» **Original Article** «

Platelet Nitric Oxide Signaling System in Patients with Coronary Artery Disease

Mahdi Garelnabi, PhD,¹ Vinod Gupta, MD,² Venkatesan Mallika, MD,² and Jayashree Bhattacharjee, MD³

Background: Coronary artery disease (CAD) is the leading cause of death worldwide, and the major cause of hospital admissions in the Western countries. The pathogenesis of CAD is closely related to nitric oxide release and formation. The purpose of this study was to investigate the status of platelets nitric oxide in patients with coronary artery disease.

Methods: We measured platelets aggregation, cGMP, NO (nitrite/nitrate level), NO synthase activity, plasma NO, and ionized Ca²⁺ in 40 healthy volunteers and 120 patients with myocardial infarction, unstable and stable angina, with 40 subjects in each group. The subjects' age mean range was from 40–51 years.

Results: Platelets aggregation, NO, cGMP, NO synthase activity, plasma NO and ionized Ca²⁺ have increased significantly ($P < 0.001$) across the patients groups compared to controls. Platelets NO synthase activity (mean $\pm$ SD / U / 10⁹ platelets) in healthy controls, MI, unstable angina and stable angina patients were 1.19 ± 0.56 , 1.21 ± 0.64 , 1.64 ± 0.98 and 1.57 ± 0.81 respectively. The cGMP (mean $\pm$ SD / pmole / 10⁹ platelets) levels were 0.95 ± 0.41 , 1.53 ± 0.64 , 3.18 ± 0.77 , and 5.12 ± 1.5 respectively.

Conclusions: The present study demonstrated that platelets aggregation, NO, cGMP, NO synthase activity, plasma NO, and ionized Ca²⁺ profoundly increased in CAD. The increases in NO-cGMP components may have resulted as a compensatory response to ameliorate platelet activity and Ca²⁺ levels in CAD patients.

Key words: cardiovascular disease, heart disease, nitric oxide, platelets, ischemic heart disease

INTRODUCTION

Coronary artery disease (CAD) is the leading cause of death worldwide and the major cause of hospital admissions in Western countries.¹⁾ The disease is a manifest of a progressive atherosclerosis disease that develops

from early childhood through a complex process mediated by inflammation and oxidative stress.^{2–4)} Platelet contributes to the pathogenesis of cardiovascular disease, and its involvement in the pathogenesis of thrombosis-related complications is well-documented.²⁾

The nitric Oxide-Cyclic GMP signal transduction system has emerged as a ubiquitous pathway for intracellular and intercellular communication.⁵⁾ Although NO release is known to improve endothelium performance and inhibit platelets function, NO may also forms the potent peroxynitrite that in turn, contributes to the oxidative damage. Nitric oxide derived reactive nitrogen species (RNS), such as nitrogen dioxide (NO₂) and peroxynitrite (ONOO⁻), are believed to mediate cellular oxidative damage. When NO is in excess of that required to activate guanylate cyclase, it can inhibit glycolysis, the mitochondria c respiratory chain and DNA replication.^{6, 7)} The pathogenesis of CAD is closely related to nitric oxide release and formation. Several studies suggest that the

¹Department of Clinical Laboratory and Nutritional Sciences, University of Massachusetts Lowell, Massachusetts, USA

²Department of Clinical Biochemistry, G. B. Hospital and MA Medical College, New Delhi, India

³Department of Biochemistry, Lady Hardings Medical College, New Delhi, India

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Corresponding author: Mahdi Garelnabi, PhD. Department of Clinical Laboratory and Nutritional Sciences, School of Health and Environment, University of Massachusetts Lowell, 61 Wilder, Lowell, Suite 319, Lowell, Massachusetts 01854, USA
Tel: +1-978-934-4528, Fax: +1-978-934-4021

E-mail: Mahdi_Garelnabi@uml.edu

basal release of NO by the endothelium contributes to the regulation of vascular tone,⁸⁾ blood flow, and blood pressure. NO inhibits platelet aggregation and adhesion to vascular endothelium. In addition, NO inhibits leukocyte adhesion to endothelium.⁹⁾ Alteration of cellular calcium homeostasis is also a critical event in ischemic heart injury. NO, released by the endothelium or synthesized by platelets, participates in the regulation of Ca^{2+} signaling. The elevation of cGMP, as a result of the activation of guanylate cyclase by NO, stimulates a number of mechanisms that actively decrease calcium levels within the cell.^{10–14)} Although the NO-cGMP signaling system is immensely investigated; sparse data are available pertaining to the role of platelet NO activity in CAD. The current study was designed to investigate the NO-cGMP system in patients with CAD.

