compound (mg/g)[b]					
chlorogenic acid	81.7	88.8 (95)	67.4 (5)	n.d.	n.d.
caffeine	48.7	3.89 (7)	104.3 (13)	581.2 (79)	154.5 (~1)
NMP	2.54	2.87 (~99)	n.d.	n.d.	n.d.
catechol	0.45	0.02 (3.6)	6.47 (87)	0.63 (9.3)	n.d.
pyrogallol	0.32	0.05 (12.7)	4.56 (86.7)	0.34 (0.7)	n.d.
C5HT	0.02	0.01 (55)	0.04 (13)	0.08 (32)	n.d.
concentration in the reconstituted solution [mg/L][c]					
chlorogenic acid	203.66	222	168	n.d.	n.d.
caffeine	121.27	9.72	260	1453	368
N-methylpyridinium	6.32	7.17	n.d.	n.d.	n.d.
catechol	1.12	0.05	16.1	1.58	n.d.
pyrogallol	0.79	0.13	11.4	0.85	n.d.
C5HT	0.04	0.03	0.1	0.2	n.d.

[a] Yield refers to solid material after lyophilization of 1 L of standard coffee beverage. [b] Concentration of the respective compound in the freeze dried fraction; percentage of the total is given in parentheses; n.d., not detected. [c] Calculated from the concentration in the freshly prepared brew and the extraction yield, based on 2.5 mg lyophilizate per mL buffer; n.d. not detected.

The latter observation, however, was probably due to the amphiphilic nature of this compound class with its apparent tenside-like structure and subsequent localization between the solvent phases. The aqueous phase was the only extract that contained more that 99% of the total amount of *N*-methylpyridinium quantitated (**Figure 1**) due to the cationic nature of the molecule.

Secretory Activity of Regular Coffee after the Addition of *N*-Methylpyridinium. Since results from the experiments with solvent fractions isolated from the commercial regular coffee blend suggested an antisecretory effect of coffee rich in water-soluble compounds and, in particular, *N*-methylpyridinium which was solely quantitated in the water extract, *N*-methylpyridinium as a single compound and as a lyophilizate prepared from a regular coffee to which *N*-methylpyridinium was added were studied for their secretory activity in HGT-1 cells.

Quantitation of *N*-methylpyridinium in a freshly prepared brew of coffee A revealed a concentration of 32.21 mg/L (**Table 2**), being equivalent to a concentration of 2.53 mg/g in the lyophilizate, calculated from the concentration and extraction yield. Since 2.5 mg of the lyophilizate was dissolved in 1 mL of buffer for testing in the cell assay, a final concentration of 6.32 mg/L *N*-methylpyridinium resulted in the test solution (cf. **Table 2**). Fortification of the reconstituted lyophilizate was performed by adding different amounts of *N*-methylpyridinium to achieve a 2-fold (12.64 mg/L), a 3-fold (18.96 mg/L), a 5-fold (31.60 mg/L), and a 10-fold (63.20 mg/L) increase in the test solution compared to that of the nonfortified reconstituted lyophilizate with 6.32 mg/L. As shown in **Figure 2**, treatment of HGT-1 cells with the nonfortified reconstituted freeze-dried coffee had a stimulatory effect on secretory activity, with a mean IPX of -0.17 ± 0.03 (two-tailed t-test, $p \leq 0.001$ vs nontreated control cells), whereas in cells treated with reconstituted freeze-dried coffee with 2- and 3-fold *N*-methylpyridinium, a significantly lower secretory activity was analyzed (IPX: -0.10 ± 0.04 and $-3.46^{-17} \pm 0.01$, respectively, two-tailed t-test, $p \leq 0.01$ vs nontreated control cells for both samples). However, with increasing amounts of *N*-methylpyridinium up to a 10-fold fortification of the initial amount (63.2 mg/L), the secretory activity of coffee A was almost the same as that of the nonfortied reconstituted freeze-dried coffee (one-way ANOVA, $p \geq 0.05$; **Figure 2**).

