Nitric Oxide Pathway Activation and Impaired Red Blood Cell Deformability with Hypercholesterolemia

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The pathophysiological effects of the activation or inhibition of the nitric oxide (NO)-mediated pathway on the deformability of red blood cells (RBC) were evaluated in the presence of hypercholesterolemia induced in rabbits fed a cholesterol-rich diet. RBC deformability was assessed using a microchannel array flow analyzer system. The maximum passage time (MPT) by flowing a suspension of RBC through the microchannels was used as an index of RBC deformability. During cholesterol feeding for 12 weeks, MPT gradually increased with no significant elevation in the serum asymmetric dimethylarginine (ADMA) and arginine/ADMA ratio. The reduction in RBC deformability associated with hypercholesterolemia was significantly improved during incubation with each of three different NO pathway activators: a NO donor, 8-bromo-cyclic GMP, and arginine; however, no additional reduction was observed with ADMA administration. The inhibition of NO synthase due to ADMA caused a significant reduction in the deformability of normal RBC, which was reversed with NO pathway activation. These results suggest that impaired RBC deformability may be associated with a dysfunction in the NO pathway that is partially dependent upon the accumulation of ADMA in RBC, and exogenous NO pathway activators may improve the microcirculation by restoring RBC deformability in the presence of hypercholesterolemia.


Key words: ADMA, Arginine, NO donor, NO synthase inhibitor

Introduction

Endothelium-derived nitric oxide (NO) plays a major role in cardiovascular regulation, with its action mainly attributed to its effects on the vascular wall; however, it has also been shown that NO acts as a potent endogenous inhibitor of the progression of atherosclerosis through its rheological contributions such as inhibiting platelet aggregability and suppressing leukocyte adhesiveness to the endothelium. The risk factors for developing atherosclerosis have been shown to cause endothelial dysfunction following NO synthesis reduction. In hypercholesterolemia, the vascular protective effects attributable to NO are decreased, leading first to a narrowing of the arteries, then to blood flow reductions in the organ, and finally to developing symptoms of ischemia. In addition to alterations in the vascular activity of NO in hypercholesterolemia, another mechanism exists whereby hypercholesterolemia influences organ circulation through its effects on various rheological factors such as serum viscosity and the states of platelet and leukocyte activation. Rheological impairment associated with these factors could predispose hyperlipidemic patients, especially those with narrowed arteries, to organ ischemia and tissue hypoxia. RBC deformability is also one of the more important rheological factors for controlling microcirculation in organs. It has been shown that RBC interact with NO synthesized in and transferred from endothelial cells, and RBC have NO synthase (NOS) capable of synthesizing their own NO. Both extracellular and intracellular sources of NO may modulate the rheological behavior of RBC; however, the association between RBC deformability and NO in the presence of hypercholesterolemia has not been...
fully evaluated in view of the microcirculation.

A decrease in the bioavailability of endothelium-derived NO has been demonstrated in patients with certain cardiovascular risk factors. Recently, the reduction in NO bioavailability has been considered to be due partially to the action of a circulating competitive inhibitor of NOS, asymmetric dimethylarginine (ADMA)\(^{12, 13, 22-25}\), which is endogenously produced in the process of protein arginine methylation\(^{26, 27}\).

There is a high prevalence of increased serum levels of ADMA in a number of cardiovascular disorders including hypercholesterolemia\(^9\), diabetes mellitus\(^{28}\), hypertension\(^{13, 22}\), and coronary artery disease\(^{23, 24}\). The intra-arterial administration of ADMA has been shown to induce marked arterial constriction via the inhibition of NOS in endothelial cells\(^{29}\). The pathophysiological action of ADMA is well known in relation to endothelial dysfunction due to its inhibition of NO synthesis in endothelial cells, but less is known about its effects in non-vascular cells, such as RBC, and especially in relation to microcirculation.

In the present study, we evaluated extra-vascular NO effects on the changes in RBC deformability with the administration of activators or inhibitors of the NO mediated pathway in rabbits with diet-induced hypercholesterolemia.

**Methods**

**Hypercholesterolemic Rabbits**

Twenty-one New Zealand female white rabbits of the same body weight (2.1 ± 0.1 kg) were fed a normal diet, and 11 rabbits were fed a diet containing 0.5% cholesterol (Oriental Yeast Co., Tokyo, Japan) for 12 weeks. Their body weight was checked and the serum levels of cholesterol, triglyceride and HDL cholesterol were determined before and every 4 weeks during cholesterol feeding. The body weights of the control and cholesterol-fed rabbits were 3.4 ± 0.1 kg and 3.7 ± 0.1 kg, respectively, at the end of the experiment.

