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Short communication

Tetrahydrobiopterin improves endothelium-dependent vasodilation in nitroglycerin-tolerant rats

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Abstract

Tolerance to nitroglycerin is caused by a nitroglycerin-mediated increase in vascular superoxide anion production. Administration of tetrahydrobiopterin (co-factor for endogenous nitric oxide (NO) formation) may potentially influence nitroglycerin tolerance in at least two different ways. Firstly, tetrahydrobiopterin may act as a scavenger of the nitroglycerin-mediated production of superoxide anions. Secondly, tetrahydrobiopterin may protect endothelial NO synthesis from the deleterious effects of increased oxidative stress. This study investigates whether in vivo nitroglycerin tolerance is affected by tetrahydrobiopterin supplementation and assesses the in vivo role of tetrahydrobiopterin in endogenous NO-mediated vasodilation in normal and nitroglycerin-tolerant rats. The results show that tetrahydrobiopterin does not affect nitroglycerin-derived, NO-mediated vasodilation, but reduces baseline mean arterial blood pressure (by 8 mm Hg, $P < 0.05$) and normalizes endothelium-dependent responses to N^G-monomethyl-L-arginine (L-NMMA) (from 7 ± 1 to 22 ± 4 mm Hg, $P < 0.05$) in nitroglycerin-tolerant rats. It is concluded that altered bioavailability of tetrahydrobiopterin is involved in the pathophysiology of endothelial dysfunction seen in nitroglycerin tolerance.

Keywords: Nitroglycerin; Tetrahydrobiopterin; N^G-monomethyl-L-arginine (L-NMMA); Nitric oxide (NO); Nitric oxide (NO) synthase

1. Introduction

Nitroglycerin is important in the treatment of patients with heart diseases. However, continuous administration comprises a clinical problem, due to the rather quickly declining vasodilatory effects of nitroglycerin, so-called "nitroglycerin tolerance." The exact mechanisms underlying this phenomenon are unknown, but nitroglycerin tolerance is accompanied by an increase in vascular superoxide anion (O_2^-) concentrations (Münzel et al., 1995), which through accelerated degradation of the nitroglycerin-derived nitric oxide (NO), may be responsible for nitroglycerin tolerance development (Münzel et al., 1995; Dikalov et al., 1998). Moreover, nitroglycerin-driven increments in vascular O_2^- may exhaust anti-oxidative defense mechanisms protecting the functional integrity of critical co-factors (e.g. tetrahydrobiopterin) regulating the activity of the endogenous arginine–NO pathway. Accordingly, several data (Laursen et al., 1996; Münzel et al., 2000) support the view that prolonged nitroglycerin administration may attenuate the endogenous NO-mediated vasodilatory capacity, and thus, favor vasoconstrictor forces involved in the regulation of vascular tone. The overall importance of this latter mechanism in relation to the phenomenon of nitroglycerin tolerance is, however, not clear.

Endothelial NO synthase (eNOS) activity depends on the presence of essential co-factors. Ex vivo administration of the co-factor, tetrahydrobiopterin, has been shown to improve endothelium-dependent, NO-mediated vasodilation (Wever et al., 1997), suggesting a critical role of this substance in the regulation of endogenous NO. More importantly, tetrahydrobiopterin is also capable of scavenging O_2^- (Schmidt et al., 1992). Based on its dual properties as an essential co-factor during endogenous NO formation and as a target of increased O_2^- levels, it is hypothesized that tetrahydrobiopterin administration may obstruct two of the important mechanisms contributing to nitroglycerin tolerance development.

The present study was carried out (1) to investigate whether in vivo nitroglycerin tolerance is affected by intravenous tetrahydrobiopterin supplementation and (2) to assess the in vivo role of tetrahydrobiopterin in endoge-

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nous NO-mediated vasodilation in normal and nitroglycerin-tolerant animals.

