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Dietary Cholesterol Induces Transient Changes in Plasma Nitrate Levels in Rabbits That Are Correlated to Microcirculatory Changes

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Dietary treatment of rabbits with 1% cholesterol resulted in a transient rise in their plasma nitrate levels. After 3 weeks of treatment the nitrate levels were about 50% higher than those of the controls ($p < 0.005$). After 10 weeks of treatment the nitrate levels were similar to those at the start of the study. In accordance with previous work (Xiu *et al., J. Clin. Invest.,* 1994, **93,** 2732–2737), the cholesterol treatment led to a decreased blood flow velocity in arterioli of the third order in the conjunctiva, and a decreased diameter of these arterioli. There was a significant correlation between plasma nitrate levels and the two microcirculatory variables ($p < 0.0001$). Nitrate is the major metabolic end product of nitric oxide (NO), and plasma nitrate levels may be used as an index of the endogenous formation of NO. The present results suggest that dietary cholesterol induces a transient increase in the synthesis of NO. Such an increased synthesis may compensate for part of a cholesterol-induced degradation of NO. © 1996 Academic Press, Inc.

Hypercholesterolemia is known to cause an early depression of the response to endothelium-derived relaxing factor (EDRF) (1,2). EDRF has been identified as nitric oxide (NO) which can be synthesized by several types of cells in the vessel wall such as endothelial cells, macrophages, and smooth muscle cells (3). Furthermore, the endothelium-dependent vasodilation mediated by NO is inhibited by oxidized LDL (4–6). We recently showed that dietary cholesterol induces marked changes in the microcirculation in rabbits and that these changes can be prevented by the antioxidant BHT (7). It is plausible that dietary cholesterol induces an oxidation that leads to an increased degradation of NO with consequences for the microcirculation. It has been shown that hypercholesterolemia increases superoxide anion production, probably in part by effects on xanthine oxides (8,9). Superoxide anions are known to reduce the endothelium-dependent vasodilation mediated by NO, and treatment of cholesterol-fed rabbits with polyethylene glycolated superoxide dismutase has been shown to improve the microvascular response to acetylcholine (10). In view of these data, superoxide anion seems to be the most likely oxidant responsible for the cholesterol-induced degradation of NO.

About 75% of an endogenous load of NO is converted to nitrate (11). The conversion takes place in blood, and the nitrate formed is slowly excreted through the kidneys (12). The plasma levels of nitrate may thus be taken to reflect the endogenous formation of NO. If the microcirculatory changes induced by cholesterol are caused by oxidation of NO to nitrate in tissues instead of in the blood, and if cholesterol does not cause any major shift in the metabolism of NO, hypercholesterolemia would not be expected to affect circulating levels of nitrate. However, in this paper it is shown that dietary treatment of rabbits with cholesterol results in markedly increased circulating levels of nitrate. Furthermore, these changes are well correlated to changes in microcirculatory variables.

MATERIALS AND METHODS

Animals and feeding. Twelve young New Zealand White rabbits with an average weight of 3.0 kg were housed

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individually under conditions of 12 h light/dark periods. Six rabbits were fed a standard chow and 6 were fed a diet supplemented with 1% cholesterol (cf. ref. 7). The project was approved by the Animal Ethical Committee in Stockholm.

Protocol. Initially, as well as after 3, 6, and 9 weeks, blood was sampled from the central artery of the ear for analysis of nitrate. In addition, the microcirculatory vessels of the conjunctival plexus of both eyes of all rabbits were examined on these occasions. At the end of the study the rabbits were killed by an intravenous dose of fluanicone and phentanyl. In addition to the study referred above, another three similar studies were made in which no microcirculatory observations were made - only measurements of nitrate.

Measurement of nitrate. A stable isotope ($^{15}NO_3$) dilution gas chromatography/mass spectrometry assay was used. After separation of plasma from the blood samples labeled nitrate was added, and proteins were separated by filtration through a 10 K filter. Subsequently, endogenous and labeled nitrate in the ultrafiltrate were converted to nitrotoluene and injected into the GC/MS system. The procedure has been described in detail elsewhere (12). The coefficient of variation was about 10% under the conditions employed.

