ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10): 881-886
http://www.chinaphar.com

Naloxone increases vascular responsiveness in chronic morphine treated rats and facilitates intracellular signaling in cultured vascular cells[1]

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KEY WORDS morphine; naloxone; norepinephrine; calcium; cultured cells

ABSTRACT

AIM: To probe the changes of vascular responsiveness and intracellular signaling during opiate withdrawal syndrome. **METHODS**: Morphine withdrawal syndrome in rats was precipitated by iv naloxone following daily injection of increasing dose of morphine for 2 weeks, the changes in mean arterial blood pressure (MBP) caused by acetylcholine (Ach) were recorded. Isolated mesenteric vascular beds were perfused with Kreb's solution containing different concentration of drugs. The cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) in Fura 2-loaded cultured bovine aortic endothelial cells (aec) and smooth muscle cells (smc) were assayed. The positive immunoreaction to the phosphorylated cyclic AMP responsive element-binding protein (phospho-CREB) in cultured smc was calculated. **RESULTS**: Naloxone 2 mg/kg iv following chronic morphine treatment precipitated severe opiate withdrawal signs in company with blunted hypotensive effect of Ach that was the same as chronic morphine treated rats before withdrawal. In the mesenteric vascular beds from chronic morphine treated rats, the EC_{50} of pressor effect of norepinephrine (NE) was decreased from (2.06 ± 0.38) to (1.14 ± 0.21) μmol/L ($n = 8$, $P < 0.01$) after the perfusion solution containing morphine 20 μmol/L was replaced by Kreb's containing naloxone 25 μmol/L. Furthermore, NE-induced perfusion pressure increases were completely prevented by Kreb's contained morphine 40 μmol/L. Morphine acutely applied to control smc produced some variable and naloxone-reversible $[Ca^{2+}]_i$ changes, but naloxone did not. However, naloxone increased $[Ca^{2+}]_i$ in two thirds of smc preincubated with morphine 0.1 or 0.5 mmol/L for 48 h from (97 ± 20) to (167 ± 29) nmol/L ($n = 9$, $P < 0.01$) and from (106 ± 19) to (225 ± 48) nmol/L ($n = 10$, $P < 0.01$), respectively, and it also increased the ratio of positive immunoreaction to phospho-CREB from (7.7 ± 3.2) % to (19.6 ± 4.7) % ($n = 6$, $P < 0.01$) in smc preincubated with morphine 0.5 mmol/L. In addition, naloxone decreased $[Ca^{2+}]_i$ from (146 ± 34) to (78 ± 24) nmol/L in one third morphine-preincubated aec ($n = 10$, $P < 0.01$). **CONCLUSION**: That naloxone enhances vascular responsiveness to NE in chronic morphine treatment rats may be relavant to $[Ca^{2+}]_i$ transient facilitation in company with cAMP-dependent phosphorylation enhancement.

INTRODUCTION

Although naloxone-precipitated cardiovascular abnormality appeared inevitably in opiate-withdrawal syndrome[1,2], the question whether peripheral vascular activity directly participates in this process remains unclear. Up to now, the morphine withdrawal *in vitro* has been found only in ileum[3], and our previous results showed isolated Langendorff hearts or tail arterial segments from morphine-dependent rats did not exhibit evident withdrawal behaviors after naloxone challenge[4], but we still wander whether these isolated organs from opiate-dependent animals have potential abnormal responsiveness. Recently, several experimental studies indicating both opioid peptides and alkaloid opiates act as cardiovascular modulators have emerged[5], such as endomorphine 1- and 2-produced vasodepression[6] in mice and morphine-induced venodilation in human hand veins[7], and the alkaloid selective μ_3 receptor was

[1] Project supported by the grant from the Ministry of Education of China for mainstays in Chinese Universities.
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Received 2001-02-06 Accepted 2001-06-18

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

identified to induced nitric oxide release from human vascular tissues[8]. Therefore, to probe vascular circulation changes and the mechanism during opiate withdrawal is prompted.

In neural preparations acute or chronic morphine exposure evoked intracellular signaling transduction changes that appeared in adenyly cyclase and G protein as well as membrane ion channels[9-12], but the changes in peripheral organs remain unclear. To shed some new light on the mechanism of opiate withdrawal syndrome, the vascular responsiveness in chronic morphine treatment rats was investigated in the present study. With a view to discover the intracellular signaling basis of opiate abstinence the intracellular calcium ($[Ca^{2+}]_i$) and the phosphorylation of cAMP responsive element-binding protein (phospho-CREB) in cultured vascular cells were also observed.

MATERIALS AND METHODS

Drugs and reagents Morphine hydrochloride and naloxone were respectively from Qinghai and Beijing Quaternary Pharmaceutical Factory, China. Acetylcholine (Ach) and norepinephrine bitartarate (NE) were from Shanghai Third Reagent Factory and Shanghai Harvest Pharmaceutical Co Ltd. Phospho-CREB antibody, specifically recognized phosphorylated CREB at Ser^{133} was from New England Biolabs of USA. Labeled streptavidin-biotin (LSAB) immunohistochemistry staining kit was from Zymed Laboratories Inc (South San Francisco, USA). Forskolin and diaminibenzidine (DAB) as well as a part of naloxone were from Sigma Co (St louis, USA). RMPI-1640 media was from Gibco, USA. Fura 2/AM from the Shanghai Institute of Physiology, Chinese Academy of Sciences.

