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European Journal of Pharmacology 439 (2002) 129–134

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Reversal of tolerance to nitroglycerin with *N*-acetylcysteine or captopril: a role of calcitonin gene-related peptide

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Received 11 February 2002; received in revised form 18 February 2002; accepted 22 February 2002

Abstract

Previous studies have shown that the development of tolerance to nitroglycerin is related to a decrease in the release of endogenous calcitonin gene-related peptide (CGRP). In the present study, we explored whether endogenous CGRP is involved in reversal of tolerance to nitroglycerin with *N*-acetylcysteine or captopril in rats in vivo and vitro. Tolerance was induced by exposure to nitroglycerin (4.4×10^{-6} M) for 10 min in vitro or by pretreatment with nitroglycerin (10 mg/kg, s.c.) three times a day for 8 days in vivo. Nitroglycerin (3×10^{-9}–10^{-6} M) caused a concentration-dependent relaxation in the isolated rat thoracic aorta, an effect that was reduced by CGRP-(8–37) (3×10^{-7} M) or capsaicin (3×10^{-7} M). Preincubation with nitroglycerin for 10 min significantly decreased its vasodilation, which was restored in the presence of *N*-acetylcysteine (10^{-5} M) or captopril (10^{-5} M). Nitroglycerin (150 μg/kg, i.v.) produced a depressor effect and an increase in concentrations of nitric oxide and CGRP, and the effects of nitroglycerin disappeared after pretreatment with nitroglycerin for 8 days. However, tolerance to nitroglycerin in vivo also was partially restored in the presence of *N*-acetylcysteine or captopril. The present results suggest that reversal of tolerance to nitroglycerin with *N*-acetylcysteine or captopril is related to the increased release of CGRP in the rat.

Keywords: Tolerance; Nitroglycerin; CGRP (calcitonin gene-related peptide); *N*-acetylcysteine; Captopril

1. Introduction

Nitroglycerin, a potent vasodilator, is widely used in treatment of angina pectoris, congestive heart failure and myocardial infarction (Reichek et al., 1984). However, long-term administration of nitroglycerin can cause tolerance development (Needleman et al., 1973; Needleman and Johnson, 1973). The mechanisms responsible for tolerance to nitroglycerin are not clearly understood. There is amount of evidence to suggest that nitrate tolerance may be attributed to sulfhydryl depletion and thereby limiting the metabolic conversion, resulting in the decreased production of vasoactive intermediates such as nitric oxide (Kowaluk et al., 1987; Munzel et al., 1992; Needleman and Johnson, 1973; Parker et al., 1987).

Calcitonin gene-related peptide (CGRP), the principal transmitter in capsaicin-sensitive sensory nerves, is widely distributed in vascular tissues and is a potent vasodilator (Franco-Cereceda, 1988). Previous studies have suggested that nitroglycerin can evoke the release of CGRP from capsaicin-sensitive sensory nerves (Booth et al., 1997; Wei et al., 1992). We and others have also shown that CGRP mediates the depressor effect and vasodilation of nitroglycerin (Booth et al., 2000; Zhou et al., 2001a). More recently, our work has suggested that the attenuated depressor effect of nitroglycerin after long-term therapy in vivo is also related to the diminished release of CGRP (Zhou et al., 2001b).

Both the sulfhydryl donor *N*-acetylcysteine and thiol-containing angiotensin converting enzyme inhibitor captopril have been shown to partially reverse the development of tolerance to nitroglycerin in vivo and in vitro (Boesgaard et al., 1994; Kukovetz and Holzmann, 1990; Salvemini et al., 1993; Torresi et al., 1985). The mechanism for thiols-reversing tolerance to nitroglycerin may be associated with the increased formation of nitric oxide (Chong and Fung, 1991). Since nitric oxide can stimulate CGRP release (Garry et al., 2000; Hughes and Brain, 1994; Zhou et al., 2001a) and CGRP is involved in the development of tolerance to nitroglycerin, in the present study we examined whether

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0014-2999/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0014-2999(02)01439-5

reversal of tolerance to nitroglycerin with *N*-acetylcysteine or captopril is related to stimulation of CGRP release.

