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In vitro formation of ethyl glucuronide and ethyl sulfate

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Aims: Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are used as markers of alcohol consumption in various clinical and forensic settings. In controlled studies, their concentration considerably varies between subjects. Knowledge on glucuronosyltransferases (UGT) and sulfotransferases (SULT) catalyzing formation of EtG and EtS formation is as moderate as diverging. A possible influence of nutritional components such as plant-derived phenols on the formation rates has not been addressed. **Methods:** Formation rates of EtG and EtS from ethanol via recombinant human UTGs (UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10, 2B15) and recombinant SULTs (SULT1A1, 1A3, 1B1, 1E1, 2A1), respective kinetics and the inhibitory potential of quercetin, kaempferol and resveratrol were determined. Analysis was performed following either solid phase extraction due to severe ion suppression of EtG or direct injection in the case of EtS by LC/MS/MS. **Results:** All enzymes under investigation formed EtG and EtS with UGT1A9 showing the highest glucuronidation rate and SULT1A1 exhibiting the highest sulfonation activity. Data for all enzymes could best be described by Michaelis-Menten kinetics. Formation of EtG was significantly reduced following co-incubation with quercetin and kaempferol, except UGT2B15. Resveratrol inhibited conjugation of ethanol via UGT1A1 and UGT1A9. All phenolic compounds decreased activity of SULTs towards ethanol. Inhibition was reversible and competitive for most enzymes; mechanism-based inhibition was evident for UGT2B7 and SULT2A1 with regard to quercetin and SULT1E1 with regard to kaempferol. **Conclusions:** Conjugation of ethanol occurs via multiple UGTs and SULTs. Beside known polymorphisms of UGT and SULT family members, common nutritional components influence formation of EtG and EtS. The results warrant further studies but may partly serve as an explanation for the variable formation of both biomarkers in man.

1. Introduction

Alcohol use is increasing worldwide as are many of the undesirable consequences such as driving injuries and fatalities, aggressive behaviour and family disruption. To prevent social problems and control health implications due to alcohol misuse, alcohol consumption markers with high sensitivity and specificity are required.

Ethyl glucuronide (EtG) as well as ethyl sulfate (EtS) have been established as reliable markers of alcohol use with both, high sensitivity and specificity. Offering a prolonged window of detection, these markers allow clinicians and forensic experts to focus on subjects with an increased risk of alcohol misuse, monitor current treatment programs more effectively and to identify even a single lapse [1-5]. In controlled drinking studies, a high variability in the production of both markers was observed [6-9]. Except a possible influence due to polymorphisms of enzymes involved in phase-II metabolism of ethanol, a further explanation does not exist.

EtG is produced by the net addition of activated glucuronic acid to ethanol via UDP-glucuronosyltransferases (UGTs). Sulfotransferases (SULTs) transfer the sulfate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to ethanol resulting in EtS. Knowledge on these phase-II reactions of ethanol is as moderate as inconsistent.

Plant derived phenolic compounds being present in foodstuff are extensively glucuronidated and sulfonated by various UGTs and SULTs, respectively [10-14]. They are common in nearly all fruits and vegetables, e.g. grapes, onions and broccoli, as well as in beer and wine.

The purpose of this study was twofold: 1) identification of UGT and SULT isoforms being responsible for EtG and EtS formation including determination of kinetic parameters via recombinant human UGTs and SULTs and 2) potential inhibition of EtS and EtG formation by quercetin, kaempferol and resveratrol. Inhibition of ethanol's phase-II metabolism might serve as a possible reason for the highly inter-individual variability of EtG and EtS.

2. Material and Methods

2.1. Chemicals and reagents

EtG, EtS, EtS-d_5 and EtG-d_5 (internal standards) were purchased from Medichem (Steinenbronn, Germany), PAPS and recombinant SULT1A1, 1A3, 1B1, 1E1 and 2A1 from R&D Systems (Wiesbaden, Germany), uridine 5'-diphosphate-glucuronic acid (UDGPA), potassium phosphate buffer pH 7.4, recombinant UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10 and 2B15 and control microsomes from BD Biosciences (Heidelberg, Germany), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), magnesium chloride, quercetin, kaempferol and resveratrol from Sigma Aldrich (Taufkirchen, Germany). HPLC-grade acetonitrile and methanol were obtained from Roth (Karlsruhe, Germany), ethanol (99.8%) from Merck (Darmstadt, Germany), double distilled water from Fresenius Kabi AG (Bad Homburg, Germany) and cartridges for solid phase extraction (Isolute NH_2 cartridges, 100 mg, 3 mL) from Biotage (Uppsala, Sweden).

