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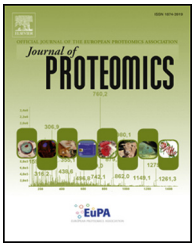

Assessment of protein modifications in liver of rats under chronic treatment with paracetamol (acetaminophen) using two complementary mass spectrometry-based metabolomic approaches

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ABSTRACT

Liver protein can be altered under paracetamol (APAP) treatment. APAP-protein adducts and other protein modifications (oxidation/nitration, expression) play a role in hepatotoxicity induced by acute overdoses, but it is unknown whether liver protein modifications occur during long-term treatment with non-toxic doses of APAP. We quantified APAP-protein adducts and assessed other protein modifications in the liver from rats under chronic (17days) treatment with two APAP doses (0.5% or 1% of APAP in the diet w/w). A targeted metabolomic method was validated and used to quantify APAP-protein adducts as APAP-cysteine adducts following proteolytic hydrolysis. The limit of detection was found to be 7 ng APAP–cysteine/mL hydrolysate i.e. an APAP–Cys to tyrosine ratio of 0.016‰. Other protein modifications were assessed on the same protein hydrolysate by untargeted metabolomics including a new strategy to process the data and identify discriminant molecules. These two complementary mass spectrometry (MS)-based metabolic approaches enabled the assessment of a wide range of protein modifications induced by chronic treatment with APAP.

Biological significance
APAP-protein adducts were detected even in the absence of glutathione depletion and hepatotoxicity, i.e. in the 0.5% APAP group, and increased by 218% in the 1% APAP group compared to the 0.5% APAP group. At the same time, the untargeted metabolomic method revealed a decrease in the binding of cysteine, cysteinyl–glycine and GSH to thiol groups of protein cysteine residues, an increase in the oxidation of tryptophan and proline residues and a modification in protein expression. This wide range of modifications in liver proteins occurred in rats under chronic treatment with APAP that did not induce hepatotoxicity.

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1. Introduction

Paracetamol also called acetaminophen or 4-hydroxy-acetanilide or N-acetyl-para-aminophenol (APAP) is the most widely used drug for treating low to moderate pain around the world [1]. APAP is eliminated progressively from the body with a half-life of 2 h [2], and its metabolism occurs within the liver to generate molecular forms that are excreted in the urine (for a review see [3]). Briefly, APAP metabolism consists mainly in phase II detoxification (up to 90%) by direct conjugation with sulfate (sulfation pathway) or glucuronide. In phase I, APAP is converted by cytochrome P450 into the strong oxidizer N-acetyl-p-benzoquinone imine (NAPQI), the toxic APAP metabolite. NAPQI formation dramatically increases when the sulfation pathway is saturated. NAPQI binds to thiol groups of cysteine (Cys) residues from glutathione (GSH) or from proteins, forming APAP–GSH and APAP-protein adducts [4,5]. APAP–GSH is rapidly converted to Cys and mercapturate conjugates and excreted in the urine [6]. Thus, NAPQI formation leads to decreased hepatic GSH [7] and protein thiols [8]. The extent of these alterations should depend on APAP dose and treatment length. Significant decrease in liver GSH content leads to oxidative stress [9]. The loss of protein thiols has been reported to be much higher than the loss expected from covalent binding of APAP to liver proteins, at least in one study [8]; suggesting an additional oxidation of Cys residue. Moreover, the formation of mitochondrial APAP-protein adducts has been related to the development of acute hepatotoxicity [10]. Indeed, these adducts induce mitochondrial peroxynitrite formation and mitochondrial permeability transition, which amplifies the oxidative stress [11,12]. Ultimately, the APAP-induced oxidative stress will lead to nitration of tyrosine [13–16] and tryptophan [17] residues of liver proteins, and leads finally to necrotic cell death [12,18,19]. However, the physiological relevance of these adverse effects of APAP metabolism in chronic therapeutic treatments remains to be evaluated as most of the literature concerning APAP-induced protein alterations is based on single administrations of hepatotoxic doses of APAP (for reviews see [20,21]). APAP-protein adducts could accumulate over time since they are more persistent than APAP metabolites. APAP-protein adduct half-life was evaluated at 1–2 days in human plasma after acute overdoses of APAP [22,23] but is still unknown in liver. To our knowledge, APAP-protein adducts and other protein modifications have never been deeply investigated in the liver after chronic treatments with non-toxic doses of APAP.

