Preservation of platelet responsiveness to nitroglycerine despite development of vascular nitrate tolerance

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Aims

Organic nitrates, via nitric oxide (NO) release, induce vasodilatation and inhibit platelet aggregation. Development of nitrate tolerance in some vascular preparations may be associated with diminished responsiveness to NO. To date it is not known to what extent vascular tolerance to organic nitrates is associated with acquired platelet hyporesponsiveness to NO. In the current study we compared the acute and chronic effects of sustained release (SR) isosorbide 5' mono-nitrate (ISMN) and transdermal nitroglycerine (TD-NTG) on blood vessels (effects on apparent arterial stiffness) and platelets (effects on responsiveness to NO donors) in patients with stable angina pectoris (SAP).

Methods

Patients ($n = 34$) with SAP entered a blinded randomized crossover study of ISMN (120 mg) vs. intermittent TD-NTG (15 mg 24 h⁻¹). Effects of each nitrate on pulse wave reflection (augmentation index (Aix)), platelet response to adenosine diphosphate (ADP 1 $\mu\text{mol l}^{-1}$), nitroglycerine (NTG 100 $\mu\text{mol l}^{-1}$) and the non-nitrate NO donor sodium nitroprusside (SNP 10 $\mu\text{mol l}^{-1}$), were measured pre-dose, 4 and 8 h post dose, on three occasions: 1) at the end of a pre-nitrate phase, 2) after dosing for 7 days and 3) following 14 days of full dose therapy with either nitrate.

Results

Acutely, both ISMN and TD-NTG markedly reduced Aix. After 14 days, these effects were significantly attenuated (ANOVA, $P = 0.018$) but not abolished, indicating development of nitrate tolerance. Neither nitrate preparation affected ADP (1 $\mu\text{mol l}^{-1}$)-induced platelet aggregation. Platelet responsiveness to NTG (100 $\mu\text{mol l}^{-1}$) and SNP (10 $\mu\text{mol l}^{-1}$) was not diminished during chronic nitrate therapy, and there was no evidence of 'rebound' hyper-aggregability during 'nitrate-free' periods.

Conclusions

Chronic therapy with either ISMN or TD-NTG is associated with development of vascular tolerance. Despite the induction of vascular tolerance, platelet responsiveness to NTG and SNP remains unaffected. Therefore, development of vascular tolerance is unlikely to compromise the anti-aggregatory effects of organic nitrates, or those of endogenous NO.

Introduction

Organic nitrates such as nitroglycerine (glyceryl trinitrate; NTG) and isosorbide mono-nitrate (ISMN) have been shown to be effective in improving exercise tolerance in patients with stable angina pectoris [1, 2]. The anti-ischaemic effects of organic nitrates result from their vasodilator actions, which improve arterial and venous compliance, as well as redistributing coronary blood flow towards the ischaemic subendocardium [3].

Organic nitrates have also been shown to exert potent anti-aggregatory effects *in vivo* [4, 5], *in vitro* and *ex vivo* [6–10]; it has been postulated that these effects may contribute to their efficacy in patients with acute coronary syndromes [11]. Agents that potentiate the haemodynamic and anti-aggregatory effects of organic nitrates, such as N-acetylcysteine [12–14], have been found to be clinically useful in patients with unstable angina pectoris [15].

The clinical efficacy of organic nitrates is potentially limited both by *de novo* tissue hypo-responsiveness to NO (NO resistance), loosely related to endothelial dysfunction, which has been observed both in blood vessels and at a platelet level [16], and by progressive diminution of responsiveness during chronic therapy ('nitrate tolerance'), which has been observed predominantly in blood vessels [17], but may also occur in platelets [18].

The predominant mechanisms underlying NO resistance involve scavenging of NO by the superoxide free radical and partial inactivation of soluble guanylate cyclase [19]. The mechanisms of nitrate tolerance induction are more controversial. 'True' tolerance (that is, demonstrable *ex vivo*) reflects a decreased release of NO from nitrates, due to impaired enzymatic biotransformation [17]. However, some studies have also shown a component of tolerance induction, which is not nitrate specific, with cross-tolerance to NO [20]. Thus it is possible that in some models, perhaps via incremental superoxide generation, nitrate tolerance may result in further impairment of the physiological actions of NO, including both vasodilatation and inhibition of platelet aggregation.

