

# Plasma Nitric Oxide Level and Its Role in Slow Coronary Flow Phenomenon

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## SUMMARY

Previous studies have suggested that microvascular abnormalities and endothelial dysfunction cause slow coronary flow (SCF). The objective of this study was to assess the plasma nitric oxide (NO) level and determine its role in the pathogenesis of SCF phenomenon. Thirty-six patients with SCF (group 1) and otherwise patent coronary arteries and 34 subjects with normal coronary flow (group 2) were included in the study. Coronary flow was quantified according to the TIMI Frame Count (TFC) method. Brachial artery endothelium-dependent flow-mediated dilatation (FMD) and nitroglycerin (NTG)-induced endothelium-independent dilatation were studied in both groups. In addition, plasma NO levels were measured and their contribution to FMD was determined. The sex, age, body mass index, arterial blood pressure, and heart rate distributions were similar in both groups. TFC was significantly higher in group 1 compared to group 2 for each artery. The plasma NO level was lower in patients with SCF than in control subjects ( $18.4 \pm 4.4$  versus  $25.2 \pm 6.3$   $\mu\text{mol/L}$   $P = 0.001$ ). FMD was significantly smaller in group 1 than in group 2 ( $4.0 \pm 3.2\%$  versus  $10.6 \pm 5.8\%$ ,  $P = 0.0001$ ). The percent NTG-induced dilatation was similar in the two groups ( $16.8 \pm 1.1\%$  versus  $17.1 \pm 1.1\%$ ,  $P = 0.42$ ). In group 1, the plasma NO level was correlated with percent of FMD. Also, the plasma NO level was inversely correlated with TFC for each artery. Reduced NO bioactivity as well as impaired FMD support the presence of endothelial damage in the pathogenesis of SCF phenomenon. (Int Heart J 2005; 46: 373-382)

**Key words:** Slow coronary flow, Endothelial dysfunction, Nitric oxide

**S**LOW coronary flow (SCF), characterized by slow antegrade progression of a dye to the distal branch of a coronary artery in the absence obstructive coronary disease is not an infrequently detected finding during routine coronary arteriography, but its clinical significance has not been well documented. In general, abnormal high small vessel resistance and increased microvascular tone have been

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suggested to be the causes of this unique angiographic finding.<sup>1-3)</sup> We have recently reported that vascular endothelial functions are impaired in these patients.<sup>4)</sup> However, the underlying mechanism of the microvascular abnormalities and endothelial dysfunction (ED) has not yet been established.

The endothelium is crucial in regulating vasomotor tone, platelet activity, leukocyte adhesion, and vascular smooth muscle proliferation through the release of several paracrine factors, including nitric oxide (NO).<sup>5)</sup> ED is characterized by increased coronary and circulating endothelin and decreased production of cyclic guanosine monophosphate, a second messenger of NO.<sup>6)</sup> Although ED has been associated with the presence of atherosclerosis, impaired endothelial-dependent NO bioactivity has also been shown in patients with atherosclerosis risk factors without overt vascular disease.<sup>7-11)</sup> It has also been shown that NO is essential for flow-mediated dilatation of large human arteries *in vivo*.<sup>12)</sup> Therefore, the purpose of this study was to measure plasma NO levels in patients with SCF and to compare them with normal coronary flow (NCF). Another aim was to determine the role of NO in modulation of vascular endothelial function in patients with SCF.

## METHODS

The study population consisted of 36 patients with SCF and otherwise patent coronary arteries (group 1), and 34 sex and age matched subjects with atypical chest pain and otherwise NCF (group 2). All participants were selected from among those who had undergone routine coronary arteriography. In both groups, coronary arteriography was performed since most of the subjects were suffering from intractable symptoms such as angina and angina-like symptoms, shortness of breath, palpitations, and so on and their symptoms could not be adequately clarified with noninvasive tests.

**The exclusion criteria were as follows:** Coronary artery disease including spasm, plaque, ectasia or obstructive lesion, positive family history of hyperlipidemia, known peripheral vascular disease, diabetes mellitus, congestive heart failure, valvular heart disease, hypertrophic, restrictive, and dilated cardiomyopathies, left ventricular hypertrophy, vasculitis, pulmonary, renal, and hematological disorders, and those on medications known to alter plasma nitric oxide levels.

