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Original Contribution

Dihomo-isoprostanes—nonenzymatic metabolites of AdA—are higher in epileptic patients compared to healthy individuals by a new ultrahigh pressure liquid chromatography–triple quadrupole–tandem mass spectrometry method

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ABSTRACT

Oxidative stress is a biochemical state in which reactive oxygen species are generated and it has been associated with pathological states including epilepsy. Therein, neuroprostanes (NeuroPs) and dihomo-isoprostanes (Dihomo-IsoPs)—a series of compounds formed nonenzymatically through free radical-induced DHA, n-6 DPA, and AdA peroxidation–are implicated in the pathophysiological status of various human neurological diseases. A new, robust, and selective analytical method for the determination of 10 NeuroPs/Dihomo-IsoPs in human urine, using solid-phase extraction and UHPLC–QqQ–MS/MS in the multiple reaction monitoring mode (using a negative electrospray ionization interface), was developed. Nine NeuroPs/Dihomo-IsoPs were identified in 15 epileptic patients, matched with healthy volunteers. Among them, 17-F_{2t}-Dihomo-IsoP, Ent-7(*R*)-7-F_{2t}-Dihomo-IsoP, and *Ent*-7-*epi*-7-F_{2t}-Dihomo-IsoP, derived from adrenic acid (AdA), were significantly higher in epileptic patients than in healthy volunteers. The validated method provided a high-throughput assay with a limit of detection and limit of quantification for each analyte of 0.10–5.90 ng mL^{-1} and 0.15–11.81 ng mL^{-1}, respectively. The intra- and interday variations were lower than 14%. Dihomo-IsoPs have been considered as potential markers of epilepsy for the first time and their measurement may increase the understanding of the role of oxidative stress in neurological diseases, in *intra vitam* studies. The present study highlights a potential role of Dihomo-IsoPs as biomarkers in persons with epilepsy, though its mechanisms and possible implications should be the subject of further investigations.

Abbreviations: OS, oxidative stress; OI, oxidative injury; IsoPs, isoprostanes; AA, arachidonic acid; NeuroPs, neuroprostanes; DHA, docosahexaenoic acid; DPA, docosapentanoic acid; Dihomo-IsoPs, dihomo-isoprostanes; AdA, adrenic acid; EIA, enzyme immunoassay; GC–MS, gas chromatography mass spectrometry; LC–MS, liquid chromatography mass spectrometry; UHPLC–QqQ–MS/MS, ultrahigh pressure liquid chromatography–triple quadrupole–tandem mass spectrometry; GC–NICI–MS, gas chromatography–negative ion chemical ionization–mass spectrometry; LC/MS–MS, liquid chromatography tandem mass spectrometry; SPe, solid phase extraction; MRM, multiple reaction monitoring; Bis-Tris, bis-(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane; AEDs, antiepileptic drugs; MS/MS, tandem mass spectrometry; PVDF, polyvinyl difluoride; FDA, food and drug administration; ICH, International Conference on Harmonization; ESI, electrospray ionization; LOD, limit of detection; LOQ, limit of quantification; S/N, signal to noise; RSD, relative standard deviation; ME, matrix effect; PE, process efficiency; HI, hypoxic–ischemic; CSF, cerebrospinal fluid; aSAH, aneurysmal subarachnoid hemorrhage; RTT, Rett syndrome; AD, Alzheimer's disease.

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Introduction

Oxidative stress (OS) is a biochemical state in which reactive oxygen species are generated. Since the 1970s, it has been associated with diverse pathological states, including epilepsy—that affects the central nervous system [1]. The human brain is particularly prone to OS because it utilizes a high amount of oxygen. Therefore, oxidative injury (OI) in the brain is a main cause of neurological disorders [2]. In the last decade, important advances have been made in the diagnosis of epilepsy. However, the understanding of the molecular mechanisms underlying epileptogenesis is still incomplete. Among various factors supposed to play a role in this disorder, the role of OS in neurological diseases has recently emerged [3,4].

One of the greatest challenges in the redox biology field has been the identification of OS biomarkers in biological samples obtained by noninvasive methods [5]. In 1990, Morrow and colleagues discovered a novel family of compounds named isoprostanes (IsoPs). These compounds are formed nonenzymatically through the free radical-induced peroxidation of arachidonic acid (AA, C20:4 n-6) *in vivo* [6]. Eight years after the discovery of IsoPs, other oxidation products were described by Roberts et al. [7], namely the neuroprostanes (NeuroPs). The F_4-NeuroPs are formed by a free radical-mediated, nonenzymatic mechanism from docosahexaenoic acid (DHA, C22:6 n-3)—a fatty acid that is very abundant in the brain, particularly in the gray matter, with its highest levels in neuronal membranes. The F_3-NeuroPs were described as the oxidation products of n-6 docosapentanoic acid (DPA, C22:5 n-6). More recently, the F_2-Dihomo-isoprostanes (F_2-Dihomo-IsoPs)—derived from radical attack on adrenic acid (AdA, C22:4 n-6), that has its highest levels in the myelin, kidney, and adrenal glands—have been described [8,9] and may be considered a very interesting marker for cerebral white matter injury [10]. Related to the nomenclature used for NeuroPs, in 2005, Taber and Roberts proposed the division of these compounds into eight classes, based on the position of the allylic alcohol. The oxidation nomenclature (A, B, C, D, F,…) was used. Similarly, the enantiomers of NeuroPs were designated "*ent*" and epimeric NeuroPs were termed as "*epi*" [11].