The aim of the present study was to quantify APAP-protein adducts in the liver from rats under chronic treatment with APAP and to analyze a wide range of the other APAP-inducible protein modifications. Two complementary mass spectrometry (MS)-based metabolomic analyses were performed on the same protein hydrolysate obtained by enzymatic digestion. The first metabolomic method consisted in a targeted quantification of APAP–Cys using liquid chromatography (LC) coupled to a triple quadrupole mass spectrometer. The second untargeted metabolomic analysis was performed with an Ultra Performance Liquid Chromatography (UPLC) coupled to a high resolution mass spectrometer aimed to assess a wide range of protein alterations. The originality of this untargeted method resides in the strategy used to process the data and identify the discriminant molecules among modified amino acids and peptides contained in protein hydrolysates. An in silico database was built up with all theoretically possible di- and tri-peptides including all oxidized forms of methionine and Cys. Indeed, these residues are known to be particularly sensitive to oxidative stress [24–26] and thiol proteins were expected to be affected by chronic treatment with APAP [8].

2. Materials and methods

2.1. Chemicals and reagents

Methanol, acetonitrile and L-tyrosine were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), ultrapure water (18.2 MΩ) from Millipore (Molsheim, France) and formic acid from Fluka (Saint Quentin Fallavier, France). The chemicals used were of analytical grade. APAP–Cys (3-(cystein-S-yl)acetaminophen) was purchased from Toronto Research Chemical (Brisbane Road, Toronto, Canada).

Leucine–enkephalin (Sigma-Aldrich) was used as reference for mass measurements.

Standard quality control (SQC) samples for untargeted metabolomics were prepared at a concentration of 10 μg/mL in water/acetonitrile (50/50, v/v) containing 0.1% formic acid. They are a mixture of L-tryptophan, L-phenylalanine, creatinine, colchicine, and 2-aminoanthracene (96%) (Sigma-Aldrich).

2.2. Animal experiment

The animal experiment was conducted in agreement with the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for Scientific Purposes. The protocol complied with the 3R (Replacement, Reduction, Refinement) rule and has already been described in Mast et al. (2014).

Briefly, male 4-month-old Wistar rats (450 g, Janvier Labs, Saint Berthevin, France) were randomly divided into three groups and housed in individual cages with standard conditions (22 ± 1 °C, 12 h light/dark cycle). The control group (0% APAP, n = 6) received a powdered standard laboratory food (A04 from SAFE, Villemoisson-sur-Orge, France) and two groups received APAP at the dose of either 0.5% w/w (0.5% APAP group, n = 7) or 1% w/w (1% APAP, n = 7) in the standard diet during 17 days. At the end of the experiment, all animals were euthanized under pentobarbital anaesthesia (50 mg/kg, injected intra-peritoneally) by aortic blood withdrawal. The liver was immediately removed, washed with saline, frozen in liquid nitrogen, and stored at −80 °C until analyses.

The two selected APAP doses (0.5% and 1%) were designed to be non-hepatotoxic, and respectively around or largely above the saturation threshold of APAP sulfation [27,28]. The cumulative APAP ingestion of 2.0 ± 0.1 and 3.7 ± 0.3 g/17 days in 0.5% APAP group and 1% APAP group respectively was not hepatotoxic according to plasma transaminase activities [29]. As expected, the GSH-dependent detoxification pathway was highly activated in the 1% APAP group and liver GSH content was strongly depleted in the 1% APAP group, but unchanged in the 0.5% APAP group compared to the control group. Liver samples from this study were suitable to study APAP-induced protein alterations in the liver under chronic treatment that was not hepatotoxic.

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