Nitroglycerin Rebound Associated With Vascular, Rather than Platelet, Hypersensitivity

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OBJECTIVES The purpose of this study was to determine whether acute withdrawal of nitroglycerin (NTG) during hemodynamic tolerance is associated with platelet hypersensitivity.

BACKGROUND Nitroglycerin is an effective antianginal medication but its use is limited by the development of tolerance and rebound. We have previously demonstrated a sustained inhibition of platelet function during continued use of NTG, but whether cessation of NTG is associated with an increase in platelet function that may contribute to rebound is unknown.

METHODS Normal porcine aortic media were exposed to flowing arterial blood from pigs (n = 8) treated continuously with NTG patches (Nitrodur 0.8 mg/h) for 48 h. Platelet function, blood pressure and the responses to angiotensin II infusion were evaluated before, during and after NTG treatment.

RESULTS Mean arterial pressure fell by 15% after 3 h of treatment compared with control, returned to baseline by 48 h and increased significantly 2 h after drug removal. Autologous 51Cr-labelled platelet deposition on the aortic media was reduced by 30% after 48 h of continuous NTG administration compared with baseline (p = 0.02) and remained decreased 2 h after cessation of NTG therapy. Platelet aggregation to thrombin decreased in parallel to the decrease in platelet deposition. Blood pressure increase after intravenous injection of 10 μg of angiotensin II was blunted during treatment with NTG but increased significantly 2 h after cessation of nitrate therapy when compared with baseline.

CONCLUSIONS Supersensitivity of the vessel wall to vasoconstrictors such as angiotensin II, but not platelet hyperactivity, may contribute to the rebound phenomenon after acute nitrate withdrawal. (J Am Coll Cardiol 2000;36:2311–6) © 2000 by the American College of Cardiology

Nitroglycerin (NTG) is an effective antianginal medication, but its clinical use is limited by the development of tolerance (1–5) and the rebound phenomenon when the medication is stopped (6–8). Angiographic studies have suggested that the rebound phenomenon after NTG withdrawal may be the result of coronary vasoconstriction and may occur even in the absence of coronary disease (9). Death has also been reported when NTG is removed abruptly in humans (10). Activation of neurohormonal feedback mechanisms during NTG use has been implicated both in the development of tolerance and in the rebound phenomenon. Plasma neurohormones such as renin and aldosterone levels, plasma vasopressin and catecholamines (11–13) are increased during administration of NTG, and abrupt withdrawal of NTG may lead to an unopposed vasoconstrictive effect mediated by these counterregulatory neurohormones. Although neurohormonal activation has been proposed to be the common and major mechanism implicated in nitrate tolerance and the rebound phenomenon, others have also shown increased superoxide anion production (14) that may inactivate nitric oxide.

In addition to its vasodilating effects, NTG has also been shown to decrease platelet function in vitro and in vivo (15–22) through the activation of the platelet guanylate cyclase enzyme thereby increasing intraplatelet cyclic guanosine monophosphate (cGMP) levels. The mechanism leading to increased cGMP intracellularly is similar to what is observed in vascular smooth muscle cells (17,21,22). However, there appears to be differences between platelet and vascular smooth muscle cell responses when NTG is given chronically. We have previously shown that there is a persistent inhibition of platelet function when NTG is given continuously, despite the development of vascular and hemodynamic tolerance (23). Thus, in contrast to acute responses, chronic responses of platelets and the vasculature to NTG therapy may differ and may account for some of the clinical responses observed during nitrate therapy or its cessation. Withdrawal of heparin in the acute coronary syndromes has been associated with a rebound increase in ischemic events that may be prevented by the use of aspirin (24), suggesting a role for platelet activation in this condition. Although a rebound phenomenon has also been described after withdrawal of nitrate therapy in the acute coronary syndromes (8), it has not been demonstrated whether platelet function may be increased after nitrate withdrawal. Platelet activation may, thus, be an attractive alternative or contributory factor accounting for the rebound phenomenon after cessation of nitrate therapy. In this study we report the effect of chronic nitrate therapy and its withdrawal on platelet function and on hemodynamic tolerance. We also examined the effect of angiotensin II on platelet function and hemodynamic parameters during continuous NTG therapy and its abrupt withdrawal.
Aortic media. The superfusion experiment was performed 2 h after the platelets were then injected intravenously into the pig, and media that was cut as 15 mm sections and placed in a Badimon superfusion flow chamber, where the media was calculated from these values as described previously (23).

