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Sodium nitroprusside modulates the fibrinolytic system in the rabbit

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1 We have investigated the effect of sodium nitroprusside (NP) and glyceryl trinitrate (GTN) on fibrinolysis in anaesthetized rabbits *ex vivo* and *in vitro* by measurement of euglobulin clot lysis time (ECLT), plasma levels of tissue plasminogen activator (t-PA) activity, plasma t-PA antigen levels and plasminogen activator inhibitor (PAI-1) activity.

2 *In vivo*, NP ($30\,\mu g\,kg^{-1}$), GTN ($30\,\mu g\,kg^{-1}$) and prostacyclin ($3\,\mu g\,kg^{-1}$) caused similar transient decreases in left ventricular systolic pressure. However, while prostacyclin induced near-maximal inhibition of *ex vivo* platelet aggregation, NP or GTN had no effect.

3 *Ex vivo*, NP caused a significant decrease in ECLT and an increase in plasma t-PA activity.

4 Intravenous co-administration of t-PA ($30\,\mu g\,kg^{-1}$) with NP caused substantial prolongation of plasma t-PA activity, without affecting t-PA antigen levels.

5 In whole blood *in vitro*, NP ($30\,\mu g\,kg^{-1}$) prevented the time-dependent increase in PAI-1 activity and inhibited inactivation of added t-PA ($10\,ng\,ml^{-1}$).

6 We propose that NP exhibited fibrinolytic activity through increased t-PA activity as a result of inhibition of PAI-1 release from platelets. These results could have important therapeutic consequences when t-PA and nitrate treatments are combined.

Introduction

Fibrinolysis is a process by which activated plasmin proteolytically degrades fibrin. Conversion of plasminogen to plasmin is stimulated by a variety of plasminogen activators (PAs), predominantly tissue-PA (t-PA). However, t-PA can be rapidly degraded by the liver, and inactivated upon binding to plasminogen activator inhibitors (PAIs) in the plasma. Therefore, a major control over the release of plasmin and fibrinolysis lies both with t-PA and PAIs.

Platelets, in addition to their procoagulant role, have an inhibitory effect on fibrinolysis, which is mainly due to the release of PAI-1 (Erickson *et al.*, 1984). Prostacyclin (PGI_2) and many of the nitrovasodilators, including sodium nitroprusside (NP) and endothelium-derived relaxing factor (EDRF)/nitric oxide (NO) inhibit platelet aggregation and adhesion (Radomski *et al.*, 1987a,b). Prostacyclin exhibits its anti-platelet activity by increasing platelet cyclic AMP (Tateson *et al.*, 1977; Gorman *et al.*, 1977) while the nitrovasodilators (NO releasing agents) inhibit platelet function by an increase in cyclic GMP (Mellion *et al.*, 1980).

Recently we have found that co-administration of NP with t-PA *in vivo* resulted in significant prolongation of plasma t-PA activity (Korbut *et al.*, 1990). In the present study we further investigate the mechanism of fibrinolytic activity induced by NP or GTN in anaesthetized rabbits.

Methods

Surgical procedure

The studies were performed on male rabbits (New Zealand White), weighing 2.1–3.2 kg, receiving a standard diet and water *ad libitum*. General anaesthesia was induced with sodium pentobarbitone ($20–30\,mg\,kg^{-1}$; Sagatal, May & Baker) administered via the left marginal ear vein and maintained with supplementary doses of anaesthetic as required. In addition, lignocaine (Xylocaine 2%) was administered for local anaesthesia. Body temperature was maintained at 37°C by means of a homeothermic blanket (BioScience, Sheerness, Kent). The trachea was cannulated and the rabbit ventilated with air at a rate of 40–45 strokes min^{-1} and a tidal volume of 14–20 ml by a miniature ventilator (Harvard, Edenbridge, Kent). Polythene cannulae were placed into the left ventricle via the right carotid artery, for withdrawal of blood samples, and the femoral vein or right marginal ear vein for i.v. injections. The left ventricular cannula was connected to a transamerica type 4-422-0001 pressure transducer to monitor left ventricular systolic pressure (LVSP) and heart rate (HR) on a Grass model 7D polygraph (Grass Instruments, Quincy, Mass., USA).

