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Identification of Organic Acids in Wine That Stimulate Mechanisms of Gastric Acid Secretion

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ABSTRACT: Wine may cause stomach irritation due to its stimulatory effect on gastric acid secretion, although the mechanisms by which wine or components thereof activate pathways of gastric acid secretion are poorly understood. Gastric pH was measured with a noninvasive intragastric probe, demonstrating that administration of 125 mL of white or red wine to healthy volunteers stimulated gastric acid secretion more potently than the administration of equivalent amounts of ethanol. Between both beverages, red wine showed a clear trend for being more active in stimulating gastric acid secretion than white wine (p = 0.054). Quantification of the intracellular proton concentration in human gastric tumor cells (HGT-1), a well-established indicator of proton secretion and, in turn, stomach acid formation in vivo, confirmed the stronger effect of red wine as compared to white wine. RT-qPCR experiments on cells exposed to red wine also revealed a more pronounced effect than white wine on the fold change expression of genes associated with gastric acid secretion. Of the quantitatively abundant organic acids in wine, malic acid and succinic acid most actively stimulated proton secretion in vitro. However, addition of ethanol to individual organic acids attenuated the secretory effect of tartaric acid, but not that of the other organic acids. It was concluded that malic acid for white wine and succinic acid for red wine are key organic acids that contribute to gastric acid stimulation.

KEYWORDS: *red wine, white wine, ethanol, organic acids, gastric acid secretion*

INTRODUCTION

Wine consumption is known to increase gastric acid secretion[1−3] and to induce reflux in patients with gastroesophageal reflux disease (GERD)[4] as well as in healthy subjects.[4,5] Chronic gastric acid secretion may cause gastric irritation such as ulcer disease,[6] heartburn, and GERD,[7] which may lead to adenocarcinomas in the lower esophagus.[8] Subjects with these diseases are often advised to refrain from drinking alcoholic beverages such as wine. Approximately 15% of the world's population suffers from GERD.[9] In an unselected population-based study in Japan, 82,894 subjects between the ages of 30 and 89 completed a questionnaire asking for symptoms of heartburn. The prevalence of heartburn, a typical GERD symptom, was high in about 20% of the subjects.[10] In another population-based study, conducted in Germany, an even higher GERD prevalence of 34% was reported.[11]

In the past few decades, several studies have investigated the influence of alcoholic beverages on gastric acid secretion using intragastric titration in humans[1−3,12] as well as in animals, such as rats[13] or dogs,[14] or in isolated gastric glands from rabbits.[15] To our knowledge, there have been only two studies comparing the effect of red wine and white wine on gastric acid secretion. In a human intervention trial, Peterson and colleagues administered 300 mL of either red or white wine to healthy subjects and did not observe any difference in gastric acid secretion.[2] In contrast, Tsukimi et al.[14] demonstrated a significantly stronger stimulating effect for red wine as compared to white wine after administering amounts ranging from 25 to 100 mL to dogs with vagally denervated Heidenhain pouches.

Apart from wine, other alcoholic beverages have been studied for their effects on gastric acid secretion. One of the major findings was that fermented alcoholic beverages are strong stimulants of gastric acid secretion, whereas spirits with a higher ethanol concentration showed very little or no effect.[1,3] These results indicate that the acid stimulatory effect of alcoholic beverages derives not just from ethanol, indicating the presence of other stimulating components.[1−3,13] Following this hypothesis, Teyssen and colleagues investigated fractions of fermented glucose and identified maleic acid and succinic acid as strong stimulants of gastric acid secretion. Other organic acids detected in the fermentation mixture, such as acetic acid, oxalic acid, and lactic acid, showed no influence. Hence, the authors hypothesized that the length of the carbon chain and the two carboxylic groups are the main determinants of a molecule's effect on stomach acid secretion[12] (Table 1). These structural characteristics are also found in other organic acids of wine, including tartaric acid, malic acid, and citric acid (Table 1). None of these organic acids has been investigated for its effects on mechanisms of stomach acid secretion in wine representative concentrations, although tartaric acid and malic acid are the predominant organic acids in wine and significantly contribute to its pH.[16]

Gastric acid secretion takes place in the parietal cells of the stomach and is regulated by a number of cell surface receptors as well as functional and signaling proteins. Activation of cell surface receptors of parietal cells leads to signal transductions in which hormones and second messengers activate the key element in the complex process of gastric acid secretion, the H^+,K^+-ATPase (coded by the gene *ATP4A*). Activation of the H^+,K^+-ATPase leads to transport of hydrogen ions into the gastric lumen in exchange for potassium ions. The histamine

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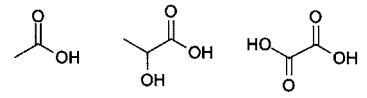
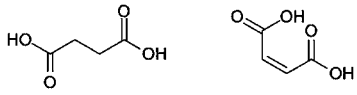
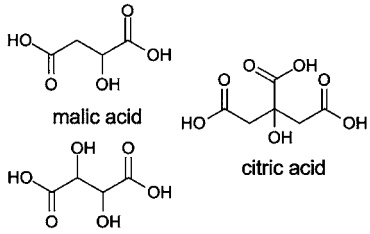
Table 1. Molecular Structures of Organic Acids as Putative Stimulants of Gastric Acid Secretion

Low effect on gastric acid secretion (12)	 acetic acid, lactic acid, oxalic acid
Strong effect on gastric acid secretion (12)	 succinic acid, maleic acid
Unknown effect on gastric acid secretion	 malic acid, citric acid, tartaric acid

H2 receptor (coded by the gene *HRH2*) and the acetylcholine M3 receptor (coded by the gene *CHRM3*) have been determined to initiate these signal transduction pathways that regulate the expression of the respective prosecretory genes further downstream. The only cell surface receptor known to inhibit secretion is the somatostatin receptor (coded by the gene *SSTR2*). These cell surface receptors and their respective ligands, histamine, acetylcholine, and somatostatin, play a crucial role in the regulation of gastric acid secretion[17,18] and are expressed in the human gastric tumor cell line HGT-1, which has been established in our group for the identification of stomach acid regulating compounds in coffee and beer.[19−24]

The aim of the present study was to identify the impact of white and red wine organic acids and ethanol on mechanisms of gastric acid secretion. We measured the intragastric pH in six volunteers after consumption of white wine, red wine, or ethanol using a noninvasive intragastric pH-probe. Mechanisms of stomach acid secretion were studied by analyzing the intracellular proton concentration as a measure of proton secretory activity in HGT-1 cells by means of a pH-sensitive dye and by determining the expression of the *ATP4A*, *CHRM3*, *HRH2*, and *SSTR2* genes by RT-qPCR. Finally, we compared the effects of wines with those of the organic acids tartaric acid, malic acid, citric acid, succinic acid, and lactic acid and of ethanol in wine representative concentrations by analyzing the intracellular proton concentration in HGT-1 cells.

