Evaluation of Pharmacological Modulation of Nitroglycerin-Induced Impairment of Nitric Oxide Bioavailability by a Catheter-Type Nitric Oxide Sensor

Tosho Imanishi; Akio Kuroi; Hideyuki Ikejima; Seiichi Mochizuki*; Masami Goto*; Takashi Akasaka

Background  The present study aimed to elucidate the effect of long-term treatment with nitroglycerin (NTG) on the bioavailability of nitric oxide (NO) examined by a catheter-type NO sensor. The study also examined whether these effects could be modified by an antioxidant, an angiotensin converting enzyme inhibitor, or an angiotensin II type 1 receptor antagonist (ARB).

Methods and Results  Male New Zealand rabbits were treated for 7 days with NTG patches, either alone or in combination with tempol, enalapril, or valsartan (ARB). The plasma NO concentration was measured with the catheter-type NO sensor. The plasma peroxynitrite concentration was measured by enzyme-linked immunosorbent assay. An increase in plasma NO concentration in response to acetylcholine (ACh) were significantly attenuated in the NTG-treated group as compared with the control. Plasma peroxynitrite concentration in NTG-treated group was significantly higher as compared with the control. The negative effects of NTG were significantly suppressed by the co-treatment with tempol, enalapril or valsartan.

Conclusions  Chronic treatment of rabbits with NTG elicits the impairment of the ACh-stimulated NO production. In addition, the negative effects of NTG might be prevented by the co-treatment with drugs attenuating nitrosative stress. (Circ J 2007; 71: 1473–1479)

Key Words: Nitrate tolerance; Nitric oxide; Nitric oxide sensor; Nitrosative stress

Coronary artery disease is associated with endothelial dysfunction (ED), an occurrence, which has been mainly attributed to decreased availability of the endothelium nitric oxide (NO). In addition, ED has been shown to be a predictor of adverse long-term outcome in patients with coronary artery disease. Thus, one might conceptualize that a treatment with an exogenous source of NO (eg, nitroglycerin (NTG)) could compensate for the diminished endothelial production of NO in atherosclerosis. However, ED was also observed in humans during prolonged NTG therapy. In large coronary arteries, Caramori et al found that continuous treatment (5 days) with NTG patches leads to enhanced acetylcholine (ACh)-induced paradoxical constriction, instead of endothelium-dependent vasodilation, which was taken as a surrogate parameter for ED. These data have suggested that chronic treatment with NTG causes ED, although the limitation of these data is that the release of NO from the endothelium was estimated on the basis of the comparison of vessel relaxation. Thus, quantitative in vivo NO measurement is a key to understand its dynamic actions. Strikingly, none of the studies answered the critical question how NTG-induced ED affect plasma NO concentrations because of the difficulty of measurement of plasma NO concentrations. There is considerable evidence that NTG-induced ED is associated with the generation of superoxide free radical. Although the endothelium represents the principal source of superoxide, the NAD(P)H oxidase system seems to contribute to superoxide generation. This finding is of potential therapeutic importance as NAD(P)H oxidase expression is stimulated by Angiotensin II (Ang II).

As a high-temporal resolution method, electronchemical measurement methods of NO, ie, NO sensors, have been developed by several groups. These sensors enable us to evaluate dynamic changes of NO concentration in solutions and tissues in response to agonists, NO-generating reagents and physical stimuli. However, electrical interference through a power line, vibration, poor durability of sensor-tip coatings and other factors make in vivo NO measurement very difficult. To overcome these drawbacks, a new NO sensor, which encloses both working and reference electrodes with a highly gas-permeable and robust enclosure, has been developed. In addition, we have developed a catheter-type NO sensor.

By using the catheter-type NO sensor we tried to elucidate the direct effect of long-term treatment with NTG on plasma NO concentration in NTG-treated rabbits. Furthermore, we investigated to clarify the effect of antioxidant, angiotensin converting enzyme inhibitor (ACE-I), or angiotensin II type 1 receptor blocker (ARB) on the impairment of bioavailability of NO induced by chronic treatment with NTG patches. The present study has shown for the first time direct evidence of impaired NO production induced by chronic treatment with NTG, whereas previous studies had used surrogate endpoints (for example, modifications in blood flow or vascular tone) to show the same concept. Ad-
ditionally, we have also demonstrated the effects of anti-
oxidants or substances which reduce nitrosative stress, such as ACE-I, and ARB, to reduce NTG-induced impairment of NO production.