**Blood Sample Preparation**

Whole blood was collected from the ear vein of rabbits using a sterile 21 gauge needle and was immediately mixed with one volume of anticoagulant consisting of 3.8% sodium citrate solution to nine volumes of blood in a plastic syringe. A 5 mL specimen of blood was centrifuged at 3000 rpm (1500 g) for 5 minutes. The supernatant containing the plasma and buffy coat was discarded to avoid the effect of leukocytes\(^{30}\) and platelets\(^{31}\) on filterability, and to evaluate the intrinsic deformability of the RBC. Next, phospho-buffered solution (PBS) at pH 7.4 was added to the sediment, which was then gently shaken to make a suspension of RBC, and again centrifuged at 3000 rpm for 5 minutes. This process was repeated three times. The washed RBC were suspended in PBS for the measurement of deformability. All RBC preparations, incubations, and deformability measurements were carried out within 2 h after blood collection.

**Incubations**

RBC suspensions were incubated at 37°C for 30 min in the presence of various chemical agents, after which RBC from these suspensions were used for the determination of deformability. This 30-min incubation period was selected based on preliminary studies that indicated a gradual increase of effects up to 15 min, with no additional change thereafter. The following agents were used for incubations:

**NOS inhibitors.** Both a nonspecific NOS inhibitor, N\(^\circ\)-nitro-L-arginine methyl ester (L-NAME) and an endogenous NOS inhibitor, ADMA were used. The effects of L-NAME were tested at concentrations between 1 × 10\(^{-6}\) and 1 × 10\(^{-2}\) M, and the most effective dose of 3 × 10\(^{-4}\) M was then selected to be used for the remaining L-NAME studies. ADMA at 1 × 10\(^{-5}\) M and 1 × 10\(^{-4}\) M were used for RBC deformability studies.

**NOS donor.** As an NO donor, 3-(2-hydroxy-1-(1-methylethyl)-2-nitroso-hydrazino)-1-propanamine (NOC5)\(^{32}\) was used at a concentration between 1 × 10\(^{-7}\) and 1 × 10\(^{-4}\) M and was employed alone or in combination with NOS inhibitors.

**NO precursor.** The effects of the NO precursor, L-arginine, were evaluated alone or in combination with an endogenous NOS inhibitor at a concentration of 1 × 10\(^{-5}\) and 1 × 10\(^{-4}\) M.

**Cyclic GMP analogue.** Since the vasodilatory effects of NO are generally thought to be mediated by cyclic GMP as the second messenger\(^2\), we also tested the effect of 8-bromo-cyclic GMP as a mimic of an intracellular physiological effector\(^{15, 34}\).

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The chemicals were dissolved in PBS for these studies.

**Deformability Measurements**

RBC deformability has been assessed by a variety of methods\(^{15, 16, 18, 35-38}\). We measured RBC deformability using a microchannel array flow analyzer system (Hitachi Haramachi Electronics Co. Ltd., Ibaragi, Japan), a negative-pressure filtration method\(^{39-42}\). Prior to the measurement of deformability, all air bubbles trapped in the apparatus were removed by flushing.
with PBS. The washed RBC were suspended in PBS at 37°C at a concentration of 2×10⁶/mL, adjusted using a cell counter, Celltac 5108 (Nihon Koden Co., Tokyo, Japan), and added to a syringe. This concentration of 2×10⁶/mL was selected based on preliminary studies which indicated that this was a suitable maximum concentration to avoid the effects of RBC aggregation. The syringe with a volume of 100 μL of sample was connected to the inlet of the apparatus consisting of a single-crystal silicon chip with 8736 microchannels, having a groove size of 5 μm width, 4.5 μm depth, and 20 μm length, to simulate the size of capillary blood vessels. A negative water pressure difference of 20 cm height was applied at room temperature and the time for 100 μL of the RBC suspension to pass through the microchannel arrays of the chip was measured to estimate their deformability. The passage time for 100 μL of PBS was measured just before blood sample measurement and each sample measurement was calibrated with the passage time of PBS adjusted to 12 seconds. The passage time through the microchannels of the silicon chip was measured for every 10 μL of the 100 μL sample by monitoring the volume in the syringe to determine the blood flow rate. RBC deformability was assessed by the maximum passage time (MPT) generated by the flow of the 100 μL RBC suspension. All measurements were made using a single silicon chip, which was cleaned between runs by ultrasonic washing with a neutral detergent and ethanol. RBC deformability was measured using RBC from hypercholesterolemic rabbits before and every 4 weeks during cholesterol feeding.