**Definition of slow coronary flow:** Coronary flow was quantified according to the TIMI frame count (TFC) method.<sup>13)</sup> All participants with a TFC greater than two standard deviations (SD) from the normal published range for the particular vessel were accepted as having SCF, while those whose TFC fell within the SD of the published normal range were considered to have NCF.<sup>13)</sup> To exclude valvular or other cardiac abnormalities, all study subjects underwent echocardiographic examination using a 3.5 MHz sector transducer (Hewlett Packard Sonos 1000

ultrasound system Andover, Massachusetts) according to the American Society of Echocardiography guidelines.<sup>14)</sup>

**Measurement of TIMI frame count:** Coronary angiography was performed using the Judkins technique in multiple angulated views. Iopromide contrast (Ultravist-370, Schering AG) was used in all patients. All arteriograms were recorded on 35-mm cine film at 25 frames/s (Kodak; Kodak-Pathé SAS, Paris, France). We measured the number of cine frames required for the contrast to first reach standard distal coronary landmarks in the left anterior coronary artery (LAD), left circumflex artery (Cx), and right coronary artery (RCA) using the Tagarno 35AX cine viewer frame counter (Tagarno A/S, Horsens, Denmark). The first frame is defined as the one where the column of nearly or fully concentrated dye is seen extending across at least 70% of the arterial lumen with antegrade dye motion, and the last frame counted is that in which contrast first appears in the distal pre-defined landmarks branch, but full opacification of the branch is not necessary.<sup>13)</sup> The distal coronary landmarks used for analysis were the distal bifurcation at the apex of the LAD (the moustache, pitchfork or whale's tail), the distal bifurcation of the major obtuse marginal or the main Cx, whichever was larger, and the site of origin of the first branch at the crux or its posterolateral extension for RCA. The cine film was run past the initial opacification of the end point branch and then moved frame by frame in reverse until the end point branch disappeared before catching the last frame. The measurement of frame count for each artery was done by subtracting the first frame from the last frame. The normal frame counts accepted were  $36 \pm 2.6$  for LAD,  $22.2 \pm 4.1$  for Cx, and  $20.4 \pm 3.0$  for RCA, as previously described.<sup>13)</sup>

**Measurement of nitric oxide level:** Blood samples were taken in the morning of the examination after overnight fasting and drawn into heparinized tubes for biochemical analysis. After immediate centrifugation ( $1000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ), plasma samples were stored frozen at  $-30^{\circ}\text{C}$ . Since NO is a very labile molecule, its direct measurement in the biological samples is very difficult. In aqueous solution, NO reacts with molecular oxygen and accumulates in the plasma as nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) ions. The stable oxidation end products of NO,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  can be readily measured in biological fluids and have been used *in vitro* and *in vivo* as indicators of NO production.<sup>15)</sup> Therefore, plasma nitrite concentration was accepted as an index of NO. For total nitrite detection, deproteinized plasma was treated with copperized cadmium granules to reduce  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . Nitrite concentrations were quantified by a colorimetric assay based on the Griess reaction.<sup>16)</sup> Briefly, a chromophore with strong absorbance at 545 nm is formed by the reaction of nitrite with a mixture of N-naphthylethylene diamine and sulfanilamide. A standard curve was established with a set of serial dilutions ( $10^{-8}$

to  $10^{-3}$  mol/L) of sodium nitrite. Results are expressed as micromoles per liter of plasma ( $\mu\text{mol/L}$ ).