Table 2. Concentrations of Major Putative Bioactive Compounds in Freshly Prepared Coffee Beverages (mg/L), and Concentrations of Reconstituted Freeze Dried Samples (mg/L) Prepared from c. Arabica Brazil (Coffee A), c. Arabica Brazil Steam-Treated (Coffee AT), c. Robusta Vietnam (Coffee R), or c. Robusta Vietnam Steam-Treated (Coffee RT)[a]

coffee beverage [mg/L]	coffee A	coffee AT	coffee R	coffee RT
extraction yield[b]	12740	12580	15800	16750
chlorogenic acid[c]	1038	1083	975	1464
caffeine[c]	618	594	1389	1326
N-methylpyridinium[c]	32.21	34.70	22.40	5.41
pyrogallol[c]	4.05	3.99	5.63	4.79
catechol[c]	5.73	5.29	9.26	4.43
C5HT[c]	0.21	0.21	0.12	0.08
concentration in the reconstituted solution [mg/L][e]				
chlorogenic acid[d]	203.66	215.22	154.27	218.51
caffeine[d]	121.27	118.04	219.78	197.91
N-methylpyridinium[d]	6.32	6.90	3.54	0.81
pyrogallol[d]	0.79	0.79	0.89	0.71
catechol[d]	1.12	1.05	1.47	0.66
C5HT[d]	0.04	0.04	0.02	0.01
ratios				
chlorogenic acid	132%	140%	**100%**	142%
caffeine	103%	**100%**	186%	167%
N-methylpyridinium	780%	851%	437%	**100%**
pyrogallol	111%	111%	125%	**100%**
catechol	170%	159%	221%	**100%**
C5HT	400%	400%	200%	**100%**

[a] In addition, the ratios of the single compounds between the reconstituted freeze dried coffees are given, where the coffee with the lowest content of a certain compound was set to 100%. Coffee A, Arabica Brazil untreated; coffee AT, Arabica Brazil, steam treated; coffee R, Vietnam Robusta untreated; coffee RT, Vietnam Robusta, steam treated. [b] Extraction yield determined by weight after lyophilization. [c] Concentration determined by HPLC-DAD/HPLC-MS/MS techniques in the freshly prepared beverage. [d] Concentrations calculated from concentrations in the beverage and the extraction yields. [e] Calculated from the concentration in the freshly prepared brew and the extraction yield, based on 2.5 mg lyophilizate per mL buffer.

When HGT-1 cells were treated with *N*-methylpyridinium as a single compound, no dose dependent effect was observed (**Figure 2**). These results demonstrate that *N*-methylpyridinium
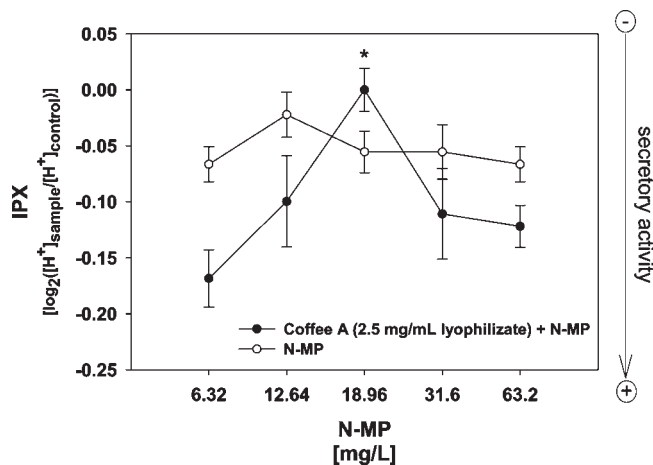
Figure 2. Intracellular proton index (IPX) of HGT-1 cells treated for 10 min with varying concentrations of *N*-methylpyridinium either as a single compound (N-MP) or in combination with reconstituted freeze-dried regular coffee (coffee (2.5 mg/mL) + N-MP), reaching the same *N*-methylpyridinium concentration in the system as was applied with the single compound (statistics: one-way ANOVA with the Holm-Sidak posthoc test, * = $p \leq 0.05$, $n = 9$).

does act as an antisecretory compound in coffee beverage, but not as an isolated substance.