Chronic morphine treatment of rats Wistar rats of both sexes (Grade Ⅱ, 243 g ± 25 g), provided by the Experimental Animal Center of Nanjing Jinling Hospital, China (Certificate № 97001), were housed at 26 ℃ ± 1 ℃ in humidity control room and allowed free access to tap water and standard laboratory diet. Morphine in daily increasing dose (24 h apart) of 5, 8, 11, 14, 17 and finally 20 mg/kg were subcutaneously injected for 2 weeks, the final dose was maintained until experiments. Control rats received daily once saline injection.

Experimental protocal *in vivo* After anaesthetized with pentobarbitone sodium 40 mg/kg ip, left common carotid artery and unilateral femoral artery of rats were cannulated respectively for measuring mean arterial blood pressure (MAP) and intra-arterial infusion (ia) of Ach, heprin 500 – 800 U was infused to prevent blood coagulation followed by 15 min's equilibrium. The hypotensive effects of ia Ach in dose of 2, 4, 8, and 16 μg/kg were tested in rats undergoing chronic morphine treatment and opiate abstinence, which was evoked by iv naloxone 2 mg/kg. The changes in MAP and the heart rate (HR) as well as opiate withdrawal signs were recorded for 1 h.

Mesenteric vascular bed perfusion Rat's superior mesenteric arteries were cannulated so that the mesenteric vascular beds was perfused by filtered Kreb's solution at the constant rate of 5 mL/min and 37 ℃. The Kreb's was composed of (mmol/L) NaCl 117.9, $NaHCO_3$ 25.0, KCl 4.7, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.3, and dextrose 11.1, and being aerated with 95 % O_2 and 5 % CO_2. The isolated preparations were mounted in a warm glass chamber, perfusion pressure was measured through a bypass duct, which linked with a transducer-coupled polygrapher (Model RM-6200c, Chengdu, China). The basal pressure in the system was (7.20 ± 0.25) kPa. The Kreb's to perfuse control preparation did not contained any drug, but that for acute or chronic drug-exposed preparations contained morphine 20 or 40 μmol/L during 30-min equilibrium, since this concentration of morphine did not affect the vascular reaction to high potassium. After then, a serial final concentration of NE in 0.3, 0.7, 1.4, and 2.8 μmol/L (15 min apart) as well as Ach were applied into perfusion system to elicit vascular responses.

Cell cultures and morphine preincubation Washed bovine aortic segments were dissected aseptically, endothelium tissue was incubated with collagenase 1 mg/mL at 37 ℃ for 2 h to dissociate endothelial cells from bovine aorta (aec). Tissues from middle layer were cut into 1 – 2 mm^2 in size and allowed adhesion to culture flasks for 2 h before adding culture media. The method for cell cultures was carried out as the previous[13]. The aortic smooth muscle cells (smc) were grown out from tissue edges in several days. The confluent cell cultures were passed every 5 d, and further grew in the medium containing 10% bovine serum. At 4 – 5 generation, cultured cells were dislodged and diluted in density of 1×10^8/L, then was planted on gelatin-coated coverslips for 48 h where they were exposed to morphine 0.1 or 0.5 mmol/L, and forskolin 20 mmol/L, respectively.

$[Ca^{2+}]_i$ assay The methods for loading cells with Fura 2/AM and $[Ca^{2+}]_i$ assay were the same as previously described[13]. Cells on coverslips were immersed in Hank's solution containing Ca^{2+} 1.3 mmol/L. In addition, the medium containing morphine 0.1 or 0.5 mmol/L was specially prepared for morphine-incubated cells.

Naloxone exposure and immunocytochemistry After preincubated with drugs for 48 h, the cell culture were washed and further incubated with Hank's solution contained naloxone 0.2 mmol/L for 30 min. Then were fixed with 3 % paraformaldehyde for 30 min. During immunocytochemistry staining, buffer containing NaCl 135 and Tris-HCl 50 mmol/L as well as 0.3% Triton X-100 (TTBS, PH 7.4) was used to wash cells. The labeled-streptavidin-biotin peroxidase complex method was carried out. In brief, non-specific binding was blocked with 3 % normal goat serum, smc were incubated with primary antibody diluted at 1:100 at 4 ℃ overnight, then reacted with biotinylated secondary antibody (at 1:200 dilution) for 1 h. After eliminated endogenous peroxidase activity with 0.6 % H_2O_2, the samples were linked with complex of streptavidin and proxidase. The mixture of DAB and 0.02 % H_2O_2 was added to reveal the presence of phosphorylated-CREB in smc, hemalum staining was applied for counter-staining. The primary antibody was omited in the light of negative control. The percentage of positive phospