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SPECIAL REPORT

Prevention of vascular nitroglycerin tolerance by inhibition of protein kinase C

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We investigated whether *in vivo* inhibition of protein kinase C (PKC) can prevent the development of vascular tolerance and restore the sensitivity of isolated vessels to nitroglycerin (NTG). Tolerance was induced in male Wistar rats by a constant i.v. infusion of NTG 1 mg kg^{-1} h^{-1}, a dose which did not alter blood pressure. After 72 h, the aorta was removed and the sensitivity of aortic rings to NTG tested. Chronic NTG infusion resulted in a 5.5 fold decrease in NTG-sensitivity as compared with controls (vehicle), indicating the development of vascular tolerance. The simultaneous *in vivo* administration of the specific PKC inhibitor N-benzoyl-staurosporine (30 mg kg^{-1} day^{-1}) prevented this decrease in NTG sensitivity. These results suggest a role for PKC activation in the development of vascular NTG tolerance.

Keywords: Nitroglycerin tolerance; protein kinase C inhibition

Introduction Despite its beneficial acute effects, the long term use of nitroglycerin (NTG) is considerably limited by the development of tolerance. Several independent mechanisms, such as systemic counterregulation, intravascular volume expansion, and impaired biotransformation of NTG in the vascular smooth muscle, are likely to be involved. For the tolerance mechanisms that directly concern the vessel, the term vascular tolerance has been coined, being first described by Needleman (Needleman, 1970). He showed that aortic strips from rats pretreated with high doses of NTG were less sensitive to NTG as compared with strips from control animals. Here we present evidence that the protein kinase C (PKC) inhibitor N-benzoyl-staurosporine prevents the development of vascular tolerance of conductance arteries in an *ex vivo* model in the rat.

Methods Male Wistar rats (~300g body weight) were anaesthetized (3% isoflurane/ oxygen), and fluid-filled femoral arterial and venous catheters inserted. The catheters were exteriorised at the neck and the animals allowed to recover for 24 h.

Tolerance was induced by a constant i.v. infusion of NTG (in 6% ethanol in 0.9% NaCl) in a dose of 1 mg kg^{-1} h^{-1} for 72 h (prepared every 24 h). N-benzoyl-staurosporine was administered orally in a single daily dose of 30 mg kg^{-1} (in 0.9% NaCl). The first dose was given immediately before the onset of the NTG infusion. Thereafter the animals were decapitated, the aorta rapidly removed, and duplicate rings mounted in an organ bath containing oxygenated Krebs-Ringer solution. After 1 h, the rings were precontracted with phenylephrine (10^{-7} M) and the sensitivity to increasing doses of NTG tested. Data were statistically evaluated by analysis of variance. Tolerance development was assessed by pairwise comparison of the following distinct groups (respective controls always examined in parallel on same day): (1) 72 h NTG treatment compared with vehicle (ethanol/0.9% NaCl); (2) 72 h NTG treatment + N-benzoyl-staurosporine compared with vehicle (ethanol/0.9% NaCl).

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Results *Blood pressure and heart rate* When compared with the respective control groups, systolic, diastolic, or mean arterial pressure (Figure 1b, d) did not change. There was only a very transient increase in heart rate after the onset of the NTG infusion. In contrast, the concomitant administration of N-benzoyl-staurosporine resulted in an overall significant sustained increase ($P<0.01$) in heart rate when compared with control animals (Figure 1c).

Isolated rings NTG had 2 main effects: firstly, aortic rings from chronically NTG treated rats developed a significantly greater absolute tension in response to phenylephrine as compared with controls (Figure 2a). Secondly, the sensitivity to NTG was 5.5 times lower ($P<0.01$) in tissues from NTG pretreated animals ($EC_{50}=107$ nM; 99% C.I. 54-212 nM) compared with controls ($EC_{50}=19.5$ nM; 11-33 nM), indicating the development of vascular tolerance (Figure 2b). When NTG treatment was combined with N-benzoyl-staurosporine, both the increase in absolute developed tension in response to phenylephrine (Figure 2c), as well as the decreased sensitivity to NTG, were abolished (Figure 2d). These 2 effects were also observed when U-46619 was used (30 nM) instead of phenylephrine to raise tone.

