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Direct myocardial anti-ischaemic effect of GTN in both nitrate-tolerant and nontolerant rats: a cyclic GMP-independent activation of K_{ATP}

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1 We have recently demonstrated that glyceryl trinitrate (GTN) exerts a direct myocardial anti-ischaemic effect in both GTN-tolerant and nontolerant rats. Here we examined if this effect is mediated by GTN-derived nitric oxide (NO) and involves guanosine 3′5′ cyclic monophosphate (cyclic GMP) and ATP-sensitive K^+ channels (K_{ATP}).

2 Rats were treated with 100 mg kg^{-1} GTN or vehicle s.c. three times a day for 3 days to induce vascular GTN-tolerance or nontolerance. Isolated working hearts obtained from either GTN-tolerant or nontolerant rats were subjected to 10 min coronary occlusion in the presence of 10^{-7} M GTN or its solvent.

3 GTN improved myocardial function and reduced lactate dehydrogenase (LDH) release during coronary occlusion in both GTN-tolerant and nontolerant hearts.

4 Cardiac NO content significantly increased after GTN administration in both GTN-tolerant and nontolerant hearts as assessed by electron spin resonance. However, cardiac cyclic GMP content measured by radioimmunoassay was not changed by GTN administration.

5 When hearts from both GTN-tolerant and nontolerant rats were subjected to coronary occlusion in the presence of the K_{ATP}-blocker glibenclamide (10^{-7} M), the drug itself did not affect myocardial function and LDH release, however, it abolished the anti-ischaemic effect of GTN.

6 We conclude that GTN opens K_{ATP} *via* a cyclic GMP-independent mechanism, thereby leading to an anti-ischaemic effect in the heart in both GTN-tolerant and nontolerant rats.

Keywords: Glyceryl trinitrate; tolerance; nitric oxide; electron spin resonance; cyclic GMP; glibenclamide

Abbreviations: cyclic GMP, guanosine 3′5′ cyclic monophosphate; GTN, glyceryl trinitrate; K_{ATP}, ATP-sensitive K^+-channel; LDH, lactate dehydrogenase; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; MGD, N-methyl-D-glucoseamine-dithio-carbamate; NO, nitric oxide

Introduction

Glyceryl trinitrate (GTN) has been widely used in the treatment of ischaemic heart disease for more than 100 years. The anti-ischaemic effect of GTN is believed to be based on the drug-induced decrease in preload and afterload, improvement of coronary collateral flow, dilation of stenotic coronary arteries, and inhibition of platelet aggregation (see for review: Harrison & Bates, 1993). It is well accepted that the enzymatic bioconversion of GTN to nitric oxide (NO) and the consequent increase in guanosine 3′5′ cyclic monophosphate (cyclic GMP) is responsible for the vascular effects of GTN (see for review: Moncada *et al*., 1991). Continuous administration of organic nitrates including GTN results in the development of tolerance to their haemodynamic effects which limits their clinical application. The mechanisms leading to vascular nitrate tolerance may include neurohormonal counterregulatory mechanisms to maintain blood pressure (see for review: Mangione & Glasser, 1994), increased production of superoxide anions (Münzel *et al*., 1995), reduced biotransformation of GTN to NO, and alterations in cyclic GMP metabolism (see for review: Axelsson & Ahlner, 1987).

We have previously demonstrated that a nonvasodilatory concentration of GTN exerts a direct myocardial anti-ischaemic effect independent of its vascular actions in isolated rat hearts (Ferdinandy *et al*., 1995a) and in conscious rabbits (Szilvássy *et al*., 1997). This effect of GTN was not diminished in vascular nitrate tolerance (Ferdinandy *et al*., 1995a; Szilvássy *et al*., 1997). The mechanism of the direct myocardial action of GTN has not been resolved yet. NO has been shown to open ATP-sensitive K^+-channels (K_{ATP}) in isolated mesenteric arteries (Murphy & Brayden, 1995) and in isolated pancreatic islets (Antoine *et al*., 1997). Activation of these channels has been found to exert protection on the ischaemic/reperfused heart (Ferdinandy *et al*., 1995b; Grover *et al*., 1989). Although the effects of GTN on vascular smooth muscle are thought to be mediated by cyclic GMP, we have recently shown that the level of NO in the heart does not correlate with myocardial cyclic GMP content in the rat *in vivo* (Csont *et al*., 1998).

Consequently, we hypothesized that activation of K_{ATP} by GTN-derived NO may play a role in the direct myocardial anti-ischaemic effect of GTN *via* a cyclic GMP-independent pathway. Therefore, in hearts isolated from experimental

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nitrate-tolerant and nontolerant rats, we investigated whether (i) GTN is converted to NO, (ii) this leads to changes in myocardial cyclic GMP level, (iii) and if the K_{ATP}-blocker glibenclamide abolishes the direct myocardial cardioprotective effect of GTN.

Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1985).

Drugs

Glyceryl trinitrate (GTN, 10% w w^{-1}, EGIS Co., Budapest, Hungary) and its vehicle lactose suspended in propylene glycol were used for the induction of nitrate-tolerance/nontolerance. GTN (Pohl-Boskamp, Hochenlockstedt, Germany) and its solvent ethanol were used for the isolated heart perfusions. Glibenclamide (dissolved in ethanol) and norepinephrine were purchased from Sigma (St. Louis, MO, U.S.A.). N-methyl-D-glucoseamine-dithio-carbamate (MGD) was synthesized as described by Shinobu *et al*. (1984).

Induction and verification of nitrate tolerance

Male Wistar rats (300–360 g) were given s.c. 100 mg kg^{-1} GTN and/or its vehicle lactose three times a day for 3 days to induce vascular tolerance to GTN (Silver *et al*., 1991). Rats were used for isolated heart preparations on the fourth day. Development of vascular tolerance to GTN was confirmed on the fourth day by testing endothelium-free thoracic aortic rings for isometric tension as described previously (Ferdinandy *et al*., 1995a; Szilvássy *et al*., 1994b). Rings of 4 mm in length were precontracted with an EC_{50} concentration of norepinephrine in addition to a resting tension of 20 mN. The rings were then exposed to cumulative concentrations of GTN in half-log increments. GTN concentrations required to produce half-maximal relaxation were 0.082 ± 0.013 μM in vehicle treated, non-tolerant rings versus 1.61 ± 0.21 μM in GTN-treated, tolerant ones ($P < 0.05$, $n = 5$ in both groups).

Isolated rat hearts

Rats were anaesthetized with diethylether and given 500 u kg^{-1} heparin. After 30 s, hearts were excised and cannulated through the aorta, and perfused in the Langendorff mode at constant pressure (73.5 mmHg) for 10 min. During this period, the left atrium was cannulated and a suture was placed around the left main coronary artery close to its origin, allowing regional ischaemia to be induced as described (Ferdinandy *et al*., 1995b). Hearts were then converted to a working preparation and perfused at 37°C with oxygenated Krebs-Henseleit bicarbonate buffer (Ferdinandy *et al*., 1995b). Preload (12.75 mmHg) and afterload (73.5 mmHg) were kept constant throughout the experiments. Heart rate, coronary flow, aortic flow, left ventricular developed pressure (LVDP), $+dP/dt_{max}$, $-dP/dt_{max}$, and left ventricular end-diastolic pressure (LVEDP) were monitored as described earlier (Ferdinandy *et al*., 1995b). Coronary effluents were assayed for lactate dehydrogenase (LDH) activity by means of an automatic analyser (Hitachi-911) which uses Boehringer-Mannheim kits. LDH release was expressed as mu min^{-1} g^{-1} (wet tissue weight). Ischaemic area of the left ventricle was determined by a dye exclusion method after each experiment (Curtis & Hearse, 1989).

Experimental design

To assess the anti-ischaemic effect of GTN, after 10 min of aerobic working perfusion, hearts of nitrate-tolerant and nontolerant rats were subjected to 10 min coronary occlusion in the presence of a nonvasodilatory concentration of GTN (10^{-7} M) and its solvent ethanol (final concentration: 2.2×10^{-3}% v v^{-1}). Measurement of cardiac function was performed before ischaemia and at the end of coronary occlusion. LDH release was determined at the end of coronary occlusion. According to our preliminary experiments and our previous studies (Ferdinandy *et al*., 1995a), the concentration of 10^{-7} M GTN was selected for the present study, since this was the highest nonvasoactive concentration of the drug which did not affect pre-ischaemic myocardial function and coronary flow in the isolated rat heart. GTN at concentrations $> 10^{-7}$ M concentration-dependently decreased LVDP, $+dP/dt_{max}$, and $-dP/dt_{max}$ in the nonischaemic heart (data not shown). In separate experiments, we examined if GTN influences myocardial NO and cyclic GMP levels in hearts isolated from GTN-tolerant or nontolerant animals. Finally, to study the involvement of K_{ATP} in the effect of GTN, the interaction of 10^{-7} M glibenclamide with GTN was examined in hearts isolated from GTN-tolerant or nontolerant rats and subjected to coronary occlusion. According to our preliminary experiments (data not shown) and our previous studies, this concentration of glibenclamide alone did not significantly affect myocardial function, however, it abolished the cardioprotective effect of the most effective concentration of the K_{ATP} activator cromakalim in this model (Ferdinandy *et al*., 1995b).