Chemical Analysis

Serum concentrations of asymmetric dimethylarginine (ADMA) were measured by high-performance liquid chromatography (HPLC) with an automated sample processing device, L-7200 (Hitachi Co., Tokyo, Japan), and a fluorescence detector, FP-2025 (JASCO Co., Tokyo, Japan). L-arginine and amino acids were also measured by HPLC. The serum total and HDL cholesterol, and triglyceride concentrations were determined enzymatically.

Statistical Analysis

All data are given as the mean ± SD. Differences between the MPT measured in RBC samples with and without any chemical agent were analyzed using the non-paired t-test. A value of p<0.05 was considered statistically significant.

Results

Effects of NOS Inhibitors or NO Pathway Activators on the Deformability of RBC Prepared from Normal Diet Control Rabbits

ADMA reduced RBC deformability, resulting in MPT increasing significantly from 16.36 ± 0.86 sec in control samples (n = 21) at 37°C at an RBC concentration of 2×10⁶/mL to 18.76 ± 1.53 sec of samples with ADMA at 1×10⁻⁵ M (n = 17, p<0.01), and 18.52 ± 1.67 sec at 1×10⁻⁴ M (n = 14, p<0.01). L-NAME at 3×10⁻⁴ M also significantly increased MPT to 18.39 ± 2.35 sec significantly (n = 9, p<0.01) (Fig. 1).

NOC5, an NO donor, had no significant effects on the MPT of control samples at concentrations of 1×10⁻⁷ M (16.67 ± 1.01 sec, n = 11), 1×10⁻⁶ M (16.66 ± 0.78 sec, n = 7), and 1×10⁻⁵ M (16.62 ± 0.44 sec, n = 7); however, NOC5 at the highest concentration of 1×10⁻⁴ M increased MPT (17.58 ± 1.57 sec, n = 7) compared to the control (p<0.05). A mimic of the second messenger of NO, 8-bromo-cyclic GMP did not shorten the MPT at 1×10⁻⁷ M (16.25 ± 0.94 sec, n = 6), 1×10⁻⁶ M (16.73 ± 0.78 sec, n = 6), and 1×10⁻⁵ M (16.59 ± 0.69 sec, n = 6). The precursor of NO, L-arginine, also did not affect MPT at a concentration of 1×10⁻⁵ M (17.07 ± 0.82 sec, n = 7) or 1×10⁻² M (16.89 ± 0.97, n = 7) (Fig. 2).

Effects of NO Pathway Activators on Impaired RBC Deformability Induced by ADMA

After 30-min incubation with 1×10⁻⁵ M ADMA, the addition of NOC5 significantly improved RBC deformability (MPT of 18.76 ± 1.53 sec (n = 17) de-
concentrations rose quickly, and the MPT gradually increased from 1.05 ± 0.06 sec at concentrations of 10⁻³ M on the maximum passage time (MPT) as an indicator of the deformability of red blood cells prepared from control rabbits.

Data represent the mean ± SD. *: p<0.05, significantly different from the control.

Blood Chemistry and RBC Deformability During Progression of Hypercholesterolemia

In 21 rabbits fed a normal diet, their serum cholesterol concentrations were 55±19 mg/dL. During cholesterol feeding in 11 rabbits, serum cholesterol concentrations rose quickly, and the MPT gradually increased, although serum triglyceride and HDL cholesterol concentrations remained normal. At 12 weeks of cholesterol feeding, a marked increase in MPT (20.65 ± 1.72 sec) was observed, with a serum cholesterol level of 1541 ± 300 mg/dL (Fig. 4).

The serum ADMA concentrations measured every 4 weeks during the cholesterol-rich diet phase were not significantly different from normal (1.05 ± 0.08 μM to 1.00 ± 0.05 μM at 4 weeks, 1.13 ± 0.18 μM at 8 weeks, and 0.97 ± 0.06 μM at 12 weeks of cholesterol-
Serum concentrations of L-arginine, the precursor amino acid of NO, as well as the L-arginine/ADMA ratio, did not significantly change during cholesterol feeding. The serum ADMA concentration did not increase with serum cholesterol elevation (Fig. 5A). MPT correlated poorly with serum ADMA concentration (Fig. 5B) and the L-arginine/ADMA ratio (Fig. 5C), but correlated well with the serum cholesterol concentration (Fig. 5D).