Methods

A Catheter Type NO Sensor

Integrated architecture and performance of the catheter-type NO sensor have been described previously[13–15]. In brief, an NO sensor (amino-700 XL, Innovative Instruments, 700 μm in diameter at the detection tip) was mounted in a 4-Fr catheter (1.200 mm long; Hirakawa Hewtech, Tokyo, Japan) and fixed with silicon adhesive. The oxidative current of NO was monitored by using an NO monitor (model inNO-T, Innovative Instruments). Each sensor was calibrated by using an NO-saturated pure water as previously described[13–15]. Briefly, NO-saturated pure water was prepared by bubbling pure NO gas in oxygen-free pure water. Using a gas-tight syringe, 5 ml was injected into a well stirred saline solution (50 ml), in which the NO sensor was immersed (final NO concentration: 190 nmol/L) as previously described[13–15]. The baseline (zero level) is set arbitrarily in the amperometric method, and thus, a change in the current form the baseline is used and is expressed as ‘change in NO concentration (nmol/L)’ because NO concentration cannot be measured as an absolute value with the amperometric method[13–15].

Animal Preparations

The study protocol was approved by the Institutional Animal Care and Use Committee of Wakayama Medical University. Male New Zealand white rabbits (2.0–2.5 kg) were maintained on tap water and standard diet. The animals were randomized into one of 7 experimental groups. Group 1 (n=6) was allocated to control, groups 2 through 6 (n=6 per group) were treated with transdermal NTG patches (Nihon Kayaku Pharma, Tokyo, Japan) to a shaved dorsal thoracic area of the body. Such patches were present continuously for a period of 3–7 days (each patch being replaced per group) were treated with transdermal NTG patches (Nihon Kayaku Pharma, Tokyo, Japan) to a shaved dorsal thoracic area of the body. These rabbits and 7 days NTG-treated rabbits and placed into chilled Krebs buffer, cleaned of excessive adventitial tissue. We cut a 3 mm long aortic ring at the mid-portion of the thoracic aorta. Two stainless steel hooks were inserted through the lumen of the aortic ring. One hook was fixed to an anchor and the other hook was connected to an isometric force transducer (Nihon Kohden TB-68417). Care was taken not to damage the endothelium. These rings were mounted in an organ chamber (volume: 3 ml) and continuously superfused with physiological salt solution, warmed at 37°C and aerated with 100% O₂, at a flow rate of 2 ml/min. The solution consisted of (mmol/L) NaCl 138, KCl 4.7, CaCl₂ 1.8, MgSO₄ 1.2, NaHPO₄ 1.2, glucose 10 and HEPES 5 aerated, and the pH was adjusted with 2 mol/L Tris-HCl to 7.40. Isometric tension was continuously monitored and recorded on a strip-chart recorder. The preparations were equilibrated until the resting tension stabilized at 2 g, which took approximately 1 h. Aortic rings were precontracted with 1×10⁻⁶ mol/L of phenylephrine. Once a stable contraction was obtained, ACh, an agent that induces vasoraxlation via stimulation of NO production from the endothelium, was added to the bath in cumulative concentrations of 10⁻⁶–10⁻⁴ mol/L. To determine endothelial function and agonist-stimulated NO production for the endothelium. Endothelium-independent relaxation by NTG (10⁻⁶–10⁻⁴ mol/L) was also examined. Once 1 experiment was completed, more than a 1 h interval was obtained before starting the next experiment. The maximal response attained at each concentration was used in the calculation.