Secretory Activity of Lyophilizates Prepared from Standardized Coffee Beverages with Varying Contents of *N*-Methylpyridinium and Their Effect on Cyclic AMP Formation. Since the experiments with *N*-methylpyridinium fortified reconstituted freeze-dried coffee prepared from coffee A suggested an antisecretory effect of those coffee beverages having a 3-fold higher *N*-methylpyridinium concentration as compared to nonfortified lyophilizates, three additional coffee samples with defined *N*-methylpyridinium contents were produced. At first, a matching sample to coffee A consisted of the same coffee beans but was mildly steam-treated (coffee AT) without effects on *N*-methylpyridinium concentration (32.2 mg/L and 34.7 mg/L, **Table 2**). For the second set of samples, a c. Robusta Vietnam (coffee R) and a c. Robusta Vietnam steam-treated (coffee RT) were prepared. Quantitation of *N*-methylpyridinium in these two samples revealed medium high and low concentrations, with 22.4 mg/L and 5.41 mg/L in the beverage, respectively (**Table 2**). The IPX analyzed in HGT-1 cells after treatment with reconstituted freeze-dried coffee A (−0.43 ± 0.05), AT (−0.49 ± 0.02), and R (−0.35 ± 0.07) demonstrates a significantly lower secretory acitvity compared to that of coffee RT (−0.60 ± 0.02) (**Figure 3A**). Thus, the high and medium high concentrations of *N*-methylpyridinium quantitated in coffee A, AT, and R, respectively, seem to be associated with a lower secretory potential compared to that of coffee RT for which the lowest *N*-methylpyridinium concentrations were quantitated.

Stimulation of gastric acid secretion in parietal cells is related to elevated cyclic AMP levels since gastrin and histamine receptor signaling increase the synthesis of cyclic AMP by adenylatcyclase due to G-protein activation (*7*). In this study, HGT-1 cells treated with histamine stimulated the secretory activity significantly to an IPX of −0.97 ± 0.02 compared to that of nontreated control cells. In accordance with this result, the cyclic AMP concentration was also increased to 7.76 ± 0.69 pmol/mL from a base level of 6.02 ± 0.36 pmol/mL in nontreated control cells (two-tailed *t*-test, $p \leq$ 0.05; **Figure 3A** and **B**). In accordance with the results from the secretory activity assay, the cyclic AMP concentration after HGT-1's treatment with coffee RT (19.69 ± 0.41 pmol/mL) was significantly higher (two-tailed *t*-test, $p \leq 0.05$) compared
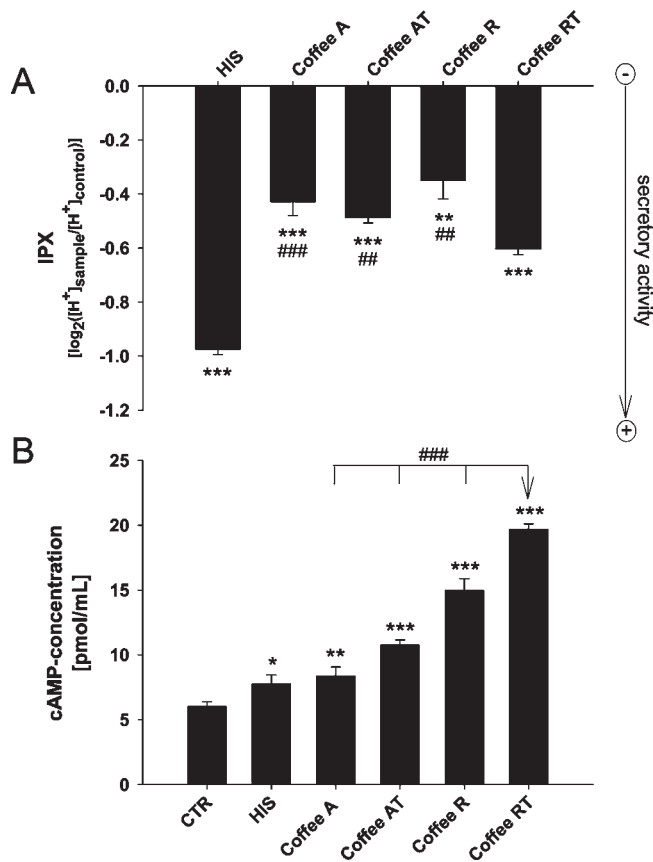

Figure 3. Intracellular proton index (IPX, exposure time: 10 min, **A**) and contents of cyclic AMP (cAMP, exposure time: 0.5 min, **B**) of non-treated HGT-1 cells (CTR) or cells treated with either histamine (HIS: 1 mmol/L) or reconstituted freeze-dried samples (2.5 mg/mL) prepared from c. Arabica Brazil (coffee A), c. Arabica Brazil steam-treated (coffee AT), c. Robusta Vietnam (coffee R), or c. Robusta Vietnam steam-treated (coffee RT) coffee, having high (coffee A, AT), medium (coffee R), and low (coffee RT) concentrations of *N*-methylpyridinium, respectively (statistics: two-tailed *t*-test vs control cells, ** = $p \leq 0.01$, *** = $p \leq 0.001$, $n = 9$; or vs coffee RT ## = $p \leq 0.01$, ### = $p \leq 0.001$).

to that of control cells and to the cyclic AMP concentrations after treatment with coffee A (8.83 ± 0,69 pmol/mL), coffee AT (10.76 ± 0.38 pmol/mL), or coffee R (14.98 ± 0.89 pmol/mL) (**Figure 3B**). Overall, compared to control cells, coffee RT stimulated the proton secretion more than any other reconstituted freeze-dried coffee tested, whereas its incubation with HGT-1 cells resulted in the highest intracellular cyclic AMP levels. Coffee R, in contrast, exhibited the least stimulatory effect on proton secretory activity but induced the second highest cyclic AMP levels in the HGT-1 cells. These results suggested that the reconstituted freeze-dried coffees tested activate gastric secretion by more pathways than cyclic AMP signaling.

Effect of Reconstituted Freeze-Dried Coffee on Signal Transduction Pathways and on Gene Expression. Signal transduction bridges the gap between receptor activation and regulation of gene transcription of functional proteins, e.g., H^+,K^+-ATPase. So far, EGFr, ERK1/2, and Akt1 signaling has been shown to be involved in gastric acid secretion (*7*). The regulation of gene transcription depends on transcription factors. Since H^+,K^+-ATPase and the somatostatin receptor both hold binding motifs for cyclic-AMP dependent transcriptions factors, we also examined the role of ATF-2 in the process of gene regulation and secretory activity. As a result, exposure of HGT-1 cells to

histamine resulted in ATF-2 activation (pathway activation index 1.18 ± 0.26 vs 0 for nontreated control cells; $p \leq 0.001$, **Figure 4**). For the reconstituted freeze-dried coffee tested, ATF-2 activation was demonstrated, with lowest $\log_2$ analyzed for coffee R (coffee R,
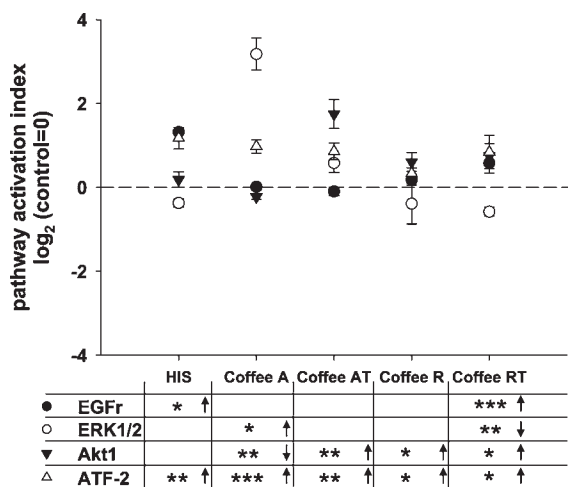

Figure 4. Pathway activation indices of EGFr, ERK1/2, Akt1, and ATF-2 in HGT-1 cells treated for 10 min with either histamine (HIS: 1 mmol/L) or reconstituted freeze-dried samples (2.5 mg/mL) prepared from c. Arabica Brazil (coffee A), c. Arabica Brazil steam-treated (coffee AT), c. Robusta Vietnam (coffee R), or c. Robusta Vietnam steam-treated (coffee RT) coffee, having high (coffee A, AT), medium (coffee R), and low (coffee RT) concentrations of *N*-methylpyridinium, respectively (statistics: two-tailed *t* test vs control cells, ** = $p \leq 0.01$, *** = $p \leq 0.001$, $n = 9$; or vs coffee RT ## = $p \leq 0.01$, ### = $p \leq 0.001$).