Discussion Several mechanisms have been proposed to account for the development of vascular NTG tolerance. NTG is a prodrug that needs to be metabolized in order to release nitric oxide (NO) as the active principle. There is now evidence that impaired biotransformation of NTG plays an important role in vascular tolerance. Husain *et al.* (1994) found lower exhaled NO concentrations after i.v. NTG injection in tolerant vs. non-tolerant lambs, supporting the hypothesis of an impaired cellular metabolism of NTG in the tolerant state. A reversible inactivation of the putative NTG metabolising enzyme(s) might be responsible for vascular tolerance. The finding that NO-donors such as Na-nitroprusside, which do not require enzymatic steps to release NO, do not show cross-tolerance with NTG, fits well with this hypothesis (Hussain *et al.*, 1994). Additionally, the evidence for the involvement of the thiol group depletion in this process has now been questioned (Boesgaard *et al.*, 1994).

Our results show that concomitant administration of N-benzoyl-staurosporine *in vivo* can prevent a decrease in NTG sensitivity of large conductance arteries in a rat model of NTG

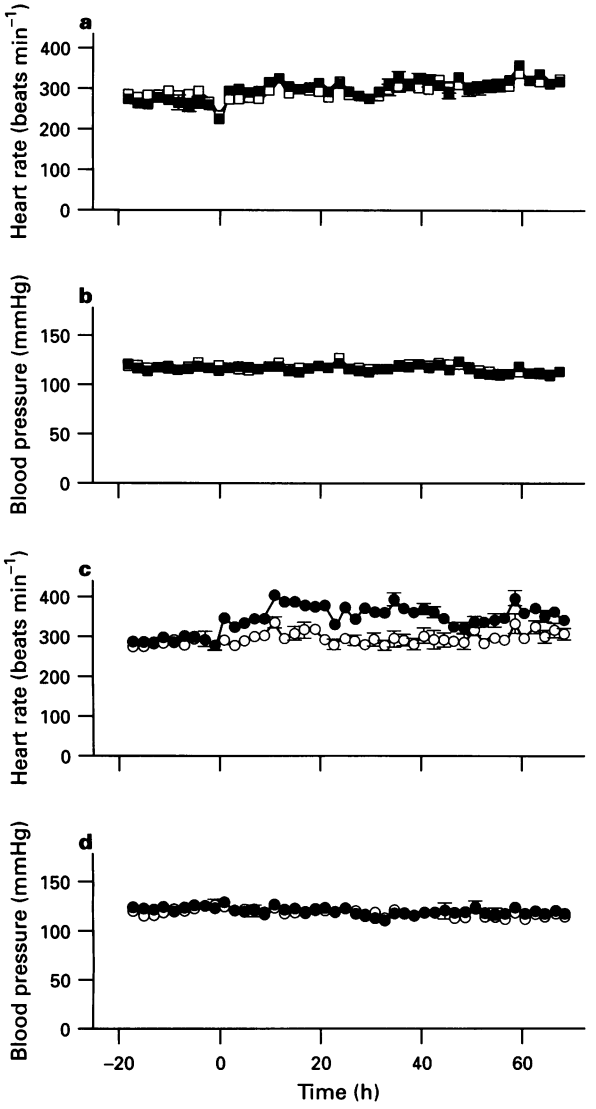

Figure 1 Effect of vehicle (ethanol/ 0.9% NaCl; (a,b) □, (c,d) ○, (a and b) nitroglycerin (1 mg kg^{-1} h^{-1}, i.v. for 72 h; ■) or (c and d) a combination of nitroglycerin (1 mg kg^{-1} h^{-1}, i.v. for 72 h) and N-benzoyl-staurosporine (30 mg kg^{-1}, p.o. daily) (●) on the heart rate (a and c) and blood pressure (b and d) of conscious rats (n = 5–9 per group).