MATERIALS AND METHODS

Chemicals. Cell culture materials such as Dulbecco's Modified Eagle Medium (DMEM), trypsin, glutamine, penicillin/streptomycin, and histamine as well as L-(+)-tartaric acid, succinic acid, and DL-lactic acid were obtained from Sigma-Aldrich. Fetal bovine serum was purchased from Invitrogen, Karlsruhe, Germany.

Citric acid and L-malic acid were included in the enzyme kits from R-Biopharm (Roche, Darmstadt, Germany) and used for their quantitative analysis. 1,5-Carboxy-seminaphtorhodafluor acetoxymethyl ester (SNARF-1-AM) and nigericin were obtained from Invitrogen. For RNA isolation, we used the RNeasy Mini Kit obtained by Qiagen, Hilden, Germany, and the SV Total RNA Isolation System obtained from Promega, Madison, WI, USA. High Capacity RNA to cDNA Master Mix was purchased from Applied Biosystems, Munich, Germany.

Samples. A total of five red wine samples of the variety "Blauer Zweigelt Klassik" and five white wine samples of the variety "Grüner Veltliner", both produced in 2009 by Wegenstein, Niederösterreich (Lower Austria), Austria, were purchased from a local store (Table 2). Edible ethanol (96%) was obtained from a local pharmacy and diluted to a concentration of 12% v/v with double-distilled water. In the cell culture experiments, samples were diluted 1:100 or 1:10 in DMEM.

Determination of Wine Buffer Capacity. The buffering capacity of 125 mL of each wine with (buffer capacity 1) and without (buffer capacity 2) 5 mL of saturated $NaHCO_3$ was determined by titration with 1 N HCl from initial pH to pH 1.5 using a pH-meter pH 211 (HANNA Instruments, BW, Germany).

Photometric and Enzymatic Quantification of Organic Acids in Wine. Tartaric acid was quantified through its reaction with vanadate and photometrically determined at a wavelength of 530 nm, as described by Matissek et al.[25] Citric acid and L-malic acid were determined using enzymatic kits from R-Biopharm (Roche). Here, citric acid quantification is based on the conversion of citrate into oxaloacetate and acetate in the presence of citrate lyase. Oxaloacetate and acetate are reduced in the presence of L-malate dehydrogenase and L-lactate dehydrogenase by reducing nicotinamide adenine dinucleotide (NADH). The decrease of NADH is photometrically determined at a wavelength of 340 nm and is stochiometric to the amount of citrate. The L-malic acid enzyme kit is based on the oxidization of L-malic acid to oxaloacetate by NADH in the presence of L-malate

Table 2. Primers Used for Gene Expression Analysis of *ATP4A*, *HRH2*, *CHRM3*, and *SSTR2* with *PPIA* as Housekeeping Gene[19,21,23]

direction	gene	sequence (5′−3′)	product length (bp)
forward	*PPIA*	CCA CCA GAT CAT TCC TTC TGT AGC	
reverse	*PPIA*	CTG CAA TCC AGC TAG GCA TGG	144
forward	*ATP4A*	CGG CCA GGA GTG GAC ATT CG	
reverse	*ATP4A*	ACA CGA TGG CGA TCA CCA GG	176
forward	*CHRM3*	AGC AGC AGT GAC AGT TGG AAC	
reverse	*CHRM3*	CTT GAG CAC GAT GGA GTA GAT GG	117
forward	*HRH2*	TGG GAG CAG AGA AGA AGC AAC C	
reverse	*HRH2*	GAT GAG GAT GAG GAC CGC AAG G	154
forward	*SSTR2*	TCC TCC GCT ATG CCA AGA TGA AG	
reverse	*SSTR2*	AGA TGC TGG TGA ACT GAT TGA TGC	189

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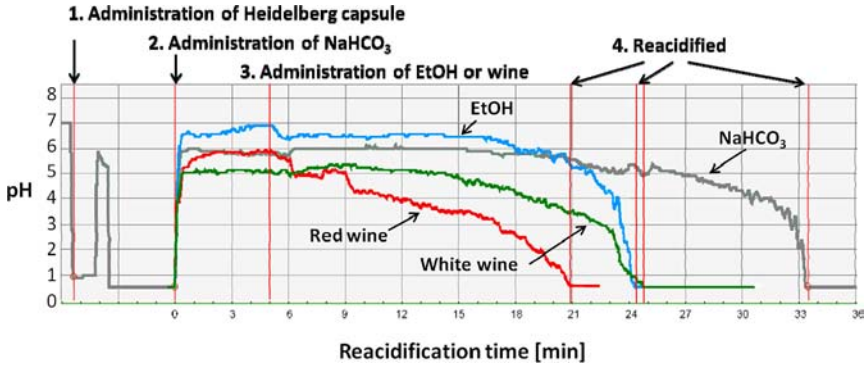

Figure 1. Gastrogram of four Heidelberg capsule measurements from one test subject. At 0 min, the pH was brought up to about 6 by administration of 5 mL of a saturated $NaHCO_3$ solution. After 5 min, either nothing (gray line) or 125 mL of ethanol (blue line), white wine (green line), or red wine (red line) was administered.

dehydrogenase. The amount of NADH formed is stochiometric to the L-malic acid content.

For succinic acid and DL-lactic acid quantification we used enzymatic test kits from Megazyme International (Wicklow, Ireland). For succinic acid quantification, the decrease of NADH indicates the conversion of succinate into succinyl-CoA measured photometrically at a wavelength of 340 nm. For DL-lactic acid quantification, also the increase of NADH is measured, which indicates the oxidation of DL-lactic acid to pyruvate. Absorptions were measured using an Infinite 200 PRO Plate Reader (Tecan, Männedorf, Switzerland). Analyses were done according to the protocols of the distributor.

Subjects. Six healthy, female volunteers between 25 and 30 years of age with a body mass index between 19.6 and 32.3 kg/m^2 were studied. None of them had been diagnosed for gastrointestinal disease, and none took any medication or antibiotics for 2 months prior to the tests. Habitual alcohol consumption was <20 g of pure alcohol per day. Each volunteer was fully informed about the test, gave written consent, and was treated following the ethical principles of the declaration of Helsinki. The trial subjects had to fast from food and liquid for 10 h prior to the intervention. During the experiment, the subjects remained in a supine left-sided position.