2. Materials and methods

2.1. Animal model

Male Wistar rats (250–300 g) were anesthetized by subcutaneous injection of Hypnorm/Dormicum for a chronic catheterization procedure. Through the right internal carotid artery, one catheter (medical-grade Tygon tubing) was placed with its tip in the ascending aorta, while the tips of the two other catheters were placed in the superior vena cava via the right jugular vein. The catheters were immediately externalized through the neck skin, and all three catheters were subsequently filled with a solution containing 0.5 ml glucose (500 g/l), heparin (100 IU) and streptokinase (5000 IU). Following the catheter implantation, the rats were housed individually (12 h light/12 h dark) with unlimited amounts of standard rat chow and tap water. Post-operatively, the rats were allowed to recover for at least 5 days in order to obtain their pre-operative weight. Details about this rat model with chronically catheterized conscious rats have been described previously (Boesgaard et al., 1991). All experiments conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

2.2. Induction of nitroglycerin tolerance

At the end of the recovery period (day 0), an osmotic minipump (Alza) was placed subcutaneously and connected to one of the intravenous catheters. Nitroglycerin or nitroglycerin/placebo (98% ethanol (nitroglycerin vehicle)) was delivered from the minipump at a constant rate of 0.2 mg/h, i.v., (10 μl/h) for 3 days (day 3). In this model, which previously has been described in detail (Boesgaard et al., 1991, 1994), nitroglycerin tolerance to the blood pressure-lowering effect of nitroglycerin develops within 24 h.

2.3. Hemodynamic response

The hemodynamic response to nitroglycerin was estimated from the blood pressure-lowering effect of intravenous bolus doses of nitroglycerin (2.5 mg nitroglycerin/kg) before implantation of the minipump (day 0) and, at day 3, in all treatment groups. The total volume of each bolus dose was 0.4 ml. At days 0 and 3, blood pressure during baseline conditions (before bolus nitroglycerin administration) and after treatment interventions (nitroglycerin, tetrahydrobiopterin/placebo, eNOS inhibition) was recorded continuously by pressure transducers (Baxter, Uden, Holland) connected to the arterial catheter and monitored via MacLab/8s system.

2.4. Treatment groups

A total of 30 conscious, unrestrained, chronically catheterized rats were studied in five different groups (three groups with nitroglycerin infusion ($n = 3 \times 6$), one group with nitroglycerin-vehicle infusion ($n = 6$) and one group of untreated animals ($n = 6$). All interventions and corresponding control experiments were done in randomized order.

2.4.1. Effect of tetrahydrobiopterin on the hypotensive response to nitroglycerin in normal and nitroglycerin-tolerant rats

Two groups of rats were investigated. Six rats received a long-term infusion of nitroglycerin (0.2 mg/h) for 3 days (nitroglycerin group). Six other rats received nitroglycerin/placebo infusion (nitroglycerin vehicle) for a similar period of time (placebo group). The hypotensive effect of a nitroglycerin bolus was examined at day 0 (before the start of the prolonged nitroglycerin infusion) and after 3 days. Finally, both groups additionally received a 2-h intravenous infusion of tetrahydrobiopterin (8 mg/kg/h). This tetrahydrobiopterin dose is well above the doses previously shown to increase eNOS activity in rats (Shinozaki et al., 2000), but has no effect on the resting blood pressure, per se. Controls, testing the effect of the tetrahydrobiopterin vehicle (isotonic saline) on nitroglycerin responsiveness, were omitted since saline has no effect on the resting mean arterial blood pressure or nitroglycerin responsiveness in this model (Boesgaard et al., 1991, 1993, 1994; Laursen et al., 1996, 1997; Bang et al., 1998, 1999). After the 2-h tetrahydrobiopterin infusion period, a third nitroglycerin bolus dose was given to all rats in both groups.

2.4.2. Effect of tetrahydrobiopterin on endogenous NO-mediated vasodilation in normal and nitroglycerin-tolerant rats

Three groups of animals were investigated. On day 3 of the constant nitroglycerin infusion and after the nitroglycerin bolus dose, two groups of rats (2×6) received an intravenous 6.5-mg/kg bolus dose of the eNOS inhibitor N^G-monomethyl-L-arginine (L-NMMA). This dose of L-NMMA has previously been shown to induce an acute rise in resting mean arterial blood pressure by 25–30 mm Hg in conscious, chronically catheterized Wistar rats (as in this study) (Filep et al., 1993). The effect of L-NMMA is short-lasting, peaking within 1–3 min. Thirty minutes later, when blood pressure was completely normalized, tetrahydrobiopterin (8 mg/kg/h, $n = 6$) or the corresponding placebo (isotonic saline, $n = 6$) was infused for 2 h and the peak hypertensive response to L-NMMA was

repeated immediately after the tetrahydrobiopterin/placebo infusion period. A group of normal, catheterized but otherwise non-medicated rats ($n = 6$) were likewise tested twice with L-NMMA, once before and once following a 2-h tetrahydrobiopterin infusion.

