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SHORT RESEARCH ARTICLE

Elevated Serum Oleic Acid Epoxide Concentration in Acetaminophen (Paracetamol) Poisoning

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ABSTRACT | *cis*-9,10-Epoxystearic acid (*cis*-EpOA) is produced from oleic acid both by hepatic cytochrome P450 (CYP) enzymes and by reactive oxygen/nitrogen species (RONS). We hypothesized that overdosed acetaminophen (paracetamol) elevates the formation of *cis*-EpOA by inducing oxidative stress. We measured the concentration of circulating *cis*-EpOA in six acetaminophen suicide subjects. Acetaminophen and *cis*-EpOA were measured by high-performance liquid chromatography (HPLC) and gas chromatography-tandem mass spectrometry (GC-MS/MS), respectively. In serum samples of the acetaminophen-suicided persons, *cis*-EpOA (nM) and acetaminophen (μM) concentrations were 47 and 132, 65 and 921, 275 and 270, 300 and 762, 319 and 166, and 3723 and 185, respectively. There was no correlation between *cis*-EpOA and acetaminophen concentration. In acetaminophen self-poisoned patients, serum *cis*-EpOA concentration is increased compared to non-medicated healthy subjects. Acetaminophen is a weak inhibitor of CYP enzymes (e.g., 30% inhibition of CYP3A4 at 1000 μM). Suicidal acetaminophen seems to induce the formation of RONS which oxidize esterified oleic acid to *cis*-EpOA which is then hydrolyzed by secretory hepatic phospholipase A_2 (PLA_2) to free *cis*-EpOA. Dihydroxy-stearic acids, the hydrolase products of *cis*-EpOA, are known to inhibit the activity of several clotting factors. We hypothesize that alterations of the coagulation cascade seen after acetaminophen administration/intoxication are in part due to *cis*-EpOA and dihydroxy-stearic acids. Being a stable lipophilic epoxide, *cis*-EpOA is likely to inhibit enzymes of the vitamin K cycle.

KEYWORDS | Acetaminophen; Cytochrome P450; Epoxidation; Oleic acid; Paracetamol

ABBREVIATIONS | *cis*-EpOA, *cis*-9,10-epoxystearic acid; CYP; cytochrome P450; GC-MS/MS, gas chromatography-tandem mass spectrometry; HPLC, high-performance liquid chromatography; NAC, *N*-acetylcysteine; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; PFB, pentafluorobenzyl; PLA_2, phospholipase A_2; QC, quality control; RONS, reactive oxygen/nitrogen species

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1. INTRODUCTION

Oleic acid, *cis*-9-octadecenoic acid, is the most abundant monounsaturated fatty acid in human blood [1]. Epoxidation of the single C9/C10 double bond of oleic acid by cytochrome P450 (CYP) enzymes leads to the formation of *cis*-9,10-epoxy-octadecanoic acid (*cis*-EpOA) [1–3]. The physiological roles of oleic acid and *cis*-EpOA are poorly investigated. Oleic acid has been proposed to exert vasoprotective actions and to play a role in immune function [1]. *cis*-EpOA lacks carcinogenic potential [4]. Oleic acid and *cis*-EpOA are mainly metabolized by β-oxidation. *cis*-EpOA is physiologically present in human blood and urine [1]. The detection of racemic *cis*-EpOA in lipids of human leukocytes suggests a free radical-catalyzed epoxidation of esterified oleic acid in lipids.

The first accurately measured basal concentration of *cis*-EpOA in human plasma was achieved by a validated GC-MS/MS after HPLC separation [5]. In ethylenediaminetetraacetic acid (EDTA)-conditioned plasma of healthy humans, *cis*-EpOA occurs in its free, non-esterified form at concentrations within the range of 30 to 50 nM. In end-stage liver disease, *cis*-EpOA plasma concentrations are lower (i.e., 10 to 40 nM). The lowest *cis*-EpOA plasma concentrations were measured in patients suffering from liver cirrhosis [3], suggesting liver as the main *cis*-EpOA-synthesizing organ. Various CYP isoforms including CYP2C9 and CYP3A4 have been shown to epoxidize oleic acid to *cis*-EpOA in vitro [3].

