2002년도 산학협동연구비
Korean Circulation J 2005;35:94-99
ISSN 1738-5520

Time-Phasic Development of Nitrate Tolerance According to the Hemodynamic Responses and the Expression of Phosphodiesterase 1A1

Dongsoo Kim, MD, Bum Kee Hong, MD, Eui-Young Choi, MD, Hyuck Moon Kwon, MD, Dong-Yun Kim, MD, Sung Woo Kwon, MD, Sung Ju Lee, MD, Jong Kwan Park, MD, Young Won Yoon, MD and Hyun-Seung Kim, MD

Department of Cardiology, Yongdong Severance Hospital, College of Medicine, Yonsei University, Seoul, Korea

ABSTRACT

Background and Objectives : Time-phasic development of nitrate tolerance in cardiovascular diseases is very important because it can contribute to the advent of blunted vasodilation or rebound ischemia even during continuous NTG treatment. In such a condition, we should change the therapeutic regimen of nitrate treatment to prevent the worsening of symptoms. **Materials and Methods** : We created a nitrate-tolerant rat model using an osmotic minipump, and we examined the hemodynamic response to bolus NTG infusion *in vivo*. We checked the phosphodiesterase (PDE)1A1 mRNA and protein level by relative quantitative RT-PCR and western blot analysis. We used 8-cpt-cGMP for investigating the development of a time-phasic nitrate tolerance mechanism after nitrate infusion. **Results** : NTG-treated rats revealed a significant decrease in NTG-induced MAP drop (nitrate tolerance) from 1-day and this continued to the third day. The mRNA and protein levels of PDE1A1 similarly increased during these periods. **Conclusion** : This study revealed the development of time-phasic nitrate tolerance from the the aspects of *in vivo* hemodynamic responses and PDE 1A1 gene expression, and our work supports the need for further investigation to come up with a different therapeutic strategy and new drugs. **(Korean Circulation J 2005;35:94-99)**

KEY WORDS : Nitrates ; Hemodynamic phenomena ; Phosphodiesterases ; Aorta.

Introduction

Organic nitrates have been used for rapid relief of angina pectoris for over 100 years. The major action of organic nitrates is to decrease the VSMC tone that leads to dilation of the peripheral and coronary arteries, as well as dilation of the peripheral veins.[1] Within the VSMCs, it is recognized that organic nitrates ($RONO_2$) are converted to short-lived S-nitrosothiols (RSNO), and they then activate guanylate cyclase for stimulating cGMP formation.[2][3] cGMP leads to vasodilation by decreasing intracellular Ca^{2+} via the reuptake of Ca^{2+} by the sarcoplasmic reticulum, and also by reducing the cell permeability to extracellular Ca^{2+}.[4] The endogenous vasodilator released from the vascular endothelium, endothelium-derived relaxing factor (EDRF or NO), also activates guanylate cyclase. The fact that nitrates may have a role as a physiological substitute for EDRF has led to a revival of interest in organic nitrates for the treatment of angina. However, the long-term therapeutic effect of nitrates is limited by nitrate tolerance. The sustained administration of organic nitrates has long been recognized to result in the rapid development of tolerance to their anti-anginal and hemodynamic effects in both human and experimental animals (nitrate tolerance).[5-7] The basis for this nitrate tolerance is still not completely understood, but various mechanisms have been proposed to explain this phenomenon. These mechanisms are 1) impaired nitrate biotransformation,[8] 2) intracellular sulfhydryl group depletion,[9] 3) neurohumoral counter-regulation,[10] 4) overproduction of ROS,[11] and 5) alteration in the activities of such key enzymes as guanylate cyclase (GC) and cyclic nucleotide phosphodiesterase (PDE), which regulate cGMP levels.[12][13] It is probable that clinical nitrate tolerance is

Received : October 19, 2003
Accepted : November 18, 2003
Correspondence : Dongsoo Kim, MD, Department of Cardiology, Yongdong Severance Hospital, College of Medicine, Yonsei University, 146-92 Dogok-dong, Gangnam-gu, Seoul, 135-270, Korea
Tel : 82-2-3497-3336, Fax : 82-2-3463-3882
E-mail : kim0426@yumc.yonsei.ac.kr

caused by several of these mechanisms. Among these mechanisms, the role of PDE to hydrolyze cGMP in nitrate tolerance has recently been investigated.[14)15)]