Analyses of Gastric pH in Healthy Subjects. The intragastric pH was analyzed by means of a Heidelberg Detection System (Heidelberg Medical Inc., Mineral Bluff, GA, USA). This test system has been approved by the U.S. Food and Drug Administration for measuring the intragastric pH. The system consists of a pH-sensitive capsule (called a Heidelberg capsule) that has to be swallowed and contains a miniature radio transmitter. A transceiver placed on the abdomen of the volunteer receives the signal and sends it to the recorder connected to a computer.

Prior to administration, Heidelberg capsules were activated for 5 min in a 0.9% sterile filtered NaCl solution (filter pore size = 0.22 μm) and calibrated using two calibration points, pH 1 and 7. Afterward, the subjects swallowed the capsule. When the intragastric pH was constant at pH <1 for at least 3 min, the capsule was considered to be in the stomach. Afterward, each trial started with the administration of 5 mL of a saturated sodium bicarbonate solution ($NaHCO_3$). This alkaline challenge triggers a rise in stomach pH and subsequently leads to the secretion of stomach acid by the parietal cells (Figure 1).

Each volunteer completed at least four interventions. In the first intervention, the volunteer was administered 5 mL of $NaHCO_3$ solely. To test the effect of the samples, the subject received, first, the alkaline solution, and, second, 5 min later, 125 mL of either white wine, red wine, or ethanol (12% v/v).

Reacidification time was analyzed with the Heidelberg Detection System software (Figure 1). The area under the curve (AUC) and the slope of the reacidification plot over time were analyzed using the software ImageJ 1.43 (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The slope of the curve was calculated from the point of the start of acid secretion to the end point at which the initial baseline pH was reached and is given as pH/min. Data are presented as AUC per minute of reacidification time normalized to the buffer capacity.

Cell Culture. The human gastric tumor cell line HGT-1 was obtained from Dr. C. Laboisse (Laboratory of Pathological Anatomy, Nantes, France). The cells were cultured under standard conditions at 37 °C, 95% humidity, and 5% CO_2. DMEM with 4 g/L glucose was used as culture medium and supplemented with 10% fetal bovine serum, 2% L-glutamine, and 1% penicillin/streptomycin.

Cytotoxicity Test. Cellular viability was tested by trypan blue staining using a hemocytometer (Brand, Wertheim, Germany). A total of about 560,000 HGT-1 cells was seeded per well in a 24-well plate (Greiner Bio-One, Kremsmünster, Austria) and allowed to settle for 24 h at 37 °C and 5% CO_2. Cells were washed once with Krebs–HEPES–buffer (KRHB; 10 mM HEPES, 11.7 mM D-glucose, 4.7 mM KCl, 130 mM NaCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4, brought to a pH of 7.4 with 5 M KOH) and treated with dilutions from 1:500 to 1:5 of red wine, white wine, or 12% v/v ethanol during incubations at 37 °C, 95% humidity, and 5% CO_2 for up to 45 min, depending on the subsequent analysis. Then, the cells were washed twice with KRHB, harvested with trypsin, and stained with trypan blue. The number of living cells as well as blue-colored dead cells was counted with a hemocytometer. The viability of treated cells was calculated and compared to the viability of nontreated cells (=100%). Three biological with two technical replicates per sample were measured.

Determination of the Intracellular pH in HGT-1 Cells. The intracellular pH (pH_i) was measured as an indicator for proton secretion with the pH-sensitive fluorescence dye SNARF-1-AM. A total of 100,000 viable cells per well was spread in a white 96-well plate and allowed to settle for 24 h at 37 °C, 95% humidity, and 5% CO_2. Cells were washed once with KRHB and incubated at previous conditions for 30 min with the fluorescence dye SNARF-1-AM at a concentration of 3 μM.[19,20,23,24] Afterward, cells were washed twice with KRHB and treated with 100 μL of the diluted sample in DMEM for 10 min. Cells treated with 1 mM histamine were used as positive control. Nontreated cells were used as control and compared to cells treated with different concentrations of the wine samples or ethanol. The organic acids tartaric acid, citric acid, malic acid, succinic acid, lactic acid, and a combination thereof as recombinate were tested in a 1:100 dilution of their respective concentration in white and red wines. In this high dilution of 1:100, any pH effects originating from tested compounds can be excluded. Furthermore, we tested the influence of ethanol on the effect induced by the organic acids by adding ethanol in the respective concentration of wine to the organic acids, the recombinate, and red and white wines to which ethanol was added to reach a 2-fold higher concentration compared to the original product.

Treatment was followed by a washing step with KRHB. Afterward, 100 μL of KRHB was added and the 96-well plate was placed into an Infinite 200 PRO Plate Reader. Fluorescence was analyzed at an excitation of 488 nm and emission wavelengths of 580 and 640 nm. The ratio of the fluorescence intensities from those two emission

Table 3. Buffer Capacity, pH, Ethanol Content, and Organic Acid Content of White Wine and Red Wine[a]

	white wine	red wine	ethanol
pH	3.5 ± 0.0	3.6 ± 0.1	6.5 ± 0.7
buffer capacity 1 (mmol HCl)	11.1 ± 0.1	11.8 ± 0.4	5.5 ± 1.4
buffer capacity 2 (mmol HCl)	15.9 ± 0.8	16.0 ± 0.3	10.2 ± 0.2
ethanol (% v/v)	11.5	13	12
malic acid (g/L)	2.42 ± 0.03	0.021 ± 0.004 ***	
tartaric acid (g/L)	1.86 ± 0.12	1.75 ± 0.19	
lactic acid (g/L)	0.44 ± 0.01	1.69 ± 0.07 ***	
citric acid (g/L)	0.30 ± 0.03	0.19 ± 0.05 **	
succinic acid (g/L)	0.27 ± 0.01	0.57 ± 0.05 ***	

[a]Buffer capacity is given as consumption of HCl determined by titration of 125 mL of the samples with (buffer capacity 1) and without (buffer capacity 2) 5 mL of saturated $NaHCO_3$ to pH 1.5 of white wine, red wine, and ethanol. Data are given as the mean ± SD from triplicate analyses (statistics: two-tailed t test; significant differences vs concentrations of white wine are indicated by ** = $p < 0.01$ and *** = $p < 0.001$).

wavelengths allows an accurate determination of pH when plotted on a calibration curve.[19−24]

A calibration curve was generated for each experiment by staining the cells in potassium buffer solutions of varying pH values, ranging from 7.2 to 8.2 adjusted with NaOH using a pH-meter pH 211 (HANNA Instruments), in the presence of 2 μM nigericin to equilibrate intracellular pH (pH_i) and extracellular pH (pH_{ex}). The potassium buffer calibration solutions for the intracellular pH measurement consisted of 20 mM NaCl, 110 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 18 mM D-glucose, and 20 mM HEPES. The pH_i calibration was fit to a linear regression. Intracellular H^+ concentration was calculated from the pH_i. The intracellular proton index (IPX) was calculated by log 2 transformation of the intracellular proton concentration ratio between treated cells and control cells.[19−21,23] The effect of ethanol addition to the organic acids on IPX values is shown as percent difference.

