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双苯氟嗪、氟桂利嗪、桂利嗪对 5-羟色胺所致离体猪基底动脉收缩的拮抗作用

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关键词 双苯氟嗪；氟桂利嗪；桂利嗪；基底动脉；血清素；血管收缩

A 目的：探索双苯氟嗪（Dip，一种我国自行合成的桂利嗪衍生物），对 5-羟色胺所致的脑动脉收缩的影响．方法：比较双苯氟嗪、氟桂利嗪（Flu）、桂利嗪（Cin）对 5-羟色胺所致离体猪基底动脉收缩的抑制及两种收缩成分的影响．结果：三者的拮抗作用强度顺序（IC_{50}）为 Dip 4.0 μmol·L^{-1} > Flu 15.6 μmol·L^{-1} > Cin 25.2 umo·L^{-1}．这三种药对 5-羟色胺所致离体猪基底动脉的两种收缩成分均有拮抗．Dip 和 Cin 抑制收缩的快速相强于持续相，而 Flu 对二者的作用无显著差异．结论：在 Dip，Flu 和 Cin 三种药之中，Dip 对脑血管的扩张作用最强，其原因主要与抑制内钙的释放有关．

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Effects of *m*-nisoldipine on aortic calcium accumulation in rats with vascular calcium overload[1]

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KEY WORDS *m*-nisoldipine; verapamil; cholecalciferol; calcium; arteries; electron probe microanalysis

AIM: To study the effects of a novel calcium channel blocker, *m*-nisoldipine, on vascular calcium overload (VCO) at both tissue and cellular levels. **METHODS**: VCO was induced in Wistar rats by treatment with colecalciferol (Col, 400 000 IU·kg^{-1}, *po*) and an aqueous mixture of ethanol and polyethyleneglycol-400 for 3 d. The tissue and subcellular calcium contents of aorta were determined by atomic absorption spectrometer and electron probe microanalysis, respectively. **RESULTS**: Chronic treatment with *m*-nisoldipine (*m*-Nis, 1 - 15 mg·kg^{-1}, *po*, bid) only had mild inhibition on the elevation of total calcium in aorta, and the dose-response relationship of *m*-Nis displayed a bell shape, with inhibition ratio of 24 % only for *m*-Nis 2.5 mg·kg^{-1}. The effect of verapamil (12.5 mg·kg^{-1}, *po*, bid) was a little better than that of *m*-Nis. The intracellular VCO in medial smooth muscle cells of aorta were remarkably inhibited by *m*-Nis (2.5 mg·kg^{-1}), with inhibition ratios of 72 % for cytoplasm and 75 % for mitochondrion. The calcium accumulation in nucleus was reduced to a lesser degree than

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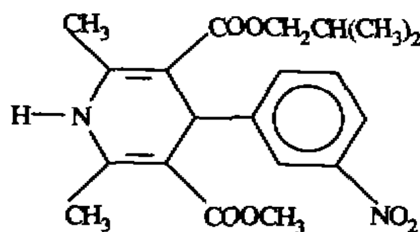
those in cytoplasm and mitochondrion. CONCLUSION: As for aorta in VCO rats, *m*-Nis mainly had conspicuous inhibition on intracellular VCO in medial smooth muscle cells, particularly in cytoplasm and mitochondrion, but with little effect on extracellular calcium deposition at tissue level.

In arteriosclerosis, the role of vascular calcium overload receives attention recently[1-5]. Our study (unpublished data), combined with previous reports[3], suggested that VCO was possibly a basic process common to certain cardiovascular disorders such as arteriosclerosis and aging. A rat model of VCO on high dose of colecalciferol (Col) was used for test of drug action[1-5]. However, one of limitations for intramuscular injection of Col in previous models was long-term retaining of Col in the body due to slow absorption. To minimize indirect influence of residual Col on the analysis of drug action, the administration of Col was changed to oral route in our study.

Several calcium channel blockers (CCB) such as verapamil, nilvadipine, benidipine, and isradipine had been tested for their effects on the evolution and functional consequences of VCO, but its effectiveness was inconsistent[1-7]. Besides that intracelluar Ca^{2+} was not measured, in some cases, only one dose of CCB was tested. *m*-Nisoldipine (*m*-Nis) was a new synthetic CCB characterized by relative light stability and vascular selectivity[8]. Its possible effect against VCO was examined in this study.