Five different families of PDEs have been identified in VSMCs from humans and animals, including Ca^{2+}/CaM-stimulated PDE (PDE1) preferentially hydrolyzing cGMP, cGMP-stimulated PDE (PDE2) hydrolyzing both cGMP and cAMP, cGMP-inhibited PDE (PDE3) preferentially hydrolyzing cAMP, cAMP-specific PDE (PDE4) hydrolyzing cAMP, and cGMP-specific PDE (PDE5) hydrolyzing cGMP.[16)] Each different PDE plays a distinct role in controlling the vascular tone. The presence of more than one PDE (PDE1 and PDE5) that are capable of hydrolyzing cGMP in VSMCs suggests a complex interplay between the enzymes. One concept is that PDE1 plays a dominant role when smooth muscle is exposed to stimuli that increase intracellular Ca^{2+}. Thus, PDE1 is the major enzyme for the hydrolysis of cGMP in rabbit aorta stimulated with norepinephrine (NE) or KCl, which increases the intracellular calcium concentration.[17)] In addition, we have already demonstrated that nitrate tolerance is associated with the upregulation of Ca^{2+}/CaM-stimulated PDE (PDE-1A1).[13)]

As a clinical concern, the time point in the development of nitrate tolerance for angina pectoris patients is very important because it can contribute to the development of blunted vasodilation or rebound ischemia even during continuous NTG administration,[18)] and in such a condition the physician should change the therapeutic regimen of nitrate to prevent rebound angina. The present study investigated the time phasic development of nitrate tolerance in relation to the *in vivo* hemodynamic responses and PDE 1A1 gene expression.

Materials and Methods

Development of nitrate tolerance *in vivo*

Male Sprague-Dawley rats (Charles River Labs, 250 g to 300 g each) were anesthetized with ketamine 40 mg/kg, xylazine 0.5 mg/kg and acepromazine 5 mg/kg i.p. plus 1/3 the loading dose as needed. An osmotic minipump (model 2ML1: Alza Corp) filled with either NTG (Zeneca Inc., Wilmington, DE, n=16) or vehicle (propylene glycol, n=6) was subcutaneously implanted at the dorsum of the neck. NTG was then infused at an average rate of 10 μg/kg/min for 1, 2 and 3 days. At the end of each infusion period, the animals were sacrificed and dissected, and their thoracic aortas were taken and snap-frozen in liquid nitrogen after removal of the adventitia, and the aortas were then stored at −80℃ until use.

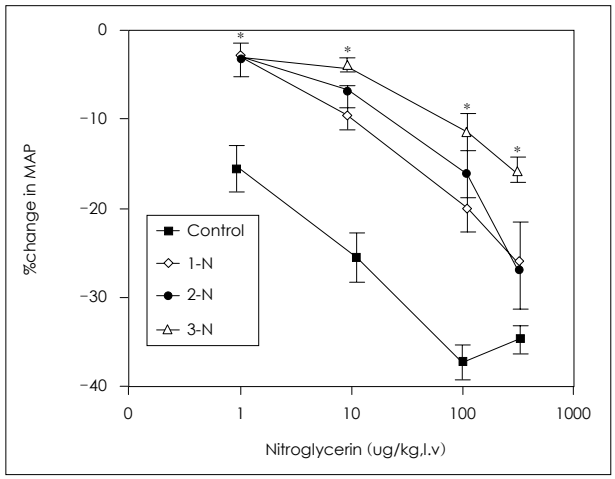

Fig. 1. Hypotensive effect of bolus i.v. doses of nitroglycerin in vehicle and nitroglycerin-pretreated rats for 1, 2 and 3 days. Values are expressed as the % change from the baseline. ∗: p<.05 vs baseline within each group. MAP: mean arterial pressure.

Hemodynamic responses to subsequent nitroglycerin bolus infusion

To assess for nitrate tolerance, the hypotensive effect of NTG was examined. The rats' mean arterial pressure (MAP) was continuously monitored by means of a catheter in the right common carotid artery and using a polygraph recorder (Grass Instruments). Bolus doses of nitroglycerin of 1, 10,100 and 300 μg/kg i.v. were administered in a volume of 0.5 mL saline through the right jugular vein. Evaluating nitrate tolerance with multiple doses of nitroglycerin took less than 20 minutes, and the maximum decrease in MAP was recorded for each dose. Hydralazine hydrochloride was then infused into both groups of rats as a NO-independent hypotensive agent (1 mg/kg, i.v.) (Fig. 1).