Because the ethanol concentration of white wine was 11.5%, whereas red wine contained 13%, we used a 12% ethanol solution as control solution in the Heidelberg capsule experiments. Prior to the Heidelberg experiment, these three ethanol concentrations (11.5, 12, and 13% v/v) were tested in a 1:100 dilution in HGT-1 cells and did not show any different effects on the IPX (data not shown).

RNA Isolation and cDNA Synthesis. In six-well plates, 100,000 HGT-1 cells/well were seeded and grown until confluence. Then, cells were treated with a 1:100 dilution of white wine, red wine, or ethanol and a 1:10 dilution of white wine or ethanol for 5, 10, 15, 20, 25, 30, and 45 min. Afterward, cells were harvested for RNA isolation using the RNeasy Mini Kit and the SV Total RNA Isolation System. Quantity and quality of RNA were checked spectrophotometrically at 260 nm and by calculation of the ratio of 260 and 280 nm using the nanoquant plate for the Infinite 200 PRO Plate Reader. All samples used were in a ratio between 1.8 and 2.2. High-capacity RNA to cDNA Master Mix (Applied Biosystems, Munich, Germany) was used for cDNA synthesis following the manufacturer's protocol.

Gene Expression Assays. Primers for the H^+,K^+-ATPase α-subunit (*ATP4A*), the histamine H2 receptor (*HRH2*), the somatostatin receptor (*SSTR2*), and the acetylcholine receptor M3 (*CHRM3*) (Table 2) were designed and validated previously.[19,21] Peptidylprolyl isomerase A (*PPIA*) was used as housekeeping gene. Real-time PCR assays were performed on a StepOne plus (Applied Biosystems) using the Fast SYBR green master mix (Applied Biosystems). Cycling conditions were set as follows: 20 s/95 °C (activation), 3 s/95 °C (denaturation), 30 s/60 °C (annealing), 15 s/72 °C (elongation with fluorescence measurement). Cycling conditions for *HRH2* were set to 20 s/95 °C, 3 s/95 °C, 30 s/62 °C, and 15 s/77 °C, respectively.

Statistical Analysis. Statistical analysis was performed using Excel 2007 (Microsoft, Seattle, WA, USA) and Sigma Plot software 11.0 (Systat Software, Erkrath, Germany). Outliers were excluded by Nalimov outlier analysis. Significant differences in the human intervention trial between samples were tested by a one-way ANOVA with Holm−Sidak post hoc analysis and a one-sided paired Student's t test. The cytotoxicity of the samples on HGT-1 cells compared to nontreated cells was determined with the two-tailed Student's t test and considered to be significant at $p < 0.05$. Significant differences in the data set of the proton secretory analysis were determined by a one-way ANOVA with Holm−Sidak post hoc analysis and the two-tailed Student's t test. For analyzing time-dependent effects on gene expression, we performed the two-way ANOVA with Holm−Sidak post hoc analysis. At least three biological replicates and two technical replicates were analyzed for each cell culture experiment. Data under Results as well as in diagrams are given as the mean ± SEM, unless indicated otherwise.

RESULTS

Effect of White Wine, Red Wine, and Ethanol on Gastric Acid Secretion in Healthy Volunteers Determined in Vivo by Gastric pH Measurement. To determine the influence of white wine and red wine on gastric acid secretion in comparison to a 12% v/v ethanol solution, we measured the stomach pH of six fasted volunteers by means of a noninvasive pH-sensitive intragastric probe. First, the subject's gastric pH was challenged by a 5 mL solution of saturated $NaHCO_3$, resulting in a stable pH of 5−7 for at least 5 min postload and a mean reacidification time of 35.4 ± 6.3 min. Intervention with the 12% v/v ethanol solution resulted in a clear trend toward a shorter reacidification time of 23.9 ± 2.6 min compared to 25.3 ± 3.5 min ($p < 0.70$) and 27.2 ± 3.3 min ($p < 0.57$) for red wine and white wine, respectively (data not shown). Next to the reacidification time, the slope of the reacidification curve is a valuable measure of a compound's effect on gastric acid secretion: the greater the slope, the faster the pH is falling. Administration of the 12% v/v ethanol solution (−0.76 ± 0.09 pH/min) caused a stronger decline compared to red wine (−0.41 ± 0.05 pH/min, p vs ethanol = 0.016), white wine (−0.48 ± 0.06 pH/min, p vs ethanol = 0.021), and the saturated $NaHCO_3$ solution (−0.54 ± 0.05 pH/min, p vs ethanol = 0.201; data not shown). Because the lower buffering capacity of ethanol compared to wine (Table 3) might affect these results, we normalized the AUC to reacidification time and buffer capacity. Reacidification parameters of the saturated $NaHCO_3$ solution administered alone were defined as control and were set to 100%. All treatments were compared to this control. Thus, a lower percent value refers to a stronger acid secretion. Administration of red wine and white wine as well as 12% v/v ethanol significantly ($p < 0.001$) increased gastric acid secretion compared to the saturated $NaHCO_3$ solution alone. Red wine (12.8 ± 1.5%) showed a clear trend for the strongest stimulation of gastric acid secretion compared to white wine

(14.5 ± 1.6%, p vs red wine = 0.054) and ethanol (22.5 ± 0.7%, p vs red wine < 0.001; data not shown). For illustration, Figure 2 shows a typical gastrogram from one study subject.

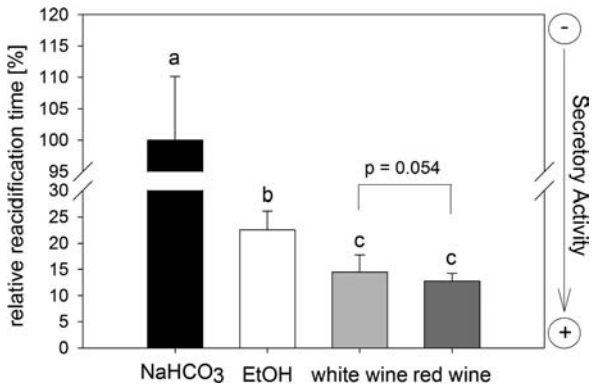

Figure 2. In vivo determination of gastric pH using Heidelberg-pH-probe. Displayed data refer to AUC/min normalized to buffer capacity results from administration of $NaHCO_3$, ethanol (EtOH) 12% v/v, white wine, and red wine; 5 mL of $NaHCO_3$ alone was set to 100%, and data are displayed as the mean ± SEM, $n = 6$ (statistics: one-way ANOVA with Holm−Sidak post hoc test; letters indicate significant differences between groups; $p < 0.001$ and a one-tailed t test between red wine and white wine).

