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Effect of metallothionein on tolerance of nitroglycerin in rats

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KEY WORDS metallothionein; drug tolerance; nitroglycerin; zinc compounds; thoracic aorta; nitroprusside; hypotension; vasodilation

ABSTRACT

AIM: To assess whether metallothionein (Met) could improve the nitroglycerin tolerance *in vivo*. **METHODS**: Nitrate tolerance was induced by 2-d treatment of nitroglycerin (Nit) patch ($0.05\ mg \cdot h^{-1}$). Endogenous Met production was induced by pretreatment of $ZnCl_2$ and coadministration of intravenous Met ($15\ mg \cdot kg^{-1} \cdot d^{-1}$) 2 d with Nit in tolerant rats. The induction of Met production was confirmed by the assay of liver and plasma Met levels. **RESULTS**: $ZnCl_2$ induced large amount of endogenous Met production in liver and plasma in Nit + $ZnCl_2$ group than that in control group, $(89 \pm 4)\ \mu g/g$ tissue *vs* $(11.0 \pm 2.4)\ \mu g/g$ tissue in liver, $P < 0.01$, and in plasma $(85 \pm 6)\ \mu g \cdot L^{-1}$ *vs* $(71 \pm 6)\ \mu g \cdot L^{-1}$, $P < 0.01$. There was no significant difference in plasma Met levels in Nit and control groups [$(75 \pm 6)\ \mu g \cdot L^{-1}$ *vs* $(71 \pm 6)\ \mu g \cdot L^{-1}$, $P > 0.05$]. The endogenous Met production enhanced the hypotensive response in Nit + $ZnCl_2$ group [(15.7 ± 0.8) kPa *vs* (11.5 ± 0.6) kPa, $n = 6$, $P < 0.05$]. The maximal vessel relaxation induced by sodium nitroprusside (SNP) was the same in 4 different groups but the highest EC_{50} (concentration which produces 50 % of the maximal response to SNP) was found in tolerant group [$(42 \pm 9)\ nmol \cdot L^{-1}$, $P < 0.01$]. **CONCLUSION**: Exogenous Met or induction of endogenous Met production antagonize the development of Nit tolerance.

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INTRODUCTION

Nitroglycerin (Nit) and other nitrovasodilators have been widely used to treat angina pectoris and congestive heart failure. The vasorelaxing effect of Nit is mediated by elevating intracellular cGMP levels, resulting from the activation of soluble guanylate cyclase by Nit metabolized active intermediate nitric oxide (NO) in vascular smooth muscle cells[1]. However, the long-term hemodynamic and anti-ischemic efficacy of organic Nit is rapidly attenuated by the development of Nit tolerance. A phenomenon related to Nit tolerance is cross tolerant to other nitrovasodilators and endothelium-dependent vasodilators[2]. The phenomena of Nit tolerance defined as increased drug requirement to produce same effect or a state in which lower effect produced by the same amount of drug, have been well known[3]. The mechanisms involved in the tolerance of Nit are not completely understood yet and probably these are multifactorial. The possible mechanisms include neuro-hormonal regulation, desensitization of the target enzyme guanylate cyclase or a decreased Nit biotransformation, increase in superoxide anion, intracellular sulfhydryl group depletion, *etc*[2,4-6]. Increase in superoxide anion and intracellular sulfhydryl group depletion are very important mechanisms involved in Nit tolerance. So it is considered that sulfhydryl group donor and antioxidant will have the preventive function for the Nit tolerance[7,8].

Metallothionein (Met) is a class of sulfur rich low-molecular weight protein. It has been demonstrated that Met has many biological effects including scavenging oxygen free radicals, especially hydroxyl group[9]. So in this study we assessed whether the Met could improve the Nit tolerance in rats by induction of endogenous Met production with Zinc chloride or administration of exogenous Met.