Measurement of cardiac NO

To study if GTN is converted to NO in the myocardium, cardiac NO content was measured with electron spin resonance spectroscopy in the GTN-treated and in the solvent-treated hearts isolated from either GTN-tolerant or nontolerant rats. An aqueous solution of the spin-trap for NO, $Fe^{2+}(MGD)_2$, was prepared freshly before each experiment. MGD (175 mg) and $FeSO_4 \cdot 7H_2O$ (33.4 mg) were dissolved in distilled water (pH = 7.4, final volume: 6 ml). After 10 min of aerobic perfusion, $Fe^{2+}(MGD)_2$ solution was infused into the heart through the aortic cannula under Langendorff perfusion for 5 min at a rate of 1 ml min^{-1}. Tissue samples from the apex of the heart (approximately 150 mg) were then placed into quartz electron spin resonance tubes and frozen immediately in liquid nitrogen. Samples were assayed for electron spin resonance spectra of the relatively stabile NO-$Fe^{2+}(MGD)_2$ adduct. The detection limit of NO by this electron spin resonance method is approximately 0.05 nmol g^{-1} (wet tissue weight) (Mülsch *et al*., 1992). Electron spin resonance spectra were recorded with a Bruker ECS106 (Rheinstetten, Germany) spectrometer operating at X band with 100 kHz modulation frequency at a temperature of 160 K, using 10 mW microwave power to avoid saturation. Scans were traced with 2.85 G modulation amplitude, 340 G sweep width, and 3356 G central field as described (Ferdinandy *et al*., 1997a,b; Mülsch *et al*., 1992). After subtraction of background signals, analysis of NO content was performed with double integration of the NO signal as described (Csont *et al*., 1998).

Measurement of cardiac cyclic GMP

To examine if GTN increases myocardial cyclic GMP content, in separate studies, cardiac cyclic GMP concentration was measured in solvent and GTN-treated hearts in both the nitrate-tolerant and the nontolerant groups. After 10 min aerobic perfusion, left ventricular tissue mass was frozen by means of a Wollenberger clamp prechilled in liquid nitrogen. Samples were then homogenized and centrifuged and the supernatants were extracted six times in water-saturated diethylether, evaporated, and assayed for cyclic GMP by radioimmunoassay using Amersham kits (Szilvássy *et al*., 1994a,b).

Statistics

Data were expressed as means ± s.e.mean and analysed with one way analysis of variance. If a difference was established, each group was compared to the solvent-treated group using a modified *t*-test corrected for simultaneous multiple comparisons according to the Bonferroni method (Wallenstein *et al*., 1980).

Results

Effect of GTN on myocardial function and LDH release

In the nontolerant, solvent-treated group, 10 min coronary occlusion resulted in a marked decrease in coronary flow (Table 1), aortic flow (Figure 1A), LVDP (Figure 2A), $+dP/dt_{max}$ (Figure 3A), and $-dP/dt_{max}$ (Table 2), an increase in LVEDP (Figure 4A), and a significant release of LDH (Figure 5A). GTN at 10^{-7} M significantly improved ischaemic aortic flow (Figure 1A), LVDP (Figure 2A), $+dP/dt_{max}$ (Figure 3A), and $-dP/dt_{max}$ (Table 2), and decreased LVEDP (Figure 4A) and LDH release (Figure 5A), however, it did not affect pre-ischaemic myocardial function (Figures 1A–5A, Table 2) and pre-ischaemic or ischaemic coronary flow (Table 1). In hearts isolated from GTN-tolerant rats, 10^{-7} M GTN showed a similar anti-ischaemic effect as seen in the nontolerant group (Figures 1B–5B, Table 2). GTN did not affect heart rate, ischaemic area (data not shown), and coronary flow (Table 1).

Effect of GTN on myocardial NO and cyclic GMP contents

Basal level of NO in the myocardium was near the detection limit in the nontolerant group, however, it was significantly higher in the GTN-tolerant group as assessed by electron spin resonance (Figure 6A). GTN-treatment significantly increased cardiac NO content in both the GTN-tolerant and the nontolerant groups (Figure 6A). In contrast to NO, cardiac cyclic GMP content measured by radioimmunoassay was not changed either by the development of GTN-tolerance or by acute GTN treatment (Figure 6B).

Effect of glibenclamide on the anti-ischaemic effect of GTN

Glibenclamide (10^{-7} M) alone did not affect pre-ischaemic and ischaemic coronary flow (Table 1), aortic flow (Figure 1),