Cytotoxicity of White Wine, Red Wine, and Ethanol in HGT-1 Cells. We conducted a trypan blue toxicity test for testing which concentrations of wine and ethanol can be used in cell culture experiments without exhibiting cytotoxic effects. Results are shown in Table 4. Red wine was toxic in a 1:10 dilution but not in a 1:100 dilution, whereas white wine and ethanol in a dilution of 1:10 showed no toxicity (Table 4). Therefore, all cell culture experiments were carried out in dilutions of 1:10 and 1:100 using ethanol and white wine, whereas red wine was tested only in a 1:100 dilution.

Table 4. Cell Viability in Percent versus Nontreated Cells (Control): 100 ± 2%[a]

dilution abs	12% v/v ethanol	white wine	red wine
1:500	nd	nd	99 ± 3
1:250	nd	nd	98 ± 1
1:100	nd	99 ± 1	95 ± 3
1:10	99 ± 2	95 ± 4	37 ± 8 ***
1:5	98 ± 2	80 ± 12 *	4 ± 1 ***

[a]Data are given as the mean ± SD, $n = 3$, tr = 2. nd, not determined (statistics: two-tailed t test vs control; significant differences vs nontreated control cells are indicated by * = $p < 0.05$ and *** = $p < 0.001$).

Effect of White Wine, Red Wine, and Ethanol on Intracellular Proton Concentrations in HGT-1 Cells. To study the influence of wine and ethanol on mechanisms of proton secretion in parietal HGT-1 cells, we measured the intracellular pH using the pH-sensitive dye SNARF-AM and analyzed the data as the IPX. The lower the proton concentration in the cell, the lower the IPX and the stronger is the proton secretion[19,20,22−24] HGT-1 cells treated with histamine (1 mM), a physiological stimulant of gastric acid secretion,[17] resulted in a significant decrease of the IPX (−0.21 ± 0.03; $p < 0.001$) compared to nontreated cells (Figure 3). Ethanol and white wine in 1:10 dilutions significantly decreased

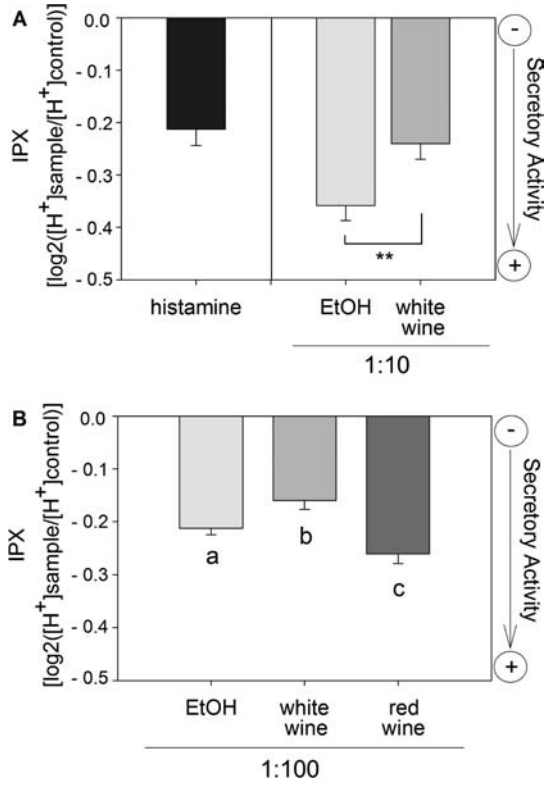

Figure 3. Intracellular proton index (IPX) of HGT-1 cells treated for 10 min with histamine (1 mM): (A) 1:10 or (B) 1:100 dilution of ethanol (EtOH, 12% v/v), white wine, or red wine. Data are displayed as the mean ± SEM, $n > 4$; tr = 3−6 (statistics: (A) two-tailed t test, ** = $p < 0.01$; (B) one-way ANOVA with the Holm−Sidak post hoc test; letters indicate significant differences between groups, $p < 0.05$).

the IPX compared to nontreated cells ($p < 0.001$) (Figure 3A). The IPX values after the cell's treatment with dilutions of 1:10 of white wine and ethanol were −0.24 ± 0.03 and −0.36 ± 0.03, respectively. Comparison of the effects of 1:100 dilutions of red wine (Figure 3B), white wine, and 12% v/v ethanol demonstrated a significantly stronger decrease of the intracellular pH, as indicator of a higher proton secretion, for red wine (IPX = −0.26 ± 0.02) compared to white wine (IPX = −0.16 ± 0.02, $p < 0.001$) and ethanol (IPX = −0.21 ± 0.01, $p = 0.02$). However, 12% v/v ethanol in dilutions of both 1:10 and 1:100 stimulated proton secretion, as indicated by a lower IPX in HGT-1 cells, more potently than white wine (1:100, $p < 0.05$; 1:10, $p < 0.01$).

Influence of White Wine, Red Wine, and Ethanol on Gene Expression of *ATP4A*, *HRH2*, *CHRM3*, and *SSTR2*. A time course experiment was performed to investigate the influence of a 1:100 dilution of white wine, red wine, and ethanol and, additionally, a 1:10 dilution of white wine and ethanol on the expression of genes involved in the regulation of gastric acid secretion.[19,21−23] Gene expression of *ATP4A*, *HRH2*, *CHRM3*, and *SSTR2* was measured by qPCR. Gene expression of *PPIA* served as the control. Results are given as relative gene expression; treated cells were compared to nontreated cells (control = 1) (Figure 4).

