Role of nitric oxide in the plasma lipid profile in the rabbits

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Submitted: 21 October 2008
Accepted: 17 March 2009

Arch Med Sci 2009; 5, 3: 308-312

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Abstract

Introduction: One important aspect of endothelial function is synthesis and release of nitric oxide (NO), which is not only a potent vasodilator but also an inhibitor of the platelet aggregation and adhesion. It also suppresses the proliferation of vascular muscle cells and possesses a crucial role in the pathogenesis of several cardio-vascular diseases. The present study communicates adverse effects of mimicking endothelial dysfunction on the plasma lipid profile *in vivo*.

Material and methods: Twenty five male rabbits were treated s.c. twice daily for 7 successive days with either saline solution, nitroglycerin (NTG, 0.1 mg/kg), L-arginine (10 mg/kg), Nω-nitro L-arginine methyl ester (L-NAME, 1 mg/kg), or methylene blue (MTB, 1 mg/kg). Blood samples were taken before treatments on days 1, 2, and 8 for the measurement of plasma triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and cholesterol levels.

Results: No time-course effects in the saline-treated (control) group was observed. Nitroglycerin and L-arginine caused an increased HDL and decreased TG level on day 2 ($p < 0.05$). Treatment with either L-NAME or MTB increased the concentration of LDL slightly. One week treatment with either of L-NAME or MTB increased LDL and TG levels significantly ($p < 0.05$). Both L-arginine and NTG elevated the HDL levels and the HDL/cholesterol ratio after 1 week treatment ($p < 0.05$).

Conclusions: These data suggest that NO donors, used routinely in cardiovascular disease therapy, may increase the levels of HDL and decrease harmful lipoprotein levels and, hence, decrease the risk of hypercholesterolemia and atherosclerosis.

Key words: LDL, HDL, nitric oxide, rabbit, triglyceride, cholesterol.

Introduction

In recent decades, special attention has been paid to the physiological and pathophysiological functions of nitric oxide (NO). The discovery that mammalian cells generate NO has provided important information about many biologic processes with great impact on clinical medicine [1]. Nitric oxide is synthesized from the amino acid L-arginine by a family of enzymes named NO synthase (NOS). Nitric oxide has important roles in the regulation of vascular tone and blood pressure, in the central and peripheral nervous system and in defense mechanisms and immunologic reactions. These actions are mediated by the activation of soluble guanylate cyclase (sGC) and the consequent increase in the concentration of cyclic guanosine monophosphate (cGMP) [2].

The vascular endothelium is now recognized as an important participant in a healthy cardiovascular system and dysfunction of this monolayer

might be an initiating event in many or most cardiovascular disease states [3]. An important feature of endothelium function is the synthesis and release of NO, which is not only a potent vasodilator, but also inhibits platelet aggregation and adherence of circulating blood components to the vessel wall and suppresses the proliferation of vascular smooth muscle cells [4].

High cholesterol level is regarded as an important factor in development of ischemic heart disease [5]. Evidence suggests that high-cholesterol diet impairs NO-cGMP signaling in the cell [6]. Oxidized low-density lipoprotein stimulates NO release by aortic endothelial cells in the rabbit [7] and the synthesis and metabolism of lipoproteins may be affected by NO. Lipoproteins are inhibitors of endothelium-dependent relaxation of rabbit aorta [8]. Endothelial dysfunction induced by low-density lipoprotein (LDL) may be attenuated by a variety of substances, including the precursor of NO, i.e., L-arginine [9].

The present study communicates adverse effects of mimicking endothelial dysfunction on the plasma lipid profile *in vivo*. For this purpose, the following substances were applied *in vivo*:

1) L-arginine – it is a substrate for nitric oxide synthase and the precursor of endogenous NO;
2) nitroglycerine (NTG) – it serves as a donor of NO in biological systems;
3) Nω-nitro L-arginine methyl ester (L-NAME) – this substance competes with L-arginine in binding to nitric oxide synthase and inhibits the production of endogenous NO;
4) methylene blue (MTB) – the chemical is an inhibitor of guanylyl cyclase and, hence, prevents cGMP production, a key process in the action of NO.

Material and methods

Animals

Twenty five healthy, male, New Zealand white rabbits weighing 1.75-2.25 kg were purchased from Pasteur Institute of Iran (Karaj, Iran) and were kept in the animal house of the Veterinary Faculty of the Urmia University (Urmia, Iran). They had free access to commercial chow and tap water and were kept at room temperature and a 12 h light-dark cycle. Rabbits were used in the study as they are easy to handle for repeated blood punctures. In smaller experimental animals, such as mice or rats, the procedure was not practical as repeated sampling of high amounts of blood for biochemical analysis was not possible.