Platelet aggregation ex vivo

Arterial blood (2 ml) was withdrawn from the left ventricle and collected into tri-sodium citrate (3.15% w/v) in a ratio of 9:1 and immediately centrifuged at $1400\,g$ (4000 r.p.m.) for 20 s (Biofuge A; Hereus) to produce platelet-rich plasma (PRP). The blood was further centrifuged at $14900\,g$ (12000 r.p.m.) for 1 min to obtain platelet-poor plasma (PPP). Platelet aggregation was measured in a Payton aggregometer with 0.5 ml PRP. In initial experiments, dose-response curves to adenosine 5'-diphosphate (ADP, $0.2–3.2\,\mu g\,ml^{-1}$) were determined to establish $1.6\,\mu g\,ml^{-1}$ ADP as a sub-maximal dose with respect to aggregation, as measured by peak increase in light transmission.

After a stabilization period of 20–30 min two blood samples were processed and the aggregation to ADP ($1.6\,\mu g\,ml^{-1}$) assessed. At time 0, vehicle or NP, t-PA, NP + t-PA or GTN (all at $30\,\mu g\,kg^{-1}$) were given intravenously. Blood samples were withdrawn at 1, 5, 15, 30 and 60 min after injection, and the PRP challenged with ADP ($1.6\,\mu g\,ml^{-1}$).

Fibrinolytic activity ex vivo

The fibrinolytic activity within plasma samples was assayed *ex vivo*, by measurement of t-PA activity and t-PA antigen levels, using commercially available kits (Biopool; Sweden). In addition, the euglobulin clot lysis time (ECLT) was assessed, based on the method of von Kaulla & Schultz (1958), as described below.

Blood containing tri-sodium citrate (3.15% w/v) in a ratio of 9:1 was centrifuged at $14900\,g$ (12000 r.p.m.) for 1 min

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(Biofuge A; Hereus) to produce PPP. Distilled water was added (14 ml ml^{-1} PPP) and the pH adjusted to pH 5.4 by bubbling with CO_2 gas (approximately 3 min). This procedure causes precipitation of the euglobulin fraction, while the acidity destroys the biological activity of PAI. After a second centrifugation at 14900 g (12000 r.p.m.) for 1 min, the supernatant was discarded and the euglobulin precipitate dissolved in 1 ml buffer (13.4 mM KH_2PO_4, 53.6 mM Na_2HPO_4).

At time 0, 2 u human thrombin (10 μl; 200 u thrombin ml^{-1} 0.05 M $CaCl_2$) was added to 0.3 ml euglobulin fraction to induce clot formation. The fraction was incubated at 37°C and the time for complete lysis to occur recorded.

Fibrinolytic activity in vitro

Rabbit blood was collected into tri-sodium citrate (3.15% w/v) in a ratio of 9:1 and centrifuged at 200 g (low speed) for 8 min (Petalfuge; OrthoDiagnostis Systems) to produce PRP. The PPP was obtained by further centrifugation of PRP at 900 g (high speed).

Whole blood, PRP or PPP (2 ml) was added to plastic tubes and incubated at 37°C with gentle agitation. At time 0, vehicle, NP (0.1–30 μg ml^{-1}), t-PA (1–10 ng ml^{-1}), or NP and t-PA was added and samples taken after 1, 5, 15, 30 and 60 min. The fibrinolytic activity was evaluated by measurement of plasma t-PA activity and PAI-1 activity (Biopool, Sweden).

Drugs

Sodium nitroprusside (Sigma Chem. Co., Poole, U.K.) and glyceryl trinitrate (Lipha, West Drayton, U.K.) were dissolved and administered in saline. Single chain tissue plasminogen activator, specific activity 600,000 i.u. mg^{-1} (Biopool, Sweden) was initially dissolved in $KHCO_3$ (1 M) and diluted and administered in saline. Prostacyclin (a gift from Dr B.J.R. Whittle, Wellcome Research Labs., U.K.) was initially dissolved in Tris buffer (1 M, pH 8.4) and diluted and administered in 1.25% $NaHCO_3$. Sagatal (May & Baker, Dagenham, U.K.) was administered in saline 1:1 and Xylocaine 2% (Astra Pharm., Kings Langley, UK) was administered directly.

Statistical analysis

Results are expressed as the mean ± s.e.mean of n experiments, and analysed by two-way analysis of variance followed by a least significance procedure to determine the nature of the response. A P value of less than 0.05 was considered statistically significant.