0.33 ± 0.14; coffee RT, 0.84 ± 0.39; coffee A, 0.98 ± 0.16; coffee AT, 0.86 ± 0.20; each $p \leq 0.05$ vs nontreated control cells; **Figure 4**). However, coffee AT did not affect the expression of the pathway related gene of the H^+,K^+-ATPase α-subunit (ATP4A), whereas cells treated with coffee R showed an enhanced gene expression of the antisecretory somatostatin receptor (0.74 ± 0.19, one-way ANOVA $p \leq 0.01$ vs nontreated control cells ($= 0$), **Figure 5D**). When steam-treated coffee R (coffee RT) was studied, no effect was demonstrated for the somatostatin receptor, but instead an increase in ATP4A gene expression was demonstrated (0.55 ± 0.05, one-way ANOVA $p \leq 0.05$ vs nontreated control cells ($= 0$), **Figure 5A**). HGT-1 cells treated with coffee A showed an increase in ATP4A gene expression (0.47 ± 0.10, $p \leq 0.05$ vs nontreated control cells ($= 0$), **Figure 6A**), whereas the gene expression of the antisecretory somatostatin receptor was decreased (-0.59 ± 0.17, one-way ANOVA $p \leq 0.01$ vs nontreated control cells ($= 0$), **Figure 6D**).

Another transcription factor that is reported to enhance gastric acid secretion and gene expression of H^+,K^+-ATPase is Akt1 (7). In this study, Akt1 signaling was activated significantly in HGT-1 cells exposed to coffee AT, R, or RT, with maximum pathway activation indices of 1.75 ± 0.40, 0.61 ± 0.23, and 0.75 ± 0.30, respectively (two-tailed *t*-test, $p \leq 0.05$–0.01 vs nontreated control cells; **Figure 4**). However, neither coffee AT nor coffee R affected the gene expression of ATP4A. Treatment of the HGT-1 cells with coffee A, in contrast, caused a slight but significant decrease of Akt1 activation compared to that of nontreated control cells (-0.22 ± 0.07, two-tailed *t*-test $p \leq 0.05$, **Figure 4**). Since coffee A increased ATP4A expression, Akt1 signaling appeared not to be the triggering factor for ATP4A expression after exposure to coffee. Nevertheless, when HGT-1
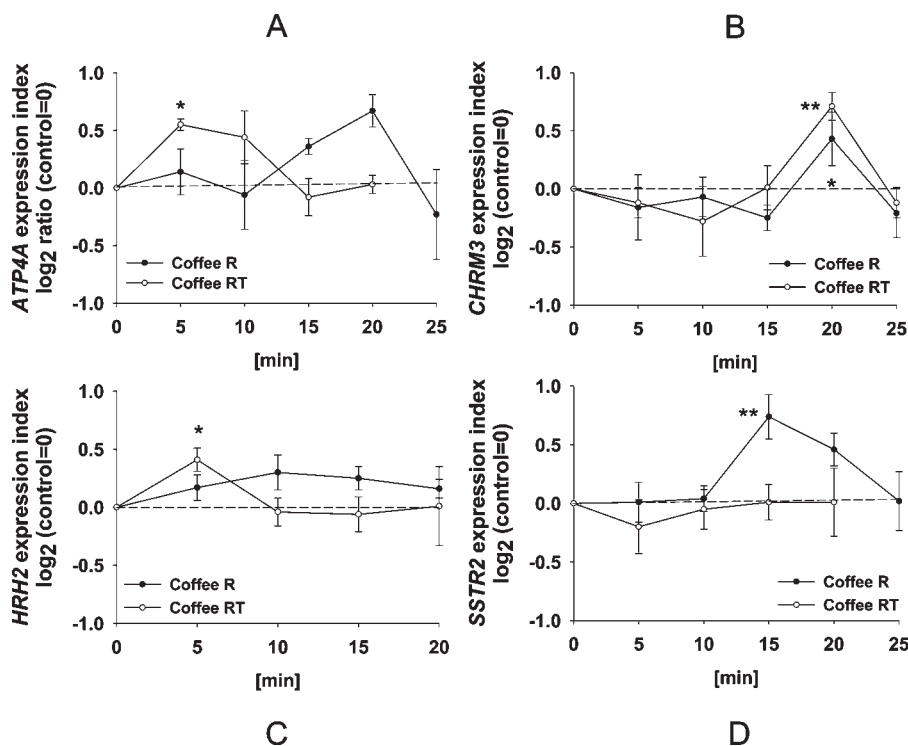