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MATERIALS AND METHODS

Materials Zinc chloride, phenylephrine, metallothionein (M7641; from rabbit liver, contain both form Ⅰ and form Ⅱ), sodium nitroprusside (SNP), bovine hemoglobin all were purchased from Sigma Chemical Co. Nitroglycerin patch (25 mg/patch) was purchased from Ciba-Geigy Limited. [^{109}Cd] CdCl (37 GBq/g) was purchased from NEN Life Sci.

Animal model Specific-Pathogen free male Wistar rats of weight 250 – 300 g were provided from Experimental Animal Center of Beijing Medical University. The rats were housed under constant temperature and humidity conditions. Before and during experimental period, all rats had free access to a standard rat chow and tap water. Total 24 rats were divided into 4 different groups (n = 6, each). (1) control group: No drugs were used. (2) Nit group: Nit tolerance animal model was prepared according to the study performed by Munzel *et al*[2]. A region on the dorsal aspect of the thorax or between scapulae was shaved and Nit patch (0.05 mg·h^{-1}) was applied to the skin. The treatment period was started between 8 and 10 AM and Nit patch changed each morning for the ensuing 2 days. (3) Nit + $ZnCl_2$ group: $ZnCl_2$ 200 μg·kg^{-1}·d^{-1} were injected ip, then 24 h after first injection with $ZnCl_2$, Nit patch was applied as same in Nit group. 4) Nit + Met group: Met 15 mg·kg^{-1}·d^{-1} was injected iv for 2 days and simultaneously Nit patch treatment was the same as group (2).

Assay of arterial blood pressure (ABP) Rat was anesthetized with sodium pentobarbital (80 mg·kg^{-1}). The left common carotid artery was cannulated with a catheter flushed with heparin for measuring ABP on polygraphy. Left internal jugular vein was also catheterized for administration of SNP (20 μg·kg^{-1}). The ABP before and after SNP administration was compared among different groups.

Assay of liver Met and plasma Met contents[10] Livers were homogenized and centrifuged. Supernatant 200 μL was mixed with 200 μL solution of ^{109}Cd in Tris-HCl buffer 10 mmol·L^{-1}, pH 7.4 (Cd concentration of 2.0 mg·L^{-1} and radioactivity of 37 MBq·L^{-1}). A 2 % bovine hemoglobin solution 100 μL was added to the sample. Heating, cooling, and centrifuging were repeated. Clear supernatant of 500 μL aliquote was transfered to a gamma counting tube and the amount of radioactivity in the supernatant fraction was then measured on Searle 1185 gamma counter. Blank samples and total counts samples were also measured on the counter for radioactivity. Heparinized blood samples were centrifuged to separate plasma. Plasma Met content was also measured with same process.

Vessel relaxation study[2] Thoracic aorta was removed and placed in Krebs' buffer, cleaned of excessive adventitial tissue, and cut into 5 mm ring segments. The vessel relaxation study was performed in organ chamber filled with Krebs' buffer at 37 ℃ and aerated with 95 % O_2 and 5 % CO_2. The preparations were allowed to equilibrate for 45 min. The vessels were preconstricted with phenlyephrine 10 μmol·L^{-1} to achieve maximal tone. Rings were then exposed to increasing concentrations of SNP for relaxation.

Statistical analysis The results are expressed as $\bar{x} \pm s$. The statistical analysis of the data was performed using one-way ANOVA followed by multiple comparison procedures.

RESULTS

Plasma Met and liver Met contents When $ZnCl_2$ 200 μg·kg^{-1} was preadministered, $ZnCl_2$ induced an increase in liver Met content in Nit + $ZnCl_2$ group compared with control group [μg/g tissue: (89 ± 4) *vs* (11.0 ± 2.4), $P < 0.01$]. There was about 3.5-fold liver Met-Cd content in Nit group higher than that in control group [μg/g tissue: (39 ± 7) *vs* (11.0 ± 2.4), $P < 0.01$]. Plasma Met content in Nit + $ZnCl_2$ group was increased by 20 % compared with control group [(85 ± 6) μg·L^{-1} *vs* (71 ± 6) μg·L^{-1}, $P < 0.01$] and it was increased by only 6 % in Nit group [(75 ± 7) μg·L^{-1} *vs* (71 ± 6) μg·L^{-1}, $P > 0.05$], compared with control group.