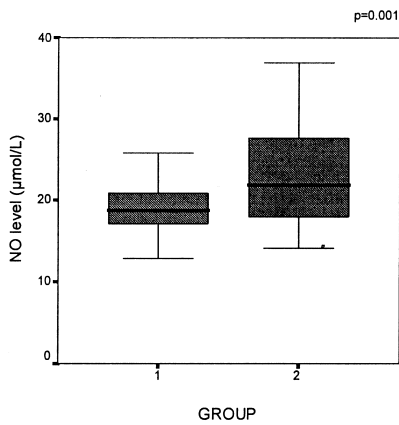
**Assessment of vascular endothelial function:** All participants underwent brachial artery ultrasonographic evaluation according to standard techniques.<sup>7)</sup> Subjects were asked to refrain from drinking alcohol- or caffeine-containing beverages for at least 12 hours before the study. Studies were performed following an overnight (10-12 hours) fast in a dimly light room at 21-23°C. A high-resolution Doppler ultrasound system (HDI-5000; ATL, Bothell, Washington) equipped with a 12-MHz linear-array transducer was used to image the right brachial artery, and vasodilator responses were measured. After baseline images of the brachial artery were obtained, a pneumatic tourniquet placed around the forearm distal to the target artery was inflated to a pressure of 250 mmHg, and the pressure was maintained for 5 minutes. Increased flow was induced by sudden cuff deflation. A second scan was performed continuously for 30 seconds before and for 90 seconds after cuff deflation. Increased blood flow after sudden cuff deflation, termed reactive hyperemia, results in endothelium-dependent flow-mediated dilatation (FMD).<sup>17)</sup> Fifteen minutes later a further resting scan was recorded to confirm vessel recovery. Sublingual nitroglycerin (NTG) 0.4 mg was then administered, and 3 to 4 minutes later the last scan was performed. The diameter of the brachial artery was measured from the anterior to posterior interface between the media and adventia ("m" line) at the end of diastole, incident with the R wave on a continuously recorded ECG. Diameters for 4 cardiac cycles were determined from images, and the measurements were averaged. Two independent observers who were unaware of the clinical state of the subject performed these measurements. FMD was calculated as the percentage increase in arterial diameter during reactive hyperemia and was used as an index of endothelium-dependent vasodilatation. The diameter change caused by NTG was expressed in the same way, as the percent change relative to that at the recovery scan used as an index of endothelium-independent vasodilatation.<sup>18)</sup> In our laboratory, interobserver variability for repeated measurements is  $0.05 \pm 0.01$  mm.

**Statistical analysis:** Statistical analysis was performed with SPSS for Windows version 10.0 (SPSS Inc. Chicago, Illinois). Data are presented as the mean  $\pm$  SD. The unpaired Student t test was used for continuous variables and the chi-square test was used for categorical changes. Linear regression analysis was conducted to investigate the association between plasma NO levels, FMD, and various factors such as TFC for each artery, age, gender, body mass index, and blood pressure. A *P* value  $< 0.05$  was considered statistically significant.

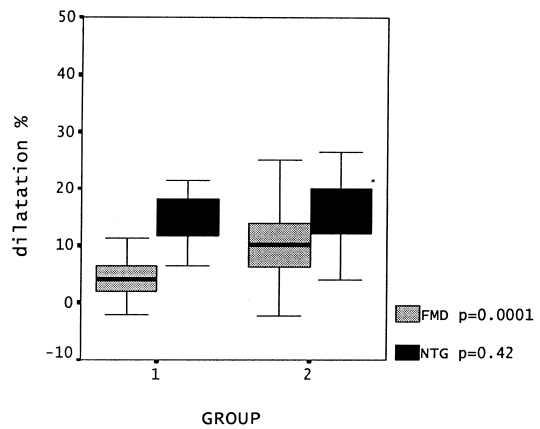
**Table.** General Characteristics of Study Subjects

Variable	Group 1	Group 2	P values
Number (male/female)	24/12	22/12	NS
Age, years	48 ± 9	47 ± 8	NS
Body mass index (kg/m <sup>2</sup> )	26 ± 4	26 ± 3	NS
Systolic blood pressure, mmHg	129 ± 11	126 ± 10	NS
Diastolic blood pressure, mmHg	83 ± 10	81 ± 9	NS
Heart rate, bpm	73 ± 9	71 ± 8	NS

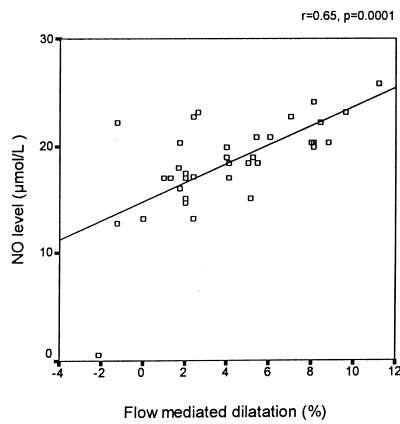
NS = statistically not significant.



**Figure 1.** Comparison of plasma NO level in patients with slow coronary flow (group 1) and normal coronary flow (group 2).