Figure 5. Time dependent indices of gene expression for the H^+,K^+-ATPase (ATP4A, A), the histamine H2 receptor (HRH2, B), the acetylcholine receptor M3 (CHRM3, C), and the somatostatin receptor (SSTR2, D) in HGT-1 cells after the exposure of reconstituted freeze-dried samples (2.5 mg/mL) prepared from either c. Robusta Vietnam (coffee R) or c. Robusta Vietnam steam-treated (coffee RT) coffee, having medium (coffee R) and low (coffee RT) concentrations of *N*-methylpyridinium, respectively (statistics: one-way ANOVA with the Holm-Sidak posthoc test, * = $p \leq 0.05$, ** = $p \leq 0.01$, $n = 6$; the p-value of the one-way ANOVA is given in the diagram; the significant changes in the time course compared to the most pronounced change of expression identified by the posthoc analysis are mentioned in the text).
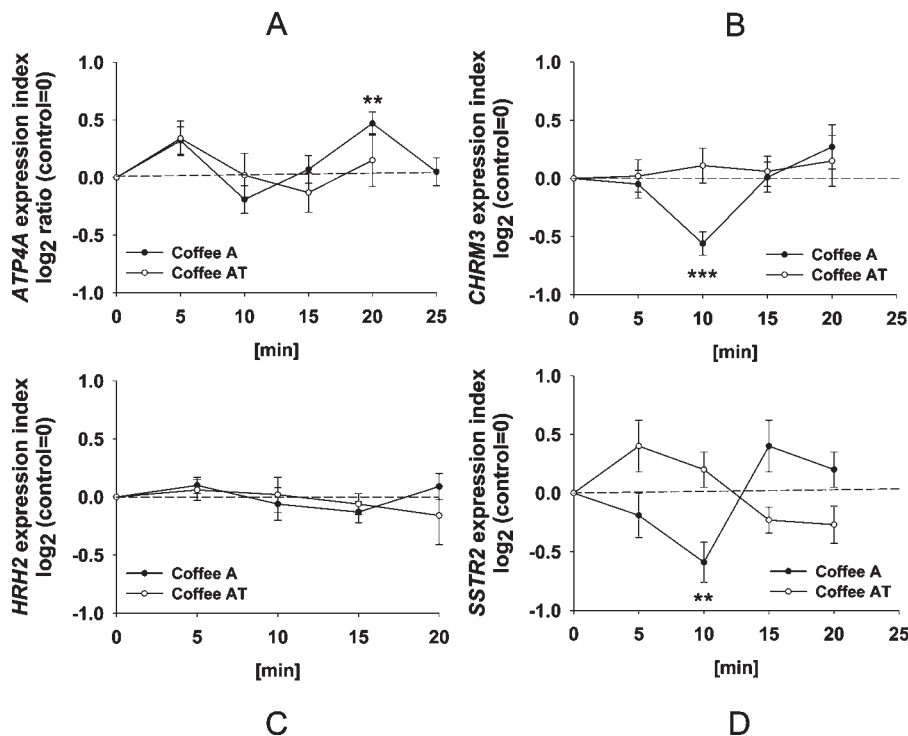
Figure 6. Time dependent indices of gene expression for the H^+,K^+-ATPase (ATP4A, A), the histamine H2 receptor (HRH2, B), the acetylcholine receptor M3 (CHRM3, C), and the somatostatin receptor (SSTR2, D) in HGT-1 cells after the exposure of reconstituted freeze-dried samples (2.5 mg/mL) prepared from either c. Arabica Brazil (coffee A) or c. Arabica Brazil team-treated (coffee AT) coffee, having high concentrations of *N*-methylpyridinium (statistics: one-way ANOVA with Holm-Sidak posthoc test, * = $p \leq 0.05$, ** = $p \leq 0.01$, $n = 6$; the *p*-value of the one-way ANOVA is given in the diagram; the significant changes in the time course compared to the most pronounced change of expression identified by the posthoc analysis are mentioned in the text).

cells were exposed to coffee A, ERK1/2 was activated (1.30 ± 0.58, two-tailed *t*-test vs nontreated controls $p \leq 0.05$, **Figure 4**). In contrast, ERK1/2 signaling was decreased after treatment with coffee RT (−0.58 ± 0.49 **Figure 4**).