Given the hepatotoxicity of acetaminophen at high doses, we hypothesized that circulating *cis*-EpOA concentrations would be altered in subjects poisoned by acetaminophen. In the present study, we report on *cis*-EpOA concentrations in the blood of six female subjects after suicide by acetaminophen.

2. MATERIALS AND METHODS

2.1. Subjects

Six females (age range: 12 to 18 years) with suspected acetaminophen poisoning were included in the study (**Table 1**). Blood samples were obtained approximately at 4 h (n = 3), 5 h (n = 1), and 28 h (n = 1) after acetaminophen ingestion. The time point of acetaminophen ingestion by one patient was unknown. Two patients had been under *N*-acetylcysteine (NAC) treatment at the time point of blood sampling. Serum acetaminophen concentration ranged between 132 and 921 µM as measured by HPLC with UV detection (236 nm). The Ethics Committee of the Hannover Medical School was consulted. The study was performed according to the Declaration of Helsinki and the recommendations of the Central Ethics Committee for Medicine of the German Medical Association regarding the further use of human body materials for medicinal research purposes.

2.2. Quantification of *cis*-EpOA

cis-EpOA was quantitated in 1 ml aliquots of biological samples by GC-MS/MS using *cis*-[9,10-$^2H_2$]-EpOA (at 50 nM) as the internal standard [5]. The procedure includes solvent extraction with ethyl acetate (2 ml), derivatization by pentafluorobenzyl (PFB) bromide, and HPLC isolation of the PFB ester derivatives of *cis*-EpOA and *cis*-[9,10-$^2H_2$]-EpOA. Quantification was performed by selected-reaction monitoring the mass transition *m/z* 297 to *m/z* 171 for *cis*-EpOA and *m/z* 299 to *m/z* 172 for *cis*-$^2H_2$-EpOA. Potential interference of *cis*-EpOA measurement by high acetaminophen concentrations was investigated by adding acetaminophen to human plasma samples at concentrations up to 1000 µM and by analyzing *cis*-EpOA by GC-MS/MS [5]. *cis*-EpOA in unspiked human plasma samples and samples spiked with acetaminophen (range: 0–1000 µM) was measured at 53.8 ± 2.9 nM, (RSD: 5.4%), suggesting no interference of *cis*-EpOA measurement by acetaminophen [5]. In parallel, we measured in triplicate the *cis*-EpOA concentration in unspiked plasma and plasma samples spiked with 16 nM *cis*-EpOA serving as a quality control (QC) sample. In the QC plasma sample, the acetaminophen concentration was less than 0.7 µM. The *cis*-EpOA concentration was determined to be 63.2 ± 4.6 nM in the unspiked plasma

TABLE 1. *cis*-EpOA and acetaminophen (APAP) concentrations in blood of six subjects after suicide by acetaminophen without and with *N*-acetylcysteine (NAC) treatment

Subject No.	Time of APAP Analysis after Ingestion (h)	NAC Treatment	APAP (μM)	*cis*-EpOA (nM)
#11608	5	Yes	132	47
#11616	4	Yes	185	3723
#11631	28	No	921	65
#11644	4	No	270	275
#11704	4	No	166	319
#11749	Unknown	No	762	300

sample and 80.6 ± 3.5 nM in the spiked plasma sample (recovery: 109 ± 10%).

