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Research Paper

Snapshot situation of oxidative degradation of the nervous system, kidney, and adrenal glands biomarkers-neuroprostane and dihomo-isoprostanes-urinary biomarkers from infancy to elderly adults

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ABSTRACT

We analyzed biomarkers of lipid peroxidation of the nervous system -F_2-dihomo-isoprostanes, F_3-neuroprostanes, and F_4-neuroprostanes- in urine samples from 158 healthy volunteers ranging from 4 to 88 years old with the aim of analyzing possible associations between their excretion values and age (years). Ten biomarkers were screened in the urine samples by UHPLC-QqQ-MS/MS. Four F_2-dihomo-isoprostanes (*ent*–7-(*R*)–7-F_{2t}-dihomo-isoprostane, *ent*–7-*epi*–7-F_{2t}-dihomo-isoprostane, 17-F_{2t}-dihomo-isoprostane, 17-*epi*–17-F_{2t}-dihomo-isoprostane), and one DPA-neuroprostane (4-F_{3t}-neuroprostane) were detected in the samples. On the one hand, we found a significant, positive correlation (Rho: 0.197, P=0.015) between the age increase and the amount of total F_2-dihomo-IsoPs. On the other hand, the values were significantly higher in the childhood group (4–12 years old), when compared to the adolescence group (13–17 years old) and the young adult group (18–35 years old). Surprisingly, no significant differences were found between the middle-aged adults (36–64 years old) and the elderly adults (65–88 years old). We display a snapshot situation of excretory values of oxidative stress biomarkers of the nervous system, using healthy volunteers representative of the different stages of human growth and development. The values reported in this study could be used as a basal or starting point in clinical interventions related to aging processes and/or pathologies associated with the nervous system.

1. Introduction

Biomarkers have been increasingly employed in empirical studies of human populations to understand physiological processes that change with age, diseases whose onset appears linked to age, and the aging process itself [1]. The free radical/oxidative stress theory of aging is the most popular explanation of how aging occurs at a molecular level in aerobic biological organisms [2]. This theory of aging consisted of age-related biochemical and physiological decline associated with cumulative oxidative damage to cellular components and tissues, promoting oxidative stress (OS) and leading to lesser longevity [3]. Nowadays, this theory is controversial since there may be interventions independent of reactive oxygen species (ROS) that promote longevity without affecting ROS or OS [2,4]. Oxidative stress is widely accepted to be a perturbation in the balance of free radicals in a cell and the cell´s ability to cope with the change by means of its antioxidant defense mechanisms [5]. The balance between ROS production and antioxidant defenses determines the degree of OS according to Finkel and Holbrook [6]. Recently, it has been reported that mild stress stimulates endogenous defense systems, which will promote health, but if the stress becomes chronic or is too extensive, it induces cellular damage and/or aging and a shortening of lifespan [7].

The brain and nervous system are prone to OS and are inadequately equipped with antioxidant defense system, which can lead to persistently increased levels of ROS and reactive nitrogen species reacting with the various target molecules (proteins, lipids, and DNA) [8]. The

Abbreviations: OS, (Oxidative stress); ROS, (Reactive oxygen species); PUFAs, (Polyunsaturated fatty acids); F_2-dihomo-IsoPs, (F_2-dihomo-isoprostanes); F_3-NeuroPs, (F_3-neuroprostanes); F_4-NeuroPs, (F_4-neuroprostanes); AdA, (Adrenic acid); DPA, (Docosapentaenoic acid); DHA, (Docosahexaenoic acid)

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lipids, especially polyunsaturated fatty acids (PUFAs), are vulnerable to oxidation by both enzymatic and non-enzymatic process. In humans, the products of lipid peroxidation have been accepted as toxic mediators, but they are also known to exert diverse biological effects [9]. Solberg et al. [10] mentioned that the determination of OS is complex and requires a quantification of the levels of free radicals or damaged biomolecules. The measurement of F_2-isoprostanes (F_2-IsoPs) by mass spectrometry has been extensively employed as a marker of oxidant stress and is widely considered to be the gold-standard index of lipid peroxidation in vivo. The measurement of free F_2-IsoPs in plasma or urine can be utilized to assess the endogenous formation of IsoPs but not to reveal the organ in which they are formed. Unless determining the levels of IsoPs in the cerebrospinal fluid, which reflects the ongoing metabolic activity of the brain, provides a great opportunity to reveal the occurrence of OS and lipid peroxidation in the brain. However, there are now some IsoPs-like compounds that might be regarded as markers of lipid peroxidation of the nervous system [11]. The F_2-dihomo-isoprostanes (F_2-dihomo-IsoPs), F_3-neuroprostanes (F_3-NeuroPs) and F_4-neuroprostanes (F_4-NeuroPs) are used to analyze the OS status of the nervous system in humans [11–13]. These biomarkers are formed by a free radical non-enzymatic mechanism from adrenic acid (C22:4 n-6, AdA) [14], docosapentaenoic acid (C22:5 n-6, DPA) [15] and docosahexaenoic acid (C22:6 n-3, DHA) [16] respectively. While DHA is an essential constituent of nervous tissue, highly enriched in neurons, and highly prone to oxidation [17], F_4-NeuroPs provide a specific quantification of the OS suffered by neural membranes *in vivo* [18] and F_2-dihomo-IsoPs are potential markers of free radical damage to myelin in the human brain [14]. Recent efforts have focused on the assessment of F_2-dihomo-IsoPs, F_3-NeuroPs, and F_4-NeuroPs as biomarkers in conditions associated with increased OS (particularly in disease conditions) and/or after dietary supplementation with antioxidants [11–13,19–21]. Despite its increasing clinical us to the best of our knowledge the biological variation of these biomarkers in healthy people of different age has not been reported yet. The ability to quantify these compounds in non-invasive samples like urine could shed light on the changes in excretion values of products of lipid peroxidation across a wide age range and may be useful for comparing these values detected in healthy individuals with those obtained diseased individuals. Therefore, the aim of this cross-sectional study was to quantify biomarkers of lipid peroxidation in the nervous system (F_2-dihomo-IsoPs, F_3-NeuroPs, and F_4-NeuroPs) in urine samples from healthy volunteers of different life stage (4–88 years), analyzing possible associations between their values and age intervals.

2. Materials and methods

2.1. Study population

This study was conducted in accordance with the Helsinki declaration. Approval was obtained from the Bioethics Committee of the University Hospital of Murcia. The participants were insured from the Hospital Virgen de la Arrixaca (Murcia, Spain) aged between 4 and 88 years of both genders (n=158). Age was reported at the time of the household interview as the age (years) at the last birthday. The assignment of the age ranges was based on social aging processes (childhood, adolescence, young adulthood, middle-aged adults, and elderly adults) according to Settersten and Mayer [22]. The age categories used in our statistical analyses were 4–12 years (childhood, n=20; mean: 8.20 ± 2.50 years), 13–17 years (adolescence, n=14; mean: 15.73 ± 1.43 years), 18–35 years (young adulthood, n=45; mean: 27.62 ± 4.97 years), 36–64 years (middle-aged adults, n=58; mean: 49.12 ± 9.03 years) and 65–88 years (old age, n=21; mean: 75.61 ± 6.62 years). All volunteers signed the informed consent document (18–88 years old). Volunteers under the age of 18 years had all referred to a doctor clinic for a routine check-up and a parent signed the informed consent document.

Regarding the exclusion criteria, individuals with chronic diseases, under drug treatments, and volunteers with overweight or obesity were excluded from the study. None of the subjects was cigarette smoker an alcoholic and pregnant. All the participants were submitted to clinical examination to confirm their health status. The health status of the participants was considered in the data analysis. The clinical parameters for determining the health status of the individuals are summarized in Supporting information 1.

2.2. Sample collection and preparation

A complete clinical analysis - consisting of hematology, chemistry, and urine chemical analysis - was performed in the volunteers. All samples (blood and urine) were collected, by a nurse at the University Hospital Virgen de la Arrixaca from the subjects early in the morning and under fasting conditions. Blood samples at rest were obtained by venipuncture and were placed in different tubes according to the analytical procedures. The samples were processed within 1 h of collection and stored at −80 °C for the analytical determinations. The hematological parameters were recorded using an automated hematological analyzer (Cell Dyn 3700 and 4000, Abbott, IL, USA) at the clinical analysis service of the University Hospital Virgen de la Arrixaca (Murcia, Spain). One-milliliter from the 24-h urine was used for analysis of the lipid peroxidation biomarkers. The metabolites were normalized as ng mg^{-1} creatinine and were assayed using the method described by Medina et al. [21]. Clinical parameters results of our volunteers (mean ± standard deviations (SD)) are summarized in Table 1.

2.3. Chemicals and Standards

Six NeuroPs (4(*RS*)−4-F_{4t}-NeuroP, 4-F_{4t}-NeuroP, 10-*epi*−10-F_{4t}-NeuroP, 10-F_{4t}-NeuroP, 4-*epi*−4F_{3t}-NeuroP and 4-F_{3t}-NeuroP); as well as, four F_2-dihomo-IsoPs (*ent*−7-(*R*)−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 analyzed in this experiment and three deuterated internal standards (d_4−4(*RS*)-F_{4t}-NeuroP, d_4−10-*epi*-10-F_{4t}-NeuroP, and d_4−10-F_{4t}-NeuroP) were used for the quality control of the analyses. All standards were synthesized using our published strategies [23–25]. β-glucuronidase, type H2 from *Helix pomatia* and BIS-TRIS (Bis-(2-hydroxyethyl)-amino-tris (hydroxymethyl)-methane) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All LC-MS grade solvents were from J.T. Baker (Phillipsburg, NJ, USA). Strata X-AW (100 mg 3 mL^{-1}) solid phase extraction cartridges were purchased from Phenomenex (Torrance, CA, USA).

2.4. UHPLC-QqQ-MS/MS analyses

The separation of NeuroPs and F_2-dihomo-IsoPs in the urine samples was performed with an Ultra High-Performance Liquid Chromatography 6460-Triple Quadrupole-tandem Mass Spectrometry (Agilent Technologies, Waldbronn, Germany), using the set up previously described by Medina et al. [21]. Data acquisition and processing were performed using Mass Hunter software version B.04.00 (Agilent Technologies, Waldron, Germany). The identification and quantification of NeuroPs and F_2-dihomo-IsoPs were carried out using the authentic markers described by Medina et al. [21].

2.5. Statistical analyses

Quantitative data are presented as mean ± SEM (standard error of the mean) or SD (Table 2). Concerning the study population, women and men were analyzed together because no difference between them was detected according to the Student's *t*-test (data not shown). The Kolmogorov-Smirnov test and Shapiro-Wilk test were applied to assess