Hypotensive response Hypotensive response after SNP 20 μg·kg^{-1} iv was different in 4 groups. The response was attenuated in Nit group, and decreased in ABP from (14.8 ± 0.9) kPa to (11.9 ± 0.7) kPa (21 % ± 4 %) compared with control group 32 % ± 7 % [from (15.7 ± 1.6) kPa to (10.7 ± 1.4) kPa], $P < 0.01$. When $ZnCl_2$ 200 μg·kg^{-1} was preadministered ip, the hypotensive response was enhanced [from (15.7 ± 0.8) kPa to (11.5 ± 0.6) kPa] in Nit + $ZnCl_2$ group (28 % ± 5 %) compared with Nit

group ($P < 0.05$). Similarly when exogenous Met was coadministered with Nit, it showed marked improvement in hypotensive response 34 % ± 4 % [(15.0 ± 1.0) kPa to (9.8 ± 1.0) kPa] compared with Nit group ($P < 0.01$). (Tab 1)

Tab 1. Changes in ABP before and after iv SNP (20 μg·kg^{-1}) in different groups. $^bP < 0.05$, $^cP < 0.01$ *vs* Nit.

Group	ABP before iv SNP (kPa)	ABP after iv SNP (kPa)	Decrease in ABP (%)
Control	15.7 ± 1.6	10.7 ± 1.4	32 ± 7^c
Nit	14.8 ± 0.9	11.9 ± 0.7	21 ± 4
Nit + $ZnCl_2$	15.7 ± 0.8	11.5 ± 0.6	28 ± 5^b
Nit + Met	15.0 ± 1.0	9.8 ± 1.0	34 ± 4^c

Vessel relaxation When isolated rat aortic ring segments preconstricted with phenylephrine 10 μmol·L^{-1} were exposed to different concentration of SNP, it produced concentration-dependent relaxations. The resting tension was 5 g for control aortic rings. The concentration dependence of SNP effects on vessel relaxations showed that there was complete maximal relaxation of vessels in 4 groups but the highest EC_{50} (concentration which produces 50 % of the maximal response to SNP) was found in Nit group [(42 ± 9) nmol·L^{-1}, 95 % confidence levels were 52.4 nmol·L^{-1} and 33.6 nmol·L^{-1}] compared with control group [(5.7 ± 0.6) nmol·L^{-1}, 95 % confidence levels were 6.3 nmol·L^{-1} and 5.1 nmol·L^{-1}], $P < 0.01$. It was also observed that when pretreated with $ZnCl_2$ to Nit group or Met was coadministered with Nit, EC_{50} were markedly decreased compared with Nit group [(7.0 ± 1.2) nmol·L^{-1}, 95 % confidence levels were 8.25 nmol·L^{-1} and 5.75 nmol·L^{-1} and (15.0 ± 3.2) nmol·L^{-1}, 95 % confidence levels were 18.3 nmol·L^{-1} and 11.6 nmol·L^{-1}, respectively], $P < 0.01$. These results indicated the Met or $ZnCl_2$ improved the vessels relaxation. (Fig 1)

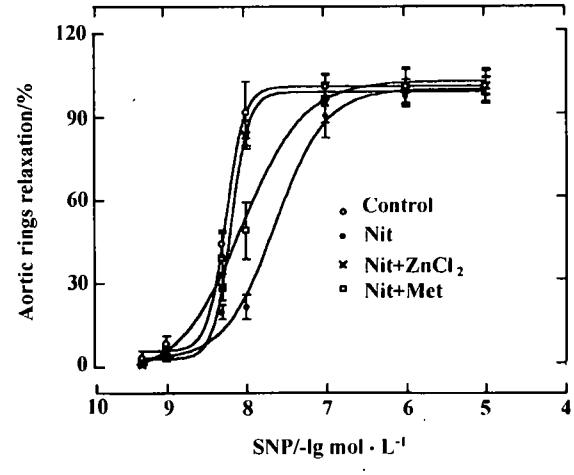

Fig 1. The dose-response curves to sodium nitroprusside for four groups in rat aortic rings preconstricted with phenylephrine 10 μmol·L^{-1}. $n = 6$. $\bar{x} \pm s$.

