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Vascular effects of oxysterols and oxyphytosterols in apoE −/− mice

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ABSTRACT

Objectives: The aim of our study was to investigate vascular effects of oxysterols and oxyphytosterols on reactive oxygen species (ROS), endothelial progenitor cells, endothelial function and atherogenesis.
Methods: Male apoE−/−mice were treated with cholesterol, sitosterol, 7-ß−OH−cholesterol, 7-ß−OH−sitosterol, or cyclodextrin by daily intraperitoneal application. The respective concentrations in the plasma and in the arterial wall were determined by gas chromatography-flame ionization or mass spectrometry. ROS production was assessed by electron-spin resonance spectroscopy in the aorta, endothelial function of aortic rings and atherosclerosis in the aortic sinus was quantitated after 4 weeks.
Results: Compared to vehicle, there was no difference in plasma cholesterol levels and arterial wall concentrations after i.p. application of cholesterol. 7-ß−OH−cholesterol concentrations were increased in the plasma (33.7 ± 31.5 vs. 574.57.2 ± 244.92 ng/ml) but not in the arterial wall (60.1 ± 60.1 vs. 59.3 ± 18.2 ng/mg). Sitosterol (3.39 ± 0.96 vs. 8.16 ± 4.11 mg/dL; 0.08 ± 0.04 vs. 0.16 ± 0.07 µg/mg, respectively) and 7-ß−OH−sitosterol concentrations (405.1 ± 151.8 vs. 7497 ± 3223 ng/ml; 0.24 ± 0.13 vs. 16.82 ± 11.58 ng/mg, respectively) increased in the plasma and in the aorta. The i.p-application of the non-oxidized cholesterol or sitosterol did not induce an increase of plasma oxysterols or oxyphytosterols concentrations. Oxidative stress in the aorta was increased in 7-ß−OH−sitosterol treated mice, but not in mice treated with cholesterol, sitosterol, or 7-ß−OH−cholesterol. Moreover, cholesterol, sitosterol, 7-ß−OH−cholesterol, and 7-ß−OH−sitosterol did not affect endothelial-dependent vasodilation, or early atherosclerosis.
Conclusion: Increased oxyphytosterol concentrations in plasma and arterial wall were associated with increased ROS production in aortic tissue, but did not affect endothelial progenitor cells, endothelial function, or early atherosclerosis.

Hypercholesterolemia is a major risk factor for the development of cardiovascular diseases [1]. High dietary intake of cholesterol together with sedentary habits have been identified as major contributors to atherosclerosis [2]. The latter has long been considered a cholesterol storage disease; however, today atherosclerosis is considered a more complex disease in which both innate and adaptive inflammatory mechanisms as well as interactions between the arterial wall and blood components play a role [3].

Supplementation of foods with plant sterols has been suggested to prevent atherosclerosis because of their cholesterol-lowering effect [4]. Similar to cholesterol, plant sterols can be oxidized and can make an important contribution to the pro-atherogenic effects

Abbreviations: EPC, endothelial progenitor cells; ESR, electron-spin resonance; i.p., intraperitoneal; MNC, mononuclear cells; PSE, plant sterol esters; ROS, reactive oxygen species.

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of both cholesterol and cholesterol oxidation oxides in relation to inflammatory disease onset and the development of plaques [5]. Several lines of evidence suggest that oxysterols (oxidized cholesterol) are atherogenic and play a role in the pathogenesis of cardiovascular disease. The presence of one or more unsaturated bonds also makes plant sterols susceptible to oxidation. Only small amounts of oxidized plant sterols (oxyphytosterols) can be found in the diet [6]; nevertheless, relatively high concentrations of oxyphytosterols are present in the serum of sitosterolemia patients and smaller amounts in the plasma of healthy individuals [7–9]. Thus, higher plasma plant sterol concentrations may translate into higher plasma oxyphytosterol concentrations. Intervention trials found conflicting results whether the consumption of a plant sterol-enriched margarine for 4 weeks increases plasma 7-ß–OH–campesterol concentrations in healthy individuals [10,12]. Differences with regard to the post-prandial state, the genetic background and different duration of the wash out periods may explain the heterogenicity of the data, however, additional research is needed to address the relation of specific diets with plasma oxyphytosterol concentrations.

The factors related to the oxidative behavior of plant sterols are unknown; however, it has been suggested for cholesterol that patients characterized by oxidative stress, such as type 2 diabetics and patients with stable coronary artery disease have increased oxysterol concentrations [11], [12]. Since the exact factors predisposing for plant sterol oxidation or oxyphytosterol formation are unknown, it is difficult to experimentally show the effects of endogenously formed oxyphytosterols on vascular wall characteristics and atherosclerotic lesion formation. Moreover, oxyphytosterol concentrations due to endogenous formation are usually rather low which makes long-term interventions necessary. Another approach is to feed oxyphytosterols, as has been done for oxysterols [13]. So far, there have been only limited data regarding the effects of an oxyphytosterol-enriched diet on atherogenesis, which was however suggestive for atherogenic effects and incorporation of oxyphytosterols in the lesion [14]. However, also in this case serum concentrations are low due to the low percent absorption of oxyphytosterol from the diet. Tomoyori et al. showed that oxyphytosterols are indeed absorbed from the diet in low amounts followed by transport to the lymph and accumulation in the serum, liver, and aorta [15]. To overcome these problems, in the present study we therefore synthesised specifically highly purified sitosterol and its respective 7b-hydroxylated metabolite and investigated the i.p. application route on different vascular phenotypes such as oxidative stress (ROS production) in the aorta, endothelial progenitor cells, and on endothelial function and atherogenesis using male apoE−/−mice.

