Human vascular smooth muscle cells inhibit platelet aggregation when incubated with glyceryl trinitrate: evidence for generation of nitric oxide

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1 The effect on platelet aggregation of glyceryl trinitrate in the presence of cultured vascular smooth muscle cells was determined turbidometrically. U46619 (a thromboxane mimetic) was used as agonist and experiments were performed in the presence of aspirin. Inorganic nitrite production from glyceryl trinitrate by vascular smooth muscle cells was also measured, to provide an indirect index of nitric oxide synthesis.

2 The combination of vascular smooth muscle cells together with glyceryl trinitrate, at concentrations that had little effect individually, profoundly inhibited platelet aggregation.

3 The inhibitory effect on platelet aggregation of vascular smooth muscle cells together with glyceryl trinitrate was markedly attenuated by haemoglobin, an inhibitor of nitric oxide.

4 These results show that vascular smooth muscle cells inhibit platelet aggregation when exposed to glyceryl trinitrate and suggest that this is due to generation of nitric oxide from glyceryl trinitrate by vascular smooth muscle.

Keywords: Nitric oxide; glyceryl trinitrate; vascular smooth muscle; platelets; aggregation

Introduction

Glyceryl trinitrate is used extensively to relieve angina pectoris, but its actions remain incompletely understood. It relaxes blood vessels by activating soluble guanylate cyclase (Katsuki *et al.*, 1977; Ignarro *et al.*, 1981), and is converted to nitrite ions when incubated with vascular tissue (Needleman & Johnson, 1973). Inorganic nitrite ions have only weak vasodilator activity, but are formed rapidly and non-enzymatically under physiological conditions from nitric oxide which is a potent vasodilator and activator of soluble guanylate cyclase (Palmer *et al.*, 1987; Ignarro *et al.*, 1981). Nitric oxide is bound avidly by haemoglobin (Haldane *et al.*, 1897), which is a potent inhibitor of the endothelium-derived relaxing factor described by Furchgott & Zawadzki (1980). Endothelial cells synthesize nitric oxide from endogenous L-arginine, accounting quantitatively for relaxing factor activity (Palmer *et al.*, 1987; 1988). Vascular smooth muscle may metabolize glyceryl trinitrate to form nitric oxide which mediates its vasorelaxant effect, before conversion to inactive nitrite (Katsuki *et al.*, 1977). There is evidence that tolerance to glyceryl trinitrate occurs as a result of failure in the enzymic synthesis of nitric oxide, at the level either of an enzyme or a cofactor such as reduced glutathione, although the exact mechanism remains obscure (Needleman & Johnson, 1973; Newman *et al.*, 1990).

Glyceryl trinitrate is a very weak inhibitor of platelet aggregation *in vitro* (Loscalzo, 1985). However, it was shown recently that nitric oxide, in addition to its vasodilator action, potently inhibits platelet adhesion and aggregation (Radomski *et al.*, 1987). Consequently, if nitric oxide is indeed formed from glyceryl trinitrate by vascular smooth muscle, it could inhibit local thrombus formation. Such an effect could be important in the therapeutic action of glyceryl trinitrate in patients with ischaemic heart disease. In the present study we have therefore measured platelet aggregation in suspensions of platelet rich plasma mixed with cultured human vascular smooth muscle cells and glyceryl trinitrate in the presence and in the absence of haemoglobin. Aspirin was used to inhibit biosynthesis of anti-aggregatory prostaglandins. Glyceryl tri-

nitrate can break down slowly to yield nitric oxide even in cell-free systems (Feelisch & Noack, 1987), so we also monitored inorganic nitrite as an index of nitric oxide production in the presence and absence of smooth muscle cells. In addition, we used vascular smooth muscle cells made tolerant to glyceryl trinitrate to determine whether anti-aggregatory activity is generated specifically by sensitive as opposed to tolerant cells.

Methods

Smooth muscle cells: culture and preparation

Human vascular smooth muscle cells were cultured by a modification of the method of Ives *et al.* (1978) from uterine artery obtained at hysterectomy and grown to confluence at passage 4–7 in 12-well plates (4.52 cm²) in Medium E199 with penicillin/streptomycin 50 iu/50 µg ml⁻¹, fungizone 2.5 µg ml⁻¹, glutamine 10 mg ml⁻¹, foetal calf serum 10%, oestradiol 0.01 µg ml⁻¹ and ITS: insulin-transferrin-sodium selenite medium supplement. Cell morphology was monitored in unstained preparations by phase contrast microscopy with a Nikon TMD inverted stage microscope. Cells stained positively with anti-smooth muscle actin using monoclonal anti-α smooth muscle actin and fluorescein isothiocyanate-coupled rabbit anti-mouse immunoglobulin (Skalli *et al.*, 1986). Suspensions of cultured vascular smooth muscle cells were prepared from the 12-well plates as follows. After removal of medium, 2 ml of trypsinizing solution (0.05% trypsin and 0.02% EDTA in phosphate buffered saline) was added to each well for 2 min and then replaced by 0.5 ml of platelet-poor plasma and 1.5 ml of phosphate buffered saline. Clumps of cells were dispersed by gentle repeated aspiration with a fine tipped Pasteur pipette. This provided a cell suspension containing approximately 5 × 10³ cells ml⁻¹. Tolerance was induced by 18-h incubation of cells with glyceryl trinitrate (220 µM).

