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# Biotransformation of glyceryl trinitrate by blood platelets as compared to vascular smooth muscle cells

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**Abstract**

The present study investigated the metabolism of glyceryl trinitrate by washed human platelets as compared to that by rat vascular smooth muscle cells. Possible changes in metabolism after induction of nitrate tolerance were also studied in both systems. Incubation of the cells with glyceryl trinitrate (0.1 mM) resulted in a time-dependent release of nitrite ($NO_2^-$) amounting to $6.30 \pm 0.63$ nmol mg protein$^{-1}$ h$^{-1}$ in vascular smooth muscle cells and $0.61 \pm 0.08$ nmol mg protein$^{-1}$ h$^{-1}$ for platelets, respectively. The nitric oxide (NO) scavenger, oxyhemoglobin (10 μM), significantly reduced $NO_2^-$ generation in both cell types studied. Nitrate tolerance was induced by incubation of the cells with glyceryl trinitrate (2 mM) for 2 h. In tolerant vascular smooth muscle cells as well as in tolerant platelets, $NO_2^-$ release was significantly reduced. The inhibitory capacity of glyceryl trinitrate on ADP (6 μM)-induced platelet aggregation and on intracellular $Ca^{2+}$ signals was significantly reduced in tolerant platelets. The data show a direct metabolism of glyceryl trinitrate by human blood platelets which is subject to a type of tolerance development similar to that in vascular smooth muscle cells.

*Keywords:* Glyceryl trinitrate; Nitrite; Nitrate tolerance; Platelet; Smooth muscle cell; Vascular

## 1. Introduction

Various organic nitroesters have been shown to inhibit platelet function (Loscalzo, 1985; De Caterina et al., 1988; Gerzer et al., 1988; Karlberg et al., 1992). In previous studies we have shown evidence that direct platelet inhibition by organic nitroesters such as isosorbide dinitrate and related compounds is based on a nitric oxide (NO)/cGMP-related mechanism (Weber et al., 1993).

In contrast to nitrovasodilators that spontaneously release NO, such as linsidomine and sodium nitroprusside, organic nitroesters, such as glyceryl trinitrate, require bioactivation, i.e. metabolic conversion to NO (for review see Ahlner et al., 1991). Conversion of glyceryl trinitrate to NO in platelets with subsequent activation of platelet soluble guanylate cyclase is still incompletely understood. While inhibition of platelet aggregation by glyceryl trinitrate was associated with some increase in cGMP levels (Loscalzo, 1985; Ahlner et al., 1991), incubation of washed platelets, even with milimolar concentrations of glyceryl trinitrate in the presence of the phosphodiesterase inhibitor, zaprinast, did not change platelet cGMP (Kuhn et al., 1991). Furthermore, neither glyceryl trinitrate nor isosorbide dinitrate was able to activate platelet soluble guanylate cyclase (Gerzer et al., 1988). The general conclusion of these studies was that, in contrast to vascular smooth muscle cells, platelets are not able to release NO directly from organic nitroesters (Gerzer et al., 1988).

The present study investigated the metabolism of glyceryl trinitrate by washed human platelets as compared to that in vascular smooth muscle cells. Evidence is presented that the metabolism of glyceryl trinitrate that occurs in platelets is susceptible to tolerance development. Thus, the data indicate a bioactivation pathway of glyceryl trinitrate in platelets which is similar to that in vascular smooth muscle cells.

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## 2. Materials and methods

### 2.1. Preparation of washed human platelets

Washed human platelets were prepared as previously described (Weber et al., 1993). Briefly, fresh citrated blood was obtained from healthy volunteers and platelet-rich plasma was prepared by centrifugation at 250 × g for 10 min at room temperature. The pH was adjusted to 6.5 with acidic citrate dextrose (Biotest, Frankfurt, Germany). The platelets were washed twice in a buffer (pH 6.5) containing (mM): 113 NaCl, 4 $Na_2HPO_4$, 24 $NaH_2PO_4$, 4 $KH_2PO_4$, supplemented with 5 mM glucose. Washed platelets were resuspended in Hepes-buffered Tyrode solution (pH 7.4) of the following composition (mM): 134 NaCl, 12 $NaHCO_3$, 2.9 KCl, 0.36 $NaH_2PO_4$, 1 $MgCl_2$, 5 Hepes, supplemented with 5 mM glucose. Cell protein was determined using the Bio-Rad protein assay according to Bradford (1976).

