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Original Contribution

Dietary polyunsaturated fatty acids and heme iron induce oxidative stress biomarkers and a cancer promoting environment in the colon of rats

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ABSTRACT

The end products of polyunsaturated fatty acid (PUFA) peroxidation, such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), and isoprostanes (8-iso-$PGF_{2\alpha}$), are widely used as systemic lipid oxidation/oxidative stress biomarkers. However, some of these compounds have also a dietary origin. Thus, replacing dietary saturated fat by PUFAs would improve health but could also increase the formation of such compounds, especially in the case of a pro-oxidant/antioxidant imbalanced diet. Hence, the possible impact of dietary fatty acids and pro-oxidant compounds was studied in rats given diets allowing comparison of the effects of heme iron vs. ferric citrate and of ω-6- vs. ω-3-rich oil on the level of lipid peroxidation/oxidative stress biomarkers. Rats given a heme iron-rich diet without PUFA were used as controls. The results obtained have shown that MDA and the major urinary metabolite of HNE (the mercapturic acid of dihydroxynonane, DHN-MA) were highly dependent on the dietary factors tested, while 8-iso-$PGF_{2\alpha}$ was modestly but significantly affected. Intestinal inflammation and tissue fatty acid composition were checked in parallel and could only explain the differences we observed to a limited extent. Thus, the differences in biomarkers were attributed to the formation of lipid oxidation compounds in food or during digestion, their intestinal absorption, and their excretion into urine. Moreover, fecal extracts from the rats fed the heme iron or fish oil diets were highly toxic for immortalized mouse colon cells. Such toxicity can eventually lead to promotion of colorectal carcinogenesis, supporting the epidemiological findings between red meat intake and colorectal cancer risk.

Therefore, the analysis of these biomarkers of lipid peroxidation/oxidative stress in urine should be used with caution when dietary factors are not well controlled, while control of their possible dietary intake is needed also because of their pro-inflammatory, toxic, and even cocarcinogenic effects.

Introduction

Replacement of dietary saturated fat by polyunsaturated fatty acids (PUFAs), especially ω-3 fatty acids, would improve health, particularly cardiovascular and cognitive functions [1]. However, PUFAs, particularly ω-3 fatty acids with a high double bond index (DBI), are prone to oxidation by dietary oxidants like heme iron, giving rise to secondary lipid peroxidation compounds.

These lipid oxidation products and their metabolites can be assayed in blood, urine, and tissues as markers of endogenous lipid peroxidation/oxidative stress. For instance, malondialdehyde (MDA) comes from the oxidation of fatty acids bearing more than two methylene interrupted double bonds [2]. Urinary MDA has been used to reflect the lipid peroxidation occurring *in vivo* in the case of oxidative stress due to, for instance, CCl_4 poisoning, vitamin E deficiency, or iron nitriloacetate administration [3].

Abbreviations: DBI, double bond index; DHN-MA, 1,4-dihydroxynonane–mercapturic acid; EIA, enzyme immunoassay; FAME, fatty acid methyl ester; HNE, 4-hydroxynonenal; HNE-His, HNE-histidine protein adduct; MDA, malondialdehyde; MPO, myeloperoxidase; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid reactive substances

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In the same way, elevated levels are associated with hypertensive disorders of pregnancy [4], ageing [5], and with exposure to polycyclic aromatic hydrocarbons and particulate matters [6]. However, Draper's group pointed out that urinary MDA could be a useful indicator of endogenous lipid peroxidation only when peroxidation of dietary lipids is precluded [3], because precursors of MDA are also found in the diet. Kanner's group reported the increase of plasmatic MDA after a red meat-rich meal that can be prevented by coffee or red wine polyphenols [7,8]. The mercapturic acid of dihydroxynonane (DHN-MA) is the major urinary metabolite of 4-hydroxynonenal (HNE), an end product of ω-6 fatty acids oxidative breakdown, which is a well-known bioactive marker of lipid peroxidation involved in cell growth regulation and signaling [9]. Thus DHN-MA has also been used as a marker of oxidative stress/lipid peroxidation in $BrCCl_3$- [10] or CCl_4-treated rats [11] and in smokers with or without vitamin C supplementation [12]. Rats given a diet containing heme iron and ω-6 fatty acids, in the form of hemoglobin or red meat and safflower oil, excrete huge quantities of DHN-MA, indicating the formation of HNE in diet or during digestion [13,14]. HNE is present in food containing heme iron and PUFAs [15]. Another group reported an important formation of HNE in thermally oxidized oils or in food fried in these oils, but these studies did not report any measurement of HNE metabolite in urine of animals fed on diets made with those oils [16,17].

In the early 1990s, the determination of isoprostanes marked a new era in the field of oxidative stress biomarkers. Their unique precursor is arachidonic acid, and their formation is believed to be independent of the lipid content of the diet [18]. They appear to be more specific of lipid peroxidation than MDA. As for MDA and urinary metabolites of HNE, their excretion increase under conditions of oxidative stress [10]. For these reasons, isoprostanes are still regarded now as gold standards of lipid peroxidation/oxidative stress biomarkers and are widely used for this purpose.