MATERIALS AND METHODS

All chemicals and reagents were of analytical grade. Naphthylendiamine, sulfanilamide, HEPES, ADP, nitrate reductase, methemoglobin and dithiothreitol were purchased from Sigma Chemical Co. USA; Radiometer, Denmark; reagents used for the determination of ionized Calcium (Ca^{2+}). cGMP ELISA kit was purchased from Biomedical Technologies Inc., USA. The remainder of chemicals were purchased from Merck, Germany.

Study design

Recruitment and analysis were completed at G. B Pant Hospital and Mulana Azad Medical College, New Delhi, India.

Study population: All subjects completed and signed a consent form explaining the voluntary nature of the study. The research protocol was approved by MA Medical College and G B Pant Hospital Institutional Review Board. The patients' population consisted of 120 subjects diagnosed with ischemic heart diseases during their visit to the New Delhi city metropolitan Emergency Unit of LNJP Hospital and the Department of Cardiology at G.B. Pant Hospital. The patients initially complained of chest pain and were later diagnosed with ischemic heart diseases, according to the criteria of the New York Heart Association.¹⁵⁾ Patients with primary coagulopathy, bleeding diathesis, diabetes, or any other disease known to alter platelet activity, were excluded from the study. In addition, those who underwent angiographic imaging studies and their findings failed to match with the criteria for the disease, or on drugs that may affect platelet

function or on prescribed lipid-lowering drugs, were dropped from the study. A group of 40 apparently healthy individuals, who belongs to similar socio-economic demography as that of the patients, and without any history suggestive of coronary artery disease were selected for the study from volunteers visiting the Blood Bank and the Out Patients Department (OPD) of G.B. Pant Hospital.

Blood samples: 20 mL blood from fasting subjects was collected in vials containing 3.8% trisodium citrate anticoagulant and mixed gently by inversion. The platelet count was performed immediately as previously described.¹⁶⁾ Blood samples in plain vials were also collected and centrifuged at 2500 rpm, and serum was obtained. Platelet Rich Plasma (PRP) was prepared by centrifugation of the citrated blood at 1000 rpm for 15 minutes at 37°C using polyethylene test tubes. The process for the PRP and platelets pellets preparation was completed within the first hour of the blood collection according to Fraser et al.¹⁷⁾

Biochemical assays: Using enzymatic methods, serum total cholesterol (TC), triglycerides (TG), and high-density lipoproteins (HDL) were determined by reagents purchased from E. Merck (India), using an Olympus AU400 chemistry analyzer.

Determination of platelets aggregation: Platelet aggregation was determined in the PRP by a previously described method¹⁷⁾ using Chrono Log aggregometer model 560-CA make of Chrono Log Corporation of USA.

Measurement of ionized calcium (Ca^{2+}): Ionized Calcium Ca^{2+} in plasma was determined using ICA2 ionized calcium analyzer, of Radiometer, Copenhagen, Denmark.

Measurement of nitric oxide: Nitric Oxide (NO) as nitrite/nitrate level in plasma or washed platelets suspensions determined indirectly, by the measurement of the stable by-products nitrate and nitrite, employing the Griess's reaction according to the method of Mathew et al.¹⁸⁾ Briefly, in an oxygenated solution NO decomposes to form NO_2^- and NO_3^- . These stable products can be determined using a spectrophotometer assay. Nitrite is measurable by observing the magenta-colored azo dye that is formed from NO_2^- and the Griess's reagent. This requires that NO_3^- be reduced to NO_2^- using nitrate reductase. Griess reagent is a mixture of equal volumes of 0.2% (w/v) naphthylendiamine and 0.2% (w/v) sulfanilamide prepared in 5% (v/v) phosphoric acid. The reagent was prepared freshly each time before use. Standard: 100 μmol Sodium nitrite stock solution was prepared and stored

at 4°C. Serial dilutions were carried out at the time of testing. 20–100 µmol/L of standard dilutions were prepared for testing NO₂ in plasma, and 0.5–2 µmol/L dilutions were prepared for the estimation of NO₂ in washed platelets suspensions. Plasma samples were brought to room temperature before starting the assay. Washed platelet suspensions were subjected to a series of freeze and thaw cycles and sonication to ensure complete platelet membrane disruption. Platelets suspensions and standard aliquots were incubated for 30 min at 37°C separately in tubes containing 0.2 U/mL nitrate reductase, 50 mmol HEPES buffer, 5 µM FAD, and 0.1 mmol NADPH in a total volume of 500 µL. Following incubation, 5 µL of 1 mmol potassium ferri-cyanide was added to each tube to oxidize any non-reacted NADPH, because reduced pyridine nucleotides (NADPH, NADH) strongly inhibit the Griess reaction. Samples were then incubated for an additional 10 min at 25°C. 1 mL of freshly prepared Griess reagent, then added to each tube, and the absorbance of each tube was determined at 543 nm, using a Syva S-111 spectrophotometer, from Gilford instrument Laboratories Inc., USA. Total nitrate and nitrite (µmol/L) were calculated from known standard concentrations.

Determination of nitric oxide synthase activity:

Nitric oxide synthase activity in washed platelets suspensions was determined by employing the reaction of NO with oxyhemoglobin, based on a modified method of Joan and Michael.¹⁹⁾ NO reacts with oxyhemoglobin to form methemoglobin which absorbs at 401 nm. 50 µL of washed platelet suspension, 20 µL HEPES (100 mmol/L, pH 7.5), 40 µL H₄B (90 µmol/L) prepared in 100 mmol/L HEPES (pH 7.5) and dithiothreitol (100 mmol), 40 µL DL Valine (37 mmol/L), 10 µL CaCl₂ (6 mmol/L), and 10 µL MgCl₂ (30 mmol/L) were added using a microplate. The plate was incubated at 37°C for 10 min. 10 µL FAD (1.2 mmol/L), 20 µL NADPH (1.5 mmol/L) and 50 µL L-arginine (6 mmol/L) were then added, and the plate was further incubated for 15 min at 37°C. At the end of the incubation, the initial readings at 405 nm were immediately noted, and the increase in absorbance after 1, 2, and 3 min. was recorded. NOS activity in platelets was then calculated; the methemoglobin molar absorption coefficient is 60000 M⁻¹ cm⁻¹.

Determination of cyclic GMP: cGMP was measured in washed platelets suspensions using an enzyme immunoassay (EIA) commercial kit supplied by Biomedical Technologies Inc., USA, an assay based on a previous method of Honma et al.²⁰⁾

Statistical analysis

Data were analyzed with computer software (S-plus 6 for Windows and SAS Software Release 8.2 packages). The results were expressed as mean ± SD, and the significance of the difference between the mean values of the groups were determined by the Student's t test employing the ANOVA program. Spearman's rank correlation was calculated to assess the association between the markers of NO-cGMP system and lipids or platelets function.

RESULTS

The subjects' age mean range was from 40–51 years. The study population has a small number of female enrollment, across all the groups.

There is a significant increase in serum total cholesterol and triglycerides (P <0.001), in all CAD groups and a significant decreases in HDL (P <0.01), compared to HC, the calculated LDL was significantly (P <0.001) higher among the CAD groups compared to the controls.

Platelets counts were notably higher in CAD patients with UA or SA than in patients with MI or healthy controls. The differences between the means of platelets counts (× 10⁹/L platelets) in HC Vs UA or SA are significant (P <0.01). Platelets aggregation in the CAD patients groups and healthy controls increased immensely compared to platelets aggregation in healthy controls. The differences between the means of platelets aggregation (%) in MI, UA, or SA, patients and the healthy controls are greatly significant (P <0.001). The respective means for the platelets aggregation (%) in HC, MI, UA and SA are 50.85 ± 10.76, 70.95 ± 15.85, 65.87 ± 20.16, and 73.85 ± 16.67. We also measured plasma-ionized calcium in all subjects. Abreast with the platelet aggregation; the plasma ionized Ca²⁺ levels in CAD patients and HC have demonstrated a similar trend. Plasma Ca²⁺ in CAD groups significantly increased (P <0.001) compared to HC. The means ± SD of Ca²⁺ (mmol/L) in the study population were, 1.25 ± 0.19, 1.41 ± 0.18, 1.40 ± 0.14, and 1.34 ± 0.15 for HC, MI, UA and SA respectively. The ionized Ca²⁺ levels in SA patients are slightly low compared to the remaining CAD groups; however, the differences between their means were not significant (**Fig. 1**).