In further experiments, nitrate tolerance was induced by incubation of platelet-rich plasma with glyceryl trinitrate (2 mM) for 2 h. Tolerant platelets were washed twice to remove any residual glyceryl trinitrate and were resuspended in Hepes-buffered Tyrode solution, as described above.

### 2.2. Preparation of vascular smooth muscle cells

Rat vascular smooth muscle cells were isolated as previously described (Sachinidis et al., 1995). Thoracic aortas from Wistar-Kyoto rats (6–8 weeks old, Charles River Wiga GmbH, Sulzfeld, Germany) were removed, transferred into phosphate-buffered solution (PBS) containing 1% penicillin/streptomycin (w/v). After removal of connective tissue, the aorta was incubated for 30 min at 37°C in 3 ml of enzyme dissociation mixture containing Dulbecco's modified Eagle medium (DMEM), 400 IU/ml collagenase type I, 0.5 mg/ml elastase and 0.5 mg/ml soybean trypsin inhibitor. Then the aorta was transferred into PBS and the adventitia was stripped off under a binocular microscope. The aorta was minced and the tissue was incubated for 2 h at 37°C in 6 ml of enzyme dissociation mixture under constant agitation. The cells were pelleted by centrifugation at 250 × g for 10 min, then resuspended in DMEM with 10% fetal calf serum, and plated in Petri dishes. Vascular smooth muscle cells were cultured over several passages in DMEM supplemented with 10% fetal calf serum, non essential amino acids, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. Confluent cells cultured in 3-cm dishes were used for the experiments. Cell protein was determined using the Bio-Rad protein assay.

In further experiments, nitrate tolerance was induced by incubation of glyceryl trinitrate (2 mM) in DMEM for 2 h. Tolerant vascular smooth muscle cells were washed 3 times with PBS to remove the nitrate.

### 2.3. Preparation of oxyhemoglobin

Oxyhemoglobin was prepared from bovine hemoglobin by means of reduction with sodium dithionite and subsequent purification by passage through a Sephadex G-25 column (Schrör et al., 1991; Weber et al., 1993).

### 2.4. Platelet aggregation

Platelet aggregation was measured as previously described (Weber et al., 1993). Briefly, 400 μl of washed platelet suspension and 90 μl of test buffer (Hepes-buffered Tyrode, see above) were incubated in a 2-channel aggregometer (Labor, Hamburg, Germany) for 2 min at 37°C. Platelets were stimulated by adding 10 μl of ADP (final concentration 6 μM). Changes in light transmission were recorded during constant stirring of the samples (1200 rpm, 37°C). When the effects of glyceryl trinitrate were studied, glyceryl trinitrate (100 μM) was incubated for 2 min prior to the addition of ADP.

### 2.5. Measurement of platelet $[Ca^{2+}]_i$

Platelet-rich plasma was incubated with 2 μM fura-2-pentaacetoxymethyl ester for 30 min at 37°C. Then, platelets were washed as described above. ADP (6 μM)-induced $Ca^{2+}$-signals were measured spetrofluorometrically (Perkin Elmer LS50, Überlingen, Germany). Fluorescence was measured at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 505 nm. Calibration was performed using 0.5% Triton X-100 for maximum fluorescence following EGTA (10 mM) for minimum fluorescence. Signals were corrected for cell autofluorescence.