The current study examined the effects of two commonly utilized long-acting nitrate preparations on vascular tone and platelet aggregation during acute and chronic therapy, in patients with SAP. The study design facilitates a comparison of tolerance induction in blood vessels (measured *in vivo*) and platelets (measured *ex vivo*) and the implications of vascular tolerance on platelet responsiveness to NO.

Methods

Study protocol

Thirty-four patients with mild to moderate SAP (Canadian cardiovascular class II–III) aged 68 ± 8 years (mean $\pm$ SD) were assessed. All had a history of angina pectoris with each receiving multiple medications at study enrolment (Table 1). No patient received an ADP or glycoprotein IIb/IIIa receptor antagonist either prior to, or during the study. Written informed consent was obtained prior to study commencement, with the protocol being approved by the Ethics of Human Research Committee of the North-western Adelaide Health Service.

Study exclusion criteria were known intolerance to long-acting nitrates, use of sulphhydryl-containing agents (e.g. captopril, gold salts and penicillamine), participation in any other study, and/or a known bleeding diathesis or myocardial infarction within the preceding 3 months.

The effects of ISMN and TD-NTG were examined in a randomized, crossover study lasting 49 days with each active treatment period of 21 days duration (Figure 1). Seven days prior to the first nitrate dose, all prophylactic

Table 1

Patient characteristics and baseline pharmacotherapy
($n = 34$)

Parameter	
Age (mean $\pm$ S.D.) (years)	68 ± 8
Males, n (%)	19 (56)
Current smokers, n (%)	7 (21)
Ex-smokers, n (%)	12 (35)
Hypercholesterolaemia, n (%)	32 (94)
Hypertension, n (%)	23 (68)
Previous MI, n (%)	16 (47)
Previous UAP, n (%)	18 (53)
Diabetes mellitus, n (%)	9 (26)
CCF, n (%)	3 (9)
<i>Pharmacotherapies</i>	
Aspirin, n (%)	30 (88)
Ca ²⁺ antagonists, n (%)	23 (68)
Perhexiline, n (%)	13 (38)
ACE inhibitors, n (%)	20 (59)
β -adrenoceptor antagonists, n (%)	7 (21)
Statins, n (%)	21 (62)
Digoxin, n (%)	3 (9)

MI = Myocardial infarction; UAP = Unstable angina pectoris; CCF = Chronic cardiac failure; ACE = Angiotensin converting enzyme.

Figure 1

Schematic representation of the study phases. Arrows denote platelet/haemodynamic assessment days with parameters being examined at 0, 4 and 8 h post nitrate administration. TD-NTG was an intermittent 12 h application (applied at 08.00 h, removed at 20.00 h)

long-acting nitrate use was ceased. Patients were allowed to continue other non-nitrate anti-anginal medications. Additional nitrate use and the number of anginal episodes experienced throughout all phases of the study were recorded.

At the end of the run in phase, a series of baseline (no nitrate) parameters were assessed. Blood samples and haemodynamic variables were obtained at 0, 4 and 8 h, corresponding to 08.00 h, 12.00 h and 16.00 h, following 10 min of physical inactivity. Each patient was then randomized to receive either ISMN (60 mg) or TD-NTG (7.5 mg 24 h⁻¹; 12 h application) for 7 days. At the end of these 7 days, patients underwent a 24 h nitrate-free period, allowing for an examination of any potential rebound in platelet/vascular function. For the remainder of each treatment period, ISMN dosage was 120 mg day⁻¹ and TD-NTG dosage 15 mg 24 h⁻¹; 12 h application. On days 15 and 36 the acute effects of these increased dosages were examined. Each nitrate preparation was administered immediately following an initial platelet/haemodynamic assessment (0 h). This therapeutic regimen was then continued for a further 14 days whereupon the chronic effects of each preparation were assessed (day 28/49). Each patient crossed over to the alternative regimen at the end of day 28.