Chemicals

Saline solution was purchased from Shahid Ghazi Pharmaceutical Company (Tabriz, Iran). Nitroglycerine and MTB were products of Merck Company (Darmstadt, Germany). L-arginine was bought from Reidel-deHaën (Hanover, Germany) and L-NAME from Sigma (St. Louis, USA).

Experimental protocol

Animals were divided into 5 groups of 5 each and received either of the following medications subcutaneously twice daily for 7 successive days at 9.00 am and 4 pm: saline solution (control), NTG (0.1 mg/kg) [10], L-arginine (10 mg/kg) [11], L-NAME (1 mg/kg) [12] or MTB (1 mg/kg) [13]. Doses were selected based on an almost average of what used by other researchers in published data.

Blood biochemistry

Blood samples for measurement of lipoproteins were drawn from the marginal vein of rabbits at 8 o'clock on days 1, 2, and 8. Fresh blood was centrifuged at 1500-RPM for 10 min, plasma was isolated and stored at –20°C for later analysis. Plasma cholesterol (CHO) and triglyceride (TG) levels were measured using a standard colorimetric-enzymatic method. The measurement was performed by means of routine spectrophotometric method (Ultra Spect 3300 Pro, UK) and commercial kits produced by Pars Azmoon (Tehran, Iran). The photospectra was obtained by means of Rigaku UV-vis-NIR spectrophotometer (Ultraspec 3300 pro, GE Healthcare, UK). For determination of VLDL, the amount of LDL and HDL were subtracted from total cholesterol.

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed using the SPSS software. The results obtained from 5 experimental groups were compared by one-way analysis of variance (ANOVA). When $p < 0.05$, differences between individual groups were evaluated by a *post-hoc* test (Tukey's *t* test). A p value < 0.05 was considered to reflect a statistically significant difference.

Results

Effect on cholesterol (Figure 1A) and triglyceride (Figure 1B) levels

On day 1, the basal levels among all groups were comparable. On day 2, cholesterol was decreased in the groups treated with NTG (6.3%) and L-arginine (2.03%) and increased in the groups exposed to L-NAME (12.3%) and MTB (4.8%). On day 8, NTG and L-arginine decreased the cholesterol levels by 15.3 and 13.2%, respectively, whereas, L-NAME and MTB increased cholesterol by 88.64% ($p < 0.005$) and 37.15% ($p < 0.001$), respectively. Basal levels of triglycerides were

similar in all groups before treatments. Nitroglycerine and L-arginine caused a slight decrease in TG levels on day 2; L-NAME and MTB caused no effect. On day 8, however, changes were prominent as NTG and L-arginine led to a declined TG level by 30.3% ($p < 0.001$) and 16.9% ($p = 0.048$), respectively. L-NAME-treated group showed a 77.71% increase ($p < 0.001$). In the MTB-treated animals also the TG level was dramatically raised by 29.7% ($p < 0.02$).

Effect on LDL (Figure 1C) and VLDL (Figure 1D) levels

On day 2, slight changes were seen in LDL levels; L-arginine-induced decrease and L-NAME-induced increase were more prominent although did not reach the level of statistical significance. On day 8, the groups treated with NTG and L-ARG showed significant decline by 55.7% ($p < 0.01$) and 70.4% ($p < 0.01$), respectively. On the other hand, L-NAME increased LDL level by 144.82% ($p < 0.002$) and MTB by 54.52% ($p < 0.01$). One day after the treatment initiation, NTG and L-arginine induced a small decline in VLDL level. On day 8, NTG- and L-arginine-treated group showed significant decrease by 30.3% ($p < 0.001$) and 16.9% ($p < 0.04$), respectively. L-NAME increased LDL level by 77.71% ($p < 0.001$) and MTB by 29.7% ($p < 0.02$).

Effect on HDL levels (Figure 2A) and HD/cholesterol ratio (Figure 2B)

On day 2, NTG increased HDL by 41.24% and L-arginine by (56.22%, $p < 0.001$). On day 8, NTG increased the HDL level by 104.26% ($p < 0.001$) and L-arginine by 99.76% ($p < 0.001$). Neither L-NAME nor MTB affected the HDL throughout the experiment. The ratio was significantly higher in NTG- and L-arginine-treated groups on day 2 ($p < 0.001$). The increase in this ratio on day 8 was even more prominent; in NTG-treated group, the rise was 152.38% ($p < 0.001$) and 138.46% ($p < 0.001$), respectively. Treatment with L-NAME and MTB resulted in a 50% ($p = 0.008$) and 28% ($p = 0.02$) decreased level of HDL/cholesterol ratio.

Discussion

Although some data can be found in the literature over interactions between NO and blood lipoproteins, this study is unique in using rabbit as an animal model. In addition, chemicals used and 7 day successive treatments twice daily with NO pathway modulators is a protocol not done in previous research work in this field.

Nitric oxide is not only a potent vasodilator, but also inhibits the platelet aggregation and adherence of circulating blood elements to the vessel wall. Therefore, it is possible that it may provide an

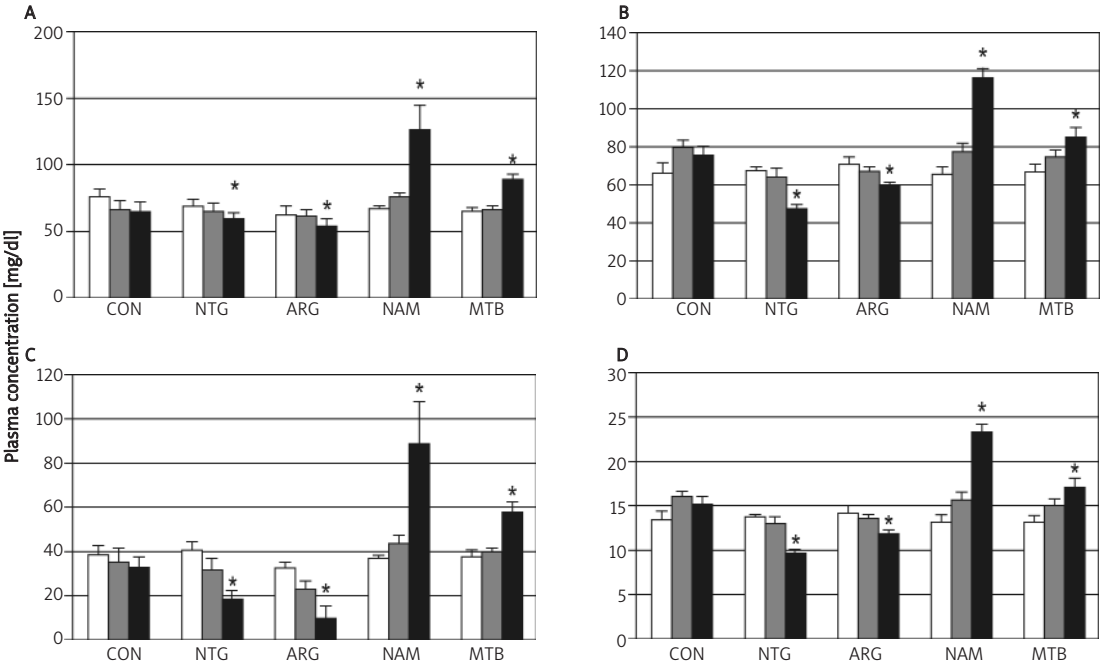

Figure 1. Role of NO pathway in the regulation of plasma levels of cholesterol (**A**), triglyceride (**B**), LDL (**C**), and VLDL (**D**) in the rabbit. Saline (CON), nitroglycerine (NTG, 0.1 mg/kg), L-arginine (ARG, 10 mg/kg), L-NAME (NAM, 1 mg/kg), or methylene blue (MTB, 1 mg/kg) were administered s.c. twice daily for 7 days. Measurements were accomplished before the treatment on day 1 (open bars), day 2 (grey bars) and day 8 (black bars)

**p < 0.05 in comparison to the corresponding control (saline-treated) group*

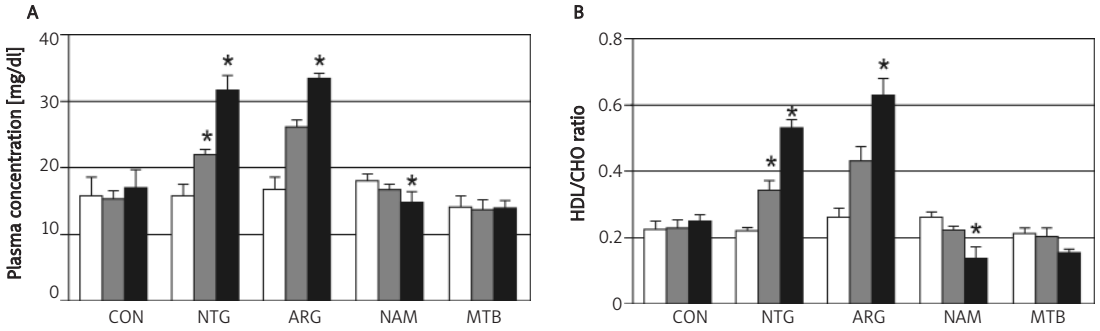

Figure 2. Role of NO pathway in the regulation of plasma level of HDL (**A**) and HDL/cholesterol ratio (**B**) in the rabbit. Saline (CON), nitroglycerine (NTG, 0.1 mg/kg), L-arginine (ARG, 10 mg/kg), L-NAME (NAM, 1 mg/kg), or methylene blue (MTB, 1 mg/kg) were administered s.c. twice daily for 7 days. Measurements were accomplished before the treatment on day 1 (open bars), day 2 (grey bars) and day 8 (black bars)

**p < 0.05 in comparison to the corresponding control (saline-treated) group*

endogenous defense against atherosclerosis. Pinelli *et al*. showed that decline in plasma NO level is one of the risk factors of cardiovascular diseases [14]. In their research, inhibition of NOS by L-NAME increased the blood lipoproteins, blood pressure and coagulation parameters.