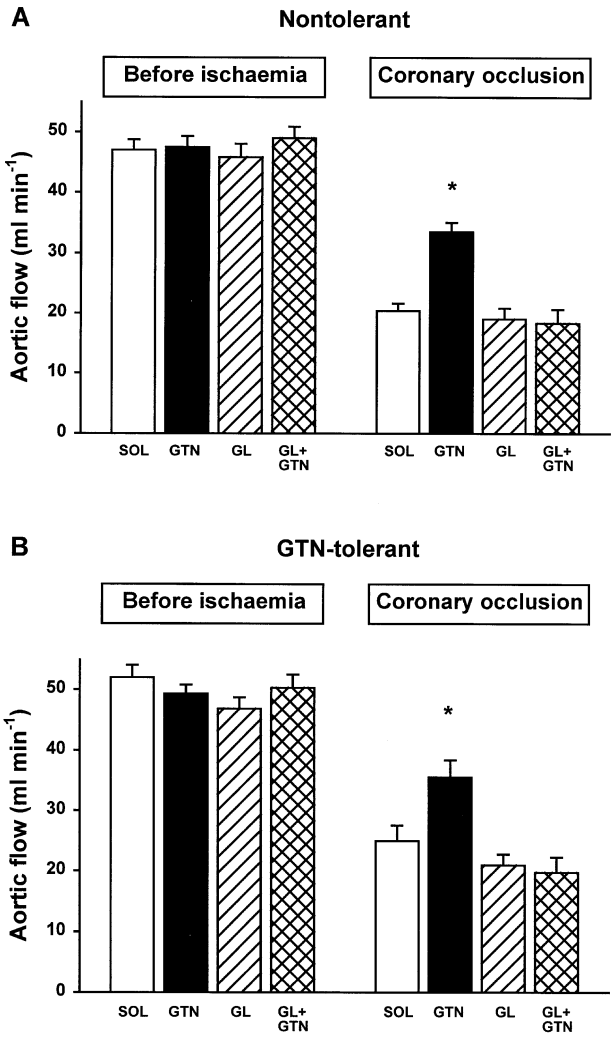

Figure 1 Aortic flow before ischaemia and at the end of coronary occlusion in working hearts isolated from rats with (B) or without (A) vascular tolerance to glyceryl trinitrate (GTN). Hearts were perfused in the presence of GTN (10^{-7} M), glibenclamide (GL, 10^{-7} M), the combination of GL and GTN, and their solvent (SOL), respectively. Data are mean ± s.e.mean. $^*P<0.05$ vs solvent-treated group ($n=7$ in each group).

Table 1 Coronary flow (ml min^{-1}) in isolated working hearts from rats with or without vascular tolerance to glyceryl trinitrate (GTN)

			Non-tolerant		*GTN-tolerant*	
Groups	*Conc.*	n	*Before ischaemia*	*Coronary occlusion*	*Before ischaemia*	*Coronary occlusion*
SOL		7	23.2 ± 0.9	15.6 ± 0.9	23.9 ± 1.1	16.6 ± 1.3
GTN	10^{-7} M	7	24.1 ± 0.8	16.0 ± 0.3	25.2 ± 1.4	16.9 ± 0.6
GL	10^{-7} M	7	22.1 ± 1.4	14.8 ± 0.4	22.9 ± 1.2	15.8 ± 0.4
GL + GTN	10^{-7} M	7	23.0 ± 1.2	15.3 ± 0.5	24.5 ± 1.0	16.4 ± 0.7

Data are mean ± s.e.mean. SOL: solvent, GL: glibenclamide.

LVDP (Figure 2), $+dP/dt_{max}$ (Figure 3), $-dP/dt_{max}$ (Table 2), LVEDP (Figure 4), and ischaemic LDH release (Figure 5) in the GTN-tolerant and the nontolerant groups. Glibenclamide, however, abolished the anti-ischaemic effect of GTN, as ischaemic myocardial function was not improved (Figures 1–4, Table 2) and LDH release was not reduced (Figure 5) when hearts were perfused in the presence of both glibenclamide and GTN either in the GTN-tolerant or the nontolerant groups.

Discussion

Our results show that GTN at 10^{-7} M, a nonvasodilatory concentration in the coronary circulation, exerts a direct myocardial anti-ischaemic effect. This effect of GTN is not diminished by the development of vascular tolerance to GTN. We have also shown that GTN is converted to NO in the hearts isolated from either GTN-tolerant or nontolerant rats, however, myocardial cyclic GMP content is not affected by