EGFr receptor activation was significant after HGT-1 exposure to histamine (1.32 ± 0.42) and coffee RT (0.58 ± 0.24; two-tailed *t*-test, $p \leq 0.05-0.001$, **Figure 4**). This result again indicates a higher secretory activity of coffee RT. Considering the expression of pro-secretory receptors, the histamine H2 receptor was not impaired by the incubation of HGT-1 cells with coffee A or AT. Coffee RT, in contrast, increased its expression significantly (0.41 ± 0.10, one-way ANOVA $p \leq 0.01$ vs nontreated control cells, **Figure 5C**). The pro-secretory acetylcholine receptor M3 was decreased in its gene expression after treatment of the HGT-1 cells with coffee A (−0.56 ± 0.10, one-way ANOVA $p \leq 0.001$, **Figure 6B**), whereas coffees R and RT showed an enhancing effect on this receptor (0.43 ± 0.23 and 0.71 ± 0.12, respectively, one-way ANOVA, $p \leq 0.01$ vs nontreated control cells, **Figure 5B**). Taken together, the treatment of parietal cells with coffee RT resulted in the highest secretory activity, most comprehensive up-regulation of pro-secretory genes, and activation of gastric acid secretion related pathways. HGT-1 cells exposed to coffee AT did not show any increase in the expression of pro-secretory genes nor could a decrease in the expression of antisecretory genes be observed. In line with that, the secretory activity of parietal cells after exposure to coffee AT was below that of coffee RT (**Figure 3**).

The least pronounced effect on secretory activity was demonstrated for coffee R (**Figure 3**), which did show an increasing effect on the gene expression of the pro-secretory acetylcholine receptor M3 but also demonstrated the highest stimulating effect on the gene expression of the antisecretory somatostatin receptor among all coffees tested (**Figure 5B**).

DISCUSSION

Consumption of coffee has often been reported to be associated with heartburn or stomach irritation (*1*, *2*, *6*) which can both be induced by, e.g., increased stomach acid secretion (*1*, *6*). The effect of coffee beverages on gastric irritation and intragastric pH in humans was first studied by Ehrlich et al. (*29*). After oral administration of 150 mL of 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. On the basis of these results, 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 (*29*). This technology was again filed for patent in 1997 (*3*) and was initially developed to remove caffeine and chlorogenic acids as the main compounds alleviating the sensory qualities of coffee beverages. Next to caffeine and chlorogenic acid, $^\beta N$-alkanoyl-5-hydroxy-tryptamides are also discussed to have ulcerogenic effects on the gastric mucosa (*30*). However, for neither of these compounds has the stomach irritating activity in humans been proven yet, and even the results on the stomach irritating potential of steam-treated vs regular coffee are conflicting (*1*, *31*). Using human 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 to lyophilizates from regular, neither steam-treated nor decaffeinated, coffees (*6*). Putative stomach irritating coffee components were quantitated in all coffee beverages tested, but no correlation between the secretory activity and one or more constituents was found (*6*).

The work presented here aimed at the compositional characterization of a coffee beverage that effectively down-regulates

