Inhibition of Development of Tolerance to Nitroglycerin by Preventive Administration of N-Acetylcysteine in Rats

Hideo Tsuneyoshi, M.D., Nobuharu Akatsuka, M.D., Minoru Ohno, M.D., Kazuhiro Hara, M.D., Masahiko Ochiai, M.D., and Masao Moroi, M.D.*

SUMMARY

The efficacy of N-acetylcysteine in reversing nitrate tolerance has been controversial. This study examined whether continuous administration of N-acetylcysteine, a sulfhydryl compound, can prevent the development of tolerance to nitroglycerin; its acute effects on developed tolerance were also assessed. Rats were treated with subcutaneous injections of 1) 100 mg/kg nitroglycerin, 2) 100 mg/kg nitroglycerin and 700 mg/kg N-acetylcysteine, 3 times a day for 3 days. The sensitivity to nitroglycerin was studied in aortic preparations. The degree of developed tolerance to nitroglycerin was partially inhibited by simultaneous injection of N-acetylcysteine. Subsequent in vitro preincubation of aortic strips with nitroglycerin (10^{-5} M) reduced the subsequent nitroglycerin sensitivity of vessels from rats treated with nitroglycerin and N-acetylcysteine; sensitivity returned to the initial control level after in vitro preincubation with N-acetylcysteine. The nitroglycerin sensitivity of vessels from rats treated only with nitroglycerin, though, was not affected by in vitro preincubation with N-acetylcysteine. In conclusion, N-acetylcysteine is not effective in reversing the high degree of tolerance developed to nitroglycerin. However, continuous administration of N-acetylcysteine is effective in preventing the development of nitroglycerin tolerance.

Additional Indexing Words:
Nitroglycerin Nitrate tolerance N-acetylcysteine

It is widely known that patients develop drug tolerance after continuous high dose administration of nitroglycerin and other nitrates. Although development of tolerance has been demonstrated both in experimental animals\(^1\)\textsuperscript{,}\textsuperscript{3} and in clinical settings,\(^4\)\textsuperscript{,}\textsuperscript{6}\ the mechanisms for the development of

\textsuperscript{1}From the Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo.
\textsuperscript{*}Third Department of Internal Medicine, Faculty of Medicine, Toho University, Tokyo.
Address for reprints: Hideo Tsuneyoshi, M.D., First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan.
Received for publication September 30, 1988.
Accepted January 6, 1989.

733
nitrate tolerance are unclear. Previous studies have revealed a reduction in tissue levels of sulfhydryl groups in tolerance-induced vessels, and have suggested that this reduction is the primary cause of nitrate tolerance.\textsuperscript{7,8} These findings led to investigation of the effects of N-acetylcysteine, a sulfhydryl compound, on nitrate tolerance,\textsuperscript{9\textendash}\textsuperscript{12} but the results of the studies have not been consistent. Furthermore, the question of whether continuous administration of N-acetylcysteine can prevent the development of nitrate tolerance has not been examined.

The purpose of this study is to elucidate whether continuous administration of N-acetylcysteine can prevent the development of tolerance to nitroglycerin in experimental animals.

**Methods**

Twenty four male Wistar rats (6\textendash}8 weeks), weighing 150\textendash}200 g, were divided into 3 groups.

1) Nitroglycerin group (TNG group, n=8 rats): Nitroglycerin was administered by subcutaneous injection (100 mg/kg), 3 times daily for 3 days.

2) Nitroglycerin with N-acetylcysteine group (TNG+NAC group, n=8 rats): Nitroglycerin (100 mg/kg s.c.) was administered simultaneously with N-acetylcysteine (700 mg/kg s.c.) 3 times daily for 3 days.

3) Control group (n=8 rats): Ethanol (100\%) (2 ml/kg s.c.) was administered simultaneously 3 times daily for 3 days.

The rats were killed 14\textendash}18 hr after the last injection, and thoracic aortas were removed. The vessels were gently cleaned of surrounding connective tissue and cut into ring segments 5 mm in length. These segments were suspended in a 10 ml organ bath containing Tyrode solution aerated with 95\% O\textsubscript{2}-5\% CO\textsubscript{2} at 37\°C. The composition of the Tyrode solution (pH 7.4) was (mM): NaCl 114; MgCl\textsubscript{2} 1.2; CaCl\textsubscript{2} 1.9; KCl 5; NaHCO\textsubscript{3} 25; NaH\textsubscript{2}PO\textsubscript{4} 1.2; glucose 11. The rings were attached to a Nihon Kohden TB-611T isometric force transducer connected to a fine control micrometer and were allowed to equilibrate for 2 hours under a resting tension of 0.5 g.

Concentration-response curves for nitroglycerin (10\textsuperscript{-9} M\textendash}10\textsuperscript{-5} M) were constructed on strips given a constant dose of norepinephrine (10\textsuperscript{-6} M). The following experiments were performed serially for ring segments from each group of rats.

1) Concentration-response curves for nitroglycerin were constructed.

2) The vessels were incubated with nitroglycerin (10\textsuperscript{-5} M) for 5 min, and washed repeatedly for 30 min.
3) Concentration-response curves were constructed again to assess the effects of nitroglycerin preincubation.

4) The vessels were washed for 30 min and then incubated for 10 min with N-acetylcysteine (10^{-5} M).

5) After washing, concentration-response curves were determined to assess the effects of N-acetylcysteine preincubation.

**Drugs and chemicals:**

The nitroglycerin used for subcutaneous injections was a 5% solution in ethanol (Nippon Kayaku Co., Tokyo). Subcutaneously injected N-acetylcysteine (176.2 mg/ml in distilled water) was obtained from Senju Seiyaku Co., Tokyo. Other drugs and chemicals used for in vitro experiments were norepinephrine (Sankyo Co., Tokyo); nitroglycerin (Nippon Kayaku Co., Tokyo); and N-acetylcysteine (Sigma Chemical Co., St. Louis).

**Statistical analysis:**

From the dose-response curve, the ED_{50} value (half maximally effective concentration) was calculated by probit analysis. The log ED_{50} was used to compare ED_{50} values. The data are expressed as the mean±SE. The data were evaluated statistically by Duncan’s multiple comparison test. A p value of less than 0.05 was considered significant.

**RESULTS**

**Initial dose-response relation for nitroglycerin: evidence for induction of nitroglycerin tolerance:**

The vessels from TNG group rats showed a reduced sensitivity to nitroglycerin as compared with control group rats, indicating the development of tolerance to the drug. The sensitivity to nitroglycerin of the vessels from TNG+NAC group rats was intermediate, indicating a partial inhibition of induced tolerance (Fig. 1). There were significant differences between the ED_{50} values (−log [molar concentration]) for the 3 groups (Table I).

**Changes in the sensitivity to nitroglycerin caused by in vitro preincubation with nitroglycerin and N-acetylcysteine:**

In the control and TNG groups, there were no differences in the ED_{50} values in the serial dose-response curves under different preincubation conditions (Figs. 2 and 3). In the TNG+NAC group, though, preincubation for 5 min with 10^{-5} M nitroglycerin reduced the sensitivity of the aortic rings to nitroglycerin. However, subsequent preincubation with N-acetylcysteine
Fig. 1. Initial dose-response relations for nitroglycerin. CONTROL = control group; TNG = nitroglycerin group; TNG+NAC = nitroglycerin with N-acetylcysteine group. Each value represents a mean±SE of 8 rat aortic strips.

Table I. Effects of in Vitro Preincubation with Nitroglycerin and N-Acetylcysteine in 3 Groups of Rats

<table>
<thead>
<tr>
<th></th>
<th>1st</th>
<th>2nd</th>
<th>pretreatment with NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>control group</td>
<td>7.526±0.067*</td>
<td>7.636±0.052</td>
<td>7.673±0.038</td>
</tr>
<tr>
<td>TNG group</td>
<td>5.596±0.213*</td>
<td>5.498±0.148</td>
<td>5.628±0.220</td>
</tr>
<tr>
<td>TNG+NAC group</td>
<td>6.513±0.179*</td>
<td>5.958±0.126**</td>
<td>6.422±0.141***</td>
</tr>
</tbody>
</table>

The data are mean±SE. TNG = nitroglycerin; NAC = N-acetylcysteine; ED$_{50}$ = half maximally effective dose.

* p<0.05 among 3 groups, ** p<0.05 vs 1st, *** p<0.05 vs 2nd.

(10$^{-5}$ M) turned the nitroglycerin sensitivity to the first control level (Fig. 4). The ED$_{50}$ values for the 3 serial dose response curves are shown in Table I.

**DISCUSSION**

The mechanism of nitrate tolerance has not been clearly elucidated. Needleman et al demonstrated a reduction in tissue levels of sulfhydryl groups in tolerance-induced vessels and proposed that it is the primary cause of tolerance.7,8 In light of this hypothesis, many investigators have studied the effects of N-acetylcysteine, a sulfhydryl compound, on nitrate tolerance. The efficacy of this compound, though, varies with the preparations tested. In
experimental animals, tolerance to nitroglycerin induced in vitro could be partially reversed by N-acetylcysteine. Clinically, the peripheral hemodynamic responses and coronary vasodilative effects of nitroglycerin have been potentiated by N-acetylcysteine. Further, Packer et al reported that orally administered N-acetylcysteine could reverse tolerance induced by con-
Fig. 4. Changes in the sensitivity to nitroglycerin of the vessels from rats treated with nitroglycerin and N-acetylcysteine. Effects of in vitro preincubation with nitroglycerin and N-acetylcysteine are illustrated. (○) First dose-response relation. (●) After preincubation with nitroglycerin. (△) After preincubation with N-acetylcysteine.

tinuous nitroglycerin infusion in patients with congestive heart failure. It was also reported that tolerance to nitroglycerin in the coronary vascular bed could be partially reversed by N-acetylcysteine. In contrast, induced tolerance to isosorbide dinitrate was not reversed by administration of N-acetylcysteine in patients with angina pectoris.

However, the question of whether N-acetylcysteine can prevent the development of nitrate tolerance has not been examined. Only a single clinical study in patients with unstable angina showed that combined use of nitroglycerin and N-acetylcysteine reduced the incidence of acute myocardial infarction, but not the frequency of episodes of chest pain. And this reduction in the frequency of acute myocardial infarction was attributed to the potentiated antiplatelet effect of nitroglycerin by N-acetylcysteine.

The present animal study demonstrated that simultaneous administration of N-acetylcysteine and nitroglycerin to rats partially prevented the development of nitrate tolerance. This inhibition of the development of tolerance was reversed by preincubation of arterial strips in a high concentration of nitroglycerin. Further, subsequent preincubation with N-acetylcysteine returned the sensitivity to the initial (prenitroglycerin) control level. On the other hand, N-acetylcysteine preincubation did not affect the nitrate tolerance of vessels from rats that had received only nitroglycerin prior to preparation of arterial strips.

A current hypothesis is that organic nitrate such as nitroglycerin reacts with sulfhydryl groups to form nitrous acid which is converted to nitric oxide.
Subsequently, nitric oxide is thought to react with another thiol forming nitrosothiol which is then able to stimulate guanylate cyclase, and resultant elevation of cyclic GMP mediates relaxation.\textsuperscript{16)--20)} In tolerance-induced vascular smooth muscle, inhibited relaxation by nitroglycerin was associated with corresponding inhibition of cyclic GMP generation.\textsuperscript{21),22)} In addition, vascular smooth muscle relaxation induced by 8-bromoguanosine-3', 5'-monophosphoric acid, a lipophilic derivative which mimics the action of cyclic GMP, was not impaired by the presence of nitroglycerin tolerance.\textsuperscript{22)} Furthermore, the sensitivity of nitroprusside, which directly yields nitric oxide, was not significantly affected by the presence of nitroglycerin tolerance.\textsuperscript{22)} Moreover, Kukovetz et al\textsuperscript{23)} reported that SIN-1, a metabolite of molsidomine which activates guanylate cyclase in the absence of cysteine, produced no significant tolerance and was fully active in nitroglycerin-tolerant strips. These results suggest that one type or part of tolerance could be due to a deficiency in sulphhydryl groups.

There is another possible mechanism of tolerance development. Recently, Kukovetz et al\textsuperscript{24)} reported that preincubation of supernatants of coronary strips with nitroglycerin diminished the activity of guanylate cyclase, and this inhibition could not be reversed by the addition of thiol. They also demonstrated that when cysteine was present during the preincubation with nitroglycerin, the inactivation of the enzyme was significantly reduced. From this result they proposed that some of the development of nitroglycerin tolerance is caused by an inactivation of guanylate cyclase which can be partially prevented, but cannot be reversed by cysteine.

The results of the present study that continuous administration of N-acetylcysteine partially prevented the development of nitroglycerin tolerance may at least in part be attributed to the protecting effect by N-acetylcysteine against inactivation of guanylate cyclase. In the case of a high degree of developed tolerance, if guanylate cyclase is inactivated, the reduced sensitivity may not be reversed by N-acetylcysteine. On the other hand, when inactivation of guanylate cyclase is partially prevented by continuous administration of N-acetylcysteine, subsequent in vitro preincubation with N-acetylcysteine might be efficient to reverse the attenuated sensitivity by elevating the tissue level of sulphhydryl groups.

In a recent animal study,\textsuperscript{25)} N-acetylcysteine did not restore nitroglycerin responsiveness in tolerant epicardial arteries or veins in vivo, and they proposed that a small tolerance-independent augmentation of nitroglycerin-induced dilation may result from N-acetylcysteine-induced extracellular formation of a stimulant of guanylate cyclase from nitroglycerin. But in our experimental design, N-acetylcysteine was not present when dose-response
curves for nitroglycerin were constructed, so the possibility of an extracellular interaction between N-acetylcysteine and nitroglycerin can be excluded.

The nitroglycerin dose administered in the present study (300 mg/kg/day) was the same as used in animal studies in rats. Clinically, continuous intravenous injection of nitroglycerin amounts at most to 15 mg/kg/day. The N-acetylcysteine dose used in this study was 2,100 mg/kg/day, which exceeds the dose used clinically (200–300 mg/kg/day). The concentration used in vitro for preincubation with N-acetylcysteine was $10^{-5}$ M, this concentration approximately corresponds to the plasma concentration associated with clinical N-acetylcysteine therapy.

A recent report showed that some patients developed early tolerance to a continuous intravenous infusion of nitroglycerin, while others had a persistent response as assessed by changes in pulmonary artery wedge pressure. The present results imply that individual variability in the basal level of sulfhydryl groups may contribute to individual differences in the development of nitrate tolerance.

In conclusion, to prevent the development of nitrate tolerance and to maintain the effectiveness of nitrate therapy, continuous supplementation of sulfhydryl groups is recommended. Because of the high dose of N-acetylcysteine and nitroglycerin used in the present study, our observation may not be directly applicable to a clinical setting. Further studies are needed to evaluate the clinical use of continuous N-acetylcysteine administration.

REFERENCES


25. Münzel T, Holz J, Mülsch A, Stewart DJ, Bassenge E: Nitrate tolerance in epicardial arteries or in the venous system is not reversed by N-acetylcysteine in vivo, but tolerance-independent interactions exist. Circulation 79: 188, 1989