Results of the various NO-cGMP system parameters studied in MI, UA, SA patients, and HC subjects are presented in **Table 1**. Plasma nitric oxide (NO) levels have increased significantly (P <0.001) in MI and SA compared to HC, also increased in MI (P <0.001) compared to the remaining CAD groups. However, the difference

Table 1 Platelets and plasma NO-cGMP variables

Groups/Variables (N = 40)	Plasma NO Mean $\pm$ SD $\mu\text{mol/L}$	Platelets NO Mean $\pm$ SD $\mu\text{mol}/10^9$ platelets	Platelets NOS Mean $\pm$ SD U/ 10^9 platelet	Platelets cGMP Mean $\pm$ SD Pmole/ 10^9 platelets
Healthy Controls	27.20 $\pm$ 8.92	1.47 $\pm$ 0.67	1.19 $\pm$ 0.56	0.95 $\pm$ 0.41
MI	56.75 $\pm$ 27.25	1.72 $\pm$ 0.60	1.21 $\pm$ 0.64	1.53 $\pm$ 0.64
Unstable Angina	31.22 $\pm$ 16.99	2.33 $\pm$ 0.83	1.64 $\pm$ 0.98	3.18 $\pm$ 0.77
Stable Angina	38.17 $\pm$ 17.69	2.25 $\pm$ 1.0	1.57 $\pm$ 0.81	5.12 $\pm$ 1.5
P	P < 0.001	P < 0.001	P < 0.005	P < 0.001

Table shows the levels of plasma NO, and platelets NO, cGMP, NO synthase activity in patients with CAD and healthy control.

Fig. 1 Platelets aggregation and plasma Ca^{2+} levels in all groups. Plasma Ca^{2+} mmol/L (left) and platelet aggregation % (right) increases in CAD compared to HC, increases in Ca^{2+} may influence the platelet aggregation

Fig. 2 NO- NOS levels in patients with stable angina. Platelet nitric oxide levels significantly correlated ($P < 0.0001$) with nitric oxide synthase activity ($r = 0.633$) in patients with stable angina.

between HC and UA is not significant. Platelets NO levels were significantly ($P < 0.001$) increased in UA and SA compared to HC or MI. The platelets NO levels, though increased in the MI group compared to HC; however, the difference was not statistically significant. Platelets NO correlated with platelets NO synthase activity ($r = 0.557$, $P < 0.001$), and cGMP ($r = 0.296$, $P < 0.05$). Platelet nitric oxide levels significantly correlated ($P < 0.0001$) with nitric oxide synthase activity ($r = 0.633$) in patients with stable angina (**Fig. 2**).

Platelets nitric oxide synthase activity levels in CAD patients and healthy controls were abreast with the NO-NO synthase activity trend. The NO synthase activity levels among the study groups were similar to that

depicted in platelets NO levels in CAD and HC groups; however, platelets NO synthase activity levels increased significantly in all CAD groups. The platelets cGMP levels in CAD patients and healthy controls as seen in **Table 1** have profoundly increased among CAD groups compared to HC ($P < 0.001$). Among the CAD groups, patients with SA have shown greater increases in platelets cGMP levels ($P < 0.001$), compared to the rest of the groups, likewise, cGMP levels in UA patients significantly ($P < 0.001$) increased compared to MI patients. Platelets cGMP levels correlated with platelets aggregation ($r = 0.461$, $P < 0.001$), and with platelets NOS activity ($r = 0.338$) in MI patients **Fig. 3**. Plasma ionized calcium (Ca^{2+}) explicitly increased ($P < 0.001$) in CAD groups compared to the healthy con-

Fig. 3 NOS- cGMP in MI patients.

Platelets cGMP significantly correlated ($P < 0.001$) with Nitric oxide synthase activity ($r = 0.338$) in MI patients.

trols.