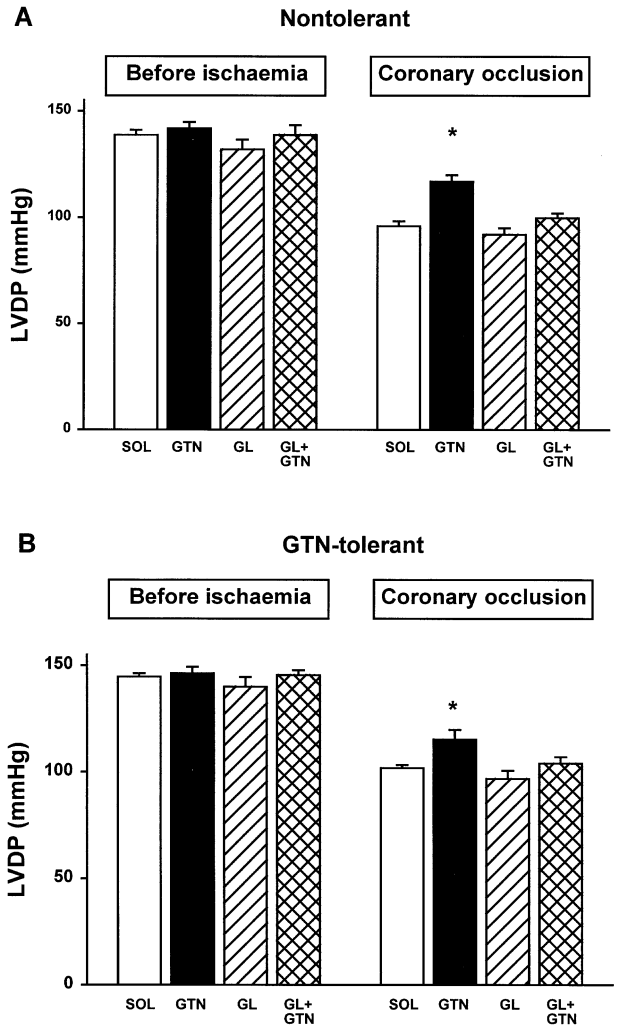

Figure 2 Left ventricular developed pressure (LVDP) before ischaemia and at the end of coronary occlusion in working hearts isolated from rats with (B) or without (A) vascular tolerance to glyceryl trinitrate (GTN). Hearts were perfused in the presence of GTN (10^{-7} M), glibenclamide (GL, 10^{-7} M), the combination of GL and GTN, and their solvent (SOL), respectively. Data are mean ± s.e.mean. *$P<0.05$ vs solvent-treated group ($n=7$ in each group).

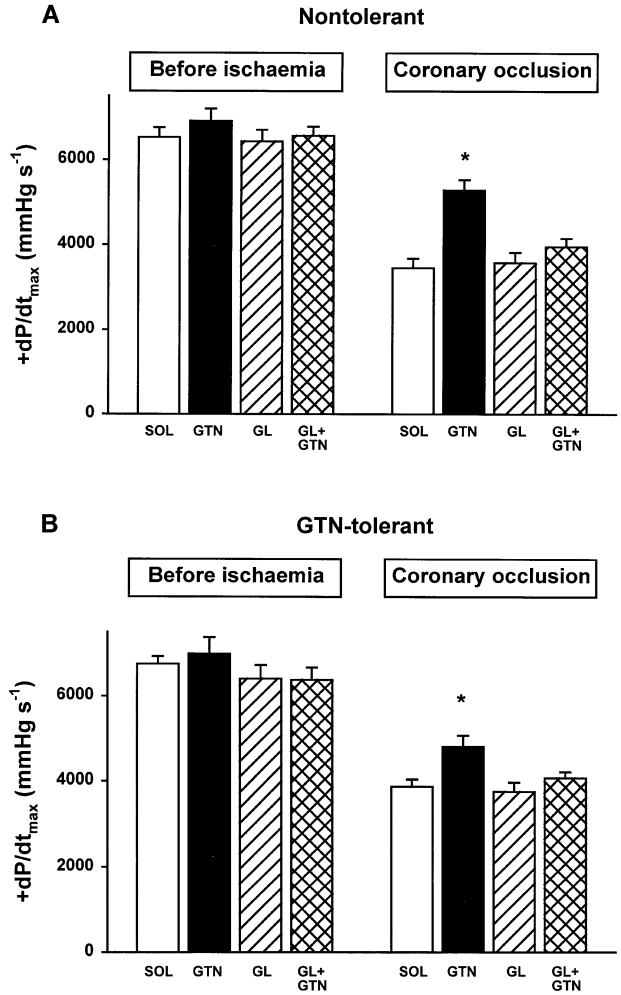

Figure 3 $+dP/dt_{max}$ before ischaemia and at the end of coronary occlusion in working hearts isolated from rats with (B) or without (A) vascular tolerance to glyceryl trinitrate (GTN). Hearts were perfused in the presence of GTN (10^{-7} M), glibenclamide (GL, 10^{-7} M), the combination of GL and GTN, and their solvent (SOL), respectively. Data are mean ± s.e.mean. *$P<0.05$ vs solvent-treated group ($n=7$ in each group).

Table 2 $-dP/dt_{max}$ (mmHg s^{-1}) in isolated working hearts from rats with or without vascular tolerance to glyceryl trinitrate (GTN)

			Non-tolerant		*GTN-tolerant*	
Groups	*Conc.*	n	*Before ischaemia*	*Coronary occlusion*	*Before ischaemia*	*Coronary occlusion*
SOL		7	2985±210	2198±135	3375±263	2588±120
GTN	10^{-7} M	7	3338±135	*2813±98	3067±218	*2842±165
GL	10^{-7} M	7	3125±195	2223±120	3280±235	2550±180
GL+GTN	10^{-7} M	7	3405±210	2377±128	3495±248	2603±113

Data are mean ± s.e.mean. * $P<0.05$ vs solvent-treated group. SOL: solvent, GL: glibenclamide.

