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Oxyphytosterol formation in humans: Identification of high vs. low oxidizers

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ABSTRACT

Animal studies suggest that oxyphytosterols are atherogenic. However, we have previously shown that plasma oxyphytosterol concentrations did not increase after consuming a diet enriched in plant sterol esters (3 g/day), while minor reductions were seen after consuming a plant stanol ester-enriched diet. Large variations in oxyphytosterol concentrations between individuals however existed. The aim of this study was to identify factors that may explain inter-individual differences in plasma oxyphytosterol concentrations. For this, 43 subjects consumed for 4 weeks in random order a plant sterol, stanol and control margarine. Plasma oxyphytosterol concentrations were determined in butylated hydroxytoluene (BHT)-enriched EDTA plasma via GC–MS and serum oxidized low-density lipoprotein (oxLDL) concentrations were analyzed via ELISA. Trolox equivalent antioxidant capacity (TEAC) values, α-tocopherol concentrations and iron/copper status were determined to assess plasma oxidative and anti-oxidative capacity. Serum (non-oxidized) sitosterol and campesterol concentrations did not correlate with plasma oxysitosterol and oxycampesterol concentrations during any of the three dietary interventions. Moreover, plasma oxyphytosterol concentrations remained relatively stable over time. Six subjects could be arbitrarily classified as having consistent low or high plasma oxyphytosterol concentrations, which was also reflected in oxLDL concentrations. However, oxidative and anti-oxidative capacity markers, such as iron/copper status, α-tocopherol concentrations and TEAC values, could not explain these differences. In conclusion, subjects seem to have consistent plasma oxyphytosterol concentrations, which resulted in the identification of 'low and high oxidizers'. Differences, however, could not be attributed to the oxidative and anti-oxidative capacity markers analyzed.

1. Introduction

Cholesterol oxidation products (oxycholesterols) can be derived from dietary intake and from *in vivo* oxidation by specific enzymes or by reactive oxygen species (ROS) [1]. Increased oxycholesterol concentrations are found in plasma of patients suffering from cardiovascular disease (CVD) [2] and in atherosclerotic plaques [3]. Further, a positive correlation has been observed between ROS-induced oxycholesterol concentrations in serum and established CVD risk factors, such as obesity and increased triglyceride concentrations [4]. In addition, recent studies have also suggested that oxycholesterols contribute to the development of degenerative diseases such as age-related macular degeneration [5]. Although these findings are suggestive, causal relations between elevated plasma oxycholesterol concentrations and pathological conditions have not been established so far.

Plant sterols are structurally related to cholesterol and can be oxidized to oxyphytosterols [6]. While the biological role of oxycholesterols has been studied into large detail [1,7], studies evaluating the formation, kinetics and (patho)physiological effects of oxyphytosterols are scarce. Oxycholesterols and oxyphytosterols exert cytotoxic and apoptotic effects *in vitro*, as indicated by an

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inhibition of cell growth and a reduction of cell viability [8]. Oxycholesterols may be more cytotoxic than oxyphytosterols [9], while pathways underlying the cytotoxicity of oxycholesterols and oxyphytosterols seem to be different, i.e. antioxidants protected against the damaging effects of oxycholesterols, but not against the toxicity of oxyphytosterols [10]. Studies in rodents have demonstrated that oxycholesterols and oxyphytosterols accumulate in tissues, but discrepancies exist regarding their potential atherogenicity [11–14].

What is clearly lacking is a comprehensive understanding of the etiology behind the formation of oxyphytosterols and their effects in humans. Circulating oxyphytosterol concentrations have only been measured occasionally in human studies [15–18] and large variations in serum oxyphytosterol concentrations are observed when comparing the results of these studies. In a previous study, we have shown that plasma oxyphytosterol concentrations in healthy volunteers did not increase after consuming a diet enriched in plant sterol esters (3 g/day) for 4 weeks, while minor reductions were seen after consuming a plant stanol ester-enriched [19]. More importantly, we identified large variations in oxyphytosterol concentrations between healthy human subjects, which remained stable over time. The aim of this study was now to identify factors that relate to these inter-individual differences in plasma oxyphytosterol concentrations.

2. Subjects and methods

2.1. Subjects, diets and design

The protocol of this placebo-controlled double-blind dietary intervention study has been reported into detail [19]. Briefly, subjects were recruited in Maastricht and surroundings. Subjects met the following criteria: 18–70 years of age; body mass index (BMI) between 20 and 30 kg/m^2; no active cardiovascular disease or severe medical condition during the past 5 years that might interfere with the study; no use of lipid-lowering medication or a medically prescribed diet; stable body weight during the last three months; and no consumption of plant sterol- or plant stanol-enriched products in the previous month. In addition, serum total cholesterol concentrations were <7.8 mmol/L; serum triglyceride concentrations <3.0 mmol/L and plasma glucose concentrations <6.1 mmol/L, as determined during two screening visits. Baseline characteristics are shown in Table 1. All participants gave written informed consent before entering the study. The protocol was approved by the medical ethical committee of the Maastricht University Medical Centre+ (MUMC+), and the study was registered at clinicaltrials.gov as NCT01559428.