2.5. Drugs

Nitroglycerin was prepared from a stock solution (100 mg nitroglycerin/ml, 98% ethanol). Tetrahydrobiopterin and L-NMMA were purchased from Sigma–Aldrich, Denmark, and were respectively dissolved in oxygen-free 0.9% NaCl and distilled H_2O.

2.6. Statistics

Mean arterial blood pressure was estimated as diastolic pressure plus (systolic minus diastolic pressure)/3, in mm Hg. The reported alterations in mean arterial blood pressure represent the difference between the baseline mean arterial blood pressure value (immediately before an intervention) and the nadir/peak on the blood pressure curve after the intervention. All data are presented as mean ± S.E.M. Differences between pre- and post-treatment means within each treatment group were determined by paired Student's *t*-test. Comparisons among experimental groups were done by unpaired Student's *t*-tests. Statistical significance was assumed when $P < 0.05$.

3. Results

3.1. Induction of nitroglycerin tolerance

In the three groups of rats infused with nitroglycerin for 3 days, development of nitroglycerin tolerance was confirmed by a 95%, 92% and 95% reduction (compared with pre-infusion) in the hypotensive effect of a nitroglycerin bolus (nitroglycerin groups: from 24 ± 5 to 1 ± 1 mm Hg ($n = 3 \times 6$), $P < 0.05$). The response to nitroglycerin in the placebo group ($n = 6$) infused with the nitroglycerin vehicle did not change during the 3-day infusion period (fall in mean arterial blood pressure before 23 ± 3 mm Hg, after 21 ± 2 mm Hg, $P > 0.05$). Baseline mean arterial blood pressure values (before nitroglycerin bolus challenges) were similar in all four infusion groups and did not change during the 3-day nitroglycerin/placebo infusion period in any of the treatment groups (day 0 vs. day 3, nitroglycerin groups: 109 ± 4 vs. 108 ± 6 mm Hg ($n = 3 \times 6$); placebo group: 100 ± 6 vs. 107 ± 6 mm Hg ($n = 6$), $P < 0.05$).

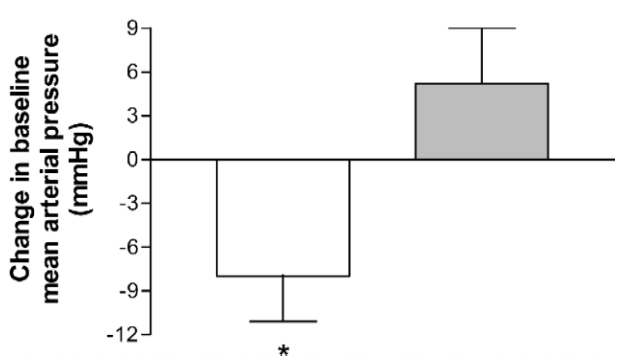

Fig. 1. Effects of a 2-h tetrahydrobiopterin infusion (8 mg/kg/h) on the baseline mean arterial blood pressure (MAP) in nitroglycerin-tolerant and non-tolerant rats ($n = 6$ in each group). □ Nitroglycerin-tolerant rats; ▨ non-tolerant rats (placebo group); * significantly different from the non-tolerant group.

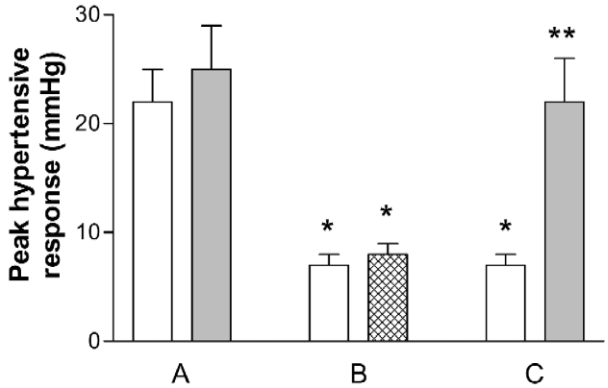

Fig. 2. Peak hypertensive response (mm Hg) to L-NMMA (6.5 mg/kg) in normal non-tolerant rats and nitroglycerin-tolerant rats before (□) and after a 2-h infusion of tetrahydrobiopterin (▨) or tetrahydrobiopterin/placebo (⊠). (A) Normal non-tolerant rats before and after tetrahydrobiopterin ($n = 6$); (B) nitroglycerin-tolerant rats before and after tetrahydrobiopterin placebo ($n = 6$); (C) nitroglycerin-tolerant rats before and after tetrahydrobiopterin ($n = 6$). * Significantly different from normal non-tolerant rats. ** Significantly different from the response before the infusion of tetrahydrobiopterin.