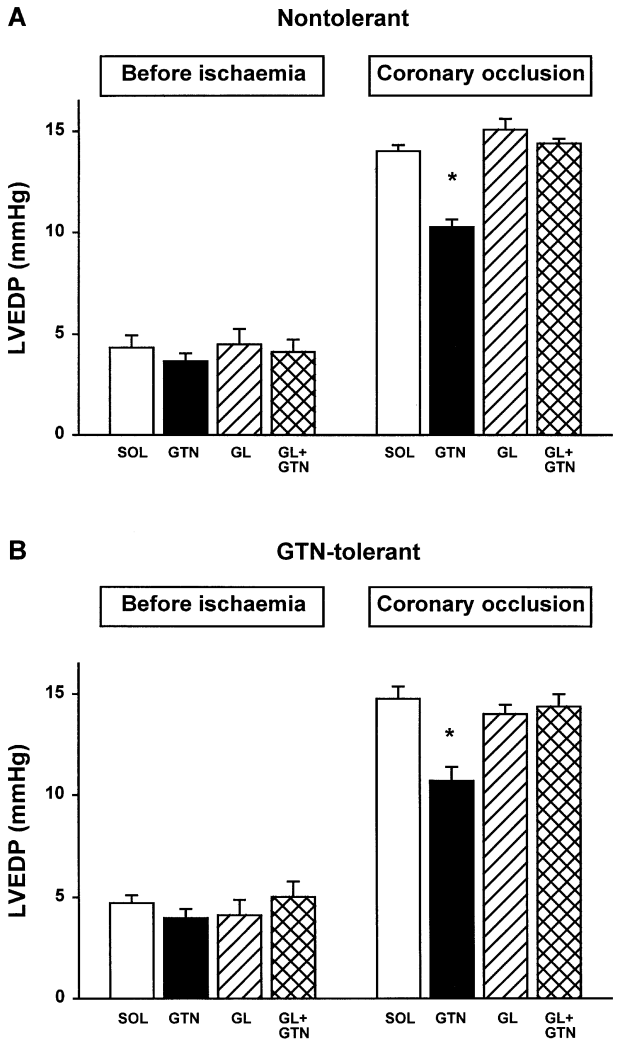

Figure 4 Left ventricular end-diastolic pressure (LVEDP) before ischaemia and at the end of coronary occlusion in working hearts isolated from rats with (B) or without (A) vascular tolerance to glyceryl trinitrate (GTN). Hearts were perfused in the presence of GTN (10^{-7} M), glibenclamide (GL, 10^{-7} M), the combination of GL and GTN, and their solvent (SOL), respectively. Data are mean$\pm$ s.e.mean. *$P<0.05$ vs solvent-treated group ($n=7$ in each group).

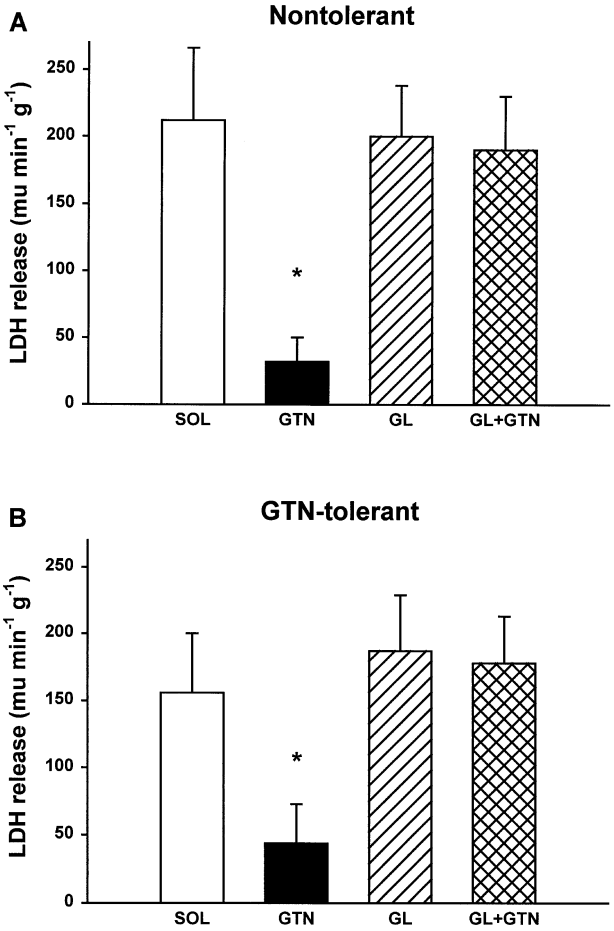

Figure 5 Lactate dehydrogenase (LDH) release at the end of coronary occlusion in working hearts isolated from rats with (B) or without (A) vascular tolerance to glyceryl trinitrate (GTN). Hearts were perfused in the presence of GTN (10^{-7} M), glibenclamide (GL, 10^{-7} M), the combination of GL and GTN, and their solvent (SOL), respectively. Data are mean$\pm$s.e.mean. *$P<0.05$ vs solvent-treated group ($n=7$ in each group).