DISCUSSION

In our experiment, we found that 2-day treatment of Nit patch (0.05 mg·h^{-1}) markedly attenuated hypotensive response, decreased vessel relaxations to SNP, and increased EC_{50}. Exogenous Met markedly enhanced hypotensive response and vessel relaxation induced by SNP *in vivo*. Endogenous Met production can be induced by multifactors such as heavy metals like Zinc, Cadmium, Copper, interleukin 1, some stress, hormones, etc[11]. In our experiment, $ZnCl_2$ induced endogenous Met production. When $ZnCl_2$ 200 μg·kg^{-1} was preadministered, liver Met was increased about 2.3 times higher in Nit + $ZnCl_2$ group compared with Nit alone group. Increased endogenous Met production also enhanced the hypotensive response and vessel relaxation. This showed that the administration of exogenous Met or induction of endogenous Met both can antagonize the development of Nit tolerance. In addition, Nit also slightly increased Met (in liver and plasma), which might be due to that Nit-induced hypotensive stress is responsible.

Sulfhydryl group depletion and increase in oxygen free radicals are important mechanisms involving in Nit tolerance[2,6]. Sulfhydryl group is necessary for Nit metabolism[7]. It has already been suggested that steady level of superoxide anion (O_2^-) in Nit tolerant vessel is approximately twice than that in control vessels[2]. Superoxide readily reacts with NO to form peroxinitrite ($ONOO^-$) which, although capable of activating guanylate cyclase, has a substantially shorter half life than NO and is likely less potent, and impairs the vessel relaxation[12]. Met contains large amount of sulfhydryl group and is one of the strongest scavenger for oxygen free radical[9,13]. In clinical practice, an-

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giotensin converting enzyme inhibitor, vitamin C, and vitamin E are frequently used to prevent Nit tolerence. It is suggested that Met could improve the Nit tolerance with supply of sulfhydryl group for Nit metabolism to form large amount of *S*-nitrosothiol, which activates the guanylate cyclase[14], and by its scanvenging action of oxygen free radical which is formed in vascular intima during development of Nit tolerance.

In conclusion, endogenous or exogenous Met could antaganize Nit tolerance.

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金属硫蛋白对大鼠硝酸甘油耐药性的影响

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关键词 金属硫蛋白; 药物耐受性; 硝酸甘油; 锌化合物; 胸主动脉; 硝普盐; 低血压; 血管舒张

目的: 评价金属硫蛋白(metallothionein, Met)在体内是否能改善硝酸甘油耐药的发生. **方法**: 大鼠给予硝酸甘油(nitroglycerin, Nit)贴剂治疗两天($0.05\ mg\cdot h^{-1}$)以产生耐药. 于耐药大鼠预先给予 $ZnCl_2$ 以诱导内源性 Met 的合成及给予外源性 Met $15\ mg\cdot kg^{-1}\cdot d^{-1}$连续 2 d. **结果**: Nit + $ZnCl_2$ 组大鼠肝脏、血浆 Met 明显高于对照组(C 组). Nit 组大鼠离体主动脉环的舒张反应最低. Nit + $ZnCl_2$ 组大鼠及 Nit + Met 组大鼠对 SNP 的降压反应明显强于 Nit 组. **结论**: 外源性 Met 或内源性诱导合成的 Met 可以改善大鼠 Nit 耐药的发生.

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