Journal of the American College of Cardiology
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Published by Elsevier Science Inc.

Vol. 37, No. 2, 2001
ISSN 0735-1097/01/$20.00
PII S0735-1097(00)01114-1

Platelet Inhibitory Effect of Nitric Oxide in the Human Coronary Circulation: Impact of Endothelial Dysfunction

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OBJECTIVES We sought to determine whether coronary vascular nitric oxide (NO) release in vivo modulates platelet activation.

BACKGROUND Nitric oxide modulates vasodilator tone and platelet activity via the cyclic guanosine monophosphate (cGMP) pathway, but whether coronary endothelial dysfunction influences platelet activation in humans is unknown.

METHODS In 26 patients, we measured coronary blood flow, epicardial diameter and coronary sinus platelet cGMP content during intracoronary infusions of acetylcholine (ACH), L-N^G monomethyl arginine (L-NMMA) and sodium nitroprusside.

RESULTS Acetylcholine increased platelet cGMP content ($p = 0.013$), but its magnitude was lower in patients with endothelial dysfunction; thus, patients with epicardial constriction with ACH had a $7 \pm 6\%$, p = ns change compared with a $32 \pm 13\%$, $p = 0.05$ increase in platelet cGMP in those with epicardial dilation. Similarly, patients with atherosclerosis or its risk factors had a smaller increase ($9 \pm 6\%$) compared with those having normal coronary arteries without risk factors ($51 \pm 22\%$, $p = 0.019$). L-N^G monomethyl arginine decreased platelet cGMP content to a greater extent in patients with epicardial dilation with ACH ($-15 \pm 7\%$, $p = 0.06$) compared to those with constriction ($+5 \pm 6\%$ change, $p = 0.5$). Sodium nitroprusside produced a similar increase in platelet cGMP content in patients with and without endothelial dysfunction ($p = 0.56$). The effects of sodium nitroprusside, but not ACH or L-NMMA, were reproduced in vitro.

CONCLUSIONS Platelet cGMP levels can be modulated by basal and stimulated release of NO. The platelet inhibitory effect of NO is reduced in patients with endothelial dysfunction, which may explain their increased risk from thrombotic events and the improved survival associated with strategies designed to improve vascular function. (J Am Coll Cardiol 2001;37:510–6) © 2001 by the American College of Cardiology

The vascular endothelium modulates smooth muscle tone by the abluminal release of endothelium-derived relaxing factors (1–5), including nitric oxide (NO) or an adduct of NO. By simultaneously diffusing into the lumen (6,7), NO potently inhibits platelet adhesion, and to a lesser extent aggregation, by increasing cytosolic levels of cyclic guanosine monophosphate (cGMP)(8–12). In whole blood, where the half-life of NO is attenuated by hemoglobin and other oxidants, the platelet inhibitory effects are less easily demonstrated and may be insignificant (13–15). Nonetheless, exogenous NO can markedly inhibit platelet adhesion, and inhibition of NO synthesis promotes platelet aggregation and cyclic flow variations (16,17).

In humans, acetylcholine (ACH) stimulates NO release from normal endothelium, but bioavailability of both basal and stimulated NO is reduced in patients with atherosclerosis or its risk factors (18–20). Although much is known about NO bioactivity in vascular smooth muscle, studies examining the in vivo effects of endothelium-derived NO on platelet function in humans are sparse, and whether any inhibitory action of NO on platelets is depressed in patients with endothelial dysfunction is unknown. Therefore, in the present study, we have investigated whether basal or pharmacologic stimulation of endothelial NO activity causes inhibition of platelet activation by increasing platelet cGMP levels, and whether atherosclerosis and its risk factors, when accompanied by abnormal dilator responses to endothelium-dependent agents, are also associated with a defect in the platelet inhibitory effects of the vessel wall. For this purpose, we studied the effects of inhibition and stimulation of coronary vascular NO activity in the human coronary circulation.