Results

Haemodynamic effects

The resting LVSP observed in this study ranged from 75 mmHg to 110 mmHg (97 ± 2 mmHg). During the 60 min experimental period, no changes in LVSP were observed in anaesthetized rabbits treated with vehicle or t-PA (30 μg kg^{-1}). Prostacyclin (3 μg kg^{-1}) caused a transient, but significant, decrease in LVSP (31 ± 5 mmHg) 20 s after administration, that returned to basal pressure within 5 min. Both NP (30 μg kg^{-1}) and GTN (30 μg kg^{-1}) caused a similar fall in LVSP (− 33 ± 3 mmHg and 38 ± 7 mmHg, respectively) at 20 s, which also returned to basal levels within 5 min, although that induced by NP was slightly more prolonged (Figure 1a).

Platelet aggregation ex vivo

Prostacyclin caused almost complete inhibition of ADP-induced platelet aggregation *ex vivo* (93 ± 7%) 1 min after administration. This effect was still significant at 5 min, but had returned to control levels by 15 min. In contrast, NP or GTN had no effect on platelet aggregation *ex vivo* (Figure 1b).

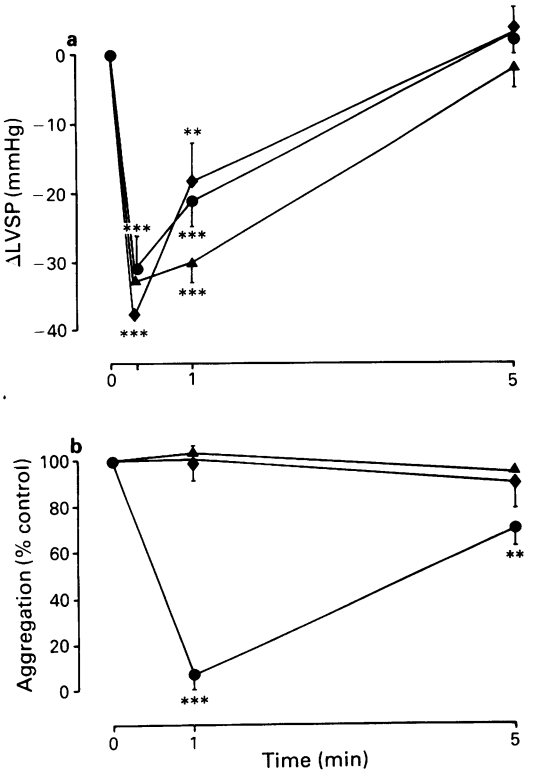

Figure 1 The effect of 3 μg kg^{-1} prostacyclin (●; n = 7), 30 μg kg^{-1} sodium nitroprusside (▲; n = 4) or 30 μg kg^{-1} glyceryl trinitrate (◆; n = 3) administration to anaesthetized rabbits on (a) left ventricular systolic pressure (LVSP) and (b) ADP-induced, *ex vivo* platelet aggregation. Results are expressed as mean and vertical lines show s.e.mean. ** $P < 0.01$; *** $P < 0.001$.

Fibrinolysis ex vivo

Intravenous administration of NP (30 μg kg^{-1}) or GTN (30 μg kg^{-1}) to anaesthetized rabbits caused a gradual decrease in ECLT measured *ex vivo*, although only that induced by NP proved significantly different from control (Figure 2a). In addition, NP (30 μg kg^{-1}), but not GTN (30 μg kg^{-1}), caused an increase in plasma t-PA activity, significantly different from the control 5 min after administration (Figure 2b).

Bolus injection of t-PA (30 μg kg^{-1}) caused an increase in plasma t-PA activity measured in a sample withdrawn after 1 min. This transient activity had returned to basal levels, just detectable by the assay, within 5 min (Table 1). However, while NP alone (30 μg kg^{-1}) had comparatively little effect on plasma t-PA activity (Figure 2b), co-administration of NP with t-PA resulted in considerable prolongation of the plasma t-PA activity measured *ex vivo*. This activity was significantly greater than that obtained with t-PA alone at 5, 15, 30 and 60 min (Table 1).

Using samples from the same experiments, exogenous t-PA caused an immediate increase in plasma t-PA antigen levels 1 min after administration, that decreased exponentially over 60 min. The co-administration of NP with t-PA did not significantly alter the t-PA antigen levels measured with t-PA alone (Figure 3).