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Tissue RNA was extracted from the aortas using a Total RNA Isolation Kit (Ambion) based on manufactured protocol. First strand cDNA was synthesized from 5 μg of total RNA by using random primers with the SuperScript Preamplification System (GIBCO BRL) according to manufacture's protocol. Quantitative RT-PCR was performed with 18s rRNA as an internal control using Ambion's competimer technology. PDE1A1 specific primers (sense 5'-AAGATGACTGGAGGGATCTTCG-3', antisense 5'-GAAAATGGAAGCCCTAATTCAGC-3') was used to generate a 281-bp PCR product for PDE1A1. The PCR products were then run on a 2%

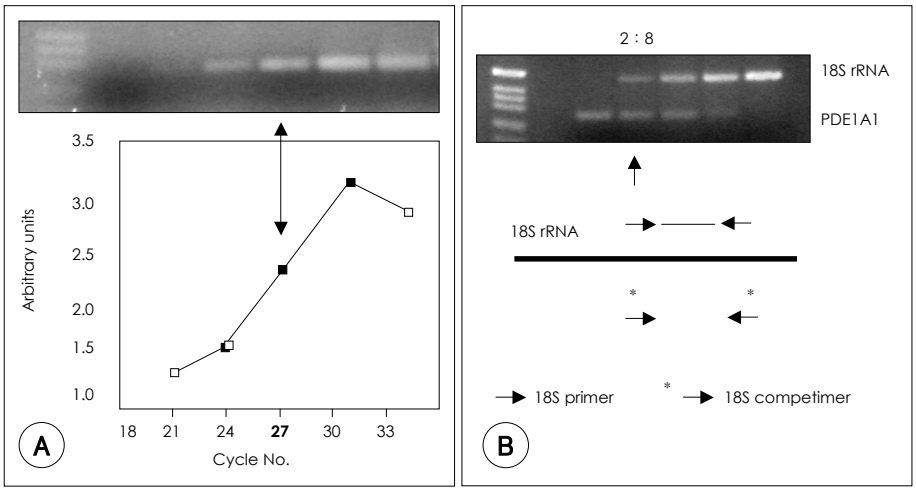

Fig. 2. Determination of linear range of PCR cycles (A) and optimal 18S rRNA ratio (B) (primer: competimer) for optimization of quantitative RT-PCR. 18S rRNA: 18S ribosomal RNA, RT-PCR: reverse transcription polymerase chain reaction, PDE: phosphodiesterase.

Table 1. Hemodynamic responses to subsequent i.v. bolus infusion of NTG

	Control (n=6)	1d-NTG (n=5)	2d-NTG (n=5)	3d-NTG (n=6)
Baseline MAP (mmHg)	104.3±3.9	100.5±8.0	103.3±3.1	100.8±4.1
Max% ⊿ MAP	−37.6±1.8	−26.8±5.1*	−27.2±0.3*	−17.5±1.4*
Max% ⊿ MAP (HDZ)	−28.7±2.0	−27.5±1.0	−31.5±0.7	−28.9±1.7

Max% ⊿ MAP: maximum % decrease in MAP after i.v. bolus NTG (1, 10, 100 and 300 ug/kg), MAP: mean arterial pressure, HDZ: hydralazine, NTG: nitroglycerin. *: p<0.01, compared to control group

agarose gel, stained with ethidium bromide and quantified using image analysis software (NIH Image 1.60) (Fig. 2).

Western blot analysis

PDE1A1 protein levels were determined by Western blot analysis. Tissue extracts were prepared as above. The tissue extract was centrifuged at 1000 g for 10 min at 4℃ to remove the cell debris. The supernatant was boiled in 1X sample buffer for 5 minutes, then loaded onto a SDS-polyacrylamide gel and electrophoresed. The separated proteins were transferred onto nitrocellulose membranes and next immunostained with PDE1-specific antibody (donated by University of Washington, Seattle, US). The immunoreactivity was detected by enhanced chemiluminescence using HRP-conjugated goat anti-rabbit IgG and a HRP-luminescent substrate mixture.

VSMC culture

Rat aortic VSMCs were isolated from 200−250 g male Harlan Sprague-Dawley rats and they were maintained in 10% bovine calf serum (BCS) with Dulbecco's modified Eagle's medium as described previously 21. The rat aortic VSMCs (passages 7 to 12) at 70% confluence in 100 mm dishes were growth-arrested by incubation in 0.1% BCS DMEM for 48 hours prior to the indicated drug treatment.