3. RESULTS

The serum concentration of acetaminophen in the poisoned subjects was 132, 166, 185, 270, 766, and 921 μM (**Table 1**). These values are higher than the maximum serum concentration (C_{max}) values obtained after oral administration of therapeutically relevant single doses of 500 to 1000 mg acetaminophen [6]. There was no correlation between *cis*-EpOA and acetaminophen concentrations in the patients' blood. The highest serum *cis*-EpOA concentration of 3723 nM is about 100 times the mean plasma *cis*-EpOA concentration measured in healthy subjects [3]. Except for two patients, circulating acetaminophen concentrations were higher than the highest acetaminophen plasma concentrations measured in four healthy subjects after ingestion of 3000 mg acetaminophen at once [7].

4. DISCUSSION

Acetaminophen (paracetamol) is one of the most commonly used and misused drugs in many countries. In the six young females of our study, we measured toxicologically relevant concentrations of acetaminophen in blood drawn 4, 5, or 28 h after drug ingestion, including one patient being treated with NAC. In four patients, the concentration of circulating *cis*-EpOA was several-fold higher compared to healthy non-medicated subjects [3, 5].

cis-EpOA may originate from non-enzymatic and CYP-catalyzed reactions (**Figure 1**) [1–3]. Acetaminophen (1000 μM) was found to inhibit CYP3A4 activity (by 30%), a major CYP isoform contributing to *cis*-EpOA formation [3]. The temporary almost 6-fold increase in *cis*-EpOA seen in two healthy subjects who ingested 3 g acetaminophen at once could be due to an acetaminophen-induced short-term release of secretory hepatic phospholipase A_2 ($sPLA_2$) [7]. This may also have occurred in the acetaminophen-suicided persons of the present study.

Acetaminophen's hepatotoxicity is associated with activity alterations of many enzymes. Diminished β-oxidation and decreased activity of microsomal and cytosolic epoxide hydrolases and glutathione *S*-transferases may lead to the accumulation of *cis*-EpOA [1]. The biological activities of *cis*-EpOA, 9,10-dihydroxy-stearic acid, and 9-hydroxy-10-glutathionyl-stearic acid are largely unknown. Acetaminophen poisoning was found to alter the activity of clotting factors and to increase the international normalized ratio (INR) [8]. *N*-Acetyl-*p*-benzoquinoneimine (NAPQI), the toxic metabolite of acetaminophen, was found to inhibit enzymes of the vitamin K cycle [9]. These observations raise safety issues in patients treated with vitamin K antagonists [10]. In rats, dietary 9,10-dihydroxystearic acid was found to induce a vitamin K-like deficiency syndrome which was reversed by 2-methyl-2,4-naphthoquinone (vitamin K_3) [11]. The similar effects caused by acetaminophen poisoning and 9,10-dihydroxy-stearic acid raise the question whether *cis*-EpOA itself or 9,10-dihydroxy-stearic acid also inhibits vitamin K cycle enzymes (**Figure 1**) and attenuates the activity of specific clotting factors [9, 10]. This remains to be elucidated in forthcoming studies. During vitamin K dependent γ-carboxylase catalysis, O_2 is transferred [12]. Such a mechanism would open the possibility of *cis*-EpOA formation