GTN. Finally, we have provided evidence that blockade of K_{ATP} with glibenclamide abolishes the direct anti-ischaemic effect of GTN. This shows that GTN activates K_{ATP} *via* a cyclic GMP-independent mechanism and thereby confers protection on the ischaemic myocardium. This effect of GTN may be mediated by GTN-derived NO.

Our present results confirmed our previous studies showing that GTN exerts an anti-ischaemic effect on the heart, which is independent from the vascular effects of GTN (Ferdinandy *et al*., 1995a). GTN selectively dilates coronary vessels >100 μm, therefore it has minimal effect on coronary resistance (Harrison & Bates, 1993). Accordingly, 10^{-7} M GTN did not change area of ischaemic zone and failed to affect CF, thus showing that GTN did not affect coronary circulation in our study. GTN was also found cardioprotective in hearts isolated from GTN-tolerant rats. Consequently, the anti-ischaemic effect of GTN involves a direct action on the myocardium in the isolated rat heart. Other NO donor compounds, such as S-nitroso-N-acetyl-DL-penicillamine, 3-morpholino-sydnonimine, and sodium nitroprusside, have been shown to exert cardioprotective action on the normal myocardium (Yasmin *et al*., 1997; Bilinska *et al*., 1996; Draper & Shah, 1997).

Acute treatment with 10^{-7} M GTN did not affect nonischaemic cardiac mechanical function parameters. GTN at $>10^{-7}$ M, however, concentration-dependently decreased both systolic and diastolic contractile function of the isolated rat heart. In contrast, Grocott-Mason *et al*. (1994a,b) reported that either exogenous (sodium nitroprusside) or endogenous NO reduces peak systolic pressure and leads to an earlier onset and increased rate of myocardial relaxation in the isolated guinea-pig heart, however, this was not confirmed by Pabla & Curtis (1996) in the rat isolated heart. These discrepancies might be attributed to species differences and different techniques for measurement of intraventricular pressure.

The physiological effects of NO are believed to be mediated by cyclic GMP, however, recent studies suggest that NO has cyclic GMP-independent actions (see for review: Balligand & Cannon, 1997). We have previously shown that changes in cardiac NO content were not reflected by changes in cyclic GMP level in the rat heart *in vivo* (Csont *et al*., 1998). Accordingly, we have found here that while cardiac NO content significantly increased by GTN administration in both GTN-tolerant and nontolerant hearts, myocardial cyclic GMP content was not changed in *ex vivo* isolated hearts. Similarly to our results, Torfgård *et al*. (1991) have shown that administration of GTN does not affect cardiac cyclic GMP content in the rat heart *in vivo*. This shows that the

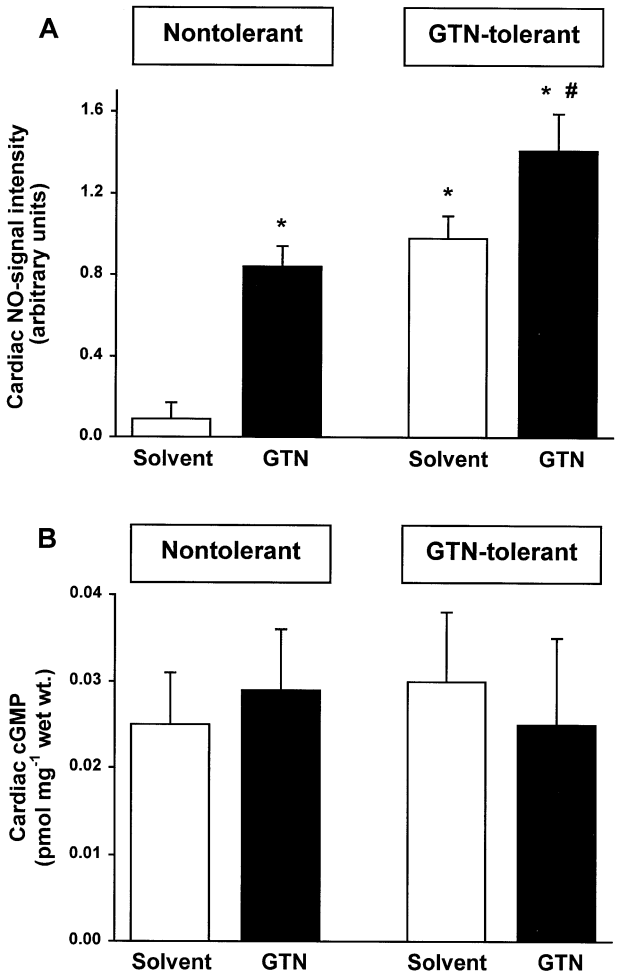

Figure 6 Cardiac NO-signal intensity (A) and cyclic GMP content (B) in hearts isolated from rats with or without vascular tolerance to glyceryl trinitrate (GTN). Hearts were perfused in the presence of GTN (10^{-7} M) or its solvent (SOL), respectively. Data are mean$\pm$ s.e.mean. *$P<0.05$ vs solvent-treated, nontolerant group. #$P<0.05$ vs solvent-treated, GTN-tolerant group ($n=4-5$ in each group).

cardioprotective effect of GTN can not be mediated by cyclic GMP in the heart and confirms that the conversion of GTN to NO is not diminished in GTN-tolerance (Laursen *et al.*, 1996; Csont *et al.*, 1998).

A specific blocker of K_{ATP}, glibenclamide, abolished the direct anti-ischaemic effect of GTN. Consequently, this effect of GTN may involve activation of K_{ATP}. This is a plausible mechanism for the direct myocardial anti-ischaemic effect of GTN, since pharmacological activation of K_{ATP} has been shown by several laboratories to mediate cardioprotection in the rat (Ferdinandy *et al.*, 1995b; Gross & Auchampach, 1992; Grover *et al.*, 1989). Although glibenclamide is widely used and accepted as the most specific blocker of K_{ATP}, it should be noted that the effects of the drug are likely not solely related to its action on K_{ATP} (Yan *et al.*, 1993; Quast, 1993). The involvement of K_{ATP}-independent mechanisms in the direct myocardial cardioprotective effect of GTN cannot be excluded in the present study. The NO donor S-nitroso-N-acetyl-DL-penicillamine at 2×10^{-7} M has been shown to reduce ischaemia/reperfusion injury *via* decreasing the endogenous formation of peroxynitrite in isolated rat hearts (Yasmin *et al.*, 1997). The direct antioxidant properties of NO or NO-generating drugs and their ability to reduce the detrimental actions of authentic peroxynitrite have also been observed by others in a variety of biological systems (Rubbo *et al.*, 1996; Villa *et al.*, 1994; Wink *et al.*, 1993).

NO has been shown to open K_{ATP} in isolated mesenteric arteries (Murphy & Brayden, 1995) and in isolated pancreatic islets (Antoine *et al.*, 1997). The mechanism by which NO may open K_{ATP} in the myocardium is not known. Similarly to our previous studies (Csont *et al.*, 1998), the present results show that basal level of NO in the myocardium was significantly increased in the GTN-tolerant hearts as compared to the nontolerant ones. These hearts, however, were not protected against ischaemia. Nevertheless, the severity of ischaemic damage diminished when NO level was increased by acute GTN-treatment either in the GTN-tolerant or the nontolerant hearts. This shows that the rapid increase in cardiac NO due to acute GTN administration may be responsible for activation of K_{ATP} rather than the increased basal level of cardiac NO. Our present study, however, does not provide direct evidence that GTN-derived NO mediates the cardioprotective effect of GTN. Use of a non-toxic, cell-permeable NO scavenger might clarify this, since GTN is mostly metabolized intracellularly, beyond the coronary vascular endothelium in the intact heart (Schror *et al.*, 1991). Such a NO scavenger has not been developed yet. Although some investigators speculated that GTN might exert NO-independent actions (Ljusegren & Axelsson, 1993) it is well accepted that the effects of GTN and other organic nitrates are mediated by NO (see for review: Abrams, 1992). Therefore, we conclude that the direct anti-ischaemic effect of GTN is most probably mediated by GTN-derived NO.

The limitations of our study include that absolute quantification of NO concentration by electron spin resonance in tissue samples is not possible (Ferdinandy *et al.*, 1997a,b; Mülsch *et al.*, 1992). Therefore, according to our previous studies, we expressed NO signal intensity in arbitrary units, which allows us to demonstrate relative changes in cardiac NO content (Ferdinandy *et al.*, 1997a,b; Csont *et al.*, 1998). Due to technical considerations, NO content in the ischaemic region of the heart cannot be measured precisely by this method. Consequently, the effect of GTN-tolerance and acute GTN treatment on cardiac NO content during ischaemia is not known. We and others have previously shown that ischaemia increases myocardial NO production (Pabla & Curtis, 1995; Wang & Zweier, 1996; Csonka *et al.*, 1999).

In summary, this is the first demonstration that the GTN-induced direct cardioprotective effect involves a cyclic GMP-independent activation of K_{ATP} in the isolated rat heart. This effect of GTN is preserved in vascular GTN-tolerance and may be mediated by GTN-derived NO. We suggest that further investigation of the direct cardioprotective effect of GTN may lead to the development of new therapeutic strategies to protect the ischaemic heart in patients with nitrate-tolerance.

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