The effects of cGMP on PDE1A1 gene expression in rat aortic VSMCs

We measured the PDE1A1 mRNA levels in rat aortas treated with cGMP analogue (8-chrolophenylthio(cpt)-cGMP) at the the indicated time. Rat aortic VSMCs were cultured and exposed to 8-cpt-cGMP (25 uM) for 0, 24, 48 and 72 hours. The total RNA was extracted and the PDE1A1 mRNA levels were assayed by quantitative RT-PCR as described above.

Statistical analysis

One-way or 2-way ANOVA was used to compare differences between the treatment means, and the differences were expressed as means±SD. After ANOVA, comparison of 2 populations was made by Students unpaired t-test. $p<0.05$ were considered as statistically significant.

Results

Induction of *in vivo* tolerance

There was no significant difference of the baseline MAP between the control group and the NTG-pretreated group (104.3±3.9 vs. 100.8±4.1 mmHg, respectively) (Table 1). For the control rats, acute NTG challenges caused dose-dependent decreases in the MAP ranging from 15.7±2.3% to 37.6±1.8% (Fig. 1). The NTG-treatment group revealed sig-

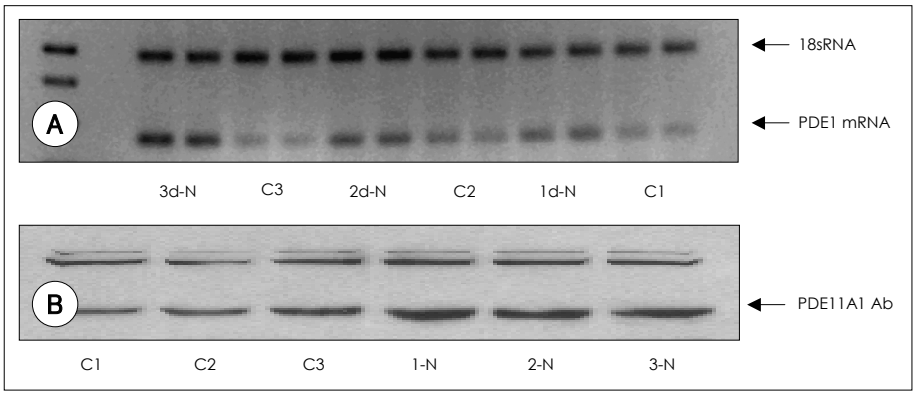

Fig. 3. PDE1A1 gene expression using PDE1A1 specific primers (A) & PDE1A1 protein expression using PDE1A1 specific antibody (B) in control and nitroglycerin-pretreated rat aorta for 1, 2 and 3 days. PDE: phosphodiesterase.

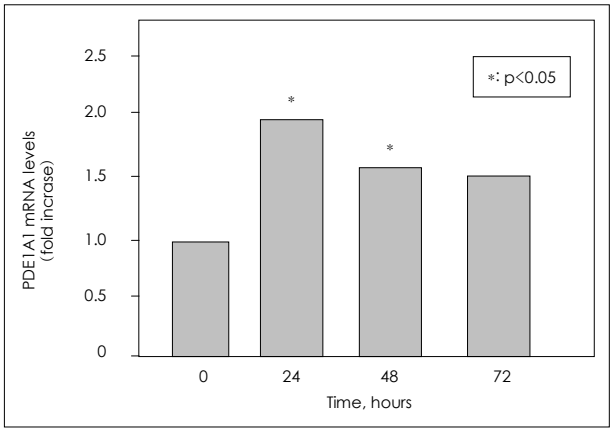

Fig. 4. Effect of *in vitro* 8-cpt-cGMP on PDE1A1 mRNA Expression in VSMC 5 ug of total RNA was amplified by RT-PCR for 27 cycles using18s rRNA as an internal control run on a 2% agarose EtBr gel (n=7). EtBr: ethidium bromide, 8-cpt-cGMP: 8-chrolophenyl-thio-cyclicGMP, PDE: phosphodiesterase, mRNA: messanger RNA, VSMC: vascular smooth muscle cell.

nificant decreases in NTG-induced MAP drop compared to control values, from 3.4±2.0% to 26.8±5.1% (the 1 day-NTG group), from 3.9±0.1% to 27.2±0.3% (the 2 day-NTG group), from 3.9±0.7% to 17.5±1.4% (the 3 day-NTG group) along with the NTG dose increment; this indicated that changes in MAP were significantly blunted in the NTG-pretreated groups from the first day after NTG treatment and the changes in MAP were remarkably blunted on day 3. The hydralazine induced decrease in MAP (1 mg/kg, bolus) was not different for both groups (Table 1), indicating that the NO-independent vasodilation was similar in both groups.