The ability to quantify these compounds in noninvasive samples like urine might provide unique markers of OI in the brain and could shed light on the role of free radicals in the pathogenesis of several diseases. Although the function of DHA in the brain is not well understood, deficiency of DHA is associated with abnormalities in brain function. Thus, the oxidation of DHA in the central nervous system has been implicated in various neurodegenerative disorders, including Alzheimer´s disease, Parkinson´s disease, Huntington´s disease, Rett syndrome, and amyotrophic lateral sclerosis, and in other diseases such as preeclampsia [4,12].

To date, NeuroPs have only been quantified by GC–MS [7]; in particular, GC–NICI–MS has been labeled as the most sensitive and suitable technique for the routine quantification of these compounds in biological samples. However, LC–MS/MS and UHPLC–MS/MS techniques are superior to GC–MS in the identification of different regioisomers and diastereomers of NeuroPs [13].

The main goal of this work was to evaluate the NeuroPs/Dihomo-IsoPs in epileptic patients and a control group in order to identify candidate clinical biomarkers of OS for the early prognosis, diagnosis, and efficient treatment of epilepsy. For this, a fast, accurate, and robust UHPLC–QqQ–MS/MS method for the identification and quantification of 10 NeuroPs/Dihomo-IsoPs in human urine, using solid phase extraction (SPE), was developed for the first time.

Subjects and methods

Chemicals and reagents

Neuroprostanes, including 4(*RS*)-F_{4t}-NeuroP; d_4–4(*RS*)-F_{4t}-NeuroP; 10-*epi*-10-F_{4t}-NeuroP; d_4-10-*epi*-10-F_{4t}-NeuroP; 10-F_{4t}-NeuroP; d_4-10-F_{4t}-NeuroP; 4-F_{4t}-NeuroP; 4-*epi*-4-F_{3t}-NeuroP; 4-F_{3t}-NeuroP; *Ent*-7(*RS*)-7-F_{2t}-Dihomo-IsoP; *Ent*-7-*epi*-7-F_{2t}-Dihomo-IsoP; 17-F_{2t}-Dihomo-IsoP; and 17-*epi*-17-F_{2t}-Dihomo-IsoP, were synthesized by Durand's team at the Institut des Biomolecules Max Mosseron (IBMM) (Montpellier, France) (Fig. 1).

The β-glucuronidase, type H2, from *Helix pomatia* and Bis-Tris (bis-(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane) used were from Sigma-Aldrich (St. Louis, MO, USA). All LC-MS grade solvents were from J.T. Baker (Phillipsburg, NJ, USA). Chlorhydric acid, hexane, trichloroacetic acid, and ethyl acetate were purchased from Panreac (Castellar del Vallés, Barcelona, Spain), and the Strata X-AW, 100 mg 3 mL^{-1} SPE cartridges from Phenomenex (Torrance, CA, USA).

Selection of volunteers

Fifteen ambulatory epileptic patients from the Epilepsy Unit of the Virgen de la Arrixaca Hospital were enrolled in this study. All these patients had been diagnosed with epilepsy, based on clinical criteria supported by electroencephalographic recordings and cerebral magnetic resonance (MRI) scanning. Their epilepsy was classified, according to the International League Against Epilepsy (ILAE) criteria, as genetic, structural, or of unknown origin. The physical characteristics and clinical profiles of the subjects are summarized in Table 1. The volunteers received classical antiepileptic drugs (AEDs) during this study, and were seizure free and without any changes in the daily dose of AEDs for the last six months prior to their inclusion. The control subjects ($n=15$) were age-matched individuals without clinical evidence of epilepsy or other neurological diseases. Importantly, all individuals in this study were nonsmokers and during the study the women were not in their menstrual days. The control group did not receive any medication or drug intake (we noted the specific absence of the acute administration of anti-inflammation drugs). All patients gave written informed consent for the experiment and the study was approved by the Bioethics Committee of the Hospital Virgen de la Arrixaca (Murcia, Spain). Urine samples were collected after 24 h, aliquoted immediately, and stored at −80 °C until posterior analysis.

Sample preparation: Calibration standards

Stock solutions of NeuroPs/Dihomo-IsoPs were diluted with methanol–water (1:1, v/v) to obtain the appropriate working solutions containing 10 analytes and three deuterated internal standards at a concentration of 1000 nmol L^{-1}. For the determination of the calibration curve, 12 successive dilutions were prepared. All solutions were stored at −80 °C. The NeuroPs/Dihomo-IsoPs concentrations were calculated from standard curves freshly prepared each day.

Also, three sets with 13 standards each were prepared to evaluate the assay accuracy and recovery and the absence or presence of the matrix effect. The first set of standards (A: external solution) was prepared to evaluate the MS/MS response of the analytes in water. The second set (B: postextraction sample) included the sample extracts spiked after SPE. The third set (C: preextraction sample) was prepared using samples spiked before SPE. Each set was spiked at two concentrations: 250 and 1000 nmol L^{-1}.