The ratios of gene expression for the target genes, compared to the housekeeping gene *PPIA*, were determined. Treatment with red wine in a 1:100 dilution for 10 min (*ATP4A*, 1.40 ± 0.35; *HRH2*, 1.62 ± 0.36; *CHRM3*, 1.88 ± 0.36; *SSTR2*, 1.85 ± 0.33) and 15 min (*ATP4A*, 1.46 ± 0.21; *HRH2*, 1.91 ± 0.21;

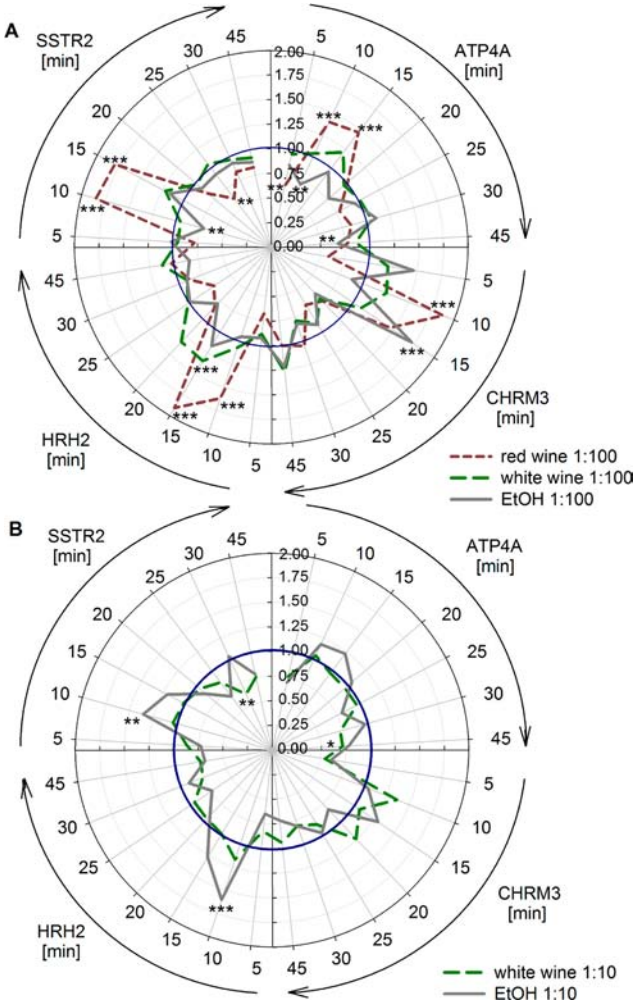

Figure 4. Time-dependent indices of gene expression for the *ATP4A*, *CHRM3*, *HRH2*, and *SSTR2* in HGT-1 cells after treatment with (A) 1% ethanol (12% v/v), white wine, and red wine or (B) 10% ethanol (EtOH, 12% v/v) and white wine compared to nontreated cells. Data are displayed as mean values, $n = 3–4$, tr = 3 (statistics: two-way ANOVA with the Holm−Sidak post hoc test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

CHRM3, 1.46 ± 0.17; *SSTR2*, 1.80 ± 0.16) resulted in the most pronounced change in gene expression of all four genes when compared to the cells' treatment with white wine and ethanol. Whereas treatment of the cells with the 1:100 dilution of ethanol increased the *CHRM3* expression after 15 min (1.72 ± 0.40, $p < 0.001$), administration of the higher concentration of 1:10 ethanol increased *HRH2* (1.60 ± 0.35, $p < 0.001$) and *SSTR2* (1.36 ± 0.21, $p = 0.002$) expression already after 10 min of exposure. Treatment of the HGT-1 cells with the 1:100 dilution of white wine significantly increased the expression of *HRH2* (1.35 ± 0.22, $p < 0.001$). Additionally, the 1:10 dilution of white wine decreased the expression of *SSTR2* (0.62 ± 0.04, $p = 0.005$) and *ATP4A* (0.72 ± 0.04, $p = 0.012$) after 30 and 45 min.

Quantification of Organic Acids in Wine. Organic acids in the wine samples were quantified to apply wine representative concentrations in the experiments. The composition of organic acids in the two wines varied considerably (Table 3). The concentration of malic acid was much higher in white wine (2.42 ± 0.03 g/L) compared to red wine (0.021 ± 0.004 g/L). In contrast, the concentration of lactic acid was higher in red wine (1.69 ± 0.07 g/L) compared to white wine (0.44 ± 0.01 g/L). The concentration of succinic acid in red wine (0.57 ± 0.05 g/L), which has been identified as a strong stimulant of gastric acid secretion,[12] was double that of white wine (0.27 ± 0.01 g/L).

Effect of Organic Acids in Wine Representative Concentrations on Intracellular Proton Concentrations in HGT-1 Cells. We tested tartaric acid, citric acid, malic acid, succinic acid, and lactic acid individually and as a recombinate in concentrations representing a 1:100 dilution of white and red wines (Figure 5). All organic acids in the respective concentration of white wine and red wine significantly ($p < 0.001$) stimulated gastric acid secretion compared to nontreated cells.

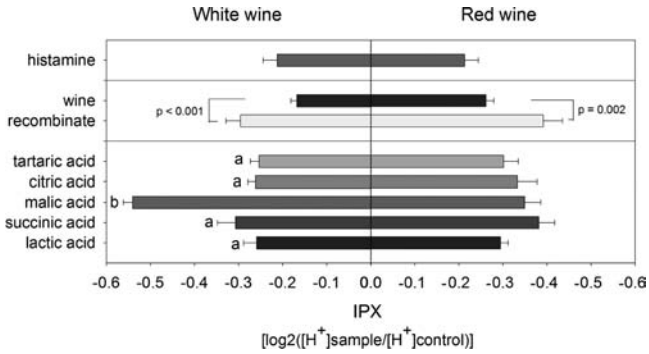

Figure 5. Intracellular proton index (IPX) of HGT-1 cells treated for 10 min with organic acids in 1:100 dilutions of white and red wine representative concentrations in the absence of ethanol. White wine representative concentrations: 18.3 mg/L tartaric acid, 3.0 mg/L citric acid, 24.2 mg/L malic acid, 2.7 mg/L succinic acid, 4.4 mg/L lactic acid, and a recombinate of those acids in these concentrations. Red wine representative concentrations: 17.5 mg/L tartaric acid, 1.9 mg/L citric acid, 0.21 mg/L malic acid, 5.7 mg/L succinic acid, 16.9 mg/L lactic acid, and a recombinate of those acids in these concentrations. The control was nontreated cells. Data are displayed as the mean ± SEM, $n \geq 3$; tr = 3–6 (statistics: wine vs recombinate, two-tailed *t* test; organic acids vs each other, one-way ANOVA with the Holm−Sidak post hoc test; letters indicate significant differences between groups, $p < 0.05$).

In white wine, malic acid was the most abundant organic acid with a concentration of 24.2 mg/L and showed the strongest stimulation of proton secretion of the tested organic acids as indicated by an IPX value of −0.54 ± 0.02. The white wine recombinate of organic acids (IPX = −0.30 ± 0.03) showed a significantly ($p < 0.001$, two-tailed *t* test) stronger effect on proton secretion, as indicated by a lower IPX, compared to white wine (IPX = −0.16 ± 0.02).