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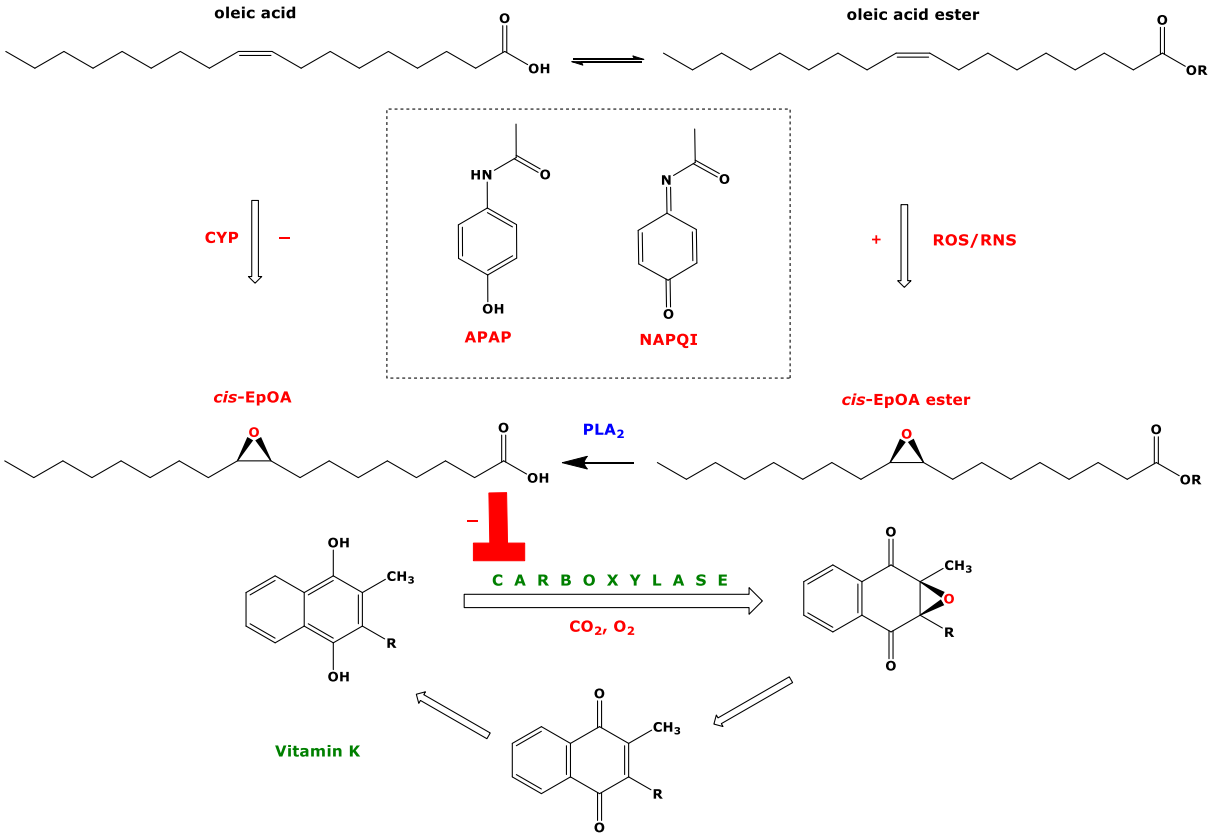

FIGURE 1. Proposed mechanism for the APAP-induced *cis*-EpOA formation and inhibition of γ-carboxylase activity in the vitamin K cycle by APAP and its toxic metabolite NAPQI, and by *cis*-EpOA. CYP enzymes catalyze the formation of *cis*-EpOA free acid, whereas ROS and RNS (such as peroxynitrite) induce the formation of *cis*-EpOA ester. The symbols + and – indicate activation and inhibition of enzymatic and non-enzymatic reactions, respectively.

from oleic acid in liver microsomes independent of CYP enzymes.

Acetaminophen causes major bleeding events at therapeutically relevant doses in patients with recent ischemic stroke [13]. Possible mechanisms could involve the participation of acetaminophen metabolites such as NAPQI [10] or *cis*-EpOA, rather than thromboxane A_2 inhibition in platelets by acetaminophen [7].

5. CONCLUSION

In acetaminophen self-poisoned young subjects, serum *cis*-EpOA concentration is several fold higher compared to non-medicated healthy subjects. Suicidal acetaminophen may induce the formation of RONS that oxidize esterified oleic acid to *cis*-EpOA which is then hydrolyzed to free *cis*-EpOA. Reported acetaminophen-induced alterations of the coagulation cascade may be in part due to *cis*-EpOA and its hydrolysis product 9,10-dihydroxystearic acid. We hypothesize that *cis*-EpOA is an inhibitor of enzymes of the vitamin K cycle, but this remains to be demonstrated.

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The author declares no conflicts of interest.

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