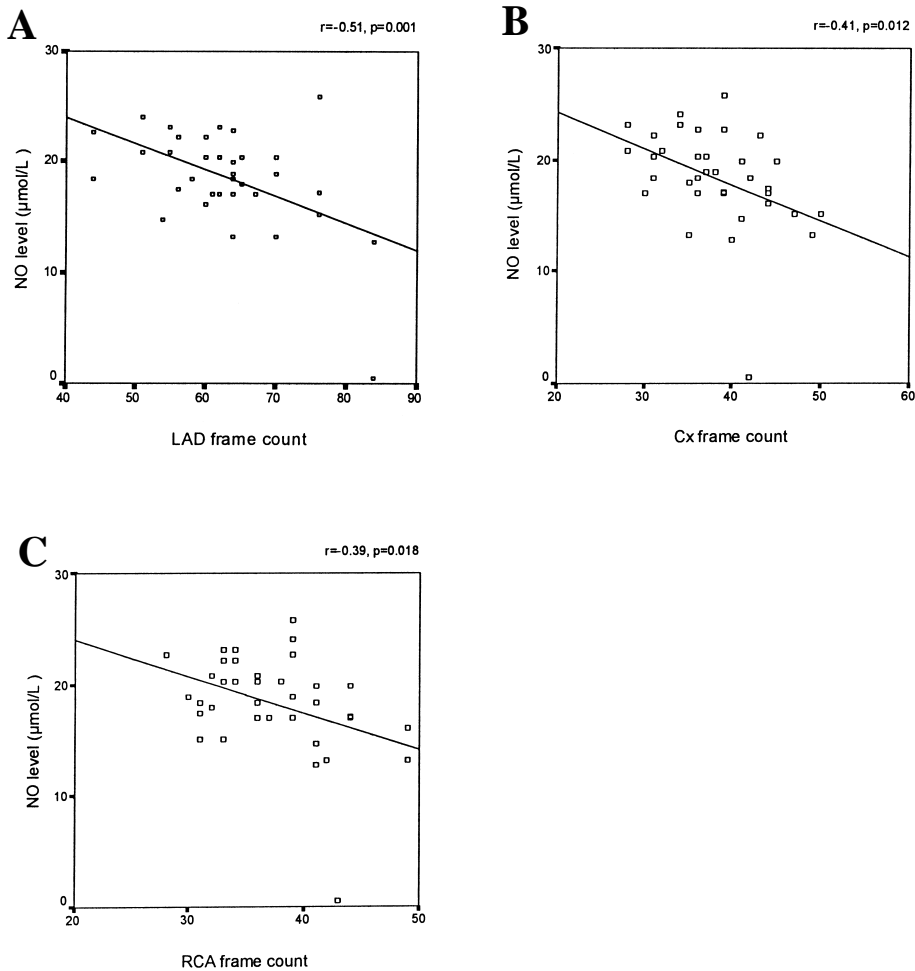
**Figure 2.** Flow-mediated dilatation (FMD) and nitroglycerin (NTG)-induced dilatation in patients with slow coronary flow (group 1) and normal coronary flow (group 2).



**Figure 3.** The relationship between NO level and the percent of flow-mediated dilatation.

**RESULTS**

The general characteristics of the study population are shown in Table I. There were no significant differences between the two groups with respect to sex, age, body mass index, blood pressure, and mean heart rate. Both groups had normal results on physical examination. None of the subjects had abnormalities on their two-dimensional or M-mode echocardiograms. In group 1, SCF was noted in



**Figure 4.** A: Negative correlation between NO level and LAD frame count. B: Negative correlation between NO level and Cx frame count. C: Negative correlation between NO level and RCA frame count.

NO = nitric oxide; LAD = left anterior descending coronary artery; Cx = left circumflex coronary artery; RCA = right coronary artery

all coronary arteries. In group 2, all coronary arteries including LAD, Cx, and RCA were entirely normal. The LAD, Cx, and RCA frame counts were significantly higher in group 1 than group 2 ( $63.2 \pm 9.4$  versus  $36.2 \pm 2.1$  frames  $P < 0.0001$ ,  $38.0 \pm 5.6$  versus  $22.1 \pm 5.0$  frames  $P < 0.0001$ ,  $37.2 \pm 5.2$  versus  $22.3 \pm 4.8$   $P < 0.0001$ , respectively). There were no significant differences in the brachial artery diameter at rest between the two groups ( $4.95 \pm 0.41$  versus  $4.82 \pm 0.39$  mm,  $P = 0.67$ ). The plasma NO level was significantly lower in patients with SCF than in control subjects (Figure 1). In group 1, FMD was significantly smaller than in group 2, while the percent of NTG-induced dilatation was similar in both groups (Figure 2). Regression analysis revealed the plasma NO level was strongly correlated with percent of FMD in group 1 (Figure 3). Also, plasma NO level was inversely correlated with TFC for each artery (Figure 4A-C).