2.2. Incubation procedures

EtG: To determine formation rates and kinetic data, recombinant UGTs (0.5 mg/mL microsomal protein) and CHAPS (2 mg CHAPS/mg protein) were placed on ice before adding potassium phosphate buffer pH 7.4 (50 mM), magnesium chloride (4 mM) and EtG-d_5 (50 ng/mL). Formation rates were determined at 50 or 200 mM ethanol. The maximum rate achieved at saturating substrate concentrations (V_{max}) and the substrate concentration at which the reaction rate is half of V_{max} (Michaelis-Menten constant; K_m) were estimated at ethanol concentrations ranging from 0 to 300 mM. Water was added up to a total volume of 200 µL to the mixture prior to incubation (37° C, 3 min). The reaction was initiated by addition of UDPGA (3 mM); the mixture was incubated (37°C, 60 min) and stopped by 100 µL ice-cold water to assess both, formation rates and kinetics. All incubations also including control microsomes were performed in duplicate.

EtS: To determine formation rates, 500 or 1000 mM ethanol was added to the incubation mixture (final volume of 200 µL) containing potassium phosphate buffer pH 7.4 (50 mM), magnesium chloride (4 mM), EtS-d_5 (50 ng/mL) and water. The reaction was initiated by addition of PAPS (85 µM) and stopped by adding isopropanol after incubation (37°C, 60 min). Incubations were conducted with 2 µg/mL protein. To determine kinetic parameters (K_m, V_{max}), ethanol concentrations ranged from 0 up to 2500 mM. All incubations including control incubations without enzyme were performed in duplicate.

2.3. Inhibition experiments

Preliminary inhibition studies at ethanol concentrations of respective K_m-values with co-addition of quercetin, kaempferol and resveratrol at different concentrations (½•K_m, K_m, 2•K_m) were conducted with each enzyme.

To identify a possible mechanism-based inhibition, incubations were performed with and without 15 min of preincubation of the mixture containing the potential inhibitor but no ethanol. If conjugation of ethanol was reduced by > 60% of the control, further experiments were performed at 6 inhibitor concentrations ranging from ½•K_m to 2•K_m to determine the half-maximum inhibitory concentration (IC_{50}). The incubation procedure was performed in duplicate at each condition following the protocol described above. The inhibitory constant (K_i) was estimated from incubations at ethanol concentrations of ½•K_m, K_m, 2•K_m with co-addition of four different phenol concentrations ranging from ¼•IC_{50} to 2•IC_{50}.

In case of a mechanism-based inhibition, the concentration required for half-maximal inactivation (K_I) and the maximum rate of inactivation at saturation (k_{inact}) were determined by conducting a time dependent experiment at four inhibitor concentrations of ¼•K_m, ½•K_m, K_m, 2•K_m and an ethanol concentration of K_m. Aliquots of a first incubation mixture (the same as described above without ethanol) were taken at 0, 10, 20, 40 and 60 min, and added to a second incubation mixture containing ethanol. This mixture was incubated (37°C, 60 min) and stopped with iso-propanol. Experiments to determine K_i, K_I and k_{inact} were performed in quadruplicate at each condition.

2.4. Analytical assay

EtG was isolated using solid phase extraction (Isolute NH_2 cartridges) referring to a previously published procedure [15]. Samples were reconstituted in 50 µL of the mobile phase, and 20 µL were injected for analysis. For EtS, 10 µL of the incubation mixture was injected into the HPLC-MS/MS system following centrifugation.