Platelet aggregation determinations were assessed by laboratory personnel blinded to the phase of the study. Haemodynamic variables were recorded by personnel blinded to the platelet aggregation data.

Haemodynamics

We utilized serial applanation tonometry in order to quantify nitroglycerine-induced changes in apparent

arterial stiffness [21], the most sensitive marker of haemodynamic effects of organic nitrates, as previously described [22]. Radial artery waveforms were recorded using a Millar micromanometer tipped pressure transducer (Millar SPT 301B, Millar instruments, Houston, TX, USA) coupled to a SphygmoCor pulse wave velocity system (Model SCOR-Vx; SphygmoCor software; Atcor Medical, Sydney, NSW, Australia) from the wrist of the dominant arm. Utilizing the SphygmoCor device, a series of radial artery pressure waveforms were recorded over a period of 8 s. A central waveform was generated using a convolutional algorithm and generalized transfer function [23, 24]. From the generated central waveform, augmentation index (AIx) corrected for a heart rate of 75 beats min⁻¹ was calculated as the difference between the second and first systolic peaks expressed as a percentage of the pulse pressure and utilized as a measure of apparent arterial stiffness [25].

Blood sampling and platelet aggregation studies

Blood was collected from the antecubital vein by venesection and transferred to a plastic tube containing acid citrate anticoagulant (1 : 10 volume ratio, two parts 0.1 M citric acid to three parts 0.1 M tri-sodium citrate). Platelet aggregation studies were then performed with whole blood samples using the dual channel impedance lumi-aggregometer (Model 560CA, Chrono-Log, Havertown, Pennsylvania, USA). Platelet aggregation experiments were performed at 37 °C with a constant stirring speed of 900 r.p.m. Blood samples were diluted two fold with normal saline (0.9% NaCl, final volume 1 ml) and pre-warmed for 5 min prior to induction of platelet aggregation. Platelet aggregation was induced

with ADP ($1 \mu\text{mol l}^{-1}$) and monitored continuously for 7 min (aggro/Link revision 4.71, Chrono-Log, Havestown, Pennsylvania, USA) corresponding to maximal aggregation. Platelet aggregation was recorded as electrical impedance in Ohms. Responses to the anti-aggregatory effects of nitroglycerine were quantified as described previously [19], utilizing percentage inhibition of ADP-induced aggregation by nitroglycerine (final concentration $100 \mu\text{mol l}^{-1}$). Tolerance to NTG at the level of platelet aggregation was measured *ex vivo* as previously described [18] utilizing paired comparisons of platelet responsiveness to NTG prior to and subsequent to initiation of nitrate therapy. Similarly, inhibition of aggregation by sodium nitroprusside (final concentration $10 \mu\text{mol l}^{-1}$) was utilized as an index of platelet responsiveness to a more direct, non-nitrate, NO donor [16]. The concentrations of NTG and SNP utilized, represent approximately the EC_{50} values for these agents to inhibit ADP-induced aggregation *in vitro* [16, 18, 26, 27].

Chemicals

Adenosine 5'-diphosphate sodium salt and SNP were obtained from Sigma (St. Louis, MO, USA). NTG was purchased from Fisons (Thornleigh, New South Wales, Australia). TD-NTG and ISMN were obtained from Schering-Plough Pharmaceuticals (Australia).

Statistical analyses

Results were expressed as mean $\pm$ SEM unless otherwise stated. Statistically significant differences were limited to $P < 0.05$. Comparisons of responses were made utilizing analysis of variance (ANOVA; repeated measures where appropriate) followed by Bonferroni's *post hoc* multiple comparison test. Differences between two groups were assessed by Student's *t*-test (paired analysis where appropriate).

Results

Clinical parameters

Thirty-four patients completed the 7 week study. Three additional patients were screened for potential study participation, but were excluded for various reasons: platelets from two patients failed to aggregate to ADP ($1 \mu\text{mol l}^{-1}$), with the remaining patient withdrawing on personal grounds prior to nitrate randomization.

Both ISMN and TD-NTG were well tolerated with no one experiencing major adverse events during the study. Ten of the 34 patients experienced mild headaches that were relieved with paracetamol. One patient experienced daily exertional dyspnea when receiving ISMN or TD-NTG. There were no significant differences in angio-

nal frequency or additional nitrate consumption between the two active treatment phases of the study (data not shown).