***m*-Nisoldipine**

MATERIALS AND METHODS

Male Wistar rats, weighing $212 \pm s$ 15 g, were given a standard diet and tap water (calcium: 0.26 $mmol \cdot L^{-1}$). Body weight was recorded daily.

Induction of VCO Rats were orally treated with Col 400 000 $IU \cdot kg^{-1}$ at 8 AM for 3 d and an aqueous mixture of 30 % ethanol and 10 % polyethyleneglycol-400 (2 $mL \cdot kg^{-1}$, *po*) at 10 AM and 10 PM. This mixture was also used as the solvent of CCB. On d 5, d 7 or d 11 after the start of Col administration, rats were killed for analysis. All rats were divided into 3 groups: (1) untreated group: no drug was given; (2) VCO group: treated with Col and the mixture; (3) treatment group: CCB was added into above mixture on basis of VCO group, and rats were treated with CCB from d 2 to d 7.

Determination of tissue and serum calcium contents[6] Thoracic aorta and caudal artery were rinsed free of blood with deionized water and heated to constant dry weight at 125 ℃ for 40 – 50 min. The dry tissues were digested in HNO_3 14 $mol \cdot L^{-1}$ at 37 ℃ for 2 h, then at about 20 ℃ for 1 d. The solution was diluted and 0.1 % $LaCl_3$ was added to minimize the chemical interference. The calcium content was measured using an atomic absorption spectrometer (Perkin-Elmer 3030, USA) at 422.7 nm.

Histological study The thoracic aorta sections of 3 μm thick were stained with $AgNO_3$ and hematoxylin-eosin to reveal deposited calcium (von Kossa staining).

Electron probe microanalysis Rats were decapitated, and the thoracic aorta was immersed in a modified Krebs-Henseleit solution bubbled with 95 % O_2 + 5 % CO_2 at 37 ℃. The aorta was wrapped on a specimen pin with inner surface out and tied at the base. After being immersed in 4 % bovine serum albumin for 4 min, the aorta was rapidly frozen in supercooled freon (– 164 ℃). The media of aorta was cut into sections of 100 – 200 nm thick at – 130 ℃ with an Ultracut E ultramicrotome equipped with an FC 4D cryosystem. The cryosections were freeze-dried, and coated with a thin carbon film, which were analyzed under an analytical electron microscope (JEM-1200 EX) attached with an energy dispersive X-ray spectroscope (Link AN 10 000). The energy spectra at nucleus and adjacent cytoplasm and mitochondria of medial smooth muscle cells were collected. The accelerating voltage 80 kV; electron beam diameter below 0.5 μm; collecting time 200 s[9,10]. The acquired spectra were processed with Quantem/FLS quantitative software for biological thin specimens (designed by Link System, UK). Data were collected from 3 – 4 rats for each group.

Materials *m*-Nis was synthesized by Prof YUAN Feng-Yan (Hebei Medical College, China), and dissolved in the above mixture under dim light just before use. The volumes of solvent were 2 $mL \cdot kg^{-1}$. Col was the recent product of Shanghai 9th Pharmaceutical Factory (within 3 months). HNO_3 (GR) and ethanol (AR) were made by Shanghai 1st Pharmaceutical Factory. Polyethyleneglycol-400 (AR) was made by Chuansha Pharmaceutical Factory (Shanghai).

Statistics Results were expressed as $\bar{x} \pm s$. Comparisons between groups were made by t-test and F test.

RESULTS

Validation of VCO model After the start of Col-treatment, the calcium accumulation in aorta progressed rapidly during the first week, then slowed down to nearly a plateau after 7 d. Serum calcium concentration decreased rapidly just from d 5, and closed (but did not return) to the normal level on d 11 (Tab 1).

Tab 1. Vascular calcium overload (VCO) after colecalciferol (400 000 IU · kg^{-1}, *po*, qd × 3 d) and solvent treatment in rats. $n = 6-7$, $\bar{x} \pm s$.
$^{b}P<0.05$, $^{c}P<0.01$ *vs* untreated rats.