In red wine representative concentrations, the effects of singly applied organic acids were not significantly different from each other. However, succinic acid was very potent, resulting in the lowest IPX value of −0.38 ± 0.04. Although this result was statistically not different from the IPX values obtained for the other organic acids, there was a clear trend for a higher proton secretory potential of succinic acid applied in a concentration of 5.7 mg/L compared to lactic acid ($p = 0.12$) and tartaric acid ($p = 0.18$), which were applied in higher concentrations of 16.9 and 17.5 mg/L, respectively. The organic acid recombinate (IPX = −0.36 ± 0.04) stimulated proton secretion more strongly than red wine (IPX = −0.26 ± 0.02; two-tailed *t* test, $p < 0.01$).

Effect of the Addition of Ethanol to Wine and to Organic Acids in Wine Representative Concentrations on Intracellular Proton Concentrations in HGT-1 Cells. To study whether ethanol, as a major compound in wine, interacts with the individual organic acids and with the organic acid recombinate to modulate proton secretion in HGT-1 cells, we added ethanol in wine representative concentrations (12% 1:100 diluted) (Figure 6). When ethanol was applied concomitantly to the individual organic acids and to the recombinate, only the effect of tartaric acid in white wine representative concentrations (18.6 mg/L) was reduced significantly by −36.6 ± 8.80% ($p < 0.01$) and by 30.4 ± 4.40% ($p < 0.05$) in red wine representative concentrations (17.5 mg/L). Addition of ethanol to organic acid recombinates of both wines did not significantly changed the IPX value. However, doubling the ethanol concentration in red wine or white wine attenuated the proton secretory effect of red wine by −31.9 ± 7.86% ($p < 0.001$), whereas the effect of white wine (0.84 ± 11.64%) remained unchanged compared to the effect demonstrated for the original sample.

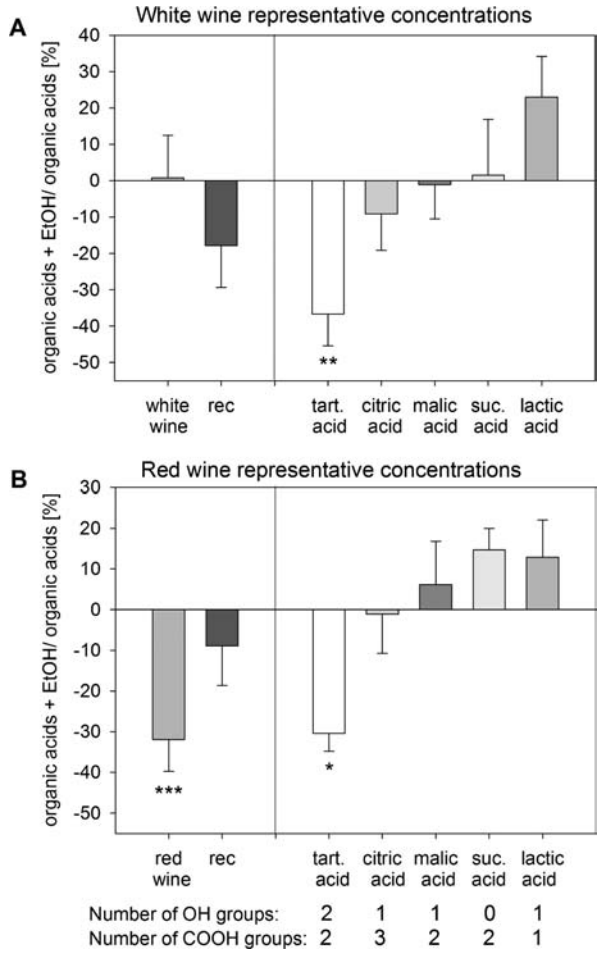

Figure 6. Intracellular proton index (IPX) of HGT-1 cells treated for 10 min with organic acids in concentrations of white wine (A) or red wine (B) with ethanol, shown as difference in percentage to organic acids without ethanol. White wine representative concentrations: 18.3 mg/L tartaric acid (tart. acid), 3.0 mg/L citric acid, 24.2 mg/L malic acid, 2.7 mg/L succinic acid (suc. acid), 4.4 mg/L lactic acid, and a recombinate (rec) of those acids in these concentrations. Red wine representative concentrations: 17.5 mg/L tartaric acid (tart. acid), 1.9 mg/L citric acid, 0.21 mg/L malic acid, 5.7 mg/L succinic acid (suc. acid), 16.9 mg/L lactic acid, and a recombinate (rec) of those acids in these concentrations. Data are displayed as the mean ± SEM, $n \geq 3$; tr = 3−6 (statistics: A and B, two-tailed t test, effect of organic acids without ethanol vs effect of organic acids with ethanol; *** = $p < 0.001$; ** = $p < 0.01$ * = $p < 0.05$).

DISCUSSION

The purpose of this study was to identify whether organic acids and ethanol in white wine and red wine contribute to their effects on gastric acid secretion. Gastric acid secretion in healthy subjects after administration of white wine, red wine, or ethanol was studied by means of pH-sensitive Heidelberg capsules. Molecular mechanisms of gastric acid secretion in the presence of white or red wine, ethanol, or organic acids were also studied in human gastric tumor cells (HGT-1), a cell line that has been established in our group for the identification of coffee and beer compounds that regulate mechanisms of stomach acid secretion.[19−24]

Organic acids have been shown to stimulate mechanisms of gastric acid secretion in humans.[12,22] The different processing technologies of white and red wine result in a characteristic, wine-specific composition of organic acids. In red wine, a second fermentation, the malolactic fermentation, is commonly used to reduce the amount of the sourer tasting malic acid by converting it into lactic acid, which has a less sour taste.[26] In white wine, by contrast, a sourer taste is preferred and the malolactic fermentation is typically not applied. Therefore, we investigated the contribution of organic acids to the stimulatory potential of white and red wine on stomach acid secretion and mechanisms thereof.

First, we conducted a human intervention study to investigate whether red and white wines have different effects on stomach acid secretion. Here, we show that administration of 125 mL of either red wine or white wine stimulated gastric acid secretion. These effects were even stronger than the effect of an equivalent amount of ethanol. The finding that red and white wine stimulate gastric acid secretion is in agreement with results from previous studies in which 300−500 mL of white[1,3] or red wine[2] instilled intragastrically by a tube also increased gastric acid secretion in healthy subjects. However, results reported for ethanol administered in beverage representative amounts are conflicting.[1,2,27,28] Lenz et al.[28] and Singer et al.[1] demonstrated that ethanol only in low concentrations ranging from 1.4 to 10% (equivalent to a total amount of 5.5−19 g ethanol) stimulated gastric acid secretion, but not when administered in higher concentrations.[1,28] Peterson et al.[2] did not find a significant effect on gastric acid secretion in healthy subjects after administration of a total amount of 28 g of ethanol, given in concentrations from 5 to 36%. In agreement with these data, we demonstrated an increase of gastric acid secretion after administration of 12% v/v (or a total amount of 12 g) ethanol to healthy subjects. We suggest that ethanol exhibits hormetic effects, being able to stimulate gastric acid secretion in lower but not in higher concentrations.