Radioactive labeling of platelets. Just before the superfusion experiment, 96 ml of blood were collected from the pig by venipuncture and anticoagulated with citrate dextrose solution. After differential centrifugation, the platelet suspension was incubated with 400 uCi of $^{51}$Cr sodium chromate (Merck Frosst) for 40 min. The suspension was then centrifuged to remove unbound $^{51}$Cr. The labeled platelets were then injected intravenously into the pig, and the superfusion experiment was performed 2 h after the injection.

Aortic media. Normal porcine aortas were dissected free of surrounding connective tissues and stored at 4°C in Hank's-HEPES buffer, at pH 7.4, containing 440,000 IU/L penicillin (Ayerst, Roga/Stb Inc.) and 125 mg of azaperone (Stresnil, Janssen Pharmaceutical) and maintained in anesthesia by mechanical ventilation with 0.5% halothane (Fluothane, Ayerst) in room air. To quantify the interaction of platelets in flowing blood with the vascular wall in superfusion experiments, platelets were radiolabeled with $^{51}$chromium (Cr).

Experimental procedure. Experiments were performed using the Badimon plexiglass superfusion flow chamber that mimics the tubelike shape of the vascular system. It consists of an upper lid and a lower block, the lower block bearing a small cylindrical hole of 1-mm diameter to allow the flow of blood. When the upper lid of this cylindrical tube is removed, it uncovers a window that permits direct exposure of flowing blood to a piece of exposed arterial media, which is held in place by the pressure of the upper lid. The lid overlying the arterial media and the lower block were secured together by a surrounding chamber holder (23,25). Femoral arterial blood was drawn into the superfusion flow chambers to interact with aortic media by means of a peristaltic pump placed distal to the flow chambers. A 3-min superfusion was performed at a constant blood flow for a calculated shear rate of 3,397 s$^{-1}$ at 37°C (23,25). This high shear rate flow corresponds to arterial flow, mimicking what can be observed in a stenosed coronary vessel. After the superfusion the aortic media was removed from the chamber, and the amount of radioactively labeled platelets in the platelet thrombus formed on the media was measured in a gamma counter. The superfusion experiment was done at baseline and was repeated after the animal has been treated continuously for 48 h with transdermal NTG patches (Nitrofur, Key Pharmaceuticals, Schering), 0.8 mg/h and again 2 h after withdrawal of NTG therapy. Mean arterial blood pressure was monitored during each superfusion experiment. Eight pigs were studied, and the NTG patches were placed on the neck area and were changed every 24 h. Nitroglycerin therapy was withdrawn after 48 h of treatment by removing the patches.

Quantitation of platelet thrombus formation. At the end of each superfusion experiment, the amount of platelet thrombus formed or deposited onto the aortic media segments was quantified by assessing the amount of $^{51}$Cr-labeled platelets ($\times10^6/cm^2$) interacting with the media. This was obtained by measuring the radioactivity in counts per minute in each segment of aortic media, using a gamma counter (Minaxi 5000, Packard Instruments). The radioactivity per milliliter of blood was also determined at the time of the experiment as well as the whole blood platelet count (Coulter counter), and the number of platelets deposited on the media was calculated from these values as described previously (23).

Platelet aggregation studies. Platelet aggregation was tested at 37°C on whole blood diluted 1:1 in physiologic saline at baseline, 48 h of NTG treatment and 2 h after its withdrawal, using an impedance aggregometer (Chronolog Corp., Pennsylvania) (23). The tests were performed in close proximity to the animal and within the first minute after blood sampling. The tests were initiated by adding a concentration of 0.075 U/ml of thrombin (Hoechst Behring). Platelet aggregation was quantified as the maximum amplitude (ohms) obtained 3 min after the addition of thrombin, and results were obtained electronically by use of the AGGRO/LINK interface software (Chronolog Corp.).

Mean arterial pressure response towards angiotensin II. Additional experiments ($n=6$) were performed to determine whether continuous nitrate administration may influence the hemodynamic effect after angiotensin II bolus infusion. A 7F introducer was placed in the femoral vein to administer a bolus dose of 10 µg of angiotensin II intravenously, while mean arterial pressure was recorded. This was done at baseline, before transdermal NTG treatment, and the measurements repeated at 3 and 48 h after continuous NTG treatment and 2 h after NTG withdrawal. Increase in mean arterial pressure after angiotensin II infusion was expressed as a percentage of baseline values. Whole blood
Platelet aggregation studies were also performed before and after angiotensin II infusion to document its effect on platelet function.