2. Materials and methods

Male Wistar rats weighing 280 to 320 g were obtained from the Xiang-Ya Medical School, Central South University Animal Center. Animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 86-23, Revised 1986).

2.1. Nitroglycerin-induced tolerance model in vitro

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and the thoracic aorta was rapidly isolated and cut into rings of 4 mm length. The rings were suspended horizontally between two stainless-steel wires and mounted in a 5 ml organ chamber filled with warmed (37 °C) and oxygenated (95% O_2 and 5% CO_2) Krebs' solution. The Krebs' solution had the following composition (mM): NaCl, 119.0; $NaHCO_3$, 25.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; $CaCl_2$, 2.5; and glucose, 11.0. One of the ring ends was connected to a force transducer. The aortic ring was stretched with 2 g resting force and equilibrated for 60 min, and then precontracted with KCl (60 mM). After a maximal response to KCl was obtained, the rings were washed repeatedly with Krebs' solution and equilibrated again for 30 min. In order to measure vasodilator responses, rings were contracted with phenylephrine (3×10^{-6} M) to 40–50% of their maximal concentration. After the constriction stabilized, an accumulative concentration–response curve to nitroglycerin (3×10^{-9}–10^{-6} M) or CGRP (3×10^{-11}–10^{-8} M) was observed. Tolerance was induced by pretreatment with nitroglycerin (4.4×10^{-6} M) for 10 min. For the studies on involvement of endogenous CGRP in the development of tolerance to nitroglycerin, the preparations were exposed to CGRP-(8–37) (3×10^{-7} M), a selective CGRP receptor antagonist, or capsaicin (3×10^{-7} M), which selectively depletes CGRP in sensory nerves, for 10 or 20 min, respectively, and then the response to nitroglycerin was tested. For *N*-acetylcysteine or captopril, the preparations were exposed to *N*-acetylcysteine (10^{-5} M) or captopril (10^{-5} M) for 10 min and then the response to nitroglycerin was tested.

2.2. Nitroglycerin-induced tolerance model in vivo

Tolerance was induced by treatment with nitroglycerin (10 mg/kg, s.c.) three times a day for 8 days and was confirmed by a reduction in hypotensive responses to intravenous nitroglycerin. After 8 days, the rats were anesthetized with sodium pentobarbital (60 mg/kg). A polyethylene (PE 50) catheter was inserted into the left femoral artery to record blood pressure. An additional catheter was inserted into the right femoral artery for the withdrawal of a reference arterial blood sample. Nitroglycerin (150 μg/kg) was administered through a cannula inserted into the right femoral vein. After surgical procedures, at least 10 min was allowed for stabilization. Blood pressure was continuously monitored. The resulting electric signals were digitized by a MacLab analog to digital converter and recorded by a Power Macintosh 7220 computer. *N*-acetylcysteine (2.5 mg/kg) or captopril (1 mg/kg) was given intraperitoneally 30 min before the experiment in the tolerant rat.

2.3. Measurement of plasma CGRP concentration

After a maximal depressor response to intravenous nitroglycerin was reached, blood samples (3 ml) were collected rapidly from the right femoral artery into tubes containing 10% Na_2EDTA 40 μl and aprotinin 500 mU/l. The plasma was obtained by centrifugation at 3000 rpm for 10 min at 4 °C. CGRP-like immunoreactivity in the plasma was measured using antisera raised against rat CGRP, ^{125}I-labelled CGRP and rat CGRP standard.

2.4. Measurement of plasma nitric oxide concentration

Blood samples (3 ml) were collected rapidly in the same way as above except that aprotinin was added. Nitrite accumulation was determined as the serum level of nitric oxide. Plasma nitrate/nitrite (NO) levels were measured as previously described (Feng et al., 2001). Briefly, nitrate was converted to nitrite with aspergillus nitrite reductase, and the total nitrite was measured with the Griess reagent. The absorbance was determined at 540 nm with a spectrophotometer.