Experimental Protocol

To measure the endothelium-dependent NO production, 20 μg/kg of Ach was administered at 1 ml/min for 5 min. To inhibit NO synthesis in the endothelium, 5 mg/kg of N⁵-methyl-L-arginine (L-NMMA, NO synthesis inhibitor) was infused at 1 ml/min for 10 min to inhibit NO synthesis. We then administered Ach injection. Plasma NO concentration in the abdominal aorta was monitored over the entire time course. We measured Ach-induced as well as L-NMMA-induced plasma NO production as the peak change in the current form the baseline, which is expressed as ‘change in NO concentration (nmol/L)’.

Measurement of Plasma Nitrotyrosine

Active NO metabolites can react with superoxide to form peroxynitrite and nitrating oxidant. Subsequent reaction of peroxinitrite with proteins results in nitrotyrosine formation. As a stable end product of peroxinitrite mediated oxidation/nitration, nitrotyrosine can be used as a surrogate index of NO dependent damage in vivo. Therefore, we investigated the effect of chronic treatment with NTG on nitrotyrosine formation. For analyzing the plasma nitrotyrosine, NWLSS Nitrotyrosine enzyme-linked immunoassay (ELISA) kit (Northwest Life Science Specialities, LLC, Vancouver, Canada) was used according to the manufacturer’s protocol. The NWLSS nitrotyrosine ELISA assay measures nitrated protein and not ‘free’ nitrotyrosine. Smaples do not need to be hydrolyzed and data should be interpreted as being inclusive of nitrotyrosine still bound to soluble proteins[16]. The kit is a simple ‘sandwich’ ELISA using a plate bound capture antibody to nitrotyrosine and a biotinylated secondary tracer antibody. Addition of streptavidine-peroxi-
dase followed by tetramethylbenzidine facilitates color development. The amount of bound protein in each well was measured by using an ELISA Plate Reader (Bionetics Laboratory, Kensington, MD, USA) at 450 nm.

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Fig 1. Effect of 7 days nitroglycerin (NTG) treatment with acetylcholine (ACh) (A) and NTG concentration-response curve (B). Data are expressed as mean±SEM (n=5). *p<0.05 vs control. Block circles, control; open circles, NTG treatment.

Fig 2. Effect of chronic treatment with nitroglycerin (NTG) either alone or in combination with antioxidant, or angiotensin converting enzyme inhibitor-I, or type 1 receptor antagonist blockers on bioavailability of nitric oxide (NO). (A) Typical tracings of the plasma NO concentration induced by acetylcholine (ACh) in aorta treated with vehicle (control), NTG (3 days), NTG (7 days), NTG + tempol, NTG + enalapril and NTG + valsartan. (B) Chronic treatment with NTG induces the decrease of ACh-induced plasma NO concentration. The decrease of ACh-induced plasma NO concentration in NTG-treated rabbits was significantly reversed by co-treatment with tempol, enalapril or valsartan. Data are expressed as an absolute value. Bars represent±SEM (n=6). *p<0.01 vs control; *p<0.01 vs NTG (7 days) treated group.
All data were expressed as mean ± SEM based on at least 6 independent experiments. Statistical analyses for comparison of changes in plasma NO concentration by ACh were conducted by paired t-test. Difference in values between groups and treatments were tested using 1-way ANOVA of repeated measurements, followed by the post-hoc Scheffe F test. A probability value of <0.05 were considered significant.

**Results**

**Calibration of Sensors**

The basic performance of the integrated catheter-type NO sensors was reported in our previous studies.13–15 The NO sensors showed no noticeable change in response to oxygen, ACh and solution mixing, indicating high specificity to NO (data not shown). The mean sensitivity of the 7 sensors used in the present study was 325±16 pA/nmol/L. This value is comparable to the values of the original in vivo sensor.12

**Effect of NTG Treatment on Endothelial Function**

In thoracic artery rings isolated from control rabbits, ACh induced a concentration-dependent vasorelaxation. In contrast, thoracic aortic rings isolated from NTG-treated rabbits showed a severe right-shifting of their dose-response curve to ACh (Fig 1A). In normal vessels, NTG produced maximal relaxations of 96±1%. In accordance with previous findings,7 in NTG-treated vessels, maximal relaxations to NTG were markedly less than that observed in normal rings.