Effects of ISMN/TD-NTG on augmentation index and other haemodynamic parameters

No significant diurnal variability in AIx was evident. Both ISMN and TD-NTG (Figure 2A/2C), in either the acute or chronic phase of the study, caused significant reductions in AIx post administration, reductions that were still apparent after 8 h (two-way ANOVA; ISMN: Time, $P < 0.01$; Acute/Chronic phase, $P < 0.01$; interaction, $P < 0.01$. Bonferroni's *post hoc* multiple comparison test $P < 0.01$ acute ISMN 4 and 8 h vs. baseline (no nitrates); $P < 0.05$ chronic ISMN 4 h vs. baseline (no nitrates): TD-NTG Time, $P < 0.01$; Acute/Chronic phase, $P < 0.01$, interaction, $P < 0.01$. Bonferroni's *post hoc* multiple comparison test $P < 0.01$ acute TD-NTG 4 and 8 h vs. baseline (no nitrates); $P < 0.05$ chronic TD-NTG 8 h vs. baseline (no-nitrates)). Examining the nitrate treatment phases only, both ISMN and TD-NTG were approximately equi-effective at reducing the AIx. However significant attenuation of effectiveness was observed across the treatment phase, indicative of tolerance development during prolonged use of either preparation (three-way ANOVA: Time, $P < 0.01$; Acute/Chronic treatment phase, $P = 0.019$; nitrate preparation, $P = 0.72$; no significant interactions). The extent of tolerance induction was marginally greater with ISMN (ANOVA $P = 0.08$: Time/Phase/Preparation interaction).

No significant diurnal variability in heart rate was observed during the baseline phase of the study. Both nitrate preparations had no significant effect on heart rate during either the acute or chronic administration (Figure 2B/2D).

Effects of ISMN/TD-NTG on platelet aggregability

ADP responses were significantly greater in female than male patients (ANOVA, $P < 0.01$), consistent with previous findings [19] and therefore, the effects of each nitrate preparation and regimen on platelet aggregability were analyzed on a gender basis. No significant diurnal variability in platelet aggregability was observed.

Neither acute nor chronic administration of ISMN (120 mg) or TD-NTG ($15 \text{ mg } 24 \text{ h}^{-1}$; 12 h application) (Figure 3A/B) had significant effects on ADP ($1 \mu\text{mol l}^{-1}$)-induced platelet aggregation. (three-way ANOVA; Figure 3A; ISMN: Gender, $P < 0.01$; Time, $P = 0.35$; Acute/Chronic phase, $P = 0.56$; no significant interactions. Figure 3B; TD-NTG: Gender, $P < 0.01$; Time, $P = 0.65$; Acute/Chronic phase, $P = 0.15$, no significant interactions).

Figure 2

Effects of acute and chronically administered ISMN (120 mg) or TD-NTG (15 mg 24 h⁻¹, 12 h application) on the haemodynamic parameters, augmentation index and heart rate. ◇—◇ = no nitrates, ▲—▲ = acute nitrate administration, ●—● = chronic nitrate administration. (A) Effect of ISMN on augmentation index, **P* < 0.05, **P* < 0.01 vs. baseline (no nitrates). (B) Effect of ISMN on heart rate. (C) Effect of TD-NTG on augmentation index, **P* < 0.05, **P* < 0.01 vs. baseline (no nitrates). (D) Effect of TD-NTG on heart rate. Data in A and C, *P* < 0.05 for acute vs. chronic nitrate effects

Figure 3

Effects of acute and chronic ISMN (120 mg) or TD-NTG (15 mg 24 h⁻¹, 12 h application) on the extent of ADP-induced whole blood platelet aggregation. ◇—◇ = no nitrates, ▲—▲ = acute nitrate administration, ●—● = chronic nitrate administration. (A) Effect of ISMN on platelet aggregation **P* < 0.01 males vs. females. (B) Effect of TD-NTG on platelet aggregation **P* < 0.01 males vs. females