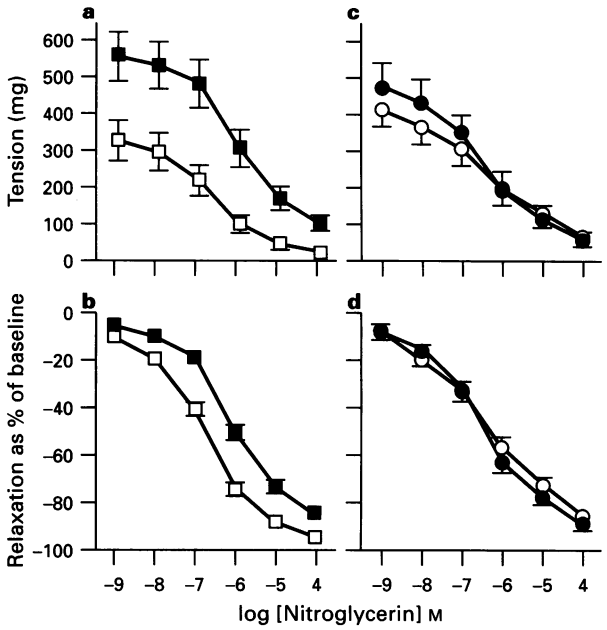

Figure 2 Effect of nitroglycerin (NTG) on the relaxation of rat isolated aortae, precontracted with 10^{-7} M phenylephrine, expressed as either absolute tension (a and c) or as a % of baseline (b and d). Rats (n = 5–9 per group) were administered either vehicle (ethanol/ 0.9% NaCl; □, ○), NTG (1 mg kg^{-1} h^{-1}, i.v. for 72 h; ■), or a combination of NTG (1 mg kg^{-1} h^{-1}, i.v. for 72 h) and N-benzoyl-staurosporine (30 mg kg^{-1}, p.o. daily) (●).

tolerance. N-benzoyl-staurosporine is a potent inhibitor of the conventional calcium-dependent subtypes of PKC (-α, -βI, -βII and -γ) with an IC_{50} between 17 and 32 nM (Marte *et al.*, 1994), being with little effect on nonconventional PKC (-δ, -ε, or -η), as well as being more selective than the parent compound. Thus, the simplest hypothesis is that activation of one or more PKC subtypes may be involved in the reversible inactivation of NTG metabolizing enzymes, leading to tolerance. Furthermore, preliminary studies with 6-benzyl-N-benzoyl-staurosporine (30 mg kg^{-1} day^{-1}), a derivative devoid of PKC inhibiting activity (see Marte *et al.*, 1994) showed that it did not prevent the elevated tension or change in EC_{50} values after NTG (data not shown). Taken together, these results support the hypothesis that PKC activation plays a major role in the development of vascular NTG tolerance, ie., the rightward shift of the NTG curve *in vitro*. Our results are also in agreement with those of Münzel (Münzel *et al.*, 1995) who found reversal of nitrate-induced supersensitivity to a variety of vasoconstrictor agents.

The absolute developed tension in response to phenylephrine of tolerant aortic rings was significantly greater as compared with control rings. Interestingly, this was also the case when structurally different NO-donors such as Na-nitroprusside were used (1 mg kg^{-1} h^{-1} over 3 days), which did not show tolerance in this assay (data not shown). Thus, the relatively high maximal response of aortic rings to phenylephrine was common to any NO-donor pretreatment, whether or not this pretreatment resulted in NTG tolerance. Therefore, this enhanced response to phenylephrine was not causally related to NTG tolerance, but one can speculate that it may be related to some consequence of chronic vasodilatation *per se*. A role for endothelin in this enhanced maximal response has been suggested (Münzel *et al.*, 1995) in rabbit studies with NTG. However, as stated above, whether this is truly specific to NTG and the phenomenon of NTG tolerance requires further examination.

It is important to note that in the present study the NTG sensitivity of large conductance arteries was restored by N-benzoyl-staurosporine. Whether or not small resistance vessels are also affected, remains to be determined. From the *in vivo* data we can conclude that at least there was no dramatic effect on the resistance vessels, since blood pressure was not affected when N-benzoyl-staurosporine was administered. In addition, N-benzoyl-staurosporine given alone at even higher doses did not affect any circulatory parameter (data not shown). However, the sustained increase in heart rate in the combination group might point to some effect on small vessels resulting in a reflex tachycardia. On the other hand, the sustained increase in heart rate may be an indicator of the lack of tolerance development in this group.

In conclusion, the administration of N-benzoyl-staurosporine *in vivo* prevented the development of NTG tolerance in rat isolated aortic rings, suggesting that PKC activation plays an important role in NTG tolerance, the precise mechanism of which remains to be elucidated. This may have important clinical implications and suggests interventions to prevent NTG tolerance.

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