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Quantitative profiling of prostaglandins as oxidative stress biomarkers in vitro and in vivo by negative ion online solid phase extraction – Liquid chromatography–tandem mass spectrometry

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ABSTRACT

Free radical-mediated oxidation of arachidonic acid to prostanoids has been implicated in a variety of pathophysiological conditions such as oxidative stress. Here, we report on the development of a liquid chromatography–mass spectrometry method to measure several classes of prostaglandin derivatives based on regioisomer-specific mass transitions down to levels of 20 pg/ml applied to the measurement of prostaglandin biomarkers in primary hepatocytes. The quantitative profiling of prostaglandin derivatives in rat and human hepatocytes revealed the increase of several isomers on stress response. In addition to the well-established markers for oxidative stress such as 8-iso-prostaglandin $F_{2\alpha}$ and the prostaglandin isomers PE_2 and PD_2, this method revealed a significant increase of 15*R*-prostaglandin D_2 from 236.1 ± 138.0 pg/1E6 cells in untreated rat hepatocytes to 2001 ± 577.1 pg/1E6 cells on treatment with ferric NTA (an Fe^{3+} chelate with nitrilotriacetic acid causing oxidative stress in vitro as well as in vivo). Like 15*R*-prostaglandin D_2, an unassigned isomer that revealed a more significant increase than commonly analyzed prostaglandin derivatives was identified. Mass spectrometric detection on a high-resolution instrument enabled high-quality quantitative analysis of analytes in plasma levels from rat experiments, where increased concentrations up to 23-fold change treatment with Fe(III)NTA were observed.

Oxidative stress describes the pathological condition where the balance between prooxidant and physiological antioxidant regulatory mechanisms is disturbed [1]. It is generally associated with the presence of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), hydroxyl radicals (•OH), superoxide anions ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2) [2]. Because living organisms have always been exposed to oxidative stressors naturally (e.g., endogenous via mitochondrial metabolism, exogenous via ultraviolet [UV] radiation), strategies to counteract with reductive enzyme systems such as superoxide dismutase [3] or via glutathione metabolism [4] have been developed. However, in some pathophysiological conditions, the defense mechanisms are disrupted, leading to oxidative stress and cellular damage caused by ROS. On the molecular level, there are three major targets of ROS: proteins, DNA, and lipids. The oxidation of proteins may lead to altered function, DNA oxidation can cause damage on the functional or genomic level, and lipid alteration may to lead to impaired membrane integrity [5].

Several pathological conditions such as metabolic syndrome, chronic obstructive pulmonary disease (COPD), cancer, neurodegenerative diseases, and ageing [6–9] are known to involve cellular oxidative stress. Furthermore, impaired oxidative balance is

Abbreviations: ROS, reactive oxygen species; UV, ultraviolet; LC–MS/MS, liquid chromatography–tandem mass spectrometry; HPLC, high-performance liquid chromatography; SRM, selected reaction monitoring; $iPF_{2\alpha}$-VI, 5-iso prostaglandin $F_{2\alpha}$-VI; $PF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PE_2, prostaglandin E_2; PD_2, prostaglandin D_2; 15*R*-PD_2, 15(*R*)prostaglandin D_2; dihydro-keto PE_2, 13,14-dihydro-15-keto prostaglandin E_2; dihydro-keto PD_2, 13,14,-dihydro-15-keto prostaglandin D_2; PD_2-d4, prostaglandin D_2-d4; Fe(III)NTA, ferric nitrilotriacetic acid; TC, trapping column; AC, analytical column; DP, declustering potential; CE, collision energy; CXP, cell exit potential; TOF, time-of-flight; SRM–HR, high-resolution SRM; EIA, enzyme immunoassay; LLE, liquid–liquid extraction; SPE, solid phase extraction; MDA, malonyldialdehyde; GSH, glutathione; RT, retention time.

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considered important in a variety of adverse reactions caused by pharmacotherapy [10]. Namely, the liver has been identified as a primary vulnerable organ due to the high metabolic load with drugs and the transformation to reactive species. Free radical-mediated lipid peroxidation or redox cycling of quinone, iminoquinone, or quinone methide type of activated drug metabolites may cause an imbalance of the cellular antioxidant system in hepatocytes [11,12]. A widely accepted hypothesis claims that the associated secondary signals (also referred to as danger signals) of oxidative stress are involved in the multifactorial mechanisms leading to liver toxicity [13]. Therefore, it is important to assess cellular oxidative stress conditions as a surrogate marker for hepatic injury early in the drug discovery and development process. Foremost, hepatocytes as primary metabolically active species have served as an experimental system to study cellular damage induced by drug metabolism [14,15].