Examination of microcirculatory vessels. The microcirculatory vessels of the conjunctival plexus of both eyes of all rabbits were examined by a long focus stereo microscope. The images were recorded with a video camera as described previously (7). Effort was made to focus on the same area of the conjunctiva on each eye of all the rabbits. Observations were always made on arterioli of the third order belonging to the anterior conjunctival artery. A total observation time of a minimum of 2 min was maintained for each rabbit eye. The observations were made without actually touching the conjunctiva under awake conditions, and without anesthesia or other drugs. The animals were placed in a standard box for sampling during the observations. The recordings and evaluations were performed without knowledge to which feeding group each rabbit belonged.

Equipment and methods used for image processing. This system has been described in detail in the previous publication (7).

Measurement of microvascular diameter. The diameter was measured by autotracking (13). Three independent measurements were made in each eye on each occasion. The arteriolar diameter (μm) was thus calculated as a mean of six measurements for each rabbit on each occasion. The coefficient of variation in the measurements was found to be less than 10% (7).

Measurement of rate of blood flow. Using the above-mentioned equipment, a computer-generated flying spot technique was used to determine blood cell flow velocity in the microvessels (arterioli of the third order belonging to the anterior conjunctival artery) (7).

RESULTS

Fig. 1 summarizes the results of the experiments in which the effect of cholesterol treatment on the plasma levels of nitrate were studied. After 3 weeks of treatment the nitrate levels increased from 112 ± 12 nmol/L to 152 ± 17 nmol/l ($n = 6$, $p < 0.005$). After 6 weeks of treatment the nitrate levels were 162 ± 14 nmol/L. After 9 weeks of treatment the nitrate levels had decreased to 116 ± 21 nmol/L, which was not significantly different from the level observed prior to treatment ($p > 0.05$).

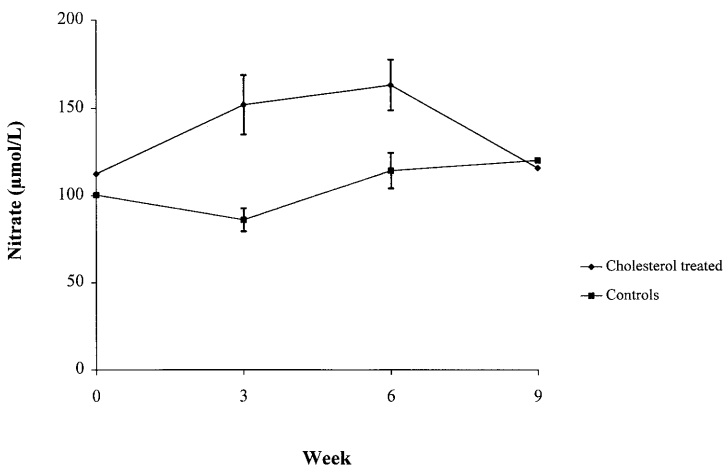

FIG. 1. Effect of dietary cholesterol (1%) on plasma nitrate levels. Each point represents mean ± S.E.M. of results from six rabbits.

In addition to the experiment summarized in Fig. 1, several experiments were made with similar results. In all these experiments there was always a highly significant rise in the nitrate levels followed by a decrease to the baseline levels. In some of these experiments the decrease in nitrate levels occurred already after 6 weeks of treatment.

In accordance with the previous study, the cholesterol treatment caused a marked decrease in arteriolar diameter and blood flow (7). These changes were more accentuated at week 3 and 6 than at week 9.

A clear inverse relation was found between the plasma nitrate levels and the flow rate in the arterioli. The correlation between plasma nitrate and flow rate was significant ($p < 0.05$) both at week 3 and at week 6. Fig. 2 shows the relation between these two variables, measured both at week 3 and 6 in the rabbits fed the control diet, and in the six rabbits fed the 1% cholesterol diet. Each rabbit is thus represented by two points-one referring to week 3 and one to week 6. The r value was 0.42 ($p < 0.0001$). Fig. 3 shows the corresponding relation between plasma nitrate levels and the arteriolar diameter. The r value was 0.48 ($p < 0.0001$).

As could be expected, there was also a highly significant correlation between the rate of blood flow and the arteriolar diameter ($r = 072$, $p < 0.0002$).