Analysis was performed on an API 4000 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with a Turbo ionization source operated in negative ionization mode, interfaced to two HPLC pumps and an auto sampler (HP 1100 series, Agilent, Waldbronn, Germany). Separation was achieved on a Synergi-Polar RP column (250 x 2.0 mm; 4 µm particle size; Phenomenex, Aschaffenburg, Germany) with 4 mM ammonium acetate pH 3.2/methanol/acetonitrile (EtG: 10:18:72 vol%; EtS: 79:1:20 vol%) as the mobile phase. To increase the ionization rate, iso-propanol was infused post column at a constant flow-rate of 100µL/min. The following settings were used: ion spray voltage, -4000 V; declustering potential, -45 V; entrance potential, - 10 V; temperature, 550 °C. Data were acquired in multiple reaction-monitoring mode: EtG, *m/z* 221.0→75.0*, 221.0→85.0, 221.0→113.0; EtG-d_5, *m/z* 226.0→75.0*, 226.0→85.0; EtS, *m/z* 125.0→96.8*, 125.0→79.8; EtS-d_5 *m/z* 130.0→96.8; transitions marked with an asterisk were used for quantification.

3. Results and Discussion

3.1. Ethyl glucuronide (EtG)

Formation rates and kinetics: All recombinant UGTs under investigation produced EtG in significant amounts. UGT1A9 and UGT2B7 exhibited the highest formation rates towards both ethanol concentrations with 10.41 pmol/min/mg and 12.96 pmol/min/mg (50 mM) and 7.38 pmol/min/mg and 10.37 pmol/min/mg (200 mM), respectively. These results are in accordance to those published by Al Saabi et al. [16]. The low yet detectable glucuronidation rate of ethanol with UGT1A1 (0.95 pmol/min/mg) contrasts with that of Foti et al. [15] and Al Saabi et al. [16]. They observed the highest glucuronidation rate or no product formation at all via UGT1A1, respectively. These discrepancies may be due to different experimental conditions such as the buffer or reagent to decrease UGTs' "latency". In addition, solid phase ex-

traction of the incubation mixture appears crucial, for its direct injection resulted in nearly undetectable, non-reproducible signals due to severe ion suppression.

EtG production followed the classic Michaelis-Menten kinetics for all enzymes; the K_m values ranged from 8.02 mM for UGT2B15 up to 40.04 mM for UGT1A3. By comparison, alcohol dehydrogenase as the main ethanol metabolizing exhibits a K_m value of 0.2-2.0 mM. Only about 0.1% of an ethanol dose is cleared via glucuronidation [17, 18], thus serving as a likely explanation for the high K_m values of UGTs towards ethanol.

Inhibition experiments: Determination of IC_{50} values enables a differentiation between competitive and mechanism-based inhibition modes. With regard to criteria proposed by Venkatakrishnan et al. [19] for CYP enzymes, IC_{50} as well as K_i values point to a potential inhibition if they do not exceed 30 µM. Due to the lack of respective established schemes for UGTs, these criteria were applied to further classify our results.

Accordingly, quercetin revealed a potential reversible competitive inhibition towards UGT1A1 and UGT1A3 that might cause problems *in vivo*. Having a closer look at kaempferol, UGT2B7 and UGT2B10 seem to be affected. In addition, the inhibition potential of resveratrol towards UGT1A9 may be assigned as moderate. The most important IC_{50} and K_i values are summarized in Table1.

Tab. 1. Most important IC_{50} and K_i values for quercetin, kaempferol and resveratrol with regard to their inhibitory potential towards the formation of EtG.

UGT isoform	IC_{50} [µM]	K_i [µM]
Quercetin		
1A1	3.04	2.0
1A3	17.84	14.10
Kaempferol		
2B7	24.40	11.10
2B10	25.23	9.93
Resveratrol		
1A9	16.47	10.04

The type of UGT inhibition was not exclusively reversible. Our data revealed a mechanism-based inhibition for UGT2B7 and quercetin (K_I= 55.92 µM, k_{inact}= 0.13 min^{-1}) which can be classified as moderate according to criteria proposed by Zhou et al. [20].

A mechanism-based inhibition causes more problems *in vivo*, because the inactivated enzyme has to be replaced by a *de novo* synthesis. UGT2B7 is one of the main enzymes catalyzing EtG formation. With this in view, the respective inhibition should not be underestimated.