Fibrinolysis in vitro

When t-PA (10 ng ml^{-1}) was incubated with citrated whole blood *in vitro* a gradual decline in plasma t-PA activity was observed over 60 min. Co-incubation of NP (3 μg ml^{-1}) with t-PA resulted in a stabilization of the t-PA activity that proved significantly higher than with t-PA alone after 60 min (Figure 4a).

Incubation of citrated whole blood at 37°C resulted in a gradual increase in PAI-1 activity in the plasma. The addition

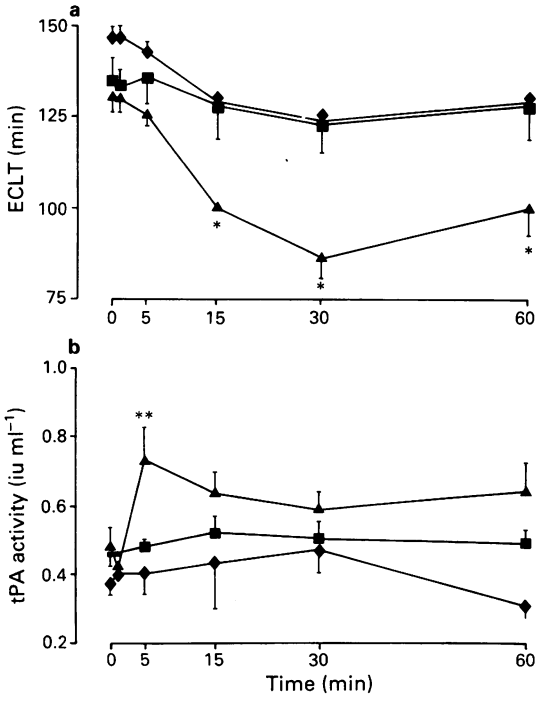

Figure 2 The effect of vehicle (■; $n = 5$), $30\,\mu g\,kg^{-1}$ sodium nitroprusside (▲; $n = 4$), or $30\,\mu g\,kg^{-1}$ glyceryl trinitrate (◆; $n = 3$) administration to anaesthetized rabbits on fibrinolysis, as measured *ex vivo* by (a) euglobulin clot lysis time (ECLT) and (b) tissue plasminogen activator (t-PA) activity in plasma. Results are expressed as mean and vertical lines show s.e.mean. * $P < 0.05$; ** $P < 0.01$.

Table 1 The time-related changes in tissue plasminogen activator (t-PA) activity measured *ex vivo*, following administration of $30\,\mu g\,kg^{-1}$ t-PA alone or in the presence of $30\,\mu g\,kg^{-1}$ sodium nitroprusside (NP) to anaesthetized rabbits at time 0

	t-PA activity (iu ml^{-1})	
Time (min)	*t-PA*	*NP + t-PA*
0	0.8 ± 0.2	0.8 ± 0.2
1	33.8 ± 2.4	32.0 ± 1
5	2.3 ± 0.9	27.0 ± 0.6***
15	0.6 ± 0.1	20.0 ± 0.6***
30	0.5 ± 0.2	8.0 ± 0.6***
60	0.7 ± 0.1	6.0 ± 0.6**

Results are expressed as mean ± s.e.mean; $n = 3$. ** $P < 0.01$; *** $P < 0.001$.

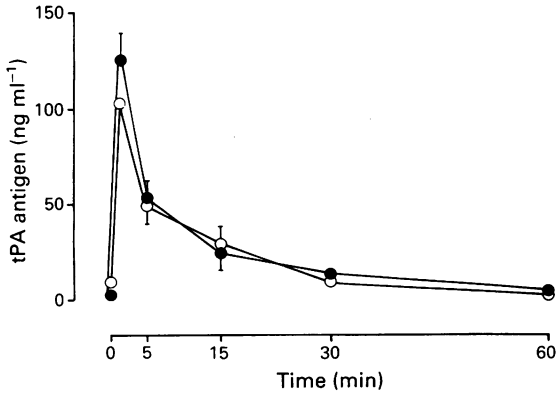

Figure 3 The time-related changes in tissue plasminogen activator (t-PA) antigen levels measured *ex vivo*, following administration of $30\,\mu g\,kg^{-1}$ t-PA alone (●; $n = 3$) or in the presence of $30\,\mu g\,kg^{-1}$ sodium nitroprusside (NP, ○; $n = 3$) to anaesthetized rabbits. Results are expressed as mean and vertical lines show s.e.mean.