Effects of acute/chronic ISMN and TD-NTG on platelet responsiveness to NTG and SNP

No significant diurnal variability in platelet responsiveness to either NTG or SNP was observed across the 8 h baseline assessment period. Despite the potential induction of nitrate-specific tolerance at the level of platelet aggregation [18], neither acute nor chronic administration of ISMN/TD-NTG had any significant effect on

platelet responsiveness to NTG. (two-way ANOVA: ISMN Figure 4A; Time, *P* = 0.17; Acute/Chronic phase, *P* = 0.28; no significant interaction: TD × NTG, Figure 4C; Time, *P* = 0.54; Acute/Chronic phase, *P* = 0.71; no significant interaction).

Similarly, neither acute nor chronic administration of ISMN or TD-NTG (Figure 4B/D) had any significant effects on platelet responsiveness to SNP. (two-way

Figure 4

Effects of acute and chronic ISMN (120 mg) or TD-NTG (15 mg 24 h⁻¹, 12 h application) on platelet responsiveness to NTG (100 μ mol l⁻¹) and SNP (10 μ mol l⁻¹). $\diamond$ = no nitrates, $\blacktriangle$ = acute nitrate administration, $\bullet$ = chronic nitrate administration. (A) Effect of ISMN on platelet responsiveness to NTG. (B) Effect of ISMN on platelet responsiveness to SNP. (C) Effect of TD-NTG on platelet responsiveness to NTG. (D) Effect of TD-NTG on platelet responsiveness to SNP.

ANOVA: ISMN Figure 4B; Time, $P = 0.54$; Acute/Chronic phase, $P = 0.18$; no significant interaction: TD $\times$ NTG, Figure 4D; Time, $P = 0.88$; Acute/Chronic phase, $P = 0.54$; no significant interaction).

Potential rebound in platelet aggregability and augmentation index following acute nitrate withdrawal

A 24 h nitrate-free period after the administration of ISMN (60 mg) or TD-NTG (7.5 mg 24 h⁻¹; 12 h application) for 1 week allowed us to examine the hypothesis that a nitrate-free interval during routine low dose nitrate pharmacotherapy is associated with a rebound in platelet aggregability and/or an increase in heart rate corrected AIx. Accordingly, for each nitrate preparation, AIx and platelet aggregability at 0 h during the acute phase (24 h nitrate free period prior to sampling, days 15 and 36) was compared with responses recorded at 0 h of the chronic phase of the trial (days 28 or 49). No significant 'rebound' in AIx was observed for either nitrate preparation (t -test Figure 5A ISMN $P = 0.16$; Figure 5B TD-

NTG $P = 0.18$). Platelet responses to ADP were analyzed according to gender and nitrate regimen. No significant rebound in platelet aggregability was observed with the discontinuation of either nitrate preparation (two-way ANOVA: ISMN Figure 5C; Gender $P < 0.01$; Acute/Chronic phase, $P = 0.9$; no significant interaction. TD $\times$ NTG Figure 5D; Gender $P < 0.01$; Acute/Chronic phase, $P = 0.94$; no significant interaction).

Discussion

The therapeutic effects of organic nitrates in patients with stable angina pectoris include, but are not necessarily limited to, prolongation of exercise duration and reduction in frequency of anginal episodes. In theory, agents such as TD-NTG and ISMN should also exert major anti-aggregatory effects, reducing the risk of thrombotic episodes.

Conversely, the haemodynamic [28] and exercise-prolonging effects [29] of organic nitrates may become attenuated during chronic therapy (induction of 'nitrate

Figure 5

Platelet aggregability and AIx following acute nitrate withdrawal of either ISMN (60 mg) or TD-NTG (7.5 mg day⁻¹, 12 h application), the '0 h' effect. ◆—◆ = females, ○—○ = males. ●—● = all subjects. (A) and (B) Effects of ISMN/TD-NTG withdrawal on augmentation index. (C) and (D) Effects of ISMN/TD-NTG on platelet aggregability