DISCUSSION

Treatment with cholesterol caused an unexpected transient increase in the plasma levels of nitrate in the present study. Such an increase is compatible with a corresponding transient augmentation of the synthesis of NO. NO is regarded to act as an endogenous regulator of vascular tone, the rate of synthesis of which is controlled by the blood flow and other factors. In line herewith the plasma levels of nitrate would, if anything, be positively correlated to flow-related variables. The plasma levels of nitrate were, however, inversely related to microvessel diameter and blood flow velocity in the present experiments. If the nitrate levels had reflected functionally important concentrations of NO in the microvessels, an opposite relation would have been expected. We interpret the currently observed increased plasma levels of nitrate as an index of an increased compensatory vascular formation of NO. Such a compensatory increase may have counteracted part of the reduction in blood flow caused by the cholesterol-induced tissue degradation of NO.

The low blood flow and reduced arteriolar diameter previously documented in cholesterol-treated rabbits (7) were, thus, associated with high nitrate levels. The results are in accordance with the finding by Minor et al. (13). These authors demonstrated that diet-induced atherosclerosis

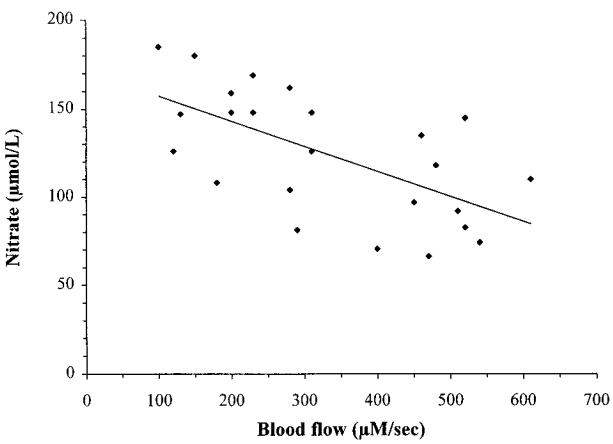

FIG. 2. Relation between plasma nitrate and blood flow in arterioli of the third order in rabbit conjunctiva. Data were obtained from both untreated and cholesterol-treated rabbits ($n = 12$) after 3 and 6 weeks.

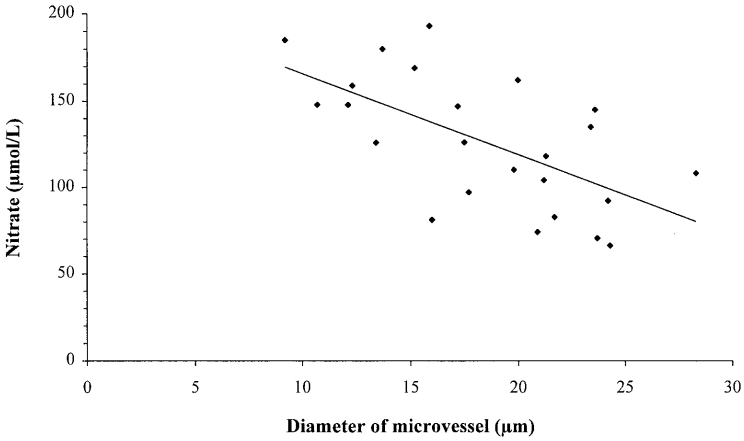

FIG. 3. Relation between plasma nitrate and arteriolar diameter in rabbit conjunctiva. Data were obtained from both untreated and cholesterol-treated rabbits (n = 12) after 3 and 6 weeks.

seemed to increase rather than decrease the total release of nitrogen oxides from rabbit aorta: In a study by Yang et al. (6), activated macrophages exposed to oxidized LDL responded with a decreased accumulation of changes in the amount of NO synthase protein, as evaluated by Western blot. The effect of hypercholesterolemia and oxidized lipids on the production of NO may thus be different in different cells.

The increased production of NO could not entirely compensate for its increased tissue degradation under the conditions employed here, as judged from the observed impairment in microcirculatory variables. The basis for this partial failure is not obvious. Furthermore, the plasma nitrate levels were normalized towards the end of the observation period. This may appear surprising taking into account that the assumed stimulus for NO formation, i.e. the hypercholesterolemia, was maintained throughout the experiments. Additional studies, in particular designed to measure the NO synthase activity in the vessels of cholesterol-treated animals, are required to further evaluate the present findings.

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