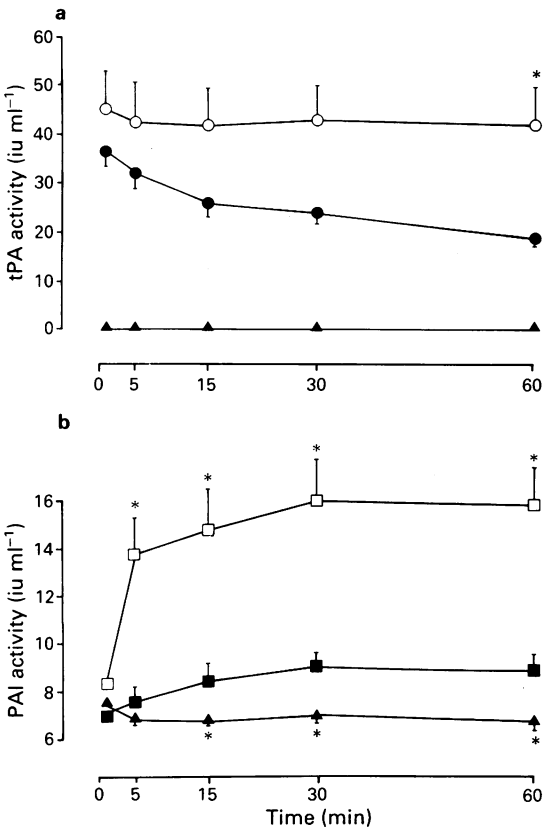

Figure 4 (a) The time-related decrease in tissue plasminogen activator (t-PA) activity following administration of $3\,\mu g\,ml^{-1}$ sodium nitroprusside (NP) (▲; $n = 3$), and $10\,ng\,ml^{-1}$ t-PA alone (●; $n = 3$) or in the presence of $3\,\mu g\,ml^{-1}$ NP (○; $n = 3$) to whole blood *in vitro*. (b) The effect of $30\,\mu g\,ml^{-1}$ NP (▲; $n = 3$) on the time-related increase in plasminogen activator inhibitor (PAI-1) activity in whole blood *in vitro* (■; $n = 3$). The increase in PAI-1 activity in platelet rich plasma (□; $n = 3$) is also represented. Results are expressed as mean and vertical lines show s.e.mean. * $P < 0.05$.

of NP ($30\,\mu g\,ml^{-1}$) to the blood inhibited this increase, maintaining basal levels of PAI-1 throughout the experimental period, and was significantly lower than control at 15, 30 and 60 min (Figure 4b). Incubation of PRP caused a considerable increase in PAI-1 activity, that was not observed with PPP.

Discussion

When administered to anaesthetized rabbits prostacyclin (PGI_2), NP or GTN all caused a transient decrease in LVSP, while only PGI_2 induced inhibition of platelet aggregation *ex vivo*. This emphasizes the weak activity that nitrates display on platelets as compared to their action on vascular smooth muscle (Schafer *et al.*, 1980). In contrast, PGI_2 was equally active on the vasculature and on platelets (Lidbury *et al.*, 1989). Despite the lack of anti-aggregatory activity, NP activated fibrinolysis, as measured by a significant decrease in ECLT and a significant increase in plasma t-PA activity *ex vivo*. To elucidate the mechanism by which NP induces fibrinolysis, exogenous t-PA was administered, in the presence or absence of NP, and the profile of t-PA removal from the circulation determined by measurement of plasma t-pA activity and t-PA antigen levels. The t-PA activity assay measures only free plasma t-PA (exclusive of that bound to PAIs), while the t-PA antigen assay measures total plasma t-PA (inclusive of that bound to PAIs). The t-PA activity measurements demonstrated the rapid removal of t-PA activity from the blood (less than 5 min) by binding to PAIs and degradation by the liver, whereas the plasma t-PA antigen levels decreased somewhat more slowly representing the loss of t-PA due to degradation by the liver only. As demonstrated recently, in the presence of NP, t-PA activity *ex vivo* was greatly prolonged,

while t-PA antigen levels remained unchanged (Korbut *et al.*, 1990). This indicates that NP prevents inactivation of t-PA by PAI, probably by inhibition of PAI-1 release from platelets, which normally accounts for more than 90% of circulating PAI (Booth *et al.*, 1985).