### 2.6. Nitrite analysis

Nitrite ($NO_2^-$) was determined according to the method of Misko et al. (1993). For nitrite measurement with washed platelets, tolerant and non-tolerant cells were incubated with glyceryl trinitrate (0.1 mM). The samples were centrifuged and each supernatant (1 ml) was allowed to react with 100 μl of 0.05 mg/ml 2,3-diaminonaphthalene dissolved in 0.62 M HCl to form the fluorescence product, 1-(*H*)-naphthotriazole. The reaction was terminated with 50 μl of 2.8 N NaOH and fluorescence was measured at excitation and emission wavelenghs of 365 nm and 450 nm, respectively (Perkin Elmer LS50, Überlingen, Germany).

For nitrite measurement with vascular smooth muscle cells, confluent cells were washed 3 times with PBS and incubated with glyceryl trinitrate (0.1 mM) in 1 ml Hepes-

buffered Tyrode solution. Supernatants were collected and nitrite was measured as described above.

Nitrite concentrations were determined using sodium nitrite standards dissolved in Hepes-buffered Tyrode. Results are expressed as nmol $NO_2^-$ mg protein$^{-1}$.

In some experiments, the cells were incubated for 60 min with glyceryl trinitrate and oxyhemoglobin (10 μM). As control, oxyhemoglobin (10 μM) was added at the end of the 60 min incubation period. Supernatants were subsequently filtered through a Centricon 10 filter ($M_r$ cut-off 10000) at 1000 × g for 60 min to remove the hemoglobin. Then, nitrite was measured as described above.

### 2.7. Materials

Acidic citrate-dextrose (Biostabil, Biotest, Frankfurt, Germany); DMEM, PBS, trypsin, non-essential amino acids, penicillin and streptomycin (Gibco, Eggenstein, Germany); ADP (Boehringer, Mannheim, Germany); linsidomine (Cassella AG, Frankfurt, Germany); glyceryl trinitrate (Schwarz-Pharma, Monheim, Germany); all other reagents were obtained from Sigma (Deisenhofen, Germany).

### 2.8. Statistics

Data are means ± S.E.M. from $n$ experiments. Statistical analysis was performed using the Mann-Whitney test. $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Release of nitrite from glyceryl trinitrate by non-tolerant and tolerant vascular smooth muscle cells

In contrast to linsidomine which dose dependently released $NO_2^-$ in a cell-free system, no spontaneous $NO_2^-$ release was observed with glyceryl trinitrate (0.1 mM) (data not shown).

Incubation of vascular smooth muscle cells with glyceryl trinitrate (0.1 mM) resulted in a time-dependent release of $NO_2^-$ (Fig. 1). The NO scavenger, oxyhemoglobin (10 μM), inhibited the formation of $NO_2^-$ from glyceryl trinitrate ($P < 0.05$, Table 1). After induction of nitrate tolerance (see Methods), $NO_2^-$ release from glyceryl trinitrate was significantly reduced ($P < 0.05$, Fig. 1).

### 3.2. Release of nitrite from glyceryl trinitrate by non-tolerant and tolerant platelets

Time-dependent $NO_2^-$ release from glyceryl trinitrate was also observed in washed platelets. After correction for the protein amount, however, the capacity of platelets to generate $NO_2^-$ from glyceryl trinitrate was about 10-fold lower, as compared to vascular smooth muscle cells (Fig. 2). Similar to vascular smooth muscle cells, oxyhemoglobin (10 μM) significantly reduced the formation of $NO_2^-$ from glyceryl trinitrate by platelets ($P < 0.05$, Table

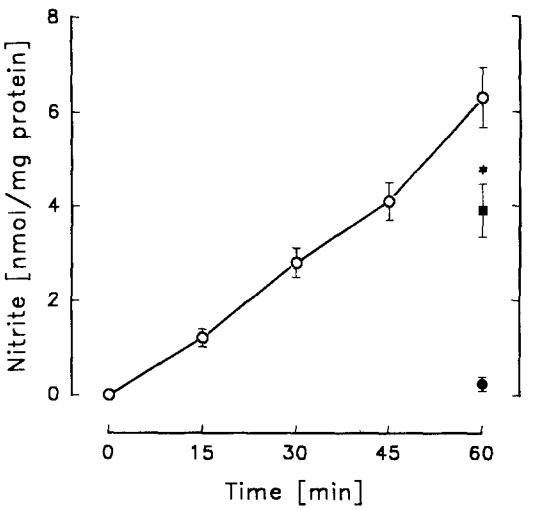


Fig. 1. Release of nitrite ($NO_2^-$) from glyceryl trinitrate (0.1 mM) by non-tolerant (○, $n = 12$) and tolerant (■, $n = 12$) vascular smooth muscle cells. ● ($n = 4$) indicates basal $NO_2^-$ release from vascular smooth muscle cells without glyceryl trinitrate. Data are means ± S.E.M., * $P < 0.05$ non-tolerant vs. tolerant cells.

Table 1
Effects of oxyhemoglobin (10 μM) on nitrite ($NO_2^-$) release (nmol mg protein$^{-1}$ h$^{-1}$) from glyceryl trinitrate (0.1 mM) by vascular smooth muscle cells and platelets

| | Vascular smooth muscle cells | Platelets |
|---|---|---|
| Control | 6.0 ± 0.3 | 0.68 ± 0.06 |
| Oxyhemoglobin | 5.1 ± 0.2 [a] | 0.48 ± 0.05 [a] |

Data are means ± S.E.M. of $n = 4$ experiments, [a] $P < 0.05$.

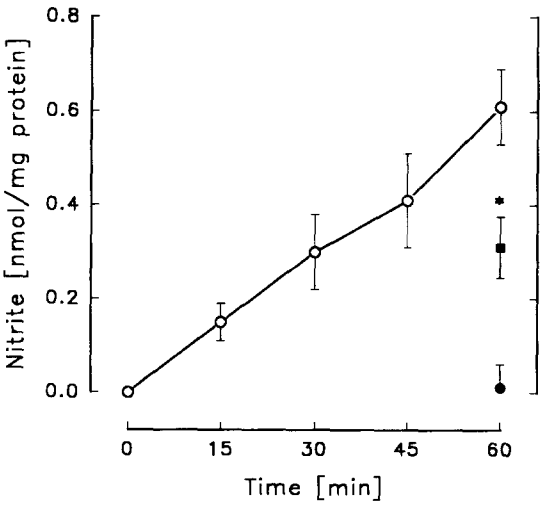


Fig. 2. Release of nitrite ($NO_2^-$) from glyceryl trinitrate (0.1 mM) by non-tolerant (○, $n = 12$) and tolerant (■, $n = 5$) platelets. ● ($n = 4$) indicates basal $NO_2^-$ release from platelets without glyceryl trinitrate. Data are means ± S.E.M., * $P < 0.05$ non-tolerant vs. tolerant cells.

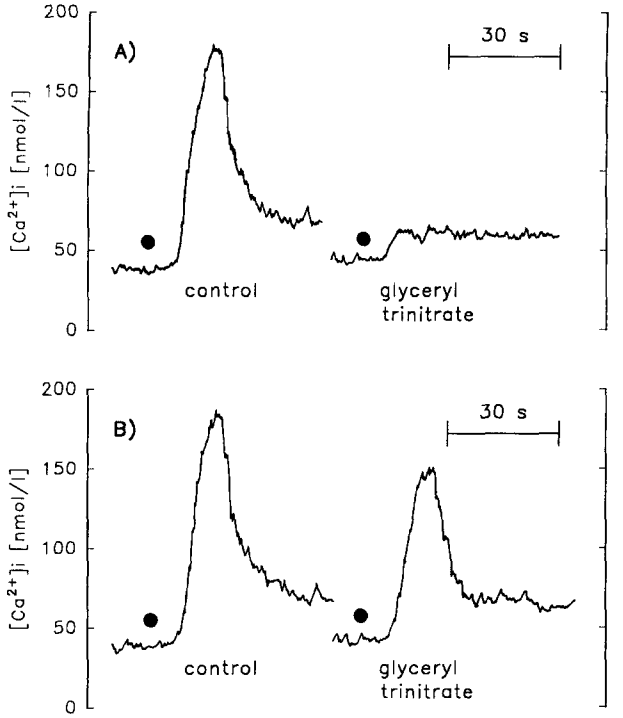


Fig. 3. Inhibition of ADP (6 μM)-induced $Ca^{2+}$ signals in non-tolerant (A) and tolerant (B) platelets by glyceryl trinitrate (0.1 mM). (●) indicates addition of ADP. Original tracing representative of $n = 3$ experiments.

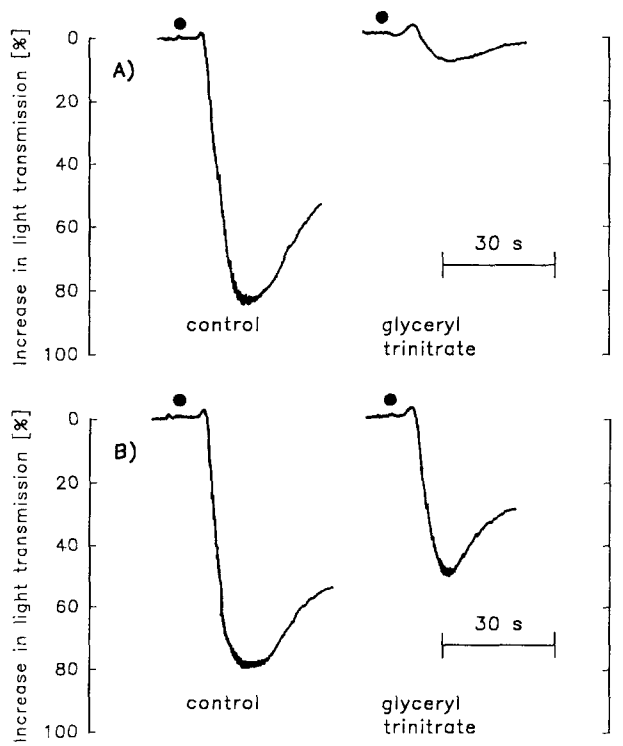


Fig. 4. Inhibition of ADP (6 μM)-induced aggregation of non-tolerant (A) and tolerant (B) platelets by glyceryl trinitrate (0.1 mM). (●) indicates addition of ADP. Original tracing representative of $n = 3$ experiments.

1). In nitrate-tolerant platelets, $NO_2^-$ release was also significantly impaired ($P < 0.05$, Fig. 2).

### 3.3. Inhibition of platelet aggregation and intracellular $Ca^{2+}$ signals by glyceryl trinitrate in tolerant and non-tolerant platelets

In non-tolerant as well as in tolerant platelets, ADP (6 μM) induced a rapid and reversible increase in intracellular $Ca^{2+}$ concentration that was inhibited by glyceryl trinitrate (0.1 mM). In tolerant platelets, however, the capacity of glyceryl trinitrate to inhibit the intracellular $Ca^{2+}$ signal was greatly reduced (Fig. 3).

Similar results were obtained for the inhibition of ADP (6 μM)-induced platelet aggregation by glyceryl trinitrate (0.1 mM). The inhibitory capacity of glyceryl trinitrate on platelet aggregation was reduced in tolerant cells (Fig. 4).

## 4. Discussion

The present study compared the conversion of glyceryl trinitrate to $NO_2^-$ in vascular smooth muscle cells and in washed platelets. Upon incubation with glyceryl trinitrate, both cell types released $NO_2^-$ in a time-dependent manner. $NO_2^-$ formation from glyceryl trinitrate was reduced by the NO scavenger, oxyhemoglobin. In addition, functional and metabolic tolerance to glyceryl trinitrate was observed in platelets.

Previous studies have shown that vascular smooth muscle cells and isolated vascular tissue release NO from glyceryl trinitrate (Schrör et al., 1991; Kurz et al., 1993) resulting in relaxation of smooth muscle cells (Ahlner et al., 1991). Nitrate-tolerant vascular smooth muscle cells metabolize glyceryl trinitrate less effectively than do normal cells as assessed from their reduced capacity to release $NO_2^-$ (Salvemini et al., 1993).

In contrast to those for vascular smooth muscle cells, the mechanisms involved in inhibition of platelet function by organic nitroesters are still a matter of controversy. There are conflicting reports on the effects of nitroesters on platelet cyclic nucleotides (Loscalzo, 1985; Kuhn et al., 1991) and on activation of platelet soluble guanylate cyclase (Gerzer et al., 1988). In previous studies we have shown evidence that organic nitroesters such as isosorbide dinitrate directly inhibit platelet function via the NO/cGMP system (Weber et al., 1993).

In the present study, we have demonstrated a time-dependent conversion of glyceryl trinitrate to $NO_2^-$ by washed human platelets. After correction for the protein content, platelets were about 10 times less potent to release $NO_2^-$ from glyceryl trinitrate than were vascular smooth muscle cells. This might reflect the lower potency of glyceryl trinitrate to inhibit platelet aggregation as compared to the vasorelaxing effects of the compound. Interestingly, a similar degree of tolerance was observed in both cell types

after incubation with a high glyceryl trinitrate concentration. In tolerant vascular smooth muscle cells, $NO_2^-$ release was reduced by about 40%, whereas in tolerant platelets, a reduction of $NO_2^-$ release by about 50% was observed. These data are in good aggreement with published data on $NO_2^-$ release from glyceryl trinitrate by tolerant and non-tolerant smooth muscle cells (Salvemini et al., 1993).

In addition to the reduced capacity of tolerant platelets to release $NO_2^-$, functional tolerance to glyceryl trinitrate was observed in the present study. Inhibition of ADP-induced platelet aggregation by glyceryl trinitrate was significantly impaired in tolerant cells. Similar results were obtained with the inhibition of the ADP-induced intracellular $Ca^{2+}$ signal by glyceryl trinitrate, which was also impaired in tolerant platelets. Previous preliminary data demonstrated the development of nitrate tolerance in platelets (Loscalzo and Amarante, 1989). In the present study, however, functional tolerance was correlated to impaired metabolism of glyceryl trinitrate by tolerant platelets.

Clearly, $NO_2^-$ formation does not necessarily represent bioactivation of glyceryl trinitrate to NO. Indeed, recent studies with vascular tissue homogenates have demonstrated that the release of NO from glyceryl trinitrate was not dependent on the formation of $NO_2^-$ (Kurz et al., 1993). Thus, bioconversion of glyceryl trinitrate to $NO_2^-$ by glutathione S-transferases has been suggested to be a degradation pathway for glyceryl trinitrate metabolism. However, since nitrate metabolism differs between intact cells and cell homogenates, tissue homogenization may alter the characteristics of glyceryl trinitrate biotransformation (Bennett et al., 1994).

In the present study, we have shown that, in both cell types studied, $NO_2^-$ generation from glyceryl trinitrate was significantly reduced upon incubation with the NO scavenger, oxyhemoglobin. The conclusion may be drawn from these data that at least part of the $NO_2^-$ generated from glyceryl trinitrate is related to the formation of NO. However, the contribution of NO for generation of $NO_2^-$ from glyceryl trinitrate by both cell types studied was rather small. Thus, measurement of $NO_2^-$ alone is not a valid parameter for NO generation from organic nitroesters, unless the relative contribution of NO to $NO_2^-$ formation is determined, e.g. by means of oxyhemoglobin.

In summary, this study demonstrated direct metabolism of glyceryl trinitrate by human blood platelets that is susceptible to tolerance development. The relative importance of direct conversion of glyceryl trinitrate to NO by platelets, as compared to NO generated from glyceryl trinitrate by adjacent vascular endothelial cells or vascular smooth muscle cells (Feelisch and Kelm, 1991) regarding the inhibition of platelet function in vivo remains to be established. Indeed, even tolerant vascular smooth muscle cells will probably still produce sufficient amounts of NO to cause platelet inhibition. On the other hand, high amounts of platelets may accumulate within an arterial thrombus. Because of the considerable lipophilicity of glyceryl trinitrate, continuous, sustained generation of NO may occur even at the site of coronary artery stenosis.

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