Statistics. All values are expressed as means ± SEM. Repeated analyses of variances were used to compare arterial pressure, platelet aggregation or platelet thrombus formation data gathered within each animal data at each time point; if significant, intergroup analysis at each time point was performed with Fisher multiple comparison test; p values of < 0.05 were considered significant.

RESULTS

Mean arterial pressure. Mean arterial pressure decreased by 15% 3 h after continuous NTG treatment (Fig. 1) from a baseline value of 58.7 ± 4.4 mm Hg to 50.0 ± 4.0 mm Hg, p < 0.05. At 48 h after continuous NTG treatment, mean arterial pressure returned to 58.0 ± 3.1 mm Hg, p = NS versus baseline value. However, 2 h after NTG withdrawal, mean arterial pressure significantly increased to 68.4 ± 3.7 mm Hg when compared with baseline, p < 0.05.

Platelet thrombus formation. Platelet thrombus formation or deposition (×10^6/cm²) on aortic media under basal conditions was 76.4 ± 7.1 as shown in Figure 2. Continuous NTG treatment for 48 h reduced platelet deposition by 37%, to 48.3 ± 7.0, p < 0.05. Two hours after NTG withdrawal, platelet deposition remained decreased at 45.3 ± 8.7, p < 0.05.

Whole blood platelet aggregation. We observed a significant 30% decrease in platelet aggregation when pigs were continuously treated with NTG for 48 h as shown in Figure 3. Platelet aggregation remained significantly decreased 2 h after cessation of NTG treatment compared with its baseline value.

Angiotensin II effects on arterial pressure and platelets. The effect of a bolus administration of angiotensin II on arterial pressure during and off NTG treatment is shown in Figure 4. It demonstrates a significant 13.3 ± 2.4% increase in blood pressure after angiotensin II infusion in animals in the basal state (time 0) before NTG treatment. However, the ability of the same intravenous infusion of angiotensin II to vasoconstrict and increase blood pressure diminished at 3 h, although not significantly, versus baseline (11.5 ± 2.3% increase) but returned to baseline values with a 15.3 ± 2.8% increase after 48 h of continuous NTG therapy. There was a supersensitive response 2 h after NTG withdrawal, with a 20.5 ± 2.4% increase in blood pressure, p < 0.05. Despite these hemodynamic changes, angiotensin II infusion had no significant effect on platelet aggregation.

DISCUSSION

Organic nitrates are widely used in the treatment of stable and unstable coronary artery disease. In addition to its vasodilator effects its beneficial effects in the setting of the...
Mechanisms of rebound. The mechanisms of the rebound phenomenon are unclear. Angiographic studies have suggested that the rebound phenomenon after NTG withdrawal may be the result of coronary vasoconstriction and may occur even in the absence of coronary disease (9). Death has also been reported when NTG is removed abruptly in humans (10). Rabbits rendered tolerant to NTG manifested myocardial ischemia and arrhythmia when NTG was subsequently withdrawn (26). The mechanism of vasoconstriction has been linked to the neurohormonal activation that occurs during chronic nitrate therapy and the development of hemodynamic tolerance (11–13). Although there is a suggestion that catecholamines may be implicated in the rebound phenomenon, because patients maintained on beta-adrenergic blocking agents while using NTG do not show a rebound phenomenon (27), a recent article has demonstrated that intermittent treatment does not improve ischemia, and rebound could occur even during beta-blocking therapy (28).

An increase in plasma renin activity has been more consistently observed during the first two days of continuous NTG therapy, suggesting activation of the renin-angiotensin system (11–13). This activation was somewhat less with intermittent therapy than it was with continuous therapy (29). Importantly, many investigators have demonstrated that treatment with angiotensin-converting enzyme (ACE) inhibitors can prevent the development of tolerance to continuous NTG therapy (30–32). However, negative results have also been reported with ACE inhibitors (33–35). Münzel and Bassen (36) have suggested that discrepancies between these studies may be related to drug dosage since a favorable effect was seen when higher doses of ACE inhibitors were used. A high dose of enalapril retarded the development of tolerance and prevented the rebound phenomenon. In addition, there is an increased sensitivity to angiotensin II infusion during chronic NTG administration that can be prevented by concomitant therapy with captopril (37), and this appears to be related to an increased expression of endothelin-1 within the vascular media (38).

