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The effect of prolonged dietary nitrate supplementation on atherosclerosis development

Elke Marsch [a], Thomas L. Theelen [a], Ben J.A. Janssen [b], Jacco J. Briede [c], Guido R. Haenen [d], Joan M.G. Senden [e], Lucas J.C. van Loon [e], Martijn Poeze [f], Jörgen Bierau [g], Marion J. Gijbels [a, h, i], Mat J.A.P. Daemen [j], Judith C. Sluimer [a, *]

[a] Department Pathology, CARIM, MUMC, Maastricht, The Netherlands
[b] Department Pharmacology, CARIM, Maastricht University, Maastricht, The Netherlands
[c] Department Toxicogenomics, Maastricht University, Maastricht, The Netherlands
[d] Department Toxicology, Maastricht University, Maastricht, The Netherlands
[e] NUTRIM, School for Nutrition, Toxicology and Metabolism, MUMC, Maastricht, The Netherlands
[f] Department Surgery/Intensive Care Medicine, MUMC, Maastricht, The Netherlands
[g] Department of Clinical Genetics, MUMC, Maastricht, The Netherlands
[h] Department Molecular Genetics, CARIM, Maastricht University, Maastricht, The Netherlands
[i] Department Medical Biochemistry, AMC, Amsterdam, The Netherlands
[j] Department Pathology, AMC, Amsterdam, The Netherlands

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ABSTRACT

Background: Short term dietary nitrate or nitrite supplementation has nitric oxide (NO)-mediated beneficial effects on blood pressure and inflammation and reduces mitochondrial oxygen consumption, possibly preventing hypoxia. As these processes are implicated in atherogenesis, dietary nitrate was hypothesized to prevent plaque initiation, hypoxia and inflammation.
Aims: Study prolonged nitrate supplementation on atherogenesis, hypoxia and inflammation in low density lipoprotein receptor knockout mice ($LDLr^{-/-}$).
Methods: $LDLr^{-/-}$ mice were administered sodium-nitrate or equimolar sodium-chloride in drinking water alongside a western-type diet for 14 weeks to induce atherosclerosis. Plasma nitrate, nitrite and hemoglobin-bound nitric oxide were measured by chemiluminescence and electron parametric resonance, respectively.
Results: Plasma nitrate levels were elevated after 14 weeks of nitrate supplementation (NaCl: 40.29 ± 2.985, $NaNO_3$: 78.19 ± 6.837, $p < 0.0001$). However, prolonged dietary nitrate did not affect systemic inflammation, hematopoiesis, erythropoiesis and plasma cholesterol levels, suggesting no severe side effects. Surprisingly, neither blood pressure, nor atherogenesis were altered. Mechanistically, plasma nitrate and nitrite were elevated after two weeks (NaCl: 1.0 ± 0.2114, $NaNO_3$: 3.977 ± 0.7371, $p < 0.0001$), but decreased over time (6, 10 and 14 weeks). Plasma nitrite levels even reached baseline levels at 14 weeks (NaCl: 0.7188 ± 0.1072, $NaNO_3$: 0.9723 ± 0.1279 $p = 0.12$). Also hemoglobin-bound NO levels were unaltered after 14 weeks. This compensation was not due to altered eNOS activity or conversion into peroxynitrite and other RNI, suggesting reduced nitrite formation or enhanced nitrate/nitrite clearance.
Conclusion: Prolonged dietary nitrate supplementation resulted in compensation of nitrite and NO levels and did not affect atherogenesis or exert systemic side effects.

1. Introduction

Ischemic heart disease and stroke remain the leading cause of death in the western world [1]. Atherosclerosis presents the main underlying cause of cardiovascular disease. Atherosclerosis is a

* Corresponding author. Maastricht University Medical Center, Department of Pathology, 6229 HX Maastricht, The Netherlands.
E-mail address: judith.sluimer@maastrichtuniversity.nl (J.C. Sluimer).

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lipid-driven inflammatory disease, initiated by endothelial dysfunction, resulting in accumulation and subsequent oxidation of cholesterol in the vessel wall. In turn, this triggers inflammatory cell infiltration and macrophage foam cell formation leading to apoptosis and secondary necrosis and plaque advancement [2]. Additionally, atherosclerotic plaques are hypoxic [3], with hypoxia playing a causal role in atherogenesis by reducing macrophage efferocytosis capacity [4]. In support, sleep apnea patients suffer from an increased risk of atherosclerosis [5,6] and continuous positive airway pressure-mediated oxygen supply in patients can improve endothelial function [7].