## DISCUSSION

The principal findings of the present study are that 1) the plasma NO level is markedly lower in patients with SCF than in those with NCF, 2) brachial artery FMD is impaired in patients with SCF, 3) there is a significant relationship between the plasma NO level and FMD, and 4) there is also a significant inverse relationship between the plasma NO level and TFC in patients with SCF.

These data confirm our recently published study in which we showed that vascular endothelial function was impaired in patients with SCF.<sup>4)</sup> However, in that study we only found a negative correlation between mean CTFC and FMD and could not define the factors contributing to ED. Also, due to the strict inclusion criteria only a small number of patients were included and those with traditional coronary artery disease risk factors were carefully excluded. In contrast, the present study included a more heterogeneous and larger patient population since some had risk factors such as hypertension and smoking. In addition, in the present study extending our search, we not only studied vascular endothelial function but also attempted to define the factors that may contribute to endothelial function.

In this study, we found that plasma NO levels were lower in patients with SCF than in those with NCF and were inversely correlated with TFC. These findings apparently support the concept that endothelial function is impaired in patients with SCF. In accordance with our findings, Zeiher, *et al*<sup>19)</sup> suggested that coronary ED might be a mechanism of exercise-induced myocardial ischemia in patients with effort angina and a normal coronary arteriogram. ED, especially a reduction in the bioavailability of endothelium-derived NO, is a key early event in atherogenesis, appearing long before the formation of structural atherosclerotic changes.<sup>7,20)</sup> It has been shown that NO may play an important role in the regula-

tion of resting blood flow, pacing-induced hyperemia, and flow-mediated dilatation in the coronary circulation.<sup>5,21-24)</sup> In this context, our findings with impaired endothelial function and reduced NO bioactivity provide new insight into the pathogenesis of SCF phenomenon.

There are only a few reports regarding the etiology of SCF. The term SCF, was first defined by Tambe, *et al*<sup>1)</sup> in 6 cases with typical or atypical chest pain. In 1986, Mosseri, *et al*<sup>2)</sup> described fibromuscular hyperplasia, medial hypertrophy, myointimal proliferation, and endothelial degeneration in the microvascular circulation from right ventricular biopsy specimens from 6 SCF patients. Later, Mangieri, *et al*<sup>3)</sup> evaluated left ventricular biopsy samples of 10 patients with SCF, none of whom had a cardiac or systemic illness, and found endothelial thickening due to cell edema, capillary damage, and decreased luminary diameter. In agreement with our results, these findings suggest the presence of endothelial damage. Mangieri, *et al*<sup>3)</sup> proposed that histopathologic abnormalities in microvascular structures cause or at least facilitate the increase in microvascular resistance. Although their patients were in part homogenous, the documentation of slow flow was done visually without any objective criteria. In contrast, we defined the SCF according to objective criteria using the TFC method.<sup>13)</sup>

From a clinical standpoint, patent coronary arteries and angina pectoris are poorly understood entities and within this spectrum, SCF and otherwise normal coronary arteries have a distinct significance. Although it is often considered to be an incidental angiographic finding, individual case reports and small clinical studies have identified some clinical consequences of SCF. Angina, myocardial ischemia, and infarction have been reported to be associated with SCF.<sup>25-28)</sup> More recently, we found that the exercise QRS score was similar in patients with SCF and significant coronary stenosis, leading us to conclude that SCF might lead to myocardial ischemia.<sup>29)</sup> We therefore speculate that the impaired peripheral endothelial function may also suggest the involvement of epicardial coronary arteries in SCF. However, the results of this study do not indicate that the patients with SCF are necessarily at risk of developing atherosclerosis. In fact, our results only indicate that NO plays a pivotal role in the modulation of vascular endothelial function in patients with SCF.

**Conclusion:** Reduced NO bioactivity as well as impaired vascular endothelial function support the role of endothelial damage in the pathogenesis of SCF phenomenon. However, to firmly establish its clinical implications, further large-scale trials and long-term follow-up are required. In addition, whether NO donors improve coronary flow remains to be determined.



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