METHODS

Patients. Twenty-six patients, 14 male, average age 50 ± 2 years (range 28 to 73 years), undergoing elective cardiac catheterization for evaluation of chest pain were recruited in the study. Those with valvular heart disease or acute coronary syndromes were excluded. Twenty patients had angiographically smooth-appearing coronary arteries and six had minimal luminal irregularities (<10% stenosis). Patients with obstructive disease in the left coronary circulation were excluded because of the potential for platelet activation with increased shear (21). Eleven patients were hypertensive (arterial pressure >140/90), five were diabetic,

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Manuscript received June 24, 1999; revised manuscript received August 12, 2000, accepted October 2, 2000.

Abbreviations and Acronyms

ACH = acetylcholine
ANOVA = analysis of variance
cGMP = cyclic guanosine monophosphate
NO = nitric oxide
L-NMMA = L-N^G monomethyl arginine

mean total cholesterol was 225 ± 9 mg/dl and two had mild impairment of left ventricular function. Risk factors for atherosclerosis, defined as age >60 years, cholesterol >240 mg/dl, hypertension, diabetes and tobacco smoking within the previous two years, were present in 19 patients. Aspirin and nonsteroidal anti-inflammatory agents were withdrawn for at least seven days and cardiac medications for 48 h prior to the study. The study was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute and all patients gave written informed consent.

Protocol. A 7-F multipurpose A2 catheter (Cordis Inc., Miami, Florida) was placed in the coronary sinus for blood sampling. Patients received 10,000 IU of intravenous heparin and all drugs were administered into the left main coronary artery. A 0.018 inch Doppler flow wire was placed in the proximal left anterior descending coronary artery to measure flow velocity (Fig. 1).

Patients with coronary atherosclerosis received 2-min infusions of ACH at 3 μg/min and 30 μg/min (10^{-7} and 10^{-6} mol/l intracoronary concentration, respectively). Patients with normal coronary arteries received 30 μg/min ACH and 12 patients who did not vasoconstrict at this dose received 100 μg/min ACH (3.3×10^{-6} mol/l intracoronary concentration). After a 10-min recovery period, intracoronary sodium nitroprusside (40 μg/min) was infused for 3 min (n = 22). After recovery sampling, 64 μmol/min (4×10^{-4} mol/l) L-N^G monomethyl arginine (L-NMMA, Clinalfa, Switzerland) was infused for 5 min (n = 23). In 11 patients, the maximum dose of ACH (mean dose 2.5×10^{-6} mol/l) was reinfused. Blood samples from the coronary sinus were obtained after each infusion (Fig. 1).

Estimation of coronary blood flow and diameter. Coronary angiograms were recorded using a cine angiographic system (Toshiba, Inc., Japan) and quantitative angiography was performed with ARTEK software (Quantim 2001, Statview, Image Comm Systems, Inc., Mountain View, California). Coronary blood flow was estimated from the flow velocity and diameter measurements using the formula ($\pi \times$ average peak velocity $\times$ 0.125 $\times$ diameter^2)(18,22). Coronary vascular resistance was calculated as mean arterial pressure ÷ coronary blood flow. For calculating flow, coronary artery diameter was measured in a 0.5 cm segment of vessel beginning 0.25 cm beyond the tip of the flow wire, and a segment of vessel distal to the flow wire was analyzed in all subjects for delineating diameter changes with each infusion. Only distal diameter changes are reported as percent change compared with the baseline before drug infusion.

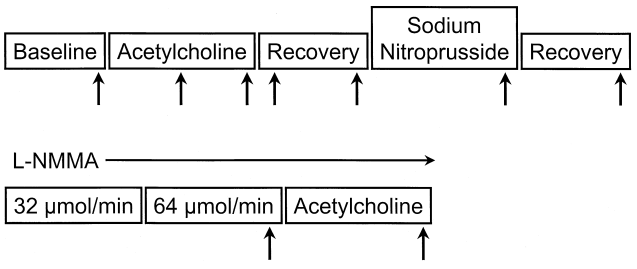

Figure 1. Protocol design. L-NMMA = L-N^G monomethyl arginine.

Measurement of platelet and plasma cGMP. Nine milliliters of blood was drawn into syringes containing 1 ml sodium citrate (14.7 mmol/l) and zaprinast (10^{-5} mol/l). Within 1 min of sampling, the blood was centrifuged at 140 *g* for 10 min. Two 1-ml aliquots of the resultant platelet-rich plasma were centrifuged at 2,500 *g* for 2 min to obtain a platelet pellet. After careful removal of all the plasma supernatant, ice-cold 6% trichloroacetic acid was added to extract the cGMP. After neutralization by solvent partition with a mixture of Freon and tri-n-octylamine, cGMP was measured by radioimmunoassay (RPA 525, Amersham International, United Kingdom). Platelet counts (Elzone particle counter, Particle Data Inc., Elmhurst, Illinois) were performed on each sample of platelet rich plasma.

Plasma cGMP levels were simultaneously measured in 11 patients. Two 100-μl aliquots of platelet-poor plasma samples were added to ice-cold 6% trichloroacetic acid; cGMP was then extracted and assayed as described in the preceding text.

Reproducibility of the coronary sinus cGMP sampling was assessed by repeat measurements in seven patients with minimal coronary atherosclerosis. Between the two measurements, the variability was 7.5 ± 5.5%, r = 0.97 (p = 0.0003), and the means were similar (0.94 ± 0.13 and 0.99 ± 0.13 pmol/10^9 plts., p = 0.8).

In vitro effects of ACH, sodium nitroprusside and L-NMMA. Blood from 10 healthy volunteers (five male) and three patients (one male) with normal coronary arteries was drawn into syringes containing Hirudin (final concentration 20 μg/ml). Within 2 min of sampling, ACH (0.01, 0.1 and 1.0 μg/ml, n = 5), sodium nitroprusside (0.125 and 0.25 μg/ml, n = 7), or L-NMMA (2×10^{-4} mol/L and 4×10^{-4} mol/L, n = 7), and a control volume of saline were added to 9-ml aliquots of blood. Following 5 min of incubation at 37°C, citrate and zaprinast (concentrations as in the preceding text) were added and the samples were processed for measurement of cGMP as in the in vivo study. In vitro concentrations were calculated to mimic intracoronary levels achieved during infusion of ACH at 3 to 100 μg/min, sodium nitroprusside at 40 μg/min, and L-NMMA at 32 and 64 μmol/min.

Statistical analysis. Data are expressed as a mean ± SEM. Difference between means were compared by the Student *t*

test or the one-way analysis of variance (ANOVA) (23) for repeated measures using SAS software (Version 6.12; SAS Institute, Cary, North Carolina). If the F value was significant, a Bonferroni multiple-comparison test was performed. A p value of ≤ 0.05 was considered statistically significant. To identify the most parsimonious multivariate model for predicting change in platelet cGMP level with ACH, we used the SAS Stepwise Procedure (stepwise technique) on the regressor's age, gender, the presence of hypertension, diabetes, cigarette use or cholesterol level; or on the diameter and flow changes with ACH (30 μg/min) or the diameter or flow changes with L-NMMA (24). The significance level for covariate entry and staying in the model was 0.15.

RESULTS

Effect of ACH on the coronary vasculature and platelet cGMP levels. There was progressive vasodilation with ACH; coronary vascular resistance was 41 ± 8% lower ($p < 0.001$) with 10^{-6} mol/l ACH. Coronary diameter changes were heterogeneous (mean 1 ± 3% change). Acetylcholine evoked an increase in platelet cGMP content ($p = 0.013$, ANOVA) with a 21 ± 8% increase with 10^{-6} mol/l ACH (Fig. 2). Plasma cGMP levels during ACH were slightly higher compared to baseline (3.9 to 4.6 nmol/l, $p = 0.04$). However, there was no correlation between changes in platelet and plasma cGMP levels ($r = 0.3$, $p = 0.1$). Platelet cGMP levels were adjusted for plasma cGMP concentrations assuming contamination of the platelet pellet samples with 10 μL of plasma. The increase in platelet cGMP with intracoronary ACH was unaffected by this correction (uncorrected 22 ± 11%, corrected 23 ± 10% increase).

To investigate the relationship between the vascular and platelet responses, we divided patients into those with and without endothelial dysfunction based on 1) epicardial coronary artery response to ACH and 2) presence or absence of atherosclerosis or its risk factors.

Eleven subjects had constriction in the distal epicardial diameter (mean −11 ± 3%) in response to 10^{-6} mol/l ACH (endothelial dysfunction) and 15 subjects vasodilated (mean 10 ± 2%) (normal endothelial function, Fig. 3). Patients who constricted with ACH had no significant change in coronary sinus platelet cGMP content (7 ± 6%, p = ns), whereas those with epicardial vasodilation with ACH had a significant increase (32 ± 13%, $p = 0.05$ by ANOVA, Fig. 3). Microvascular vasodilation in response to ACH was also reduced in patients with epicardial constriction: coronary blood flow in response to 10^{-6} mol/l ACH increased by 166 ± 25% in those with epicardial dilation compared with a 52 ± 20% ($p = 0.04$) increase in those with epicardial constriction.

Impact of atherosclerosis and its risk factors on platelet function. For this analysis, patients with coronary atherosclerosis and those with risk factors for atherosclerosis but normal coronary arteries were combined (atherosclerosis group, n = 19) and compared to those with normal coronary arteries without risk factors for atherosclerosis (normal group, n = 7). Compared to the atherosclerosis group, the normal group had greater microvascular ($p = 0.017$) and epicardial dilation ($p = 0.047$) with 10^{-6} mol/l ACH (Fig. 4). The increase in platelet cGMP level in the normal group (51 ± 22%) was significantly greater than the change in the atherosclerosis group of patients (9 ± 6%, $p = 0.019$ between groups) (Fig. 4).

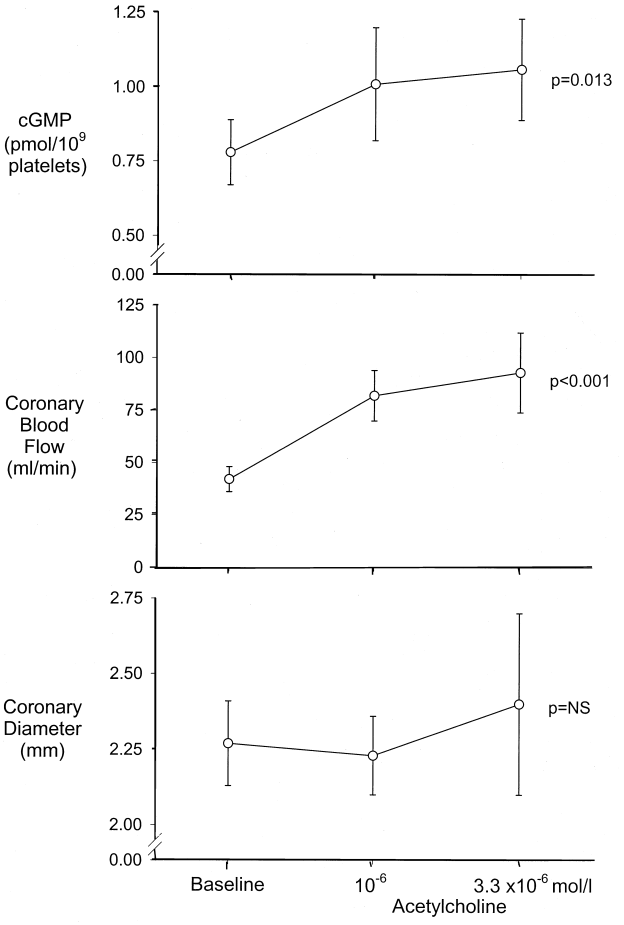

Figure 2. Changes in coronary sinus platelet cGMP content, coronary blood flow and epicardial diameter during intracoronary infusion of acetylcholine (n = 26). All tests use one-way analysis of variance. cGMP = cyclic guanosine monophosphate.

Multivariate regression analysis investigated whether the change in platelet cGMP content with ACH was related to the presence of risk factors (age, presence of hypertension, diabetes, smoking history, atherosclerosis, cholesterol or HDL levels), or the diameter or coronary vascular resistance responses to 10^{-6} mol/l ACH. The change in cGMP content with ACH was independently related to the change in epicardial diameter with ACH (a measure of endothelial dysfunction, $r^2 = 0.31$, $p = 0.009$).

Effect of sodium nitroprusside. Intracoronary sodium nitroprusside evoked significant epicardial and microvascular dilation; coronary vascular resistance decreased by 58 ± 5% and epicardial arteries dilated by 10 ± 6% (both $p < 0.001$).

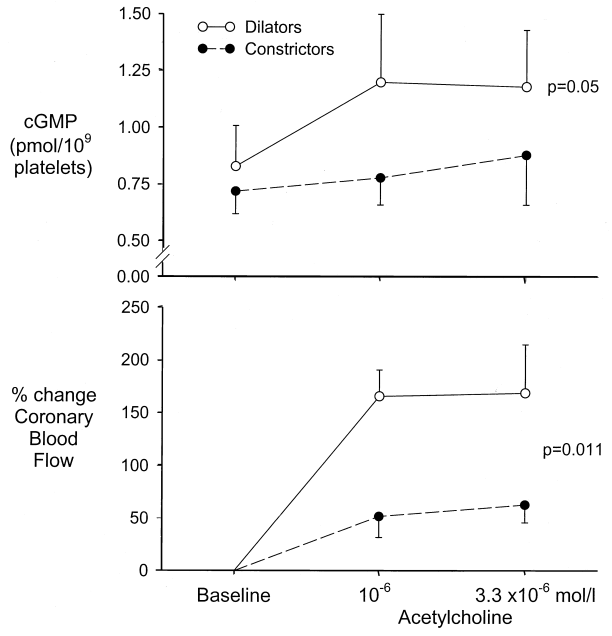

Figure 3. Acetylcholine-mediated changes in coronary sinus platelet cGMP content and coronary blood flow in patients with (n = 11) **(dashed lines)** and those without (n = 15) **(solid lines)** epicardial coronary constriction with 10^{-6}mol/l acetylcholine. Baseline coronary blood flow (40.7 ± 9 vs. 43.6 ± 8 ml/min, p = 0.8) and platelet cGMP content (0.72 ± 0.1 vs. 0.83 ± 0.2 pmol/10^9 platelets, p = 0.6) were not significantly different between patients with epicardial constriction compared to those with dilation in response to acetylcholine. Differences between groups by analysis of variance. cGMP = cyclic guanosine monophosphate.

Platelet cGMP content in coronary sinus blood increased by 247 ± 38% from 0.78 ± 1.12 to 2.4 ± 0.4 pmol/10^9 platelets (p < 0.0001), with similar increases in normal controls and the group with atherosclerosis, or in patients with epicardial constriction (endothelial dysfunction) with ACH (214 ± 68%) and those with epicardial dilation (264 ± 47%, p = 0.56) (Fig. 4). There was no change in plasma cGMP with sodium nitroprusside (4.4 ± 1.3 nmol/l with and 4.8 ± 0.8 nmol/l before, p = 0.8).

Effect of L-NMMA. Intracoronary L-NMMA produced coronary epicardial and microvascular constriction; coronary vascular resistance was 12 ± 6% (p = 0.016) higher and epicardial diameter −6 ± 2% narrower (p = 0.018). Compared with baseline, platelet cGMP content was lower after L-NMMA; 0.92 ± 1.5 to 0.75 ± 1 pmol/10^9 platelets, p = 0.05. Patients with constriction of epicardial coronary arteries with ACH (endothelial dysfunction) had no significant change in cGMP with L-NMMA (5 ± 6% change compared with baseline, p = 0.5), whereas those who dilated with ACH (normal endothelium) had a trend toward reduction in cGMP with L-NMMA (−15 ± 7% reduction compared with baseline, p = 0.06).

Patients in the normal group tended to have greater epicardial and microvascular constriction with L-NMMA compared with those in the atherosclerosis group; coronary blood flow was −14 ± 5% lower compared with +7 ± 6% change respectively, p = 0.07, and coronary diameter constricted by −13 ± 3% versus −4 ± 2% respectively, p = 0.04. Similarly, coronary sinus platelet cGMP content with L-NMMA was 22 ± 10% lower compared with baseline in the normal group, but no change (−1 ± 6%) was seen in the atherosclerosis group (p = 0.08 between groups).

There was a negative correlation between the magnitude of change in platelet cGMP level with ACH and L-NMMA (R = −0.66, p = 0.002), indicating that patients with greater increase in cGMP with ACH had greater decrease with L-NMMA and vice versa. Finally, there was no change in plasma cGMP with L-NMMA (4.8 ± 0.8 before vs. 4.9 ±0.7 nmol/l after, p = 0.8).

Effect of L-NMMA on the response to ACH. As expected, L-NMMA inhibited the vascular response to ACH; coronary vascular resistance was 1.18 ± 0.2 mm Hg.min.ml^{-1} with ACH before and 2.3 ± 0.6 mm Hg.min.ml^{-1}, p = 0.05,

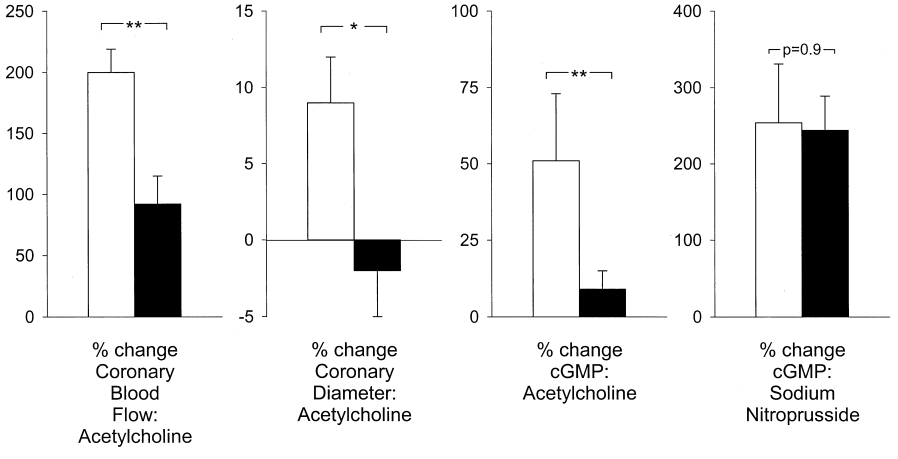

Figure 4. Changes in coronary sinus platelet cGMP content, coronary blood flow and epicardial diameter during intracoronary acetylcholine (10^{-6} mol/l) and sodium nitroprusside (40 μg/min) infusions in patients with (n = 19) **(shaded bars)** and those without (n = 7) **(open bars)** atherosclerosis or its risk factors. Baseline coronary blood flow (30.7 ± 5 vs. 43.6 ± 7 ml/min), coronary diameter (2.3 ± 0.6 vs 2.2 ± 0.6 mm) and platelet cGMP content (0.76 ± 0.2 vs. 0.79 ± 0.1 pmol/10^9 platelets) were not significantly different between patients with and those without atherosclerosis or its risk factors, p > 0.2. **Open bars** = normal; **solid bars** = atherosclerosis or risk factors. *p < 0.05, **p < 0.02. cGMP = cyclic guanosine monophosphate.

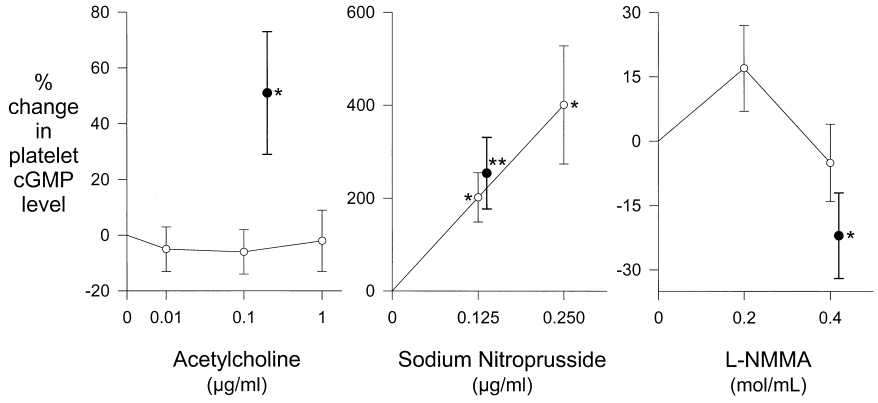

Figure 5. Percent change in platelet cGMP content during in vitro incubation with acetylcholine, sodium nitroprusside and L-NMMA is shown in open circles. For comparison, the effect of these agonists at estimated intracoronary concentrations achieved during in vivo administration in the normal subjects are shown in closed circles. *$p \leq 0.05$, **$p < 0.001$ compared to baseline. cGMP = cyclic guanosine monophosphate; L-NMMA = L-N^G monomethyl arginine.

after L-NMMA. The 12 ± 8% increase in platelet cGMP with ACH in the control study tended to be lower after L-NMMA (−8 ± 8%, $p = 0.08$) in the group as a whole. However, the seven patients in the normal group who had an increase in cGMP with ACH in the control study had no change after L-NMMA (26 ± 8% increase vs. −6 ± 12% change, $p = 0.05$ respectively), indicating that the increase in platelet cGMP content with ACH was due to stimulation of NO by ACH.

In vitro effects of ACH, L-NMMA and sodium nitroprusside. To determine whether the observed effects on cGMP levels were due to a direct intraluminal action of these vasoactive agents on platelets, or indirectly via their action on the vascular endothelium, we performed in vitro experiments where whole blood was incubated with ACH, L-NMMA, and sodium nitroprusside in concentrations achieved in vivo during intracoronary delivery. There was no effect of ACH or L-NMMA on platelet cGMP content at concentrations achieved in vivo (Fig. 5). Incubation of whole blood with sodium nitroprusside produced a 202 ± 53% ($p = 0.013$) increase in platelet cGMP level, a change that is comparable to the 254 ± 77% increase observed during intracoronary administration (Fig. 5).

DISCUSSION

Our study demonstrates that: 1) stimulation of endothelium-derived NO release by ACH into the vascular lumen increases platelet cGMP content, 2) luminal NO availability is reduced in patients with coronary vascular endothelial dysfunction (those with risk factors for atherosclerosis and angiographic atherosclerosis, or those with epicardial constriction with intracoronary ACH), and 3) inhibition of NO synthase by L-NMMA decreases platelet cGMP content, a change that was not observed in patients with endothelial dysfunction. Thus, the human coronary endothelium modulates platelet cGMP levels and hence platelet passivation in vivo, both at rest and during pharmacologic stimulation. Because NO appears to be the only agent capable of enhancing platelet cGMP activity (6) and the effects of ACH were inhibited by L-NMMA, it is likely that the observed action of ACH was due to stimulation of NO activity. Finally, the actions of ACH and L-NMMA in vivo were primarily via their effect on the endothelium and could not be explained by a direct action of these agents on platelets in in vitro studies.

The significant correlation between vasomotor and platelet cGMP responses highlights the close relationship between the effects of the vascular endothelium on smooth muscle tone and platelet function. Thus, endothelial dysfunction not only impairs vasodilation by the deficient abluminal activity of NO, but also depresses the antithrombotic properties of the vascular endothelium.

Effect of ACH on platelets. Acetylcholine, by promoting NO release, causes smooth muscle relaxation by increasing soluble cGMP levels (1,6–15,25) a mechanism that is also responsible for inhibition of platelet aggregation and adhesion (8–12,26). Intravenous carbachol inhibits platelet aggregation and increases platelet cGMP content (14) and adducts of NO inhibit platelet aggregation in vivo (27,28). Despite these apparently consistent results in experimental models, it is not known whether endogenous NO has a significant in vivo effect on platelets in humans, especially because the half-life of NO is dramatically shortened in whole blood by hemoglobin and other oxidants (29–32). We previously demonstrated inhibition of ex vivo platelet aggregation with ACH infusion (33), and together with the current findings provide convincing evidence that endothelium-derived NO can inhibit platelet activation in vivo.

Ex vivo measurement of cGMP may underestimate the effect on guanylate cyclase, either because of decay of cGMP (34,35) or dilution from the nonocclusive sampling technique used. Moreover, the platelet-inhibitory effect of NO may not be entirely mediated by cGMP because NO may

form an adduct with hemoglobin (S-nitrosohemoglobin) that releases NO at low oxygen tensions and therefore have platelet inhibitory effects independent of platelet cGMP (36,37).

Endothelial dysfunction and platelet activation. Coronary endothelial dysfunction is characterized by epicardial constriction and reduced microvascular vasodilation in response to ACH (18–20,38–40). In this study, patients with endothelial dysfunction had an insignificant effect of ACH and L-NMMA on platelet cGMP levels compared with those having normal endothelial function in whom an increase in platelet cGMP level was elicited with ACH and a reduction observed during blockade of NO synthesis. However, platelets from both groups of patients were capable of increasing cGMP levels in response to the NO donor, sodium nitroprusside, indicating that the differences observed with ACH and L-NMMA were not due to an intrinsic abnormality of platelet function.

Comparison of platelet responses with ACH and sodium nitroprusside. Compared to ACH, sodium nitroprusside produced greater increases in platelet cGMP level for similar increases in coronary blood flow. This may be due to continued action of sodium nitroprusside during the extraction process because sodium nitroprusside, unlike ACH, directly increased platelet cGMP levels in vitro (41–47). Alternatively, sodium nitroprusside is capable of acting on all platelets in the lumen, whereas NO released from the endothelium in response to ACH is likely to be available only to a minority of platelets adjacent to the vessel wall before being metabolized rapidly in blood. Finally, unlike sodium nitroprusside, ACH causes vasodilation by release of other NO-independent dilators (4).

Limitations. Because the effects of NO on platelets are transient and likely to involve platelets close to the vessel wall, we may have underestimated of the action of ACH and L-NMMA on platelets (34,35). Cyclic guanosine monophosphate may also be stimulated by heme oxygenase-dependent carbon monoxide production, a pathway that can also be stimulated with ACH and that requires further study (48,49).

Conclusions. Basal and stimulated release of endogenous NO from the human coronary endothelium modulates platelet activity by a cGMP-mediated mechanism. This platelet-inhibitory effect is lacking in patients with risk factors for atherosclerosis or those with established atherosclerosis who have abnormal endothelial function, which may explain the increased risk of thrombosis in these patients (50,51). Because adhesion of platelets to the vessel wall with subsequent release of growth factors has been hypothesized as an important mechanism underlying progression of atherosclerosis (52), our observations regarding the potential role of endothelium-platelet interactions in this phenomenon are important and require further study.

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