	Calcium contents in		
	Aorta, μmol/g dry wt	Caudal artery, μmol/g dry wt	Serum, mmol·L^{-1}
Untreated	17 ± 3	23 ± 4	2.54 ± 0.15
VCO			
d 5	189 ± 20^{c}	46 ± 15^{c}	4.67 ± 0.34^{c}
d 7	260 ± 56^{c}	90 ± 41^{c}	3.84 ± 0.28^{c}
d 11	270 ± 58^{c}	105 ± 27^{c}	3.15 ± 0.20^{b}

So the following analyses were carried out at d 7, and serum calcium level was served as a monitor of each VCO model. In VCO rats, the calcium deposition was most conspicuous in upper aorta, and commonly decreased in severity along the direction of blood flow. Light microscopy revealed massive calcium depositions in the media of aorta. The walls of coronary arterioles were usually outlined by thin medial calcium, but in some cases, they were diffusely impregnated with calcium, whereas myocardium remained less affected (Fig 1). Similar results were found in 5 rats of same group.

Effects of CCB on tissue calcium content Chronic treatment with m-Nis 1 – 15 mg·kg^{-1} had only mild inhibition on the increases of total calcium contents in aorta. The dose-response relationship for m-Nis displayed a bell shape, with inhibition ratio of only 24 % at dose of 2.5 mg·kg^{-1}. As for caudal arteries, the effect of m-Nis varied from rat to rat, and in 3 rats, the increases of total calcium were aggravated by m-Nis. On the other hand, m-Nis had no effect on serum calcium level. The effects of verapamil (Ver) 12.5 mg·kg^{-1} was a little better than that of m-Nis 2.5 mg·kg^{-1} (Tab 2).

Electron probe microanalysis On the cryosection of aortic media, nucleus and adjacent mitochondria of vascular smooth muscle cell (VSMC) were recognized (Fig 2).

Fig 1. Thoracic aorta (A) and left ventricle (B) of rats treated with colecalciferol and solvent for 3 d. von Kossa staining, × 125.

Tab 2. Effects of *m*-nisoldipine (*m*-Nis) and verapamil (Ver, 12.5 $mg \cdot kg^{-1}$) on tissue calcium contents (μmol/g dry wt) in rats with VCO. $n = 6-7$, $\bar{x} \pm s$.
$^bP<0.05$ vs VCO group, $^eP<0.05$ vs untreated group.

	Aorta	Caudal artery
Untreated	17 ± 3	23 ± 4
VCO	260 ± 56^b	90 ± 41^b
VCO + *m*-Nis, $mg \cdot kg^{-1}$		
1	212 ± 28^e	102 ± 27^e
2.5	198 ± 41^{be}	103 ± 27^e
5	213 ± 50^e	108 ± 39^e
10	238 ± 57^e	95 ± 38^e
15	313 ± 77^e	57 ± 32^e
VCO + Ver	182 ± 33^{be}	74 ± 27^e

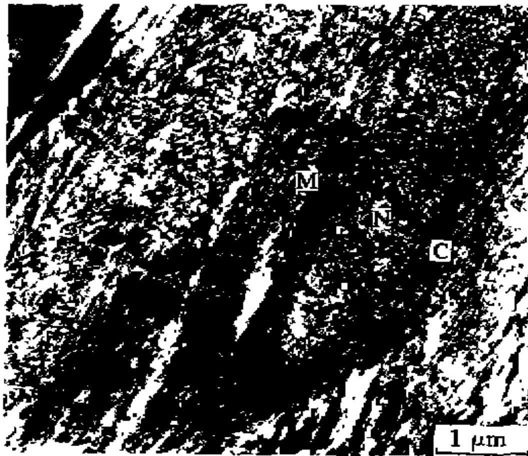


Fig 2. Media of rat aorta after VCO.
C: cytoplasm; M: mitochondrion; N: nucleus. × 20 000.

Different regions showed their special energy spectra with different ratios of P to Cl peak. In most cases, the ratios of P to Cl peak were much greater in nucleus than that in cytoplasm. The CaKα peak in the energy spectrum was at 3.7 keV and the overlapped part of K peak could be subtracted from each spectrum by the computer. In untreated rats, the intracellular Ca^{2+} concentrations in VSMC were so low that near the sensitivity of detection for EPMA technique. But in VCO rats, CaKα peak became very prominent, and intracellular Ca^{2+} concentration elevated remarkably. The cytoplasmic Cl^- was also increased along with the elevation of Ca^{2+} (Fig 3).