DISCUSSION

The major findings of this study are profound increases in NO-cGMP activity accompanied with increased Ca^{2+} levels in ischemic heart diseases. There is a paucity of data regarding NOS activity and the levels of platelet NO in CAD. There is no reported study to our knowledge, that compares the platelets NOS activity and NO levels among patients of CAD. There are several reports on NO levels in preeclampsia, hypertension, atherosclerosis, and heart failure showing a large spectrum of ambiguity on the subject. Experiments conducted in hypertensive animals have shown a similar diversity of findings, with NO generation reported to be normal, decreased, or enhanced.²¹⁻²⁹⁾ Studies with reduced NO basal production in patients with essential hypertension were reported.^{30, 31)} The data were largely attributed to the reduced bio-activity of NO. Our results are in accordance with previously reported findings^{32, 33)}; however, in contradiction with the study of Minamino et al.,³⁴⁾ whose observations suggested that the bio-availability of nitric oxide may decrease in patients exposed to multiple risk factors, and, therefore, contributing to myocardial ischemia and thrombotic vascular events. The discrepancy between the results of this study and that of others may be attributed to the fact that the plasma and platelets in this study were prepared from e venous peripheral blood; however, other studies have focused either on plasma NO

levels taken from the femoral artery, spastic sites, or evaluated the basal NO release indirectly in the arterial and coronary sinus venous blood by the measurement of the blood flow velocity, oxygen saturation, and the endothelium dependent vasodilation, after infusion of NO donors and inhibitors.

Increased NO levels observed in the present study may support the hypothesis that increases in nitric oxide production may be a compensatory response to improve blood flow in the circulation, as suggested in a preeclampsia study mentioned earlier.²⁸⁾ It is also possible that the increased NO levels may have been a response to increased platelet aggregation and adhesion in the circulation. In contrast, the increase in platelets NO levels demonstrated in this study could be due to the NO diffusion from the plasma, as it is reported that the released endothelial NO enters adjacent platelets and inhibits its activation.³⁵⁾ Increased platelet NOS activity is noted in this study, and the significant correlation between platelet cGMP and platelets NO levels could provide a plausible explanation to the increased platelets NO, which may have resulted from the increased NOS activity in CAD (**Fig. 3**). In addition, the positive correlation between NOS and cGMP seen in MI patients can explain the differences between the three types of CAD episodes investigated in this study and may also indicate that the cGMP-NO system is activated at the initial stages of the disease. The turn on of the NO-NOS system during the initial stages of the disease may continue to compensate for the NO imbalance with the development of the disease as evident in the positive relationship between the NO-NOS that is seen in patients with stable angina. This may suggest an enhanced NO-cGMP system because of the increased NO basal release, in response to the elevated platelets activity and increased Ca^{2+} influx. The molecular mechanisms by which NO influences myocardial performance and platelets function are the subject of much investigation but remain largely unexplored. Much attention has been paid to cGMP pathways; the elevation of intra-platelet cGMP levels was suggested to mediate the anti-aggregating activity of exogenous and endogenous NO.³⁶⁾ In platelets, cGMP inhibits agonist-induced increases in ionized Ca^{2+} ; this event could be a result of inhibition of both Ca^{2+} influx and Ca^{2+} mobilization and/or a result of stimulation of Ca^{2+} sequestration. An elevation in plasma ionized Ca^{2+} may have contributed to the increased platelets Ca^{2+} (data not shown) as earlier suggested³⁷⁾ that the free calcium concentration in the extracellular fluid mediate and propagate the intracellular

Ca²⁺ influx. It has been hypothesized that the inhibition of Ca²⁺ influx by NO is secondary to its initial effects on sequestration of Ca²⁺ into the stores³⁸⁾; this agrees with the previous findings of Okamoto, et al.³⁹⁾ who studied the effects of NO on platelets Ca²⁺ inhibition.

The major strength of this study is the ability to control for a number of factors that influence platelets function, including antioxidants, vitamins, and other related drugs. A limitation of this study relates to its cross-sectional nature as we cannot determine whether the NO-cGMP system negatively influenced CAD or vice versa. In this analysis, we investigated the current NO-cGMP system markers in CAD, and these measurements may not be representative of the long-term NO status in platelets or endothelial cells. Longitudinal studies would better elucidate how the NO-cGMP system and CAD are related.

In conclusion, the present study demonstrated that the NO-cGMP signaling system concomitant Ca²⁺ fluxes profoundly enhanced in CAD. NO and cGMP though implicated previously in inhibiting Ca²⁺ influx, but seems to have an obscure or ancillary role to play to ameliorate the Ca²⁺ status, particularly in CAD patients. A further investigation is needed to elucidate distinctly the influence of NO-cGMP in Ca²⁺ platelet influx associated with CAD.

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