Time-dependent inhibition by glyceryl trinitrate of platelet aggregation caused by U46619 (a thromboxane/endoperoxide receptor agonist)

G. KAMPF & J. M. RITTER

Department of Clinical Pharmacology, United Medical and Dental Schools of Guy's and St Thomas' Hospitals, London, United Kingdom

Glyceryl trinitrate is a weak inhibitor of platelet aggregation *in vitro*. Its effect on platelet aggregation in response to U46619 (a thromboxane/endoperoxide receptor agonist) was studied turbidometrically in platelet-rich plasma from healthy volunteers. The object was to determine whether inhibition was influenced by a period of preincubation between preparation of platelet-rich plasma and addition of glyceryl trinitrate. Incubation was performed at 37° C and 22° C. Samples were removed at intervals and transferred to an aggregometer cuvette at 37° C. Glyceryl trinitrate (100 µM) or an equal volume of distilled water was added 5 min before U46619 (2 µM), and aggregation recorded as change in light transmission. Inhibition by glyceryl trinitrate was markedly time and temperature dependent, with a progressive increase in inhibitory potency between 120 and 300 min preincubation at 37° C but not at 22° C. The explanation of this is unknown but the effect was not influenced by lipopolysaccharide or by cycloheximide, so it does not appear to be due to exposure to endotoxin or to enzyme induction *in vitro*.

Keywords glyceryl trinitrate nitric oxide platelets aggregation

Introduction

Glyceryl trinitrate is a weak inhibitor of platelet aggregation *in vitro* [1], possibly because it breaks down slowly to yield nitric oxide (NO), even in cell free systems [2]. NO potentially inhibits platelet aggregation [3]. We observed considerable variability in the inhibitory potency of glyceryl trinitrate on platelet aggregation, which appeared to relate to the interval between preparation of platelet-rich plasma and addition of this antagonist (Figure 1). The object of the present study was to establish more precisely the relationship between inhibition by glyceryl trinitrate and conditions of preincubation of platelet-rich plasma.

Methods

Venous blood samples (60–80 ml) from six healthy drug-free volunteers were collected with a 19-gauge needle (Butterfly™, Abbott, Sligo, Ireland) and anti-

coagulated with trisodium citrate (final concentration 0.3% w/v). Platelet-rich plasma and platelet-poor plasma were prepared by differential centrifugation at room temperature, aspirated with a plastic pipette and transferred to sterile plastic containers (Sterilin Ltd, Teddington, UK). Three such containers were incubated simultaneously in a water bath at 37° C, one untreated, one with *Escherichia coli* lipopolysaccharide (10 µg ml⁻¹, Sigma, Poole, Dorset, UK) and one with cycloheximide (10 µg ml⁻¹, Sigma, Poole, Dorset, UK). A fourth tube containing untreated platelet-rich plasma was incubated at 22° C. The time from venepuncture to the start of incubation was 20 min. Samples of platelet-rich plasma (0.5 ml) were removed with a plastic tipped pipette (Gilson Medical Electronics, Villiers-le-Bel, France) at 30 min intervals up to 300 min and transferred to cuvettes in an aggregometer (Payton) at 37° C and stirred with a magnetic bar at 800 rev min⁻¹. Glyceryl trinitrate (final concentration 100 µM, Lipha, West Drayton,

UK) or an equal volume of distilled water was added after 1 min in the cuvette. Aggregation was induced by a concentration ($2\ \mu\text{M}$) of 11,9-epoxymethano-prostaglandin H_2 (U46619; Sigma, Poole, Dorset, UK) that caused submaximal aggregation. U46619 was added 5 min after glyceryl trinitrate or control. Aggregation was followed for a further 5 min. Aggregation was recorded as change in light transmission relative to platelet-poor plasma, and percentage inhibition was calculated as:

(aggregation in absence of glyceryl trinitrate – aggregation in presence of glyceryl trinitrate/aggregation in absence of glyceryl trinitrate) $\times 100\%$.

Data were summarised as means $\pm$ s.e. mean. Differences were sought using repeated measures analysis of variance and considered significant if $P < 0.05$.

Results

Figure 2 shows mean percentage inhibition of platelet aggregation to U46619 ($2\ \mu\text{M}$) by glyceryl trinitrate ($100\ \mu\text{M}$) as a function of the duration of preincubation before addition of glyceryl trinitrate. When preincubation was performed at 37°C inhibitory activity appeared at 120 min and rose steadily to $64 \pm 6\%$ inhibition at 300 min ($P = 0.013$), whereas when preincubation was performed at 22°C the same concentration of glyceryl trinitrate was without significant inhibitory effect up to 300 min. Lipopolysaccharide ($10\ \mu\text{g ml}^{-1}$) and cycloheximide ($10\ \mu\text{g ml}^{-1}$) had no significant effect on inhibitory activity at 37°C at any time: $46 \pm 19\%$ inhibition in the presence of lipopolysaccharide, and $56 \pm 10\%$ inhibition in the presence of cycloheximide, each at 300 min (other times not shown).

Discussion

The main finding of this study is that the inhibitory potency of glyceryl trinitrate on U46619 induced platelet aggregation increases with the duration of preincubation of platelet-rich plasma at 37°C before exposure to antagonist. Responses to aggregating agents can vary with time, so it was essential to express the inhibitory effect of glyceryl trinitrate relative to aggregation in the absence of glyceryl trinitrate after the same duration of preincubation in experimental and control tubes, rather than relating it to baseline effect before incubation. Vascular-smooth muscle and endothelial cells metabolise glyceryl trinitrate to NO [4, 5]. Such metabolism is inducible by lipopolysaccharide, and induction is prevented by cycloheximide (an inhibitor of protein synthesis) [5]. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing NO, and metabolism of glyceryl trinitrate to NO in macrophages is induced by *Escherichia coli* lipopolysaccharide [6, 7]. We questioned whether the temperature-dependent nature of

Figure 1 Superimposed aggregation traces from a sample of platelet-rich plasma incubated at 37°C for the times shown before addition of glyceryl trinitrate ($100\ \mu\text{M}$) followed after 5 min by U46619 ($2\ \mu\text{M}$); (representative of 10 experiments).

Figure 2 Temperature-dependent inhibition of platelet aggregation caused by glyceryl trinitrate ($100\ \mu\text{M}$) after preincubation of platelet-rich plasma (0–300 min) at 22°C (●) and 37°C (○); agonist: U46619 ($2\ \mu\text{M}$).

the progressive increase in sensitivity to glyceryl trinitrate that we observed could be attributed to induction of glyceryl trinitrate metabolism in monocytes or other cells present in small numbers in platelet-rich plasma. However, the process was not augmented by exogenous lipopolysaccharide nor prevented by cycloheximide, arguing against this explanation. It has been shown [2] that glyceryl trinitrate can be converted to nitric oxide by reaction with cysteine, and it is possible that sulphhydryl donating groups are being generated during incubation of platelet rich plasma at 37°C but not at 22°C , and allow conversion of glyceryl trinitrate to nitric oxide. Whatever the mechanism underlying this phenomenon, awareness of its existence will be important in future studies of the effects of glyceryl trinitrate on platelet function.

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