Isoprostanes, which are isomers of prostaglandins, are one group of potential cellular biomarkers for oxidative stress. They derive from a cascade of free radical-catalyzed oxidations of arachidonic acid, the physiological precursor of prostaglandins, prostacyclins, and thromboxans. Isoprostanes have been shown to be involved in diseases such as oxidant injury and atherosclerosis [16,17] as well as in degenerative syndromes and cardiovascular diseases [18,19]. Consequently, the assessment of their levels has been suggested as biomarkers of oxidative stress or inflammation. In the past, various methods for the detection and quantification of prostaglandins have been reported. One representative of the prostaglandin family is the group of the F_2 isoprostanes, which are regarded as the “gold standard” noninvasive biomarker for oxidative stress measurement in vivo, mainly in plasma and urine. In addition, liver and brain were investigated as target tissues for prostaglandin derivatives [20,21]. Mainly immunoassays for specific isomers as well as gas chromatography coupled to mass spectrometry, mostly via negative ion chemical ionization (NICI), were applied to purify and quantify F_2 isoprostanes [22–24]. Due to increasing evidence of their role in inflammatory and oxidative stress-related processes, there has been a rising interest in the analysis of other classes of prostaglandins and isoprostanes during past years. Published comprehensive reviews give an overview of the different analytical approaches that have been established [25,26].

This study reports on the development of an integrated liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the direct analysis of isoprostanes from biological samples. It is fully automated on a 96-well plate format. An online column-switching high-performance liquid chromatography (HPLC) setup allows for enrichment of analyte by large volume injection and omits laborious a priori sample workup. Besides classical MS detection via selected reaction monitoring (SRM) on a triple quadrupole instrument, this method was also shown to be translatable as high-resolution SRM to a time-of-flight mass spectrometer. This post-acquisition selection of suitable parent–daughter ion pairs allows for the unbiased detection of a broad variety of prostaglandins because those representatives described to be the most reliable renal and plasma markers might not be the most sensitive hepatic ones. The method was applied for in vitro studies with primary rat and human hepatocytes and can also be useful for the detection of plasma analytes.

Materials and methods

Chemicals

Williams' medium E, ferric nitrate, formic acid p.a., nitrilotriacetic acid disodium salt, insulin, streptomycin, penicillin, and hydrocortisone were obtained from Sigma–Aldrich (St. Louis, MO, USA). Glutamine and gentamycin were purchased from Life Technologies Invitrogen (Lucerne, Switzerland), and acetonitrile (LC–MS grade) was purchased from Fisher Scientific (Wohlen, Switzerland). Water (chromatography grade) was obtained from Merck (Darmstadt, Germany). The prostaglandin derivatives 5-iso prostaglandin $F_{2\alpha}$-VI ($iPF_{2\alpha}$-VI) (**1**), prostaglandin $F_{2\alpha}$ ($PF_{2\alpha}$) (**2**), prostaglandin E_2 (PE_2) (**3**), prostaglandin D_2 (PD_2) (**4**), 15(*R*)prostaglandin D_2 (15*R*-PD_2) (**5**), 13,14-dihydro-15-keto prostaglandin E_2 (dihydro-keto PE_2) (**6**), 13,14,-dihydro-15-keto prostaglandin D_2 (dihydro-keto PD_2) (**7**), and prostaglandin D_2-d4 (PD_2-d4) (**8**) were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Preparation of reagents

Incubation medium was prepared by supplementation of Williams' medium E with 4 mg/L insulin, 50,000 U/L penicillin, 50 mg/L streptomycin, 10 mg/L gentamycin, 0.4 mg/L glutamine, and 2.4 mg/ml hydrocortisone.

Ferric nitrilotriacetic acid [Fe(III)NTA] stock solution (20 mM) was prepared by mixing 5 ml of 0.1 M $Fe(NO_3)_3$ in 0.1 M HCl and 0.2 M NaNTA, filling up to 30 ml, and adjusting the pH to 7.4 with 1 M $NaCO_3$.

Hepatocyte incubation and workup

Primary cells were prepared freshly in-house by a two-step procedure for liver perfusion [27,28] and used in suspension. Incubation was performed at 37 °C in humidified atmosphere (5% CO_2/95% air) in 96-well plates at a cell concentration of 1 million/ml and stopped after 1 or 3 h by precipitation with acetonitrile that contained the internal standard (PD_2-d4 at a concentration of 1 ng/ml to reach a final concentration of 0.42 ng/ml). As positive control for oxidative stress conditions, cell suspensions were spiked with diluted Fe(III)NTA stock solution to reach final concentrations of 50 and 150 μM.

Quenched hepatocyte incubations were centrifuged at 5000 *g* for 11 min at 4 °C, and the transferred supernatant (500 μl diluted with 100 μl of acetonitrile/water containing 0.1% formic acid) was directly injected onto the LC–MS/MS system.

Animal study and sample workup

Animal studies were carried out in accordance with Swiss animal welfare law and the guide for the care and use of laboratory animals published by the National Institutes of Health. The animal test facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. For the experiment, 9 male Fischer 344 rats (240–269 g) were obtained from Charles River (Sulzfeld, Germany) and maintained at 23 °C on a 12-h light:12-h dark cycle with food and water available ad libitum. Two days prior to treatment, animals were adjusted to metabolic cages. For the experiment, animals were divided into three groups ($n = 3$) and administered a single dose of Fe(III)NTA (15 and 65 mg/kg body weight i.p. treated groups 2 and 3, respectively) or 0.9% sodium chloride (control group 1). Animals were sacrificed after 3 h of treatment by CO_2 asphyxiation followed by cervical dislocation. Total blood was collected on ethylenediaminetetraacetic acid (EDTA), and plasma was prepared and stored at −20 °C.

Plasma samples were precipitated with 2 volumes of ethanol containing internal standard (PD_2-d4 at a concentration of 0.63 ng/ml to reach a final concentration of 0.42 ng/ml).