The mRNA & protein level of PDE1A1 Is increased in nitrate-tolerant rats aorta

To determine the mRNA levels of PDE1A1 for each of the treatment periods, quantitative RT-PCR using PDE1A1 specific primers was performed (Fig. 2). PDE1A1 mRNA levels in rat aorta of NTG-treated group according to the time periods were increased by 1.9±0.2, 2.0±0.3 and 2.4±0.2 fold, respectively, compared to the values of the control group (Fig. 3A). The PDE1A1 protein level was also increased about 2-2.3 fold in the tolerant vessels at the similar periods (Fig. 3B).

8-cpt-cGMP upregulates PDE1A1 mRNA gene expression

The cGMP analogue (8-cpt-cGMP, 25 uM) increased PDE1A1 mRNA by 2.0-2.1 fold, suggesting that the chronic cGMP increase stimulated by NTG treatment induced the PDE1A1 gene expression, and this protein subsequently hydrolyzes the cGMP. These findings suggest that the upregulation of PDE1A1 (and the decreased cGMP) is an important mechanism for the development of nitrate tolerance (Fig. 4).

Discussion

Organic nitrates have been safely used as vasodilators for more than 100 years for the treatment of hypertension, ischemic heart disease and heart failure. Unfortunately, the therapeutic effect of nitrates is limited by the rapid development of tolerance (nitrate tolerance). Nitrate tolerance has been documented as the significant blunting of the hypotensive, vasodilatory capacity and also the supersensitivity to vasoconstrictors, which leads to vasoconstriction.

The present study supports the results of a previous study that *in vivo* NTG treatment of rats induces nitrate tolerance and this is caused by the increase of PDE1A1 gene expression.[13] The upregulation of PDE1A1 provided a new mechanism to explain, in part, the decreased sensitivity of the vasculature to NTG and the enhanced vasoconstriction observed in response to chronic NTG treatment; in addition, it

explains the rebound phenomenon observed in response to the acute interruption of chronic NTG therapy.[13)20)]

Mechanisms underlying nitrate tolerance

Contraction studies with isolated aorta rings pretreated with NTG for several days have also shown the decreased sensitivity to NTG in the absence of the neurohormonal environment, and this suggests the intrinsic abnormalities of the tolerant vasculature itself (called true vascular tolerance). Another phenomenon that is frequently encountered during chronic NTG treatment is enhanced sensitivity of the vasculature to several vasoconstrictors. In these studies, it was demonstrated that the sensitivity of the tolerant vasculature to vasoconstrictors such as NE, Ang II, ET-1, and serotonin[21)22)] is greatly enhanced in response to NTG treatment; this is a phenomenon that's been linked to a protein kinase C-mediated mechanism.[23)]

Our previous study[13)] suggested a new mechanism, that the induction of PDE1A1 activity would lead to an increased sensitivity of vasoconstrictors via the attenuation of cGMP accumulation in the nitrate tolerant vessels.

Time-phasic development of nitrate tolerance

Stewart et al[24)] first described nitrate tolerance to the antihypertensive effect of NTG despite a 160-fold dose increase. Many studies have revealed that nitrate tolerance effect on heart rate and blood pressure occurred within several hours to several days.[10)25-33)] These wide time variations of tolerance development did not depend on the methods of administration. Many reports have shown the development of tolerance within 24 to 48 hrs of the initiation of therapy in approximately half of patients with CHF who received an infusion of IV NTG.

Our study has also shown similar results for the attenuation of MAP drop and the upregulation of PDE1A1 gene expression associated with nitrate tolerance. Significant attenuation of the MAP drop and the upregulation of PDE1A1 gene expression developed within 24 hours of chronic infusion and these effects were slightly increased till 72 hours. We already knew that nitrate tolerance develops after continuous 3 days NTG treatment.[13)] Our current study showed the much earlier development of nitrate tolerance for the hemodynamic responses and PDE1A1 gene expression.