3.2. Effect of tetrahydrobiopterin on the hypotensive response to nitroglycerin in normal and nitroglycerin-tolerant rats

Infusion of tetrahydrobiopterin for 2 h in nitroglycerin vehicle-treated rats (placebo group) and nitroglycerin-tolerant rats (nitroglycerin group) did not affect the hemodynamic response to a nitroglycerin bolus dose. In the nitroglycerin-tolerant group, the fall in mean arterial blood pressure to a bolus dose of nitroglycerin was 1 ± 1 mm Hg before, and 0 ± 0 mm Hg after tetrahydrobiopterin infusion. The comparable responses in the placebo group were 21 ± 2 and 21 ± 2 mm Hg, respectively, suggesting that tetrahydrobiopterin, neither in normal nor in nitroglycerin-tolerant rats, influences the response to exogenous nitroglycerin-derived NO. In contrast, baseline mean arterial blood pressure in the nitroglycerin-tolerant group (but not the placebo group) was significantly lowered (−8 ± 3 mm Hg) by tetrahydrobiopterin (Fig. 1). This finding is compatible with the assumption that nitroglycerin tolerance,

through inhibition of endogenous NO formation, affects the basal regulation of vascular tone.

3.3. Effect of tetrahydrobiopterin on endogenous NO-mediated vasodilation in normal and nitroglycerin-tolerant rats

A tetrahydrobiopterin-mediated effect on vascular NO formation in nitroglycerin-tolerant animals was further substantiated by the response to L-NMMA, both before and following tetrahydrobiopterin/placebo infusions. In these experiments, the diminished peak hypertensive response to L-NMMA in the nitroglycerin-tolerant rats (7 ± 1 mm Hg ($n = 2 \times 6$) vs. normal non-medicated controls 22 ± 3 mm Hg, ($n = 6$), $P < 0.05$) was significantly increased and completely normalized by tetrahydrobiopterin infusion (before, 7 ± 2 mm Hg, after tetrahydrobiopterin, 22 ± 4 mm Hg ($n = 6$), whereas a 2-h infusion of tetrahydrobiopterin placebo did not affect the response to L-NMMA (before, 8 ± 1 mm Hg, after, 8 ± 1 mm Hg, ($n = 6$), $P < 0.05$) (Fig. 2). Tetrahydrobiopterin did not change the effect of L-NMMA in normal rats (Fig. 2).

4. Discussion

Nitroglycerin-induced vascular formation of O_2^- appears to be the major mechanism underlying the development of nitroglycerin tolerance (Münzel et al., 1995). Because NO and O_2^- rapidly interacts to produce peroxynitrite ($ONOO^-$), the bioavailability of vascular NO is reduced, attenuating the vasorelaxant effect of both exogenous and endogenous nitroglycerin-derived NO (Laursen et al., 1996). The major finding of this study is that in vivo nitroglycerin tolerance is also associated with impaired endogenous NO formation, which can be counteracted by infusion of tetrahydrobiopterin.

The production of NO is catalyzed by NOS. The constitutively active form of NOS in endothelial cells eNOS requires arginine as a substrate and several co-factors, including tetrahydrobiopterin, for normal activity. Tetrahydrobiopterin is synthesized through two different pathways. The de novo pathway utilizes guanosine triphosphate (GTP) as its precursor, while the salvage pathway utilizes dihydropterins (Nichol et al., 1985). Biosynthesis of tetrahydrobiopterin depends on a normal cellular redox state, and oxidative stress impairs the endothelial recycling of tetrahydrobiopterin (Komori et al., 1995). Moreover, tetrahydrobiopterin's scavenging effect on O_2^- and other toxic radicals may result in enhanced oxidation of tetrahydrobiopterin, and thus, interact with the role of tetrahydrobiopterin as a redox agent in the synthesis of NO (Nichol et al., 1985; Kojima et al., 1995). In addition, $ONOO^-$, produced by the interaction between NO and O_2^-, is a rapid tetrahydrobiopterin oxidizer, whereby tetrahydrobiopterin looses its co-factor capability (Milstien and Katusic, 1999). Under physiological conditions, there is a balance between the endothelial production of NO and the vascular oxygen-derived free radicals. In various conditions characterized by endothelial dysfunction (e.g. smokers, hypercholesterolemia, atherosclerosis) (Stroes et al., 1997; Shinozaki et al., 2000; Ueda et al., 2000; Tiefenbacher et al., 2000), this balance cannot be maintained and the production of vasotoxic oxygen species, including O_2^- (Ohara et al., 1993; Shinozaki et al., 2000), is increased. This phenomenon has been linked to the reduced availability of tetrahydrobiopterin (Shinozaki et al., 2000) and supports the assumption that there is a close relationship among the vascular concentrations of O_2^-, $ONOO^-$ and tetrahydrobiopterin (Milstien and Katusic, 1999).