Results of the present study showed that injection of NTG caused a decrease in plasma TG and LDL, while increasing HDL levels. Indeed, Laroia *et al*. reported that infusion of NO by its donors leads to a decrease in plasma total cholesterol and reversal of endothelial dysfunction [15]. Our study also shows that administration of L-arginine decreases TG and cholesterol, and increases HDL and HDL/total cholesterol ratio. In agreement with these results, Dhawan *et al*. observed that administration of L-arginine increase the plasma nitrite and decrease the number and size of atherosclerotic lesions [16]. These authors suggested that beneficial effect of L-arginine in blockade of atherosclerotic lesions is due to increased NO and decreased LDL oxidation. On the contrary, Marra *et al*. reported that, in addition to decrease in total cholesterol, administration of L-arginine caused a decline in HDL levels [17]. Thus, they suggested that more attention should be paid in chronic administration of L-arginine.

Our data showed that administration of L-NAME increased the total cholesterol, LDL, VLDL, and TG levels, and decreased HDL concentration. In line with these results, it had been shown that L-NAME lowered plasma NO, increased plasma total cholesterol and decreased HDL. In addition, chronic inhibition of NOS caused myocardial infarction in rats [14]. The blockade of NOS by L-NAME seems to be involved in lipid metabolism alterations, leading to an increase in serum cholesterol levels in the rat and, hence, impairing endothelial function and atherosclerosis in hypercholesterolemic rabbits. In the experiments accomplished by Pinelli *et al*., the animals whose NO levels were reduced by L-NAME, showed a dose-dependent decrease in HDL and total cholesterol. The reverse relation between NO and cholesterol in the present study is in agreement with observations of Pinelli *et al*. They demonstrated that reduced NO availability increased the incorporation of labeled precursors in cholesterol and decreased its catabolism as a result of the inhibition of 7α-hydroxylase activity [14].

Wenjuan *et al*. reported that inhibition of NOS in rats causes an elevation of blood lipids. They suggested that the effect was probably due to a cGMP-dependent pathway [18]. Also, NO leads to inhibition of acetyl CoA carboxylase and decrease of malonyl CoA. Besides, there is a report showing that administration of NOS inhibitors increases the expression of oxLDL-C receptors in endothelial cells [19]. Nitric oxide, therefore, can be a beneficial factor for inhibition and improvement of endothelial disorders.

In this study, we also investigated the effects of MTB on blood lipids. Like L-NAME, MTB increased total cholesterol, harmful lipoprotein and TG levels. Methylene blue is an inhibitor of guanylate cyclase and blocks some effects of NO which are cGMP-dependent. Abdullah *et al*. reported that there are differences between NOS inhibitors and GC inhibitors [20]. This may suggest that NO owes actions beyond activation of GC. Therefore, L-NAME may be more effective than MTB.

The mechanism by which NO caused a beneficial balance in the lipoprotein profile in blood was not studied in this experimental work. However, based on the report of Giricz *et al*., inhibition of the HMG CoA reductase by statins may lead to an increased levels eNOS activity [5]. Interestingly, simvastatin of this antihyperlipidemic drugs was shown to enhance eNOS activity by 100-200% in the mouse

aorta [21]. Our findings support well those findings with the evidence that NO may serve as a beneficial mediator in relieving vascular lesions, irrespective of the source it is initiated, i.e., exogenous or endogenous source, direct or indirect, and basal or drug-induced production.

It should be stated that the interaction between NO and lipids is not a unilateral relation, i.e., lipids may be involved in the production and actions of NO as well. In the rat macrophages, VLDL and LDL but not HDL stimulated NO production at 12-48 h of incubation with macrophages. These effects of lipoproteins could be of interest in the pathophysiology of lipoproteins and NO mediated diseases [22]. In spite of this bilateral interaction and the importance of both NO and lipid metabolism, not sufficient studies have been performed in human subjects. It is highly recommended to extend similar studies to clinical settings in both healthy and hyperlipidemic subjects. It should note that we are reporting effect of NO on normal lipid profile not hyperlipidemic status, thus explanations in this paper should not be taken as what may necessarily happen under pathologic conditions.

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