Many strategies have been suggested to prevent the phenomenon of nitrate tolerance such as the administration of sulfhydryl donors (N-acetylcysteine, methionine and sulfhydryl containg ACE inhibitors), but the only clinical approach that has gained acceptance is a nitrate free interval. However, by using intermittent therapy, the increased frequency of anginal attacks (termed rebound angina) has been observed.[34)] In some cases, the rebound phenomenon can be a serious problem and may even result in sudden cardiac death.[35)] This rebound effect is believed to be associated with the increased sensitivity to vasoconstrictors in the nitrate-treated vessels.[36)] From the results of our study we should consider using other therapeutic strategies to prevent or modify nitrate tolerance such as intermittent dosing, and we should also further investigate possible new drugs such as PDE1A1-specific inhibitors.

Study limitations

This *in vivo* study was not performed for earlier time periods, like for 6 to 12 hours of NTG treatment, and it was also not performed for longer periods of more than 3 days.

■ Acknoewledgments

This work was supported by the Korean Society of Circulation (Industrial-Educational cooperation; 2002).

REFERENCES

1) Corwin S, Reiffel JA. *Nitrate therapy for angina pectoris: current concepts about mechanism of action and evaluation of currently available preparations. Arch Intern Med 1985;145:538-43.*
2) Ignarro LJ, Gruetter CA. *Requirement of thiols for activation of coronary arterial guanylate cyclase by glyceryl trinitrate and sodium nitrite: possible involvement of S-nitrosothiols. Biochim Biophys Acta 1980;631:221-31.*
3) Ignarro LJ, Lippton H, Edwards JC, et al. *Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. J Pharmacol Exp Ther 1981;218:739-49.*
4) Elkayam U. *Tolerance to organic nitrates: evidence, mechanisms, clinical relevance, and strategies for prevention. Ann Intern Med 1991;114:667-77.*
5) Bogaert MG. *Tolerance towards glyceryltrinitrate (trinitrin) in rabbits. Arch Int Pharmacodyn Ther 1968;172:228-30.*
6) Bogaert MG, de Schaepdryver AF. *Tolerance towards glyceryl trinitrate (trinitrin) in dogs. Arch Int Pharmacodyn Ther 1968;171:221-4.*
7) Needleman P. *Tolerance to the vascular effects of glyceryl trinitrate. J Pharmacol Exp Ther 1970;171:98-102.*
8) Brien JF, McLaughlin BE, Breedon TH, Bennett BM, Nakatsu K, Marks GS. *Biotransformation of glyceryl trinitrate occurs concurrently with relaxation of rabbit aorta. J Pharmacol Exp Ther 1986;237:608-14.*
9) Needleman P, Johnson EM Jr. *Mechanism of tolerance development to organic nitrates. J Pharmacol Exp Ther 1973;184:709-15.*
10) Elkayam U, Roth A, Henriquez B, Weber L, Tonnemacher D, Rahimtoola SH. *Hemodynamic and hormonal effects of high-dose transdermal nitroglycerin in patients with chronic congestive heart failure. Am J Cardiol 1985;56:555-9.*
11) Munzel T, Sayegh H, Freeman BA, Tarpey MM, Harrison DG. *Evidence for enhanced vascular superoxide anion production in*