The study had a crossover design and consisted of three intervention periods of 4 weeks, separated by washout periods of 4 weeks. Subjects were stratified for gender and allocated to the intervention periods in a randomized order, based upon a computer-generated table with random numbers. During each intervention period, subjects were asked to replace their own spread with the test margarine (70% fat) of which 20 g had to be consumed on a daily basis. The daily consumption of 20 g of margarine provided no, or 3.0 g of plant sterols or stanols (provided as fatty-acid esters), referred to as control, sterol or stanol condition. Margarine composition has previously been described into detail [19].

2.2. Blood sampling

Blood was sampled after an overnight fast after 3 and 4 weeks of intervention. A clotting tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) was sampled and serum was obtained by low-speed centrifugation at 1300 × *g* for 15 min at room temperature, at least half an hour after venipuncture. Serum was stored at −80 °C and used for analysis of lipid and lipoprotein concentrations, of plant sterol and plant stanol concentrations, of oxidized low-density lipoprotein (oxLDL) concentrations, of α-tocopherol (vitamin E) concentrations, of iron status parameters, i.e. total iron, ferritin and transferrin and of copper status parameters, i.e. copper and ceruloplasmin. An EDTA and NaF tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were sampled. EDTA and NaF plasma was obtained by low-speed centrifugation at 1300 × *g* for 15 min at 4 °C, and then stored at −80 °C. To avoid auto-oxidation, oxyphytosterol concentrations were determined in butylated hydroxytoluene (BHT)-enriched EDTA plasma; for this 10 μl BHT (25 mg/mL ethanol) was added per 1 mL of EDTA plasma, immediately after centrifugation. The trolox equivalent antioxidant capacity (TEAC) assay was performed in de-proteinated NaF plasma; for this 10% TCA was added to the plasma (1:1) followed by centrifugation (13,000 rpm, 5 min at 4 °C).

Table 1
Baseline characteristics.

Age (y)	41 ± 18
Male/female (*n*)	17/26
BMI (kg/m^2)	24.8 ± 2.8
Total cholesterol (mmol/L)	5.72 ± 1.12
LDL cholesterol (mmol/L)	3.53 ± 1.06
HDL cholesterol (mmol/L)	1.69 ± 0.37
Triacylglycerol (mmol/L)	1.14 ± 0.46

Values are means ± SD

2.3. Analyses

All analyses were performed in samples from week 4 of each intervention period, and samples from one subject were always analyzed within one run to avoid bias caused by between run variations. Serum total cholesterol, plant sterol (sitosterol, campesterol) and plant stanol (sitostanol, campestanol) concentrations were analyzed by gas–liquid chromatography–mass spectroscopy (GC–MS) as described previously [17,20]. Plant sterol and plant stanol concentrations were expressed as 10^2x μmol/mmol total cholesterol. The measured oxyphytosterols were 7α-hydroxy(OH)-sitosterol, 7α-OH-campesterol, 7β-OH-sitosterol, 7β-OH-campesterol, 7-keto-sitosterol, and 7-keto-campesterol by GC–MS according the procedure as described by Husche et al. [17] and expressed as ng/mL. Oxyphytosterol concentrations were also standardized for cholesterol concentrations and expressed as nmol/mmol cholesterol. OxLDL concentrations were analyzed by a commercially available sandwich ELISA (Mercodia, Uppsala, Sweden) and α-tocopherol concentrations by reversed-phase HPLC as described before [21]. The TEAC value is a measurement for the total antioxidant status, relating the free radical scavenging properties of a solution or a compound to that of the synthetic antioxidant trolox. The assay was performed as previously described [22]. Total iron concentrations were determined with a fixed-timed endpoint assay on a Beckman Coulter LX20 PRO Clinical Chemistry analyser (Beckman Coulter, Fullerton, USA). Transferrin and ceruloplasmin concentrations were analyzed by particle-enhanced immunonephelometry on the BN Prospect from Siemens Healthcare Diagnostics (Siemens, Munich, Germany). The transferrin saturation (%) was calculated as Iron (μmol/)/(transferrin (g/L) × 25) × 100%. Ferritin concentrations were determined using an immunoturbidimetric assay on a Cobas 6000 system (Roche Diagnostics, Basel, Switzerland) and copper