Nitrite production

Medium from 12-well plates containing confluent vascular smooth muscle cells was replaced with 2 ml phosphate buffered saline at pH 7.4. Glyceryl trinitrate (final concentration

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220 μM) was added and incubated at 37°C. Supernatant was removed at 0, 5, 15, 60 and 120 min and assayed for nitrite by the Greiss reaction, as described by Rider & Melon (1946).

Platelet aggregation studies

Venous blood samples (20 ml) from healthy drug-free volunteers were collected with a 19-gauge needle and anticoagulated with trisodium citrate (0.31% w/v). Platelet-rich plasma and platelet-poor plasma were prepared by differential centrifugation at room temperature. Aspirin (final concentration 1 mM) was added to platelet-rich plasma to prevent thromboxane and prostaglandin biosynthesis. Platelet aggregation was studied at 37°C in a Payton aggregometer (Model 600 B) as described by Born & Cross (1963). The output from the aggregometer was processed by a MacLab™ system connected to an Apple Macintosh SE computer which allowed automated analysis. Platelet-rich plasma (0.5 ml) was added to each cuvette and the volume made up with phosphate buffered saline (final volume 650 μl). Aggregation was induced by a sub-maximal dose of U46619 (11 α ,9 α -epoxymethano- prostaglandin H₂) (2 μM). Additions of glyceryl trinitrate (220 μM), cell suspension (100 μl), haemoglobin (5 μM), superoxide dismutase (15 units ml⁻¹) or combinations of these were made immediately before addition of U46619. Saturated nitric oxide solution was prepared by bubbling helium then nitric oxide (each for 45 min) through 0.9% sodium chloride solution. Measurements were made in duplicate. Aggregation was recorded as % change in light transmission 2 min after agonist; 100% aggregation was taken as the light transmission of platelet poor plasma.

Materials

All materials were obtained from Sigma, UK, except glyceryl trinitrate (Tridil, Du Pont, Stevenage, Herts), foetal calf serum, Medium E199 and phosphate buffered saline Dulbecco's Formula (modified) without calcium and magnesium (ICN Flow, High Wycombe, Bucks.) Fluorescein isothiocyanate coupled rabbit anti-mouse immunoglobulin was obtained from DAKO (High Wycombe, Bucks). Nitric oxide and helium were obtained from BOC, Brentford, Middlesex.

Analysis

Results are expressed as the mean $\pm$ s.e.mean. Differences were evaluated by the Wilcoxon signed rank test and considered significant when $P < 0.05$.

Results

Nitrite production

Nitrite was generated by cultured smooth muscle cells following addition of glyceryl trinitrate, 220 μM (Figure 1). In cells previously exposed to glyceryl trinitrate, production of nitrite was considerably reduced (Figure 1). Exposure of cells to glyceryl trinitrate did not affect their morphology as seen under light microscopy immediately after exposure and for up to six days subsequently. Nitrite was not detected in solutions of glyceryl trinitrate (220 μM) at 37°C in the absence of cells.

Platelet aggregation studies

U46619 (2 μM) caused platelet aggregation in the presence of aspirin (1 mM). Glyceryl trinitrate (220 μM) or 100 μl of vascular smooth muscle cell suspension added separately did not inhibit the response to U46619, whereas in combination they did (Figure 2). In 12 separate experiments this combination of glyceryl trinitrate with cell suspension inhibited aggregation to U46619 from $66.1 \pm 1.7\%$ in the presence of glyceryl trinitrate alone, and $66.1 \pm 1.0\%$ in the presence of cell suspension alone, to $4.0 \pm 2.8\%$ in the presence of both ($P < 0.001$).

Figure 1 Generation of nitrite by cultured vascular smooth muscle cells incubated with glyceryl trinitrate (220 μM). Cells not previously exposed to glyceryl trinitrate (○) produced nitrite rapidly compared to cells preincubated for 18 h with this concentration of glyceryl trinitrate (●).

Suspensions of cells which had been made tolerant to glyceryl trinitrate, as shown by their impaired ability to metabolize glyceryl trinitrate to nitrite, lost the ability to inhibit platelet aggregation in combination with glyceryl trinitrate (Figure 3). In 8 further experiments, the combination of glyceryl trinitrate (220 μM) with 100 μl of a suspension of non-tolerant vascular smooth muscle cells inhibited platelet

Figure 2 Representative traces showing platelet aggregation in response to U46619, alone (a), and in the presence of glyceryl trinitrate (b), smooth muscle cell suspension (c), and both glyceryl trinitrate and cell suspension (d). The arrow indicates additions of U46619.