selected mechanisms of gastric acid secretion. First, a regular coffee beverage, neither steam-treated nor decaffeinated, was subjected to solvent fractionation in order to identify the polarity of compounds that affect the proton secretory activity in HGT-1 cells. In addition, each of these solvent fractions obtained from the extraction of the coffee beverage with water, ethyl acetate, dichloromethane, or pentane was analyzed for its contents of putative stomach irritating compounds, namely, caffeine, chlorogenic acid, $^\beta$N-alkanoyl-5-hydroxytryptamides, pyrogallol, and catechol as well as N-methylpyridinium as another recently identified bioactive compound in coffee beverage (*23*). The functional assays revealed a stimulating effect on the proton secretory activity for the ethyl acetate, the dichloromethane, and the pentane extracts, whereas no effect on the proton secretion was demonstrated for the water extract. This result was somewhat unexpected, as the water extract contained the majority of chlorogenic acid (95%) and $^\beta$N-alkanoyl-5-hydroxytryptamides (55%) present in the total coffee beverage. Both compounds are hypothesized to promote stomach acid secretion (*4*, *22*, *29*). However, we hypothesize that the absence of a stimulating effect on proton secretion in HGT-1 cells is due to the content of N-methylpyridinium in the water fraction which solely contained this compound. Although we could not prove this effect in experiments in which HGT-1 cells were treated with N-methylpyridinium as a single compound, addition of N-methylpyridinium to a reconstituted freeze-dried regular coffee beverage (neither steam-treated nor decaffeinated) clearly demonstrated an antisecretory effect for this compound at a concentration of about 18 mg/L in the reconstituted freeze-dried beverage (**Figure 2**). These differences between the single compound and the complex beverage seem reasonable since a coffee beverage contains a variety of components that may act as competitors for receptor binding, act as inhibitors or enhancers of functional proteins as well as for gene regulation, and finally may exhibit synergistic or antisynergistic effects.

To further evaluate the effect of coffee beverages with varying concentrations of N-methylpyridinium on cellular mechanisms of stomach acid secretion, reconstituted freeze-dried coffee beverages with high (c. Arabica, 32 mg/L; and c. Arabica Brazil mildly steam-treated, 34 mg/L), medium (c. Robusta Vietnam, 22 mg/L), and low (c. Robusta Vietnam steam-treated, 5 mg/L) N-methylpyridinium concentrations were obtained from a local coffee manufacturer. Steam-treatment was applied according to the procedure used for the production of commercial stomach-friendly-labeled coffees (*3*). The results from the functional experiments investigating the effect of the reconstituted freeze-dried coffee beverage on the proton secretory activity revealed a significantly lower secretory activity for those coffee beverages with medium or high concentrations of N-methylpyridinium as compared to the beverage with the lowest concentration. However, the least secretory activity was demonstrated for c. Robusta Vietnam, with an N-methylpyridinium concentration of 22 mg/L beverage. This result is in accordance with the findings obtained from experiments in which a reconstituted freeze-dried commercial regular coffee was fortified with N-methylpyridinium up to a final concentration of 18 mg/L beverage. Also, results on the molecular mechanisms of stomach acid secretion, such as gene regulation, signal transduction pathways, and related transcription factors demonstrate the least pro-secretory effect among the coffee beverages studied for c. Robusta Vietnam. In particular, an inducing effect was shown on the gene expression of the antisecretory receptor somatostatin (SSTR2), whereas no effect on the gene expression of the H^+,K^+-ATPase was observed. Surprisingly, the least secretory activity among the coffee samples tested was correlated with an increased gene expression of the

acetylcholine receptor (CHRM3). This result indicates that the pathways regulating stomach acid secretion differ in their effectiveness. Up-regulation of pro-secretory genes or transcription factor proteins does not necessarily result in an increased proton secretion and vice versa. This is also demonstrated by the results for the other coffee beverages studied in this work. Steam-treated c. Robusta Vietnam, for example, showed the most stimulating effect on proton secretory activity, up-regulation of the gene expression of H^+,K^+-ATPase, the key player of gastric acid secretion and of the gene expression of the pro-secretory histamine (HRH2) and acetylcholine receptor (CHRM3), and also increased cyclic AMP levels. In contrast to these pro-secretory effects, HGT-1 cells treated with c. Robusta Vietnam showed decreased activation of ERK1/2, but increased concentrations of cyclic AMP, both involved in pro-secretory pathways.

In conclusion, we have shown for the first time that regulation and function of the proton secretory activity of human stomach cells induced by coffee beverages depend on their concentrations of N-methylpyridinium. The impact of other components such as caffeine, chlorogenic acid, or $^\beta$N-alkanoyl-5-hydroxytryptamides has to be elucidated in future multiparametric studies.

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