**Effect of NTG Treatment on ACh-Induced and Basal NO Production**

In a preliminary study, by using male New Zealand white rabbits, we measured 20 μg/kg ACh-induced increase in plasma NO concentration in the rabbit aorta. ACh-induced increase in plasma NO concentration in the rabbit aorta was observed (8.3±0.1 nmol/L; repeated 3 times using 1 rabbit), which has shown the reproducibility of this method. Plasma NO concentration was successfully measured in the aorta by
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the catheter-type NO sensor in all rabbits (Fig 2). Treatment of rabbits with NTG for 7 days resulted in a significant reduction in the increase in plasma NO concentration induced by ACh (control: 8.3±0.1 nmol/L vs 1.9±0.1 nmol/L [NTG], p<0.01) (Fig 2B). The effect of NTG on basal plasma NO concentration was evaluated with a NO synthesis inhibitor, L-NMMA. NTG-treatment for 7 days affected basal plasma NO concentration; the decrease in the basal plasma NO concentration by L-NMMA infusion was significantly lower in NTG-treated rabbits than in untreated (control) rabbits (control: –3.1±0.1 nmol/L vs –1.2±0.1 nmol/L [NTG], p<0.01), indicating a lower basal NO bioavailability (Fig 3).

**Effect of Enalapril or Valsartan Treatment on NTG-Induced Impairment of ACh-Induced and Basal NO Production**

An antioxidant, tempol, significantly reversed NTG-induced impairment of NO production induced by ACh (NTG + tempol: 7.9±0.1 nmol/L, p<0.01 vs NTG only) (Fig 2). Either a ACE-I, enalapril or an ARB, valsartan, significantly reversed NTG-induced impairment of NO production induced by ACh (NTG + enalapril: 6.9±0.1 nmol/L, NTG + valsartan: 5.7±0.2 nmol/L, p<0.01 vs NTG only) (Fig 2). The inhibitory effects of NTG on basal plasma NO concentration was significantly reversed by co-treatment with tempol (–2.2±0.1 nmol/L), enalapril (–2.8±0.1 nmol/L) or valsartan (–2.0±0.1 nmol/L) (Fig 3).

**Effect of NTG on Plasma Nitrotyrosine**

The NTG-treated rabbits had significantly higher plasma nitrotyrosine concentrations compared with that of the control. As shown in Fig 4, tempol, enalapril or valsartan significantly abolished the increase in plasma nitrotyrosine.

**Discussion**

There is considerable evidence that nitrate tolerance is associated with incremental impairment of endothelial function.17,18 This cross-tolerance to endogenous NO would imply that nitrate therapy might be potentially harmful to long-term clinical outcomes. In the present study, we have shown direct evidence of the decrease of the basal and ACh-induced plasma NO concentrations induced by chronic treatment with NTG. We have also shown the effects of antioxidants or substances which reduce nitrosative stress, such as ACE-I and ARB, to reduce NTG-induced impairment of NO production.

From an analytical point of view, detection of NO in biological fields is a challenging topic. NO produced by endothelial cells diffuses into the flowing blood, where it reacts with blood components, such as erythrocytes and proteins. It has been assumed that NO is rapidly oxidized by its reaction with the ion-containing heme groups of oxyhemoglobin. The short biological half-life of NO means that it is most likely that the ion-containing heme groups of oxyhemoglobin. The short biological half-life of NO means that it is most likely that the bioactivity of NO is confined to the vicinity of its production sites. However, Stamler et al have suggested that S-nitrosohemoglobin is a source of bioactive NO and a crucial component of the cardiorespiratory cycle.19 They propose that S-nitrosohemoglobin forms in tissues at high oxygen tensions, where at low oxygen saturations it decomposes and releases NO, which dilates blood vessels. Whatever mechanisms turn out to lie behind the role of NO in the cardiovascular cycle, it is now most possible that NO might be transported in the body in the manner of a hormone. In fact, growing experimental and clinical evidence suggests that NO, which modulates vascular tone by its diffusion to the underlying vascular smooth muscle cells, also diffuses to the bloodstream, causing remote vasodilatory responses.19,20 In the present study, we also found that NO concentration increased in blood during ACh infusion. However, we could not examine NTG-induced impairment in NO bioavailability in its producing sites, because of the technical difficulties of the measurement of NO in vessel wall itself. Further studies will be needed on the unsolved problems.