Figure 3 Representative traces showing platelet aggregation in response to U46619, alone (a), in the presence of glyceryl trinitrate (b), in the presence of glyceryl trinitrate and tolerant vascular smooth muscle cells pre-incubated with glyceryl trinitrate (c), and in the presence of glyceryl trinitrate and untreated vascular smooth muscle cells (d). The arrow indicates additions of U46619.

aggregation to U46619 ($2\mu\text{M}$) from $68.4 \pm 0.5\%$ to $17.4 \pm 9.0\%$ whereas in paired measurements tolerant cells had significantly less effect, inhibiting aggregation from $66.8 \pm 0.8\%$ only to $58.4 \pm 1.6\%$.

Addition of haemoglobin ($5\mu\text{M}$) to platelet-rich plasma had no effect on aggregation caused by U46619, but antagonized the inhibitory effect of the combination of vascular smooth muscle cell suspension and glyceryl trinitrate. In further experiments ($n = 8$), U46619 ($2\mu\text{M}$) in the presence of cells alone caused $77.3 \pm 1.6\%$ aggregation. Paired measurements in the presence of both vascular smooth muscle cells and glyceryl trinitrate confirmed that these inhibited aggregation, to $13.8 \pm 3.2\%$, and showed that haemoglobin substantially restored the aggregation response to $56.3 \pm 8.5\%$. Superoxide dismutase (15 units ml^{-1}), did not influence the inhibitory effect of a sub-maximally effective combination of cells with glyceryl trinitrate ($n = 8$; $P > 0.3$). However, the inhibition of aggregation observed with authentic nitric oxide solution ($0.2\text{--}4\mu\text{l}$) was similarly unaffected by the same concentration of superoxide dismutase ($n = 4$).

Discussion

The main finding of this study is that platelet aggregation is inhibited by a combination of glyceryl trinitrate with a suspension of vascular smooth muscle cells, under conditions when neither glyceryl trinitrate nor cell suspension alone appreciably inhibits aggregation. Glyceryl trinitrate does have weak anti-aggregatory activity in its own right (Loscalzo, 1985), but in the present experiments we selected a dose that was approximately one tenth that needed to cause 25% inhibition of platelet aggregation. Vascular smooth muscle cells synthesize prostacyclin (Baenziger *et al.*, 1980) which is a potent inhibitor of platelet aggregation (Moncada *et al.*, 1976), so the present experiments were performed in the presence of aspirin to prevent prostacyclin synthesis, and cell suspension alone did not inhibit platelet aggregation. Vascular tissue is known to generate nitrite from glyceryl trinitrate (Needleman & Johnson, 1973) and more recently, in a cell-free system, Feelisch & Noack (1987) have demonstrated the formation of nitric oxide as an intermediate in the metabolism of organic nitrates. The most likely explanation for the present findings is

therefore that vascular smooth muscle cells synthesize nitric oxide from glyceryl trinitrate, and it is this nitric oxide that inhibits platelet aggregation.

This explanation is supported by the findings that the inhibitory effect is prevented by low concentrations of haemoglobin, a potent inhibitor of nitric oxide (Martin *et al.*, 1984), and that the smooth muscle cells used in these experiments caused inorganic nitrite production from glyceryl trinitrate. Superoxide dismutase failed to enhance the inhibitory effect of the combination of glyceryl trinitrate with cells but it is possible that superoxide anion (which accelerates destruction of nitric oxide) is present only in low concentrations under these experimental conditions. This is consistent with our finding that superoxide dismutase was similarly ineffective in altering the sensitivity of platelet aggregation to authentic nitric oxide.

The finding that vascular smooth muscle cells previously exposed to a high concentration of glyceryl trinitrate were unable either to synthesize inorganic nitrite or to inhibit platelet aggregation when re-exposed to glyceryl trinitrate demonstrates that generation of platelet inhibitory activity is not a non-specific effect, and is consistent with the proposal (Needleman & Johnson, 1973; Newman *et al.*, 1990) that tolerance to glyceryl trinitrate is due to loss of the ability of smooth muscle cells to convert organic nitrate to nitric oxide.

The concentration of glyceryl trinitrate used in these experiments with cell suspensions *in vitro* was much higher than concentrations achieved therapeutically. We were limited as to the density of suspended vascular smooth muscle cells that we could use, because of the optical method used to measure platelet aggregation. It is possible that in the presence of an intact layer of smooth muscle in the media of small and medium sized arteries that sufficient nitric oxide could be produced from glyceryl trinitrate to have clinically important effects on platelet activation *in vivo*. Such an action could contribute to the therapeutic effect of glyceryl trinitrate, for instance in acute myocardial infarction where intravenous nitrates have a substantial beneficial effect on mortality (Yusuf *et al.*, 1988).

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