tolerance). Nitrate tolerance can also be induced at the level of platelet aggregation in patients with angina pectoris, although this has only been shown conclusively with exposure to large doses of NTG [18]. Previous studies from our laboratory revealed that tolerance is nitrate-specific in both human blood vessels [17] and platelets [18], suggesting that the major mechanism of tolerance induction in blood vessels (impairment of enzymatic release of NO from nitrates) may also apply in platelets. On the other hand, a number of investigators have proposed that under some circumstances tolerance induction is associated with increased oxidative stress and release of superoxide (O_2^-) anion, resulting in 'scavenging' of NO, irrespective of its exogenous/endogenous origin and therefore cross-tolerance between nitrates, 'direct' NO donors such as sodium nitroprusside, and NO of endothelial origin [20]. Thus there is a theoretical possibility that induction of vascular nitrate tolerance might be associated with desensiti-

zation of platelets to NO, with potentially deleterious consequences.

In the current study, we examined the effects of a common dosage regimen for TD-NTG and ISMN on arterial 'stiffness' (measured by AIx), as well as on platelet function. Several previous studies have shown that AIx may be reduced in response to small doses of organic nitrates [21, 25] and thus it is a convenient and highly reproducible means of examining possible changes in vasomotor response during chronic therapy, for comparison with changes in platelet reactivity. The results of the current study demonstrated that both nitrate preparations induced substantial falls in AIx during initial dosing. During chronic therapy, there was attenuation of responses, constituting development of nitrate tolerance. Nevertheless, important residual effects on AIx were present for both nitrate preparations. There was approximately 40% attenuation of the effect of ISMN, and only minor attenuation of TD-NTG effect

(Figure 2A,C). Statistical evaluation was consistent with a borderline differential effect (degree of tolerance induction). Previous studies [1, 29] are consistent with the development of clinically significant attenuation of nitrate effect in some patients treated chronically with TD-NTG, but this is the first study to raise the possibility that ISMN in equi-active doses may induce greater attenuation of response.

Despite the induction of nitrate tolerance at the vascular level, there was no detectable effect of nitrate therapy on platelet responses to ADP. The utilization of low concentrations such as $1 \mu\text{mol l}^{-1}$ in the current study, represents an optimal environment for detection of the anti-aggregatory effects *ex vivo* [18, 27]. However, these results in no way exclude the possibility that both ISMN and TD-NTG exert significant anti-aggregatory effects *in vivo*, particularly with relatively high doses of each agent: the utilization of *ex vivo* methods for measuring aggregation response is likely to cause some attenuation of detectable nitrate effects. Nevertheless, it must be observed that every previous study, in which anti-aggregatory effects of organic nitrates were detectable *ex vivo*, utilized comparatively high doses of nitrates administered either by sublingual [18] or by intravenous [8, 9] route.

Despite the uncertainties regarding the presence/absence of significant anti-aggregatory effects of the nitrate preparations, the quantification of responsiveness to NTG *in vitro* excluded the induction of tolerance at the level of platelet aggregation. This is the first human study to demonstrate differential susceptibility to tolerance induction at the vascular vs. platelet levels, but a previous investigation by Booth *et al.* 1996 [30] in rats, reached similar conclusions. The fact that platelets are less susceptible to tolerance induction than blood vessels is of potential clinical importance, as it would suggest ongoing protective effects against thrombosis in acute coronary syndromes irrespective of continuous nitrate administration. It also has some theoretical importance, given the recent suggestion that vascular tolerance is induced by inhibition of the bio-converting enzyme aldehyde dehydrogenase [31, 32]: these findings would raise the possibility that other bioconversion mechanisms may predominate in blood.

A further important conclusion from the current study is that chronic nitrate exposure dose not significantly affect platelet responsiveness to the more direct NO donor SNP. That is, there was no induction of 'NO resistance', as a consequence of incremental superoxide generation. Similarly, the finding that there was no 'rebound' hyper-aggregability of platelets during

'nitrate-free' periods supports the lack of development of pseudo-tolerance at the platelet level during chronic nitrate exposure, such as might occur if plasma concentrations of pro-aggregating agents such as norepinephrine increased during chronic therapy.

In conclusion, both ISMN and TD-NTG induce the development of partial vascular tolerance without any tolerance at the level of platelet aggregation. These findings suggest a basis for long-term safety of both preparations.

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