In general, the calcium levels in cytoplasm and mitochondria were increased to much greater degree than that in nucleus. Treatment with *m*-Nis (2.5 $mg \cdot kg^{-1}$) reduced the cytoplasmic and mitochondrial [Ca^{2+}] with inhibition ratios of 72 % and 76 %, respectively. Cytoplasmic [K^+] was also decreased after chronic treatment with *m*-Nis. The effects of *m*-Nis on the calcium overload in nucleus was relatively small (Tab 3).

Tab 3. Effects of *m*-nisoldipine (*m*-Nis, 2.5 $mg \cdot kg^{-1}$) on intracellular calcium concentrations of vascular smooth muscle cells of thoracic aorta in rats with VCO. $\bar{x} \pm s$ (number of fields measured from 3 – 4 rats for each group). $^cP<0.01$ vs VCO rats.

Groups	Subcellular [Ca^{2+}], μmol/g dry wt		
	Cytoplasm	Mitochondrion	Nucleus
Untreated	<5	<5	<5
VCO	81 ± 41 (17)	70 ± 34 (11)	45 ± 20 (10)
VCO + *m*-Nis	23 ± 11^c(22)	17 ± 8^c(21)	22 ± 16^c(17)

DISCUSSION

Our results indicated that *m*-Nis, at tissue level, exhibited only slight inhibition on VCO of conductance arteries such as aorta and caudal artery. As majority of calcium was presumably located at extracellular sites, it was logical that total calcium content mainly reflected extracellular calcium upon matrix. Intracellular VCO was also involved in this model as confirmed by EPMA technique. The inhibitory effect of *m*-Nis on intracellular VCO appeared to be more potent than that on total calcium content with respect to inhibition ratios. To our knowledge, this is the first report dealing with intracellular VCO in aorta. Previous reseachers did not study intracellular VCO simultaneously, so it was more likely to get confusable results about the effects of CCB.

It has been proposed that imbalance in the calciferol endocrine system such as chronic Col excess play a role in the etiology of atherosclerosis[11]. Treatment with large dose of Col produced pronounced calcium overload mainly in cardiovascular system, which, to some degree, showed tissue selectivity. VCO was not a direct result of an increase of serum calcium in this model.