In addition, secondary lipid oxidation reactive aldehydes are cytotoxic and genotoxic compounds, while some of them, especially HNE, are known also as second messengers of free radicals [1,2,19]. Their presence in the diet could have a toxic effect, especially on the digestive tract, which is the first target of such dietary compounds. In a previous work, we had observed that, after initiation of colorectal carcinogenesis, animals -fed a heme iron and ω-6 fatty acid-rich diet, presented more preneoplastic lesions in their colon than rats -fed a control diet without heme iron. These results demonstrate the promoting effect of heme iron on colorectal cancer in these animals [20,21]. The cytotoxic activity of the fecal extracts from those rats on immortalized, but not cancerous, mouse colon epithelial cells was much more important when rats were given a heme iron-rich diet than control diet. Moreover a heme iron-rich diet induced a selective selection of preneoplastic cells [22]. This could be related to the epidemiological link existing between red meat, containing an important concentration of heme iron, and colorectal cancer in humans.

In the present study, we have tested the effect of two edible oils, containing predominantly ω-3 fatty acids for fish oil and ω-6 fatty acids for safflower oil, combined with heme iron or ferric citrate, with different pro-oxidant properties, on MDA, DHN-MA, and 8-iso-$PGF_{2\alpha}$ urinary excretion in rats, in order to study the impact of a "peroxidable" diet on oxidative stress biomarkers. We used in a control diet coconut hydrogenated oil containing no polyunsaturated fatty acids. Hemin was used in a concentration that is in the same range as the one used in studies representing a meat-based Western diet by our group [20] or by Van der Meer and co-workers [23,24]. In addition, the cytotoxic activity of the fecal extracts of rats given the diets was evaluated on two colon/rectal cell lines.

Materials and methods

Unless specified elsewhere in this section, reagents were purchased from Sigma (Saint-Quentin-Fallavier, France).

Animals and diets

Four-week-old female Fisher 344 rats (6 rats/group) were purchased from Charles River. Animal care was in accordance with our local ethic committee (number TOXCOM/0006/FG). They were housed individually in plastic metabolic cages and allowed for a week of acclimatization to their cage and the acclimatization diet before the start of the experimental diets. The room was kept at a temperature of 22 °C on a 12 h light–dark cycle. Animals had free access to tap water and to their respective diet. Diets were given each day at the end of the afternoon in order to limit oxidation. Feces and urine were collected each day. Food consumption and rat weight were recorded at Days 0, 1, 2, 3, 4, 7, and 10 and at Day 17, when the animals were euthanized. Colon and liver were excised and placed in liquid nitrogen before being stored at −80 °C.

The acclimatization and the five experimental diets were based on a modified AIN-76 diet prepared and formulated in a powdered form by UPAE (INRA, Jouy-en-Josas, France). All diets were low-calcium diets (0.8 g/kg) and contained 5% of oil: either corn oil for acclimatization diet (MP Biomedicals 901414) or hydrogenated coconut oil (MP 901404), safflower oil (MP 102888), and Menhaden fish oil (MP 960120), for experimental diets. In addition, experimental diets contained either hemin (0.94 g/kg) (Sigma H5533) or ferric citrate (0.36 g/kg) (F6129) purchased from Sigma and had a similar content of iron (80 mg/kg). Major fatty acid composition of the oils used in the different diets is shown in Table 1.

Oxidative stress biomarker assays

Twenty-four hour urine was collected in plastic tubes placed on ice during collection. Tubes contained 1 ml of 360 mM ethanolic solution of butylated hydroxytoluene (BHT). Urine was stored at −20 °C until use.

MDA determination

Urinary MDA was measured after a derivatization procedure using diaminonaphtalene as previously described for HPLC-UV quantitation [25], except that the detection was carried out with a single quadrupole mass spectrometer in the positive electrospray ionization mode. This enables the introduction of phenyl-benzimidazole (PBI) as an internal standard. PBI, which has the same molecular weight as the MDA derivative, exhibited a very similar chromatographic and ionization behavior as the diazepinium of MDA. Measurements were made with a P4000 liquid chromatograph connected to an autosampler AS 3000 and a single quadrupole mass Navigator Aqua detector (Thermo

Table 1
Major fatty acid (> 10%) composition of the PUFA containing dietary oils.

	16:0	16:1n-9	18:1n-9	18:2 n-6	20:5n-3	22:6 n-3	DBI
Corn oil	11%		25%	60%			146
Safflower oil			11–13%	70–80%			170
Menhaden fish oil*	15%	12%	9%		16%	11%	244

DBI (double bond index – % of each fatty acid × number of double bond). Hydrogenated coconut oil contained 98.4% of saturated fatty acids (mainly lauric acid), its DBI was 3.

* A total composition of ω-3 and ω-6 fatty acids in Menhaden fish oil was: ω-3: 32.15 and ω-6: 9%.