Onset of rebound. The rebound phenomenon has been described to occur at an average time of 128 min after the removal of NTG patches (39). Significant, although modest, rebound in blood pressure has also been observed 1 h after stopping NTG infusion (40). Our study, showing a rebound effect at 2 h, is consistent with the above findings. While showing a rebound hemodynamic response at this time, we were unable to show a concomitant platelet hyperresponsiveness, either in the platelet aggregation studies or in platelet thrombus formation on an injured arterial wall media. The persistent platelet inhibitory effect observed is likely related to the active dinitrate metabolites of NTG, which have a much longer half-life than NTG itself (1). If activation of vasoconstrictor neurohormones may explain the hemodynamic rebound response, however, vasoconstrictors such as adrenaline or endothelin have not been shown to significantly influence platelet aggregation (41,42). In

Acute coronary syndromes may also be related, in part, to its platelet inhibitory or antithrombotic properties. Acute withdrawal of antithrombotic therapy, such as heparin, in the unstable or acute coronary syndromes has been associated with a rebound increase in recurrent ischemic events that may be mediated by platelet activation as it is prevented by aspirin use (24). Withdrawal of NTG treatment has also been associated with an increase in ischemia and anginal symptoms (8–10). Although platelet activation could have been one of the underlying mechanisms explaining nitrate rebound, our results suggest that the nitrate rebound phenomenon is more likely related to a hypersensitivity of the vessel wall towards unopposed vasoconstrictors, such as angiotensin II, than to a hypersensitivity of platelets or to enhanced platelet thrombosis. Thus, the increase in angina and ischemia after heparin and NTG withdrawal in the acute coronary syndromes may involve different mechanisms. Platelet hyperactivity was not observed at a time when hemodynamic rebound was clearly demonstrated after NTG cessation in this study.

Acute studies have documented the vasodilating and antiplatelet properties of NTG even at clinically relevant doses (16–19). These acute effects on vascular smooth muscle cells and on platelets seem to be mediated by the same mechanism, involving the biotransformation of NTG to nitric oxide, which activates the guanylate cyclase enzyme (17,22) producing cGMP. However, their chronic responses to nitrate therapy show important differences. We have previously shown that platelets remain inhibited during continuous NTG treatment, even when hemodynamic tolerance has occurred (23). The current results also show a differential response of platelets and the vasculature during withdrawal of NTG therapy. At a time when hemodynamic rebound has occurred after nitrate withdrawal and there was a supersensitive response of the vessel wall to vasoconstrictors, platelet hyperactivity was not observed. This lack of hypersensitivity of platelets suggests that the mechanisms involved may be different. The differential hemodynamic and platelet responses to angiotensin II infusion also support this.
this study angiotensin II infusion also has no effect on platelet aggregation.

**Clinical relevance.** These findings may be clinically relevant. They suggest that hemodynamic tolerance may be related to the effects of counterregulatory hormones activated during NTG therapy. When NTG treatment is withdrawn abruptly, there may be an unopposed effect of these vasoconstrictive agents that have been activated, thereby inducing vasoconstriction that may contribute to the rebound phenomenon. Our data are in support of these observations and suggest that rebound may not be related to enhanced platelet activity. On the contrary, platelets remain inhibited during periods of increased vasoconstrictor activity. Therefore, it is tempting to speculate that rebound ischemia after NTG withdrawal in the acute coronary syndromes may be related more to a vascular effect than to an enhanced platelet activity. As such, perhaps vasodilator therapy may have a greater impact on the treatment of NTG rebound than antithrombotic therapy although this remains to be shown.

**Conclusions.** Thus, this study shows that abrupt withdrawal of NTG therapy after 48 h of continuous use was not associated with a rebound increase in platelet reactivity, even though hemodynamic rebound had occurred, and is associated with a supersensitivity of the vasculature to vasoconstrictors. This supersensitivity may favor or facilitate the development of vasoconstriction to circulating vasoconstrictors and thereby contribute to the rebound phenomenon. Activation of neurohormones during continuous NTG therapy, while beneficial in counteracting the lowering of blood pressure induced by NTG, may have an unopposed vasoconstrictive effect that may lead to adverse consequences, once NTG therapy is withdrawn.

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