2.5. Reagents

CGRP, CGRP-(8–37), capsaicin, *N*-acetylcysteine and captopril were purchased from Sigma (St. Louis, MO, USA). Nitroglycerin was purchased from Beijing Yiming Pharmaceutical Factory (Beijing, PR China). Nitroglycerin was dissolved in 99% ethanol and further diluted in 0.9% saline (in vivo) or in Krebs' solution (in vitro) to the proper final concentration. Capsaicin was initially dissolved in ethanol and further diluted in Krebs' solution to proper final concentration. Radioimmunoassay kits for the measurement of CGRP were obtained from Dongya Immunity Technology Institute (Beijing, PR China). Supplies for the nitric oxide assay were obtained from Nanjing Ju-Li Biological Medical Engineering Institute (PR China).

2.6. Statistics

All values were expressed as means±S.E.M. Statistical analysis was carried out by analysis of variance and the Newman–Keuls test. The level of significance was chosen as $P<0.05$.

3. Results

3.1. Vasodilator responses

In presence of phenylephrine, nitroglycerin (3×10^{-9}–10^{-6} M) caused a concentration-dependent relaxation in the isolated rat thoracic aorta (Fig. 1). Vasodilator responses to nitroglycerin were reduced in the presence of CGRP-(8–37) (3×10^{-7} M), the CGRP receptor antagonist, or capsaicin (3×10^{-7} M), which selectively depletes CGRP in sensory nerves. CGRP-(8–37) or capsaicin (3×10^{-7} M) itself had no effect on constrictor responses to phenylephrine (data has not been shown).

Preincubation of the preparations with nitroglycerin (4.4×10^{-6} M) for 10 min markedly decreased vasodilator responses to nitroglycerin. To test whether the sulfhydryl group is involved in the development of tolerance to nitroglycerin, *N*-acetylcysteine or captopril was used. *N*-acetylcysteine (10^{-5} M) or captopril (10^{-5} M) significantly enhanced vasodilator responses to nitroglycerin in the tolerant ring (Fig. 2).

As shown in Fig. 3, CGRP (3×10^{-11}–10^{-8} M) caused a concentration-dependent relaxation in the isolated rat thoracic aorta, an effect that was unaltered after preincubation with nitroglycerin (4.4×10^{-6} M).

3.2. Depressor effects

There were no differences in basic values of blood pressure among groups (Table 1). As has been reported previously (Zhou et al., 2001a), nitroglycerin significantly decreased blood pressure in a dose-dependent manner. After pretreatment with nitroglycerin for 8 days, the depressor effect of nitroglycerin (150 μg/kg, i.v.) was almost completely abolished. However, tolerance to nitroglycerin was partially restored in the presence of *N*-acetylcysteine or captopril in the tolerant rat (Figs. 4 and 5A).

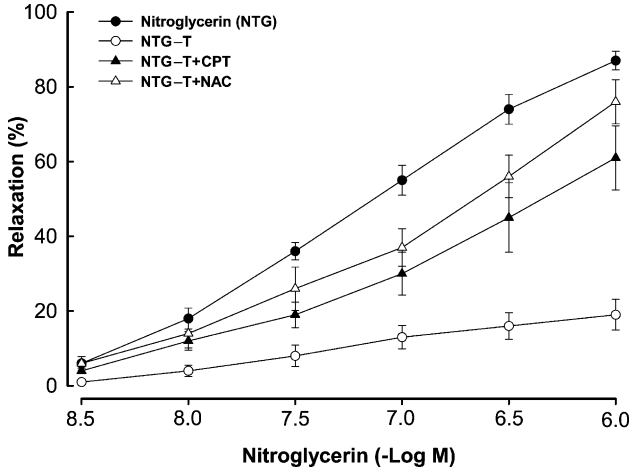

Fig. 2. Effect of *N*-acetylcysteine or captopril on vasodilator responses to nitroglycerin. Tolerance was induced by pretreatment with 4.4×10^{-6} M nitroglycerin for 10 min. Preparations were exposed to *N*-acetylcysteine (10^{-5} M) or captopril (10^{-5} M) for 10 min, and then exposed to nitroglycerin in the presence of *N*-acetylcysteine or captopril. Values are means±S.E.M ($n=7$).

3.3. The plasma nitric oxide and CGRP content

Nitroglycerin (150 μg/kg, i.v.) caused a significant increase in plasma concentrations of nitric oxide. However, nitroglycerin no longer produced the elevated concentration of nitric oxide after prolonged treatment, and this phenomenon was also partially reserved in the presence of *N*-acetylcysteine or captopril in the tolerant rat (Fig. 5B).

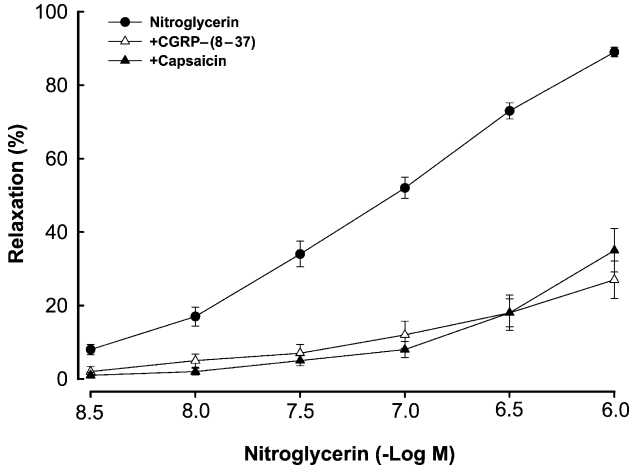

Fig. 1. Concentration–response curve of vasodilator responses to nitroglycerin. Preparations were exposed to CGRP-(8–37) (3×10^{-7} M) or capsaicin (3×10^{-7} M) for 10 or 20 min, respectively, and then exposed to nitroglycerin in the presence of CGRP-(8–37) or capsaicin. Values are means±S.E.M ($n=7$).

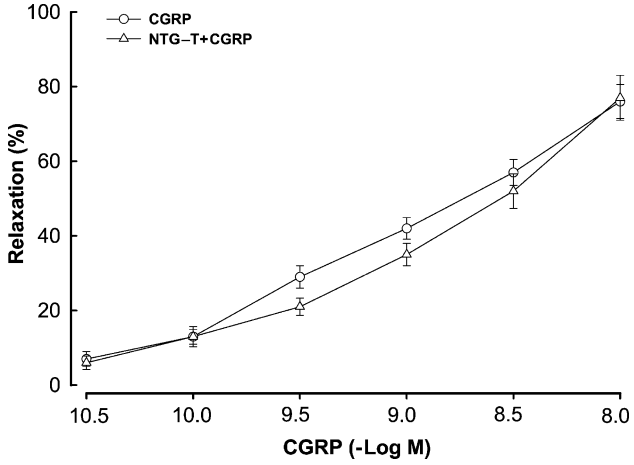

Fig. 3. Concentration–response curve of vasodilator responses to CGRP. NTG-T: tolerance was induced by pretreatment with nitroglycerin (4.4×10^{-6} M) for 10 min. Values are means±S.E.M ($n=7$).

Table 1
Basal values of blood pressure (mm Hg)

	n	Mean arterial pressure
NTG	7	135±7.5
NTG tolerance	7	130±10.5
N-acetylcysteine	7	137±4.7
Captopril	7	132±7.1

Tolerance was induced by pretreatment with nitroglycerin (10 mg/kg, s.c.) three times a day for 8 days. NTG: nitroglycerin. *N*-acetylcysteine (2.5 mg/kg) or captopril (1 mg/kg) was given intraperitoneally 30 min before the experiment in the tolerant rat. Values are means±S.E.M.

Nitroglycerin significantly increased concentrations of CGRP. However, the release of CGRP stimulated by nitroglycerin was reduced in the tolerant rat. The decreased release of CGRP was also partially reversed in the presence of *N*-acetylcysteine or captopril in the tolerant rat (Fig. 5C).

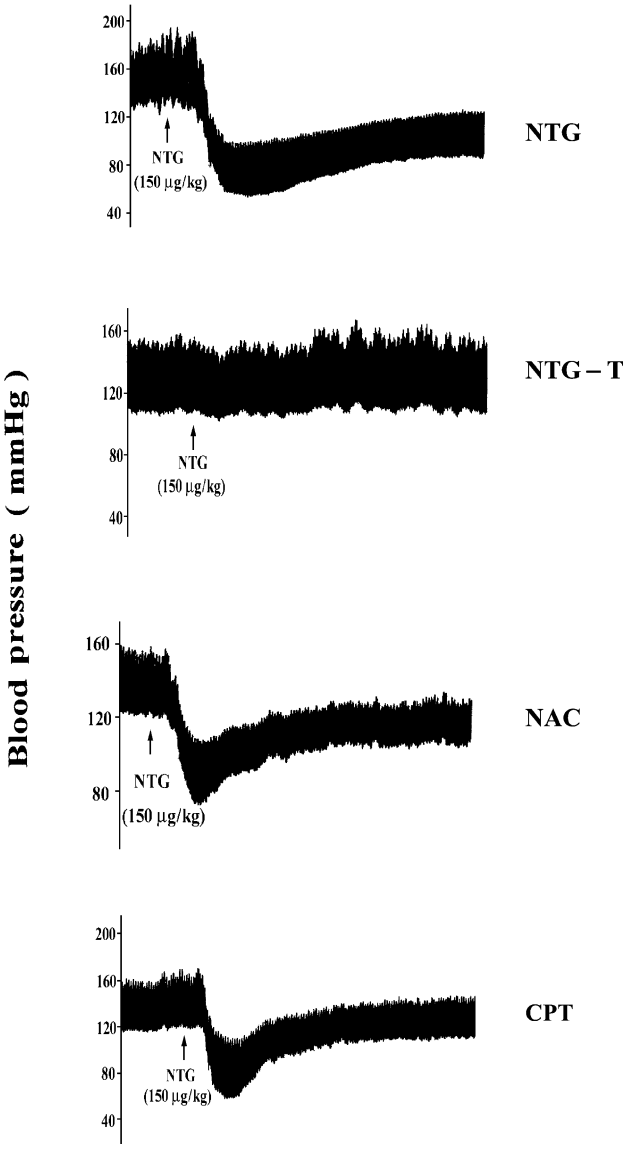

Fig. 4. Typical records of depressor effect induced by nitroglycerin in rats. NTG: nitroglycerin; NTG-T: tolerance was induced by pretreatment with nitroglycerin (10 mg/kg, s.c.) three times a day for 8 days. NAC and CPT: *N*-acetylcysteine (2.5 mg/kg) or captopril (1 mg/kg) was given intraperitoneally 30 min before the experiment in the tolerant rat.

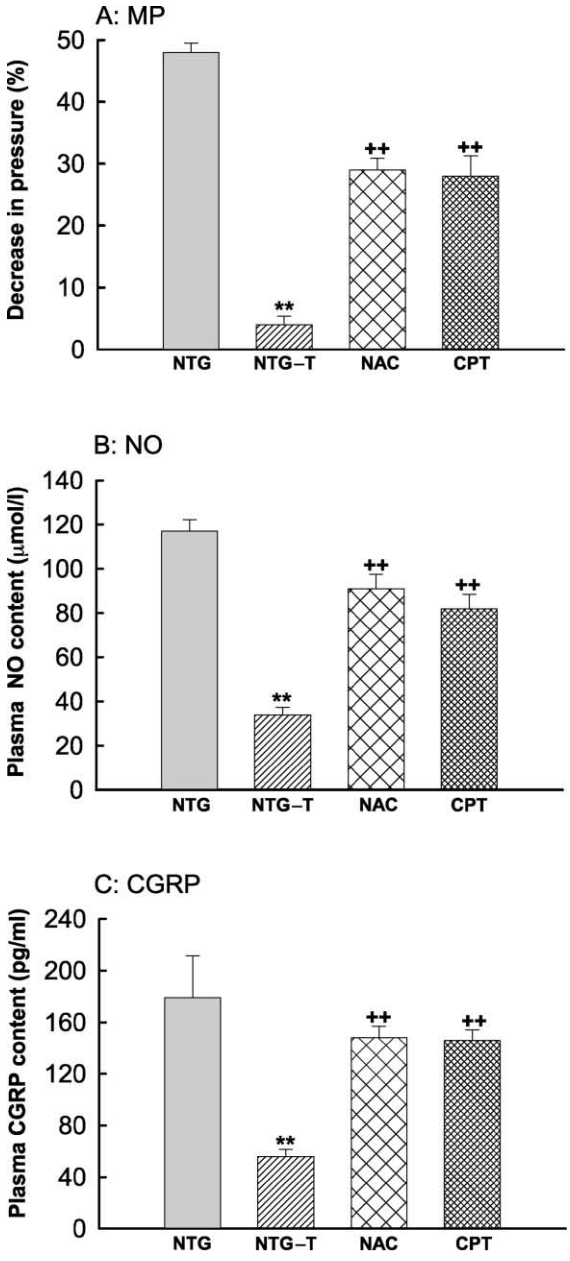

Fig. 5. Effect of nitroglycerin on blood pressure (A) and plasma concentrations of nitric oxide (B) and CGRP (C). Nitroglycerin (150 μg/kg) was injected intravenously. MP: mean arterial pressure; NTG: nitroglycerin; NTG-T: tolerance was induced by pretreatment with nitroglycerin (10 mg/kg, s.c.) three times a day for 8 days. NAC and CPT: *N*-acetylcysteine (2.5 mg/kg) or captopril (1 mg/kg) was given intraperitoneally 30 min before the experiment in the tolerant rat. Values are means±S.E.M ($n=7$). $^{**}P<0.01$ vs. NTG; $^{++}P<0.01$ vs. NTG-T.

4. Discussion

It has been suggested that nitroglycerin activates sensory nerves fibres to release CGRP from vascular tissues in both

the central and peripheral nervous systems (Booth et al., 1997; Wei et al., 1992). The present results showed that nitroglycerin (3×10^{-9}–10^{-6} M) caused a concentration-dependent relaxation in the isolated rat thoracic aorta, an effect which was abolished by CGRP-(8–37), the CGRP receptor antagonist, or capsaicin, which selectively depletes CGRP in vascular sensory nerves. Others have demonstrated that nitroglycerin (5×10^{-10}–5.8×10^{-5} M) releases CGRP in sufficiently high amounts to induce vasorelaxation, an effect that is also blocked by CGRP-(8–37) (Booth et al., 2000). A similar phenomenon has also been seen in a variety of vessels (Wei et al., 1992). As has been reported previously (Zhou et al., 2001a), the depressor effect of nitroglycerin is also decreased by pretreatment with capsaicin, which depletes neurotransmitters in sensory nerves. These results together with above-mentioned findings suggest that vasodilator responses to nitroglycerin are, at least, partially mediated by endogenous CGRP.

It has been demonstrated that frequently repeated or continuous exposure to high doses of organic nitrates leads to a marked attenuation in the magnitude of most of their pharmacological effects. However, the mechanism responsible for tolerance to organic nitrates is not fully understood. Proposed mechanisms include reduced intracellular metabolic (such as nitric oxide) activation of the organic nitrate, possibly as a result of depletion of critical intracellular sulfhydryl groups (Kowaluk et al., 1987; Munzel et al., 1992; Needleman and Johnson, 1973; Parker et al., 1987), and/or reduced nitric oxide bioavailability due to increased production of reactive oxygen species (such as superoxide, $\cdot O_2^-$) that rapidly inactivate the nitric oxide (Mihm et al., 1999; Munzel et al., 2000). The results of this study confirmed previous observations that the sulfhydryl donor *N*-acetylcysteine and the thiol-containing angiotensin converting enzyme inhibitor captopril partially reverse tolerance of nitroglycerin through increasing the generation of nitric oxide.

CGRP, a 37-amino acid peptide, is a potent vasodilator. Previous investigations have reported that vasodilator responses to CGRP are related to stimulation of cAMP production (Jansen et al., 1992), or activation of K^+ channels (Kitazono et al., 1993) and/or nitric oxide synthase III (Gray and Marshall, 1992). As mentioned above, vasodilator responses to nitroglycerin are mediated by endogenous CGRP (Booth et al., 2000; Wei et al., 1992; Zhou et al., 2001a). Thus, we postulate that the diminished vasodilator response to nitroglycerin in the tolerant rats may be secondary to a reduction of CGRP release. The results in this study revealed that vasodilator responses to nitroglycerin in vitro as well as the depressor effect and release of CGRP induced by nitroglycerin in vivo were decreased in the tolerant rat, and the decreased vasodilator responses and the attenuated release of CGRP in the tolerant rat were reversed in the presence of *N*-acetylcysteine or captopril. These results suggest that reversal of tolerance to nitroglycerin with *N*-acetylcysteine or captopril is associated with the increased release of CGRP.

We and others have found that nitric oxide evokes the release of CGRP from vasodilator sensory nerves in vascular tissues (Garry et al., 2000; Hughes and Brain, 1994; Zhou et al., 2001a). The release of substance P, which coexists with CGRP in capsaicin-sensitive sensory nerves, is regulated by nitric oxide through the activation of guanylate cyclase and the increase in cGMP levels (Kamisaki et al., 1995). Our recent works have shown that the depressor effect and elevated concentrations of CGRP induced by nitroglycerin were significantly reduced by methylene blue, an inhibitor of the soluble guanylate cyclase (Zhou et al., 2001a). There is evidence to suggest that methylene blue prevents the ability of *N*-acetylcysteine or captopril to restore responses to nitroglycerin in the tolerant rat (Salvemini et al., 1993). In the present study, *N*-acetylcysteine or captopril partially restored vasodilation and stimulation of nitric oxide and CGRP release induced by nitroglycerin in the tolerant rat. Previous study has shown that after pretreatment with isosorbide-5-mononitrate for 7 days, nitroglycerin still produces the hypotensive effect in the presence of captopril or *N*-acetylcysteine rather than enalaprilat, the non-SH-containing angiotensin converting enzyme inhibitor (Salvemini et al., 1993). Others have shown that captopril prevent the development of tolerance to nitroglycerin in arterial and venous circulation but not increase its vasodilator response (Pizzulli et al., 1996), suggesting that reversal of tolerance to nitroglycerin with *N*-acetylcysteine or captopril is by compensating sulfhydryl but not by inducing NO-release from NTG directly. These findings support the hypothesis that reversal of tolerance to nitroglycerin with thiol-containing compounds is related to stimulation of CGRP release via increasing formation of nitric oxide in the tolerant rat. However, the precise mechanism for the enhanced release of CGRP induced by *N*-acetylcysteine or captopril in the tolerant rat needs to be further investigated.

In summary, the present results suggest that reversal of tolerance to nitroglycerin with *N*-acetylcysteine or captopril is related to stimulation of CGRP release in rats.

Acknowledgements

This study was supported by a grant from the National Natural Science Foundation of China, No. 30070870.

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