Another finding of our human intervention was a clear trend for the red wine ($p = 0.054$) being more potent than the white wine in stimulating gastric acid secretion. Furthermore, in our parietal cell model, we measured a significantly stronger effect for red wine on the IPX, as an indicator of proton secretion in HGT-1 cells, compared to white wine. To further elucidate the differential effects of red wine and white wine on mechanisms of proton secretion, we conducted gene expression analyses in HGT-1 cells after treatment with white wine, red wine, or

ethanol. We performed a time course experiment to analyze the expression of the prosecretory genes *ATP4A*, *HRH2*, and *CHRM3* and the antisecretory gene *SSTR2*. Here, we demonstrated for the first time that red wine strongly increased the expression of all tested genes in HGT-1 cells after 10 and 15 min of exposure, but also inhibited the expression of the antisecretory receptor *SSTR2* after 25 min of treatment. In contrast, white wine solely stimulated the expression of *HRH2*. This suggests that red wine acts more effectively and through different mechanisms of proton secretion in the parietal cell compared to white wine. Tsukimi et al.[14] also reported a stronger stomach acid secretion after administration of red wine to six dogs with vagally denervated Heidenhain pouches compared to white wine.

Next, we wanted to know whether the different effects of white and red wine could be attributed to their individual contents of organic acids. Therefore, we quantified the most common organic acids in wine: succinic acid, tartaric acid, citric acid, malic acid, and lactic acid. All concentrations quantified were in accordance with previously published data.[16,29] The red and white wine samples contained similar concentrations of tartaric acid (Table 3). Due to the malolactic fermentation typically applied to red wine,[26] the most abundant organic acid in red wine was lactic acid, whereas malic acid was quantitatively dominating in white wine. Additionally, the concentration of succinic acid was twice as high in red wine as in white wine. For answering the question which of the organic acids contributes the most to the effect of red wine and white wine, we tested the organic acids for their effects on the IPX in HGT-1 cells in wine representative concentrations. In a red wine representative concentration of 5.7 mg/L, there was a clear trend for succinic acid to decrease the intracellular proton concentration, as an indicator of proton secretion, more potently compared to lactic acid ($p = 0.12$) and tartaric acid ($p = 0.18$), which were applied in higher concentrations of 16.9 and 17.5 mg/L, respectively (Figure 5). In white wine representative concentrations, the most abundant organic acid, malic acid, exhibited the strongest stimulation of proton secretion. Teyssen et al.[12] also showed a stimulatory effect of organic acids on gastric acid secretion. In their study, the effects of acetic acid, oxalic acid, lactic acid, maleic acid, and succinic acid, produced by glucose fermentation, were tested, and only maleic acid and succinic acid showed a significant stimulation of gastric acid secretion in six healthy volunteers. Thus, a structure-dependent effect was hypothesized by Teyssen et al.,[12] suggesting that the length of the carbon chain and the presence of two carboxylic groups are necessary for a compound to stimulate gastric acid secretion (Table 1). We here identified malic acid and succinic acid as the most potent acids in wine, which not only supports the findings by Teyssen et al.[12] but also is in agreement with our own previous results, showing that malic acid and succinic acid contribute to the stomach acid secretory potential of beer.[22] However, we also observed a strong effect by lactic acid on mechanisms of proton secretion and can, therefore, not confirm the hypothesis that two carboxylic groups are necessary for a compounds' stimulatory effect on gastric acid secretion.

Furthermore, we tested the effects of biomimetic organic acid recombinates compared to white and red wine in the respective wine representative concentrations. As a result, both recombinates stimulated mechanisms of proton secretion more potently than red wine or white wine. We then questioned whether a wine component reacts with, for example, the hydroxyl or carboxyl groups of the organic acids as structural elements hypothesized to be responsible for the ability to stimulate mechanisms of gastric acid secretion. Because ethanol is one of the predominant compounds in wine and has been demonstrated to stimulate stomach acid secretion in our Heidelberg experiment with healthy subjects in a less pronounced manner than wine, and is known to esterify organic acids, we tested whether the addition of ethanol to the individual organic acids and their recombinate could have an effect on the intracellular proton index. Here, we could show that the addition of ethanol to tartaric acid resulted in a significantly attenuated proton secretion compared to the effect of the tartaric acid alone and compared to the other organic acids tested. Because tartaric acid bears the highest number of hydroxyl groups among these organic acids, we hypothesize that esterification in the presence of ethanol may lessen the effect of tartaric acid on proton secretion. However, addition of ethanol to the recombinate of organic acids in white and red wine representative concentrations did not lead to a significant attenuation of the recombinate's stimulatory effect. This result was also observed for malic acid and succinic acid, the two most active organic acids in white and red wine. Interestingly, when ethanol was added to red wine, the stimulatory effect of red wine was also reduced, whereas addition of ethanol to white wine did not change proton secretion. However, this may be a result of white wine's already reduced ability to stimulate acid secretion. However, we cannot exclude that ethanol interacts with red wine components other than organic acids to lessen its effects on proton secretion.

For white wine, the results from the Heidelberg experiment in healthy subjects, as well as those obtained from the cell culture experiments in HGT-1 cells, indicate a less pronounced effect on mechanisms of stomach acid secretion compared to red wine. Because the addition of ethanol to white wine did not affect its proton secretory potential, other compounds in white wine must be responsible for its less stimulating effect on stomach acid secretion compared to red wine.

In conclusion, we could show that red wine enhances gastric acid secretion by regulation of the prosecretory genes coding for H^+,K^+-ATPase, histamine H2 receptor, and acetylcholine M3 receptor and the antisecretory somatostatin receptor more potently than white wine. Furthermore, we found that organic acids, especially malic acid and succinic acid, are potent gastric acid stimulants in wine. Ethanol is also a potent stimulant, but we also show that ethanol can lower the stimulatory potential of tartaric acid and red wine. Identification of wine components responsible for the less pronounced effect of white wine compared to red wine on stomach acid secretion has to be addressed in future studies.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

GERD, gastroesophageal reflux disease; NADH, nicotinamide adenine dinucleotide; SNARF-1-AM, 1,5-carboxyseminaphtorhodafluor-acetoxymethyl ester; IPX, intracellular proton index; HGT-1, human gastric tumor cell line 1; *ATP4A*, H^+,K^+-ATPase α-subunit; *HRH2*, histamine H2 receptor; *SSTR2*, somatostatin receptor; *CHRM3*, acetylcholine receptor M3; *PPIA*, peptidylprolyl isomerase A; *n*, biological replicate; tr, technical replicate.

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