That NP inhibited PAI-1 release was supported by *in vitro* experiments with whole blood. They showed that the time-related decrease in t-PA activity following incubation with t-PA, presumably due to binding to PAIs, was significantly maintained in the presence of NP. Furthermore, the time-related increase in PAI-1 activity seen in whole blood, probably due to release of PAI-1 from platelets, was significantly inhibited by NP.

While we have shown that NP can induce fibrinolysis at lower concentrations than those required for inhibition of platelet aggregation (Lidbury *et al.*, 1989), PGI_2 exhibits fibrinolytic activity (Korbut *et al.*, 1983) at concentrations very similar to anti-aggregatory concentrations (Gorman *et al.*, 1977; Moncada, 1982). Furthermore, we found that the time course of NP-induced fibrinolysis differs from that of PGI_2 (Moore *et al.*, 1988; Korbut *et al.*, 1989). Thus, the mechanism of fibrinolytic activity induced by activators of guanylate cyclase, such as NP, is different from that of activators of adenylate cyclase. Although various hypotheses have been proposed to explain the fibrinolytic activity induced by PGI_2 (Moore *et al.*, 1988), it is accepted that activators of adenylate cyclase produce a long-lasting stimulation of fibrinolysis by an unknown mechanism. Furthermore, due to the lack of effect of NP on platelet aggregation *ex vivo*, these results indicate that NP may induce fibrinolysis while producing minimal changes in platelet cyclic GMP levels. Although our evidence is indirect, fibrinolysis may be stimulated by small increases in cyclic GMP, as with platelet adhesion, whereas larger increases in cyclic GMP are required for inhibition of platelet aggregation. It is tempting to speculate that the fibrinolytic action of NP may be related to the NO-induced anti-adhesive effect on platelets (Radomski *et al.*, 1987b) rather than anti-aggregatory properties. Alternatively, NP may stimulate fibrinolysis via an unknown, cyclic GMP-independent mechanism. Although both GTN and NP cause smooth muscle relaxation via NO-mediated stimulation of soluble guanylate cyclase, only NP activated fibrinolysis *ex vivo*. This may occur because GTN can only generate formation of NO in the presence of thiols, such as cysteine (Feelish & Noack, 1987), whereas NO release from NP is regarded as non-enzymatic/spontaneous. On the other hand we believe that GTN is likely to express fibrinolytic activity, similar to that of NP, at higher concentrations or with repeated administration over a longer period of time. The possibility of a GTN-induced fibrinolytic response is presently under investigation.

NP exhibits fibrinolytic activity through increased t-PA levels that probably result from inhibition of PAI-1 release from platelets. Several studies have shown that platelets, when stimulated by a variety of stimuli such as collagen, thrombin, ADP or adrenaline, are able to release PAI-1 (Murray *et al.*, 1974; Erickson *et al.*, 1984). Irrespective of the mechanism of platelet activation, such release of PAI-1 appears to be quantitative, and in human blood a greater amount of PAI is associated with platelets than with plasma (Kruithof *et al.*, 1986). To our knowledge, no inhibitors of PAI-1 release have been investigated in non-activated platelets. It is important to stress, therefore, that in our experiments the platelets were not stimulated. However, as we observed, the conditions prevailing in experiments *in vitro*, both in whole blood or PRP, might easily result in signficantly raised levels of PAI-1. Moreover, activation of the fibrinolytic system by NP observed in anaesthetized animals, presumably due to inhibition of PAI-1 release from platelets, could suggest that platelets constantly release PAI-1 *in vivo* following surgical procedures. The physiological role of this activation could be to prevent premature lysis of primary, platelet-rich, haemostatic plugs.

Finally, the administration of anti-platelet nitrovasodilators in combination with t-PA may have important therapeutic consequences due to the prolongation of the efficacy of t-PA, perhaps allowing a reduction in the total administered dose. As we have suggested previously (Korbut *et al.*, 1990) the bleeding tendencies and a reduced t-PA requirement associated with angina pectoris patients treated with t-PA (Gold *et al.*, 1987), could result from the prolongation of t-PA activity due to combined treatment with nitrates.

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