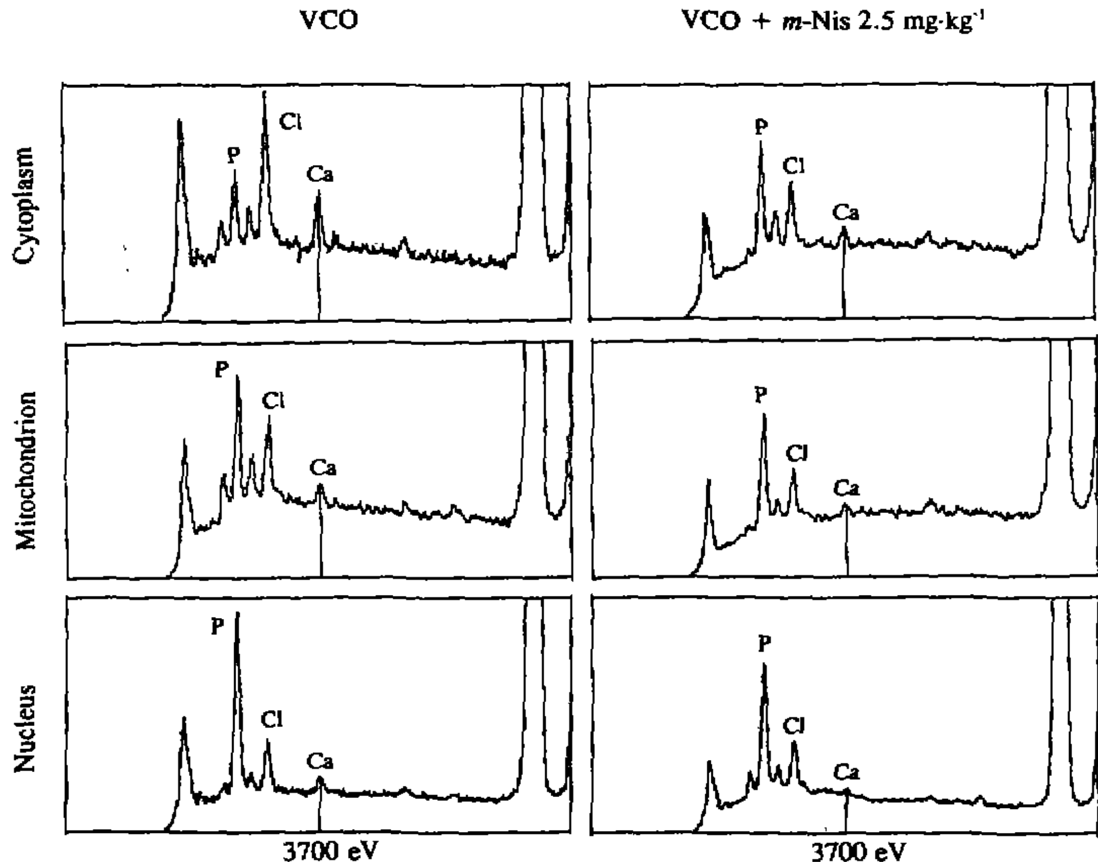


Fig 3. X-ray spectra from media of thoracic aorta. To better illustrate the Ca peak, K peaks (one of which partially overlapped Ca peak) were subtracted by computer.

The solution of ethanol and polyethyleneglycol alone did not induce VCO, but enhance the effect of Col. One limitation of our VCO model was that chronic process of VCO in human[1,3] had been densified to a short period of about 1 wk, then the progress of VCO was so rapid that many pharmacological interventions showed little effect.

It is unclear why *m*-Nis at high doses aggravate the elevation of total calcium in VCO rats. The possible causes were the intermittent reflex increase in sympathetic activity induced by dihydropyridines, and the proischemic effect due to pronounced hypotension with large dose. The mechanisms of effect on VCO that CCB showed in this study might be quite complex, which could not be explained by calcium channel blocking action alone. Other mechanisms such as endothelial protection[12], enhancement of PGI_2 release[13] and possible antiperioxidant effect similar to nisoldipine[14] might be involved. Failure of *m*-Nis to completely inhibit intracellular VCO suggested that other routes rather than *L*-type calcium channel responsible for calcium influx. Our results also indicated that nondihydropyridine verapamil was superior to *m*-Nis with respect to the effects on VCO. In addition, the focal distribution of calcium deposition upon elastic matrix of aorta was, in most cases, changed to a relatively diffuse pattern after treatment with CCB, though the total calcium content was slightly decreased only.

Mitochondrion was a "slow" compartment and served as a buffer for excess calcium accumulation in cytoplasm[15]. This idea was supported by our finding that the magnitude of calcium accumulation at mitochondrion was comparable with that of cytoplasm in VCO rats, and both of them were reduced by *m*-Nis. On the other hand, the increase of calcium in nucleus was much smaller than

that in cytoplasm, and was relatively less affected by *m*-Nis. These results indicated that nuclear envelope limited but did not block the passage of calcium accumulation from cytosol to nucleus. Intracellular VCO is also important as it may be more relevant to arterial proliferative response. At above new aspects, the role of dihydropyridine CCB in the treatment of cardiovascular diseases remained to be revaluated.

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间尼索地平对血管钙超负荷大鼠主动脉钙积累的作用[1]

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关键词 间尼索地平; 维拉帕米; 胆骨化醇; 钙; 动脉; 电子探针微分析

目的: 在组织和细胞水平探讨间尼索地平(*m*-Nis)对血管钙超负荷(VCO)大鼠主动脉钙积累的作用 **方法**: 建立大鼠 VCO 模型, 用原子吸收分光光度法和电子探针微分析法分别测定主动脉总钙和动脉平滑肌亚细胞钙. **结果**: *m*-Nis (1 - 15 mg·kg^{-1})对 VCO 大鼠主动脉总钙升高作用弱, 且量效关系呈钟罩形 *m*-Nis 2 5 mg·kg^{-1}对总钙升高抑制为 24 %. 维拉帕米 12 5 mg·kg^{-1}疗效略优于 *m*-Nis. *m*-Nis 2 5 mg·kg^{-1}对主动脉中膜平滑肌细胞的胞浆和线粒体钙升高有明显抑制, 抑制率分别为 72 %和 76 %, 对胞核钙升高抑制稍小. **结论**: *m*-Nis 对 VCO 大鼠主动脉主要抑制血管平滑肌细胞内尤其是胞浆和线粒体钙超负荷, 而对组织间质钙沉积作用较弱.

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