1. Methods

1.1. Animals and diets

Male apoE−/−(C57/Bl6 genetic background) mice, 8–12 weeks of age, weighing 20–25 g were purchased from Charles River, Sulzfeld, Germany. The 50 apoE−/− mice were randomized to 5 treatment groups, n = 10 per group, treated for 4 weeks. All groups were fed a “Western-type” diet (40 kcal% butterfat, 0.15% (w/w) cholesterol). Animal experiments were performed in accordance with the German animal protection law. Over the 4 week study period mice had ad libitum access to water and chow. Diets were prepared by the SNIFF Company (Soest, Germany).

1.2. Cholesterol, oxysterols, plant sterols, and oxyphytosterols

All commercial reagents were used without further purification unless otherwise stated. The starting materials cholesterol (>95%) and stigmasterol (>95%) were both purchased from Sigma Aldrich. β-Sitosterol (**4**) was produced in 4 steps (in 40% yield) from Stigmasterol (**3**) and was converted to 7β-OH β-Sitosterol (**6**) in 4 steps *via* acetate formation, allylic oxidation, hydrolysis of the acetate, and stereoselective borohydride-mediated ketone reduction. 7β-OH Cholesterol (**2**) was synthesized in 4 steps from cholesterol (**1**) *via* a similar method. For further information refer to Supplementary material. All sterols were diluted in sodium chloride with 30% cyclodextrin and applicated intraperitoneally 1 mg per day per mouse.

1.3. 7-β-Hydroxy-β-Sitosterol (6)

mp 137–139 °C (from EtOAc/hexane) (Found: C, 79.30; H, 11.43. Calc. for $C_{29}H_{50}O_2$.(½H_2O): C, 79.21; H, 11.69%). υmax/cm^{-1} 3400, 2959, 2871, 1465, 1384, 1056; δ_H 0.70–2.32 (47H, m), 3.51–3.58 (1H, m, 3α-H), 3.85 (1H, bd, J = 2.6, H-7), 5.29 (1H, bs, H-6); δ_C (75.5 MHz) 11.82 (CH_3), 11.98 (CH_3), 18.84 (CH_3), 19.03 (CH_3), 19.16 (CH_3), 19.81 (CH_3), 21.09 (CH_2), 23.08 (CH_2), 26.13 (CH_2), 26.39 (CH_2), 28.55 (CH_2), 29.16 (CH), 31.59 (CH_2), 33.99 (CH_2), 36.10 (CH), 36.46 (quaternary C), 36.95 (CH_2), 39.57 (CH_2), 40.93 (CH), 41.74 (CH_2), 42.94 (quaternary C), 45.86 (CH), 48.28 (CH), 55.39 (CH), 55.97 (CH), 71.44 (CH), 73.36 (CH), 125.46 (CH), 143.47 (quaternary C); *m*/*z* (ESI^+): 431 [$(M + H)^+$].

1.4. 7-β-Hydroxy-Cholesterol (*2*)

mp 182–183 °C (from EtOAc/hexane) υmax/cm^{-1} 3400, 2959, 2871, 1465, 1384, 1056; δ_H 0.70–2.37 (43H, m), 3.50–3.60 (1H, m, 3α-H), 3.85 (1H, bd, J = 2.6, H-7), 5.29 (1H, bs, H-6); δ_C (75.5 MHz) 11.83 (CH_3), 18.78 (CH_3), 19.17 (CH_3), 21.08 (CH_2), 22.57 (CH_3), 22.84 (CH_3), 23.84 (CH_2), 26.39 (CH_2), 28.03 (CH), 28.56 (CH_2), 31.57 (CH_2), 35.74 (CH), 36.20 (CH_2), 36.44 (quaternary C), 36.94 (CH_2), 39.50 (CH_2), 39.56 (CH_2), 40.90 (CH), 41.73 (CH_2), 42.93 (quaternary C), 48.25 (CH), 55.44 (CH), 55.95 (CH), 71.44 (CH), 73.36 (CH), 125.44 (CH), 143.48 (quaternary C); *m*/*z* (ESI^+): 403 [$(M + H)^+$].

1.5. Plasma and arterial wall concentrations of sterols, phytosterols, and their respective oxides

After 4 weeks, blood samples were drawn from the abdominal vena cava and the descending aorta was excised on sacrifice. Blood samples were centrifuged immediately and plasma and arterial wall tissue were stored at − 70 °C. The sterol content of plasma and arterial wall tissue samples was analyzed by gas liquid chromatography-mass spectrometry with epicoprostanol as the internal standard, the amount of 7b–OH–cholesterol and 7b–OH–sitosterol using deuterium labeled internal standards as described previously [12,13,29].

1.6. Reactive oxygen species in aortic tissue and the spleen

The production of ROS in aortic tissue was analyzed by ESR spectroscopy as described previously [16]. Briefly, aortic rings were incubated in Krebs HEPES buffer containing 25 μmol/L deferoxamine (Noxygen) and 55 μmol/L diethyldithiocarbamic acid (DETC, Noxygen) together with the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, 500 μM) for 1 h at 37 °C. Afterwards, supernatants were immediately analyzed by ESR spectroscopy. ESR spectra were recorded using a Bruker e-scan spectrometer (Bruker Biospin) with the following settings: center field, 3484.5 g; microwave power, 18.11 mW; modulation amplitude, 2.3 G; sweep time, 5.24 s; field sweep, 16 G. Results were normalized to the dry weight of the aortic rings or the