**Effects of NO Pathway Activators on the Deformability of RBC Prepared from Hypercholesterolemic Rabbits**

The MPT of samples prepared from hypercholesterolemic rabbits showed a significant decrease after incubation at 37°C for 30 min with NOC5 at concentrations of 10⁻⁶ M and 10⁻⁵ M, as well as with 10⁻⁷ M, 10⁻⁶ M, and 10⁻⁵ M cyclic GMP. The addition of L-arginine at a concentration of both 10⁻³ and 10⁻² M to the samples also reversed the impaired RBC deformability caused by hypercholesterolemia (Fig. 6).

**Effects of ADMA or L-NAME on the Deformability of RBC Prepared from Hypercholesterolemic Rabbits**

Feeding cholesterol to rabbits for 12 weeks sig-

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Fig. 5.** Correlation analysis (A) between the serum concentrations of asymmetric dimethylarginine (ADMA) and total cholesterol (TC), (B) between ADMA and the maximum passage time (MPT), (C) between arginine/ADMA and MPT, revealed no significant relationship. A significant correlation was found between TC and MPT (D).

\[ y = 247.9x - 3572.8, \quad r = 0.64, \quad p < 0.01. \]

![Graph E](image5.png)

**Fig. 6.** Effects of the nitric oxide (NO) donor NOC5, the second messenger of NO, cyclic GMP (cGMP), and the NO precursor, arginine (Arg), on the maximum passage time (MPT) as an indicator of the deformability of red blood cells prepared from hypercholesterolemic rabbits (HL). Data represent the mean ± SD. **: \( p < 0.01 \), compared with the MPT of HL.
significantly reduced RBC deformability as demonstrated by increasing passage times through the microchannels (Fig. 7A). The administration of $1 \times 10^{-5}$ M or $1 \times 10^{-4}$ M ADMA did not further increase the passage times of samples obtained from hypercholesterolemic rabbits (Fig. 7B), nor did $3 \times 10^{-4}$ M L-NAME (Fig. 7C), although both ADMA ($1 \times 10^{-5}$ M) and L-NAME ($3 \times 10^{-4}$ M) significantly increased the passage times of samples from control rabbits given a normal diet, indicating impaired deformability (Fig. 7D).

**Discussion**

The microcirculatory system is crucially important for organs and tissues in order for them to extract their required oxygen. The ability of RBC to deform is an effective factor in the maintenance of normal circulation that delivers oxygen to the cells, which allows the easy passage of RBC through narrow capillaries in the microcirculation. Hypercholesterolemia is known to disturb the rheological properties of blood, by increasing blood viscosity, and changing the composition of the cell membranes to a more cholesterol-rich composition, thereby resulting in decreased membrane flexibility and increased surface area. The major determinants of RBC deformability are cell geometry, cell shape, cytoplasmic viscosity, and membrane mechanical properties such as membrane rigidity. Our data showed that an increase in blood cholesterol concentrations is associated with a decrease in RBC deformability, as indicated by increased MPT, using a microchannel method. Although the cholesterol content of the RBC membrane was not determined in the samples, a 30-fold higher blood cholesterol concentration for 12 weeks could change the lipid component of the membrane. Both the cholesterol-enriched lipid bilayer and membrane cytoskeleton may affect RBC membrane mechanical behavior; however, we showed

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**Fig. 7.** Measurements of the time taken for every 10 μL of 100 μL of (A) a suspension of red blood cells from normal rabbits (double circles) and hypercholesterolemic (HL) rabbits (open squares) through microchannels, (B) a suspension of red blood cells from HL rabbits incubated with ADMA at $1 \times 10^{-5}$ M (closed squares) and $1 \times 10^{-4}$ M (closed triangles), (C) a suspension of red blood cells from HL rabbits incubated with L-NAME at $3 \times 10^{-4}$ M (closed circles), (D) a suspension of red blood cells from normal rabbits (double circles) incubated with ADMA at $1 \times 10^{-5}$ M (closed squares) and with L-NAME at $3 \times 10^{-4}$ M (closed circles).
that three types of NO pathway activators markedly improved the reduced RBC deformability in hypercholesterolemia. The results of this study suggest that hypercholesterolemia-augmented RBC deformability is not only dependent on the membrane’s physiochemical properties but also the RBC intracellular metabolic state associated with the NO-mediated pathway.