Although previous studies had used surrogate endpoints, such as endothelium-dependent vasodilation by ACh, to show the same concept, there are still many uncertainties remaining in relation to the bioavailability of NO. First, it is unclear whether factors that result in ED also lead to abnormalities in the function of vascular smooth muscle cells. Second, besides NO, other endothelium-derived relaxing factors and constricting factors, such as prostacyclin, endothelium-derived hyperpolarizing factors and endothelin might contribute to the vascular tone after injection of ACh through direct or indirect effects. Indeed, recent studies show that prostacyclin synthase is a highly vulnerable target of peroxynitrite and that chronic NTG application in vivo inhibits this enzyme via peroxynitrite-induced tyrosine nitration.

The long-term use of NTG resulted in increased induc-
tion of reacted oxygen species, such as superoxide? Because NTG releases NO, NTG-induced stimulation of superoxide production should increase vascular nitrotyrosine concentrations, compatible with increased formation of peroxynitrite, the reaction product from NO and superoxide. Indeed, Skatchkov et al have shown that NTG treatment stimulates vascular production of peroxynitrite. A stable metabolite of peroxynitrite, nitrotyrosine, is formed by nitration of free or protein-bound tyrosine. In vitro and in vivo data indicated that NTG treatment increased urinary nitrotyrosine and vascular nitrotyrosine content compatible with increased peroxynitrite formation. Interestingly, Hink et al have shown that in vivo exposure to NTG leads to increased vascular nitrotyrosine formation, which was restricted to the endothelial and subendothelial space. We have also confirmed that NTG treatment stimulates the production of peroxynitrite. As pointed out by Gori et al, peroxynitrite is a strong stimulus for the oxidation of the NOS III cofactor tetrahydrobiopterin (BH4) to dihydrobiopterin (BH2). The resulting intracellular BH4 deficiency might switch NOS III from a NO to a superoxide-producing enzyme, which could further increase oxidative stress in vascular tissue in a positive feedback fashion. This finding is in line with our recent findings where exogenous NO suppresses the flow-induced, endothelium-derived NO production by superoxide released from uncoupled NOS because of intracellular BH4 depletion. Taken together, a NO inactivation because of increased nitrosative stress might explain a number of the abnormalities during long-term NTG therapy, including NTG-derived NO quenching, NOS uncoupling and thus further reduced NO. In fact, in the present study, an antioxidant, tempol, significantly reversed the basal as well as ACh-induced NO production. In contrast, recent studies have shown that mitochondrial aldehyde dehydrogenase (ALDH-2) might play a central role in NTG tolerance and cross-tolerance. In the present study, we could not provide mechanistic insight how the antioxidants prevent NTG-induced impairment of NO bioavailability. Thus, it remains unknown whether preventive effects of antioxidants on NTG-induced impairment of NO bioavailability is because of prevention of oxidative inhibition of ALDH-2 and/or NADPH oxidase activation. Further studies will be needed to clarify the mechanistic insights into how antioxidants prevent NTG tolerance and cross-tolerance.

Regarding the development of nitrate tolerance, attention has been paid to the possible role of circulating, as well as local, Ang II. Indeed, in vivo treatment with NTG activates the renin-angiotensin-aldosterone axis as a neurohormonal counter-regulatory mechanism in both animals and humans. In the present study, we have shown the direct evidence, where either ACE-I or ARB, similar to tempol, significantly reversed the basal as well as ACh-induced NO production by the NO sensor.

In conclusion, taken together, the present study shows that long-term treatment of New Zealand white rabbits with NTG increases nitrosative stress in plasma leading to the decrease of basal, as well as ACh-induced plasma secretion by endothelial cells. We have also shown for the first time the protective effects of antioxidant, ACE-I or ARB, to reduce NTG-induced impairment of NO bioavailability.

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