In the present study, marked tolerance to the hemodynamic effects of nitroglycerin developed after the intravenous infusion of nitroglycerin in conscious rats. However, not only the response to nitroglycerin but also the response to the L-arginine analog, L-NMMA, was significantly attenuated, suggesting the existence of an interaction between nitroglycerin tolerance and endogenous NO-mediated responses. Infusion of tetrahydrobiopterin significantly increased the hemodynamic effect of L-NMMA and reduced resting mean arterial blood pressure in nitroglycerin-tolerant animals only.

Tetrahydrobiopterin administration may potentially influence nitroglycerin tolerance in at least two different ways. Firstly, tetrahydrobiopterin may act as a scavenger of the nitroglycerin-mediated increased production of reactive oxygen species. Secondly, tetrahydrobiopterin administration may protect endothelial NO synthesis from the deleterious effects of increased oxidative stress. In vitro administration of nitroglycerin may inhibit endothelial NO synthase (Buga et al., 1993) and it is tempting to speculate that a prolonged nitroglycerin-induced increase in vascular O_2^-, through negative feed-back regulation on endothelial NO synthase, decreases endogenous vasodilator forces—an effect which can be counteracted by tetrahydrobiopterin administration. This hypothesis may account for both the blood pressure-lowering effect of tetrahydrobiopterin in nitroglycerin-tolerant rats and the observed reduction in endothelium-dependent, NO-mediated vasoreactivity (L-NMMA), which could be reversed by tetrahydrobiopterin. The reduced response to L-NMMA suggests that nitroglycerin tolerance inhibits endogenous NO synthase activity and/or that basal-produced NO is rapidly inactivated. It is known that the bioconversion of nitroglycerin to NO remains unaltered despite development of nitroglycerin tolerance (Laursen et al., 1997). Thus, the lack of any tetrahydrobiopterin-mediated effect on responses to exogenous nitroglycerin-derived NO (nitroglycerin bolus doses) supports a primary and specific role of tetrahydrobiopterin on the endogenous NO synthesis. This is in line with previous experiments (Shinozaki et al., 2000) in insulin-resistant rats (characterized by endothelial dysfunction and increased vascular oxidative stress), showing unaltered effect

of NO, per se, but increased eNOS activity after oral tetrahydrobiopterin administration.

By comparing experimental findings in nitroglycerin tolerance, insulin resistance, hyperlipidemia and human atherosclerosis, it is interesting to note that these conditions share several characteristics, e.g. increased vascular O_2^- formation, endothelial dysfunction and restoration of endothelial function after tetrahydrobiopterin administration (Ohara et al., 1993; Shinozaki et al., 2000; Tiefenbacher et al., 2000). The present data suggest that nitroglycerin tolerance not only reduces the nitroglycerin-mediated hemodynamic effects but may also have detrimental effects on intrinsic vascular homeostasis. Because endothelial dysfunction occurs early in the development of cardiovascular disease, attempts to improve endothelial function during nitroglycerin therapy may be of significant importance in the prevention of this shift in an "atherosclerotic" direction.

In summary, this study shows (1) that hemodynamic tolerance to nitroglycerin is associated with an in vivo impairment of the L-arginine–NO pathway and (2) that intravenous infusion of tetrahydrobiopterin to nitroglycerin-tolerant animals restores endogenous NO-mediated vasodilation in vivo. We conclude that altered bioavailability of tetrahydrobiopterin is involved in the pathophysiology of endothelial dysfunction seen after the development of tolerance to nitroglycerin.

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