Seeing this highly complex disease, we aimed to target atherosclerosis on multiple levels: ameliorating hypertension, hypoxia and inflammation. Inorganic dietary nitrate supplementation may present such a multi-targeted approach, as it has been shown to dampen several processes involved in atherosclerosis: hypertension, hypoxia, endothelial dysfunction and inflammation. Short term dietary nitrate supplementation has been shown to lower resting blood pressure in humans [8]. Furthermore, dietary nitrate supplementation improves mitochondrial function, thereby reducing oxygen requirement during exercise [9–11]. This reduced cellular oxygen consumption may potentially restore plaque oxygenation and alleviate plaque progression. Additionally, one week dietary nitrate and nitrite supplementation reduced infarct size in a murine myocardial ischemia-reperfusion model, by replenishing nitrate and nitrite stores during infarction [12]. Also, dietary nitrate resulted in enhanced cardiorespiratory function [13] and improved glucose tolerance in diabetic rats [14]. Nitrate-rich beetroot juice intake prior to exercise achieved similar results, in particular improving exercise tolerance to high-intensity exercise [15], but also in patients with chronic obstructive pulmonary disease [16]. Further, an anti-inflammatory effect has been linked to dietary nitrate and nitrite consumption [17], overall hinting towards an atherosclerosis-protective role of dietary nitrate supplementation. Mechanistically, nitrate is reduced to nitrite by commensal bacteria of the gastrointestinal tract and body surfaces [18]. The resulting nitrite can be further reduced to NO via numerous pathways (reviewed in [19]). In addition to the well-known vasodilatory and anti-hypertensive action of NO [20,21], NO inhibition enhanced leukocyte rolling and adhesion [22]. This suggests an anti-atherogenic leukocyte action of NO itself. Also, NO production rate is decreased in atherosclerosis in patients [23,24] which may contribute to the general pro-inflammatory profile and hampered vasodilatation seen in this disease. Furthermore, nitrate supplementation via drinking water reduced triglyceride levels, body weight gain and glucose intolerance in endothelial nitric oxide synthase (eNOS) deficient mice [25], thus reversing symptoms of the metabolic syndrome. This suggests also anti-atherosclerotic effects of nitrate.

Dietary nitrate supplementation has entered clinical trials in cardiovascular disease, however, the results are variable [26–28]. Most human and murine studies have applied short nitrate supplementation regimens ranging from 2-6 days–15 days in humans, reporting many health benefits and improved functional performance [10,29–31]. Additionally, conflicting evidence about possible side-effects associated with long term nitrate supplementation [32] has prompted us to study the effects of prolonged dietary nitrate supplementation in hypercholesterolemic low density lipoprotein receptor deficient mice ($LDLr^{-/-}$). Based on the atheroprotective effects of nitrate described, we hypothesized that prolonged dietary nitrate reverses plaque hypoxia, lowers hypertension and reduces systemic inflammation, thereby alleviating plaque burden.

2. Methods

An expanded Methods section is available in the Online Appendix.

Low density lipoprotein receptor knockout ($LDLr^{-/-}$, n = 15 per group, 9 weeks of age) were fed a Western-type diet (WTD, 0.25% cholesterol) for 14 weeks and received either sodium-nitrate ($NaNO_3$, 1 g/L) or equimolar sodium-chloride (NaCl) in drinking water ad libitum. Blood pressure and heart rate were measured one week prior to sacrifice by tailcuff method and PE-10 catheter in the thoracic aorta. Plasma nitrate and nitrite were measured as described earlier [33] and plasma hemoglobin-bound HbNO was analyzed using electron parametric resonance.

2.1. Statistical analysis

All data are presented as mean ± SEM. All parameters were analyzed using independent sample tests and were tested for normal distribution using Shapiro–Wilk normality test. Parameters with two groups were compared with student's t-test or Mann–Whitney rank-sum test. Correlations of plasma nitrate and nitrite levels with time of last nitrate consumption were tested using Pearson or Spearman correlation (GraphPad Prism4).

3. Results

3.1. Dietary nitrate supplementation elevates plasma nitrate levels

First the effect of a western-type diet (WTD) and equimolar NaCl [34] on plasma nitrate levels was measured. Plasma nitrate concentrations were not affected by WTD with NaCl drinking compared to normal drinking water plus a chow diet (Fig. 1A).

Dietary nitrate at a dose of 1 g/L is the most common dose described to increase plasma nitrate levels, and corresponds to a physiological nitrate intake achieved by a vegetable-rich diet in humans [34]. Also in our hands, 14 weeks of dietary nitrate drinking increased plasma nitrate levels compared to sodium drinking controls (Fig. 1B), similarly to levels described [34]. No differences were observed in body weight gain and liquid consumption suggesting comparable eating and drinking behavior between groups (Fig. 1C, D).

3.2. Prolonged dietary nitrate does not cause systemic side-effects

Dietary nitrate supplementation has been linked with tumorigenesis. We therefore investigated the systemic effects of prolonged nitrate ingestion.

Morphologically, we did not observe organ tumor development, as analyzed by a certified veterinary pathologist. Nitrates have also recently been suggested to affect platelets and thrombosis [35]. However, neither total red blood cell count nor platelet count (Fig. 2A) was affected by dietary nitrate supplementation. Additionally, red blood cell composition, as measured in red blood cell mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) and hematocrit, did not differ between nitrate and NaCl treatments (Fig. 2B–E). Together, these data suggest that neither hematopoiesis nor erythropoiesis were affected following prolonged dietary nitrate supplementation.

Additionally, dietary nitrate and NO have been shown to reduce circulating and adhering leukocytes [17,22,23,36,37]. Therefore, we compared innate and adaptive immune cells in blood, spleen and lymph nodes between nitrate supplemented mice and mice receiving the control treatment. However, dietary nitrate supplementation did not modulate circulating white blood cell count (Fig. 3A) or differentiation and distribution of inflammatory cells in blood or lymphoid organs (blood: Fig. 3B–H, Supplementary Figure 1, spleen and lymph nodes: data not shown), suggesting