The maintenance of normal RBC deformability depends critically on the metabolic state of the cells, which is affected by ion pumps located in the membrane such as Na\(^+\)K\(^-\)ATPase and Ca\(^{2+}\)ATPase that use metabolic energy. Na\(^+\)K\(^-\)ATPase is the primary regulator of the intracellular volume of RBC, and cytoplasmic viscosity is regulated via maintaining osmotic balance across the cell membrane. Ca\(^{2+}\)ATPase maintains the low intracellular Ca\(^{2+}\) concentration essential for normal RBC deformability. Both the Na\(^+\)K\(^-\)ATPase and Ca\(^{2+}\)ATPase activities of RBC have been demonstrated to be stimulated by NO donors, and NO affects ion transport across the RBC membrane.

In the present study, the endogenous NOS inhibitor ADMA and the non-specific pharmacological NOS inhibitor L-NAME independently reduced the deformability of RBC prepared from normal rabbits, although activators of the NO pathway showed no additional increase in deformability over that of RBC prepared from normal rabbits. These results suggest that NO plays an important role in maintaining normal RBC deformability, and that additional NO does not further increase deformability.

Impaired RBC deformability developing in the setting of hypercholesterolemia was restored by three different NO pathway activators: NOC5 as a strong NO donor, cyclic GMP as a second messenger of NO, and L-arginine as the precursor amino acid of NO. Among these, it is interesting to note that a high concentration of NOC5 actually impaired RBC deformability, with this biphasic effect especially obvious in experiments where endogenous NO production was inhibited by the addition of endogenous NOS inhibitor ADMA. This reverse effect of high concentrations of NOC5 on the mechanical properties of RBC was also observed in untreated normal RBC. Impaired RBC deformability was reversed with the addition of NOC5 in a dose-dependent manner up to a certain concentration (10\(^{-5}\) M); however, concentrations above this caused a worsening of RBC deformability, suggesting that the presence of NO at a critical concentration is crucial for maintaining the normal deformability of RBC. The reason for higher concentrations of NO having an adverse effect on mechanical behavior is uncertain but may be related to the pathophysiological effects induced by excessive NO, such as increased oxidant stress.

It is known that endothelium-dependent, NO-mediated vasodilation is impaired in hypercholesterolemia associated with no vascular symptoms. The serum concentrations of ADMA, an endogenous competitive inhibitor of NO synthesis, are elevated in hypercholesterolemia, even in the absence of overt atherosclerotic vascular disease. This suggests that NO inhibition by ADMA plays an important role early during atherosclerosis development, and elevated ADMA may be a potential marker of certain vascular diseases. The higher serum ADMA concentrations were almost 2-fold higher in hypercholesterolemia, as well as in hypertension, coronary heart disease, and diabetes mellitus. In this study, serum ADMA concentrations in rabbits with diet-induced hypercholesterolemia remained at the control concentrations during 12 weeks of cholesterol feeding, during which time RBC deformability became significantly more impaired. An enzyme, dimethylarginine dimethylaminohydrolase, that can break down ADMA, is widely present, so that endogenous ADMA is metabolized to citrulline and cleared from the plasma rapidly. It was also considered that circulating concentrations of endogenous ADMA were the result of a spillover from ADMA that had accumulated in the cell. Small changes in the circulating concentration may indicate large changes in intracellular ADMA concentrations that clearly affect NO production and NO function in the cell. In this study, the addition of ADMA as well as L-NAME to samples prepared from hypercholesterolemic rabbits revealed no further worsening of RBC deformability. A period of 12 weeks of hypercholesterolemia may be enough to accumulate sufficient endogenous ADMA to maximally alter RBC deformability, but may be too short to cause a large amount of ADMA to spill out of the cell.

The administration of both oral and intravenous L-arginine has been shown to improve endothelial dysfunction related to decreasing NO production, to reduce monocyte adhesion, and to inhibit platelet aggregation in rabbits and humans with hypercholesterolemia. This study showed that exogenously administered L-arginine itself did not affect the deformability of RBC prepared from normal rabbits, but competed with the deleterious effects of administered ADMA to restore NO synthesis. In addition, the impaired deformability of RBC prepared from hypercholesterolemic rabbits was also restored by L-arginine administration. Although intracellular ADMA concentrations were not evaluated in the RBC of hypercholesterolemic rabbits, the effect of L-arginine, the precursor amino acid of NO, is likely to counteract the
inhibitory effects of ADMA and to restore intracellular NO synthesis.

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