nitrate tolerance: a novel mechanism underlying tolerance and cross-tolerance. J Clin Invest 1995;95:187-94.
12) Axelsson KL, Andersson RG. *Tolerance towards nitroglycerin, induced in vivo, is correlated to a reduced cGMP response and an alteration in cGMP turnover. Eur J Pharmacol 1983;88:71-9.*
13) Kim D, Rybalkin SD, Pi X, et al. *Upregulation of phosphodiesterase 1A1 expression is associated with the development of nitrate tolerance. Circulation 2001;104:2338-43.*
14) de Garavilla L, Pagani ED, Buchholz RA, et al. *Zaprinast, but not dipyridamole, reverse hemodynamic tolerance to nitroglycerin in vivo. Eur J Pharmacol 1996;313:89-96.*
15) Pagani ED, VanAller GS, O'Connor B, Silver PJ. *Reversal of nitroglycerin tolerance in vitro by the cGMP-phosphodiesterase inhibitor zaprinast. Eur J Pharmacol 1993;243:141-7.*
16) Saeki T, Saito I. *Isolation of cyclic nucleotide phosphodiesterase isozymes from pig aorta. Biochem Pharmacol 1993;46:833-9.*
17) Chiu PJ, Tetzloff G, Ahn HS, Sybertz EJ. *Comparative effects of vinpocetine and 8-Br-cyclic GMP on the contraction and 45Ca-fluxes in the rabbit aorta. Am J Hypertens 1988;1:262-8.*
18) Munzel T, Glaid A, Kurz S, Stewart DJ, Harrison DG. *Evidence for a role of endothelin 1 and protein kinase C in nitroglycerin tolerance. Proc Natl Acad Sci USA 1995;92:5244-8.*
19) Laws GL. *Effects of nitroglycerin on those who manufacture it. JAMA 1898;31:793.*
20) Paker JD, Gori T. *Tolerance to the organic nitrates, new ideas, new mechanisms, continued mystery. Circulation 2001;104:2263-5.*
21) Rydell EL, Axelsson KL. *Adrenaline toxicity in mice: sensitization of alpha 1 adrenoreceptors by nitroglycerin. Acta Pharmacol Toxicol 1984;55:73-7.*
22) Heitzer T, Just H, Brockhoff C, Meinertz T, Olschewski M, Munzel T. *Long-term nitroglycerin treatment is associated with supersensitivity to vasoconstrictors in men with stable coronary artery disease: prevention by concomitant treatment with captopril. J Am Coll Cardiol 1998;31:83-8.*
23) Matsushima N, Hayashi N, Jinbo Y, Izumi Y. *Ca2+-bound calmodulin forms a compact globular structure on binding four trifluoperazine molecules in solution. Biochem J 2000;347:211-5.*
24) Stewart DD. *Tolerance to nitroglycerin. JAMA 1905;44:1678-9.*
25) Crandal LA Jr, Leake CD, Loevenhart AS, MulhbergerCW. *Acquired tolerance to and cross tolerance between the nitrous and nitric acid esters ad sodium nitrite in man. J Pharmacol Exp Ther 1931;41:103-20.*
26) Zimrin D, Reichek N, Bogin KT, et al. *Antianginal effects of intravenous nitroglycerin over 24 hours. Circulation 1988;77:1376-84.*
27) Thadani U, Hamilton SF, Olson E, et al. *Duration of effects and tolerance of slow-release isosorbide- 5 -mononitrate for angina pectoris. Am J Cardiol 1987;59:756-62.*
28) Olivari MT, Caryle PF, Levine TB, Cohn JN. *Hemodynamic and hormonal response to transdermal nitroglycerin in normal subjects and in patients with congestive heart failure. J Am Coll Cardiol 1983;2:872-8.*
29) Rajfer SI, Demma FJ, Goldberg LI. *Sustained beneficial hemodynamic responses to large doses of nitroglycerin in congestive heart failure and comparision with intravenous nitroglycerin. Am J Cardiol 1984;54:120-5.*
30) Roth A, Kulick D, Freidenberger L, Hong R, Rahimtoola SH, Elkayama U. *Early tolerance to hemodynamic effects of high dose transdermal nitroglycerin in responders with severe chronic heart failure. J Am Coll Cardiol 1987;9:858-64.*
31) Packer M, Lee WH, Kessler PD, Gottlieb SS, Medina N, Yushak M. *Prevention and reversal of nitrate tolerance in patients with congestive heart failure. N Engl J Med 1987;317:799-804.*
32) Dupuis J, Lalonde G, Lebeau R, Bichet D, Rouleau JL. *Susteined beneficial effect of a seventy-two hour intravenous infusion of nitroglycerin in patient with severe chronic congestive heart failure. Am Heart J 1990;120:625-37.*
33) Dakak N, Makhoul N, Flugelman MY, et al. *Failure of captopril to prevent nitrate tolerance in congestive heart failure secondary to coronary artery disease. Am J Cardiol 1990;66:608-13.*
34) Ferratini M. *Risk of rebound phenomenon during nitrate withdrawal. Int J Cardiol 1994;45:89-96.*
35) Lange RL, Reid MS, Tresch DD, Keelan MH, Bernhard VM, Coolidge G. *Nonatheromatous ischemic heart disease following withdrawal from chronic industrial nitroglycerin exposure. Circulation 1972;46:666-78.*
36) Munzel T, Mollnau H, Hartmann M, et al. *Effects of a nitrate-free interval on tolerance, vasoconstrictor sensitivity and vascular superoxide production. J Am Coll Cardiol 2000;36:628-34.*