Our experiments revealed that different phenolic plant compounds inhibit UGTs. Considering that the main isoforms being responsible for EtG formation are influenced by either quercetin, kaempferol or resveratrol, it can be assumed that phenolic plant compounds in foodstuff have an influence on the formation of EtG. This may partly serve as an explanation for the high variability of EtG's formation.

3.2 Ethyl sulfate (EtS)

Formation rates and kinetics: All SULT isoforms under investigation are capable of producing EtS. SULT1A1 exhibited the highest formation rates at both ethanol concentrations, followed by SULT2A1, SULT1E1, SULT1B1 and SULT1A3, in decreasing order. Therefore,

SULT1A1 is obviously the main isoform of EtS production. SULT1A1 is the mainly expressed isoform in the liver, too [21]. This finding is in line with a study of Kurogi et al. [22] who reported on SULT1A1 being the most active isoform in EtS production. In contrast to our findings, they detected the second lowest activity for SULT2A1 towards ethanol. This discrepancy may be due to the use of in-house prepared, purified enzymes, different buffers used for incubation and an analytical procedure involving thin layer chromatography and a scintillation detector.

The SULT-mediated sulfonation of ethanol follows the typical Michaelis-Menten kinetics in all enzymes with no evidence of substrate inhibition or activation. The K_m values ranged from 190 mM for SULT1B1 up to 972 mM for SULT2A1. These high K_m values show a very low affinity of SULTs towards ethanol being in line with the observation that less than 0.1% of an ethanol dose is cleared via sulfonation [7].

Tab. 2. Most important IC_{50} and K_i values for quercetin, kaempferol and resveratrol with regard to SULT isoforms. * Evidence for mechanism-based inhibition and determination of K_I and k_{inact}.

SULT isoform	IC_{50} [µM]	K_i [µM]	K_I [µM]	k_{inact} [min^{-1}]
Quercetin				
1A1	3,77	4,43		
1A3	1,55	2,58		
1B1	5,25	5,23		
1E1	4,38	4,38		
2A1	0,55/0,19*	--	0,084	0,031
Kaempferol				
1A1	2,95	3,86		
1A3	2,89	4,34		
1B1	8,82	14,5		
1E1	8,16/3,61*	--	4,53	0,035
2A1	5,32/2,30*	--	1,45	0,026
Resveratrol				
1A1	4,81	7,70		
1A3	1,51	2,05		
1B1	5,97	8,23		
1E1	3,37	6,72		
2A1	7,20	11,53		

Inhibition experiments: Inhibition experiments were assessed according to the criteria developed for CYP enzymes by Venkatakrishnan et al. [19] and Zhou et al. [20] due to the lack of established criteria for SULT enzymes. According to these criteria, inhibition of nearly all SULT isoforms was reversible and competitive suggesting a moderate *in vivo* influence. Quercetin and kaempferol showed a mechanism-based inhibition of SULT2A1; in addition, kaempferol seems to inhibit SULT1E1 in a mechanism-based mode. Results of mechanism-based inhibition could be classified as weak. However, considering the fact that this inhibition mode is only terminated by enzyme re-synthesis, the present findings may be of significance. Most important results are summarized in Table 2.

A closer look at the results reveals that phenolic compounds influence all enzymes under investigation. This means that if one isoform will be inhibited, another isoform will not be capable to compensate the lack of the activity, too. Having this in mind one can assume that

polyphenolic plant compounds in foodstuff will influence EtS production despite the overlapping selectivity of SULTs towards ethanol.

4. Conclusions

UGT1A9 and UGT2B7 appear to be the main isoforms producing EtG; SULT1A1 and SULT2A1 exhibit the highest EtS formation rates. Phenolic plant compounds from foodstuff inhibit both enzymatic pathways with sulfonation being more affected than glucuronidation. Beside the known polymorphisms of members from both enzyme families, the present findings could serve as an explanation for the high inter-individual variability in both, EtG and EtS production. The present results may serve as a basis for further experiments to evaluate the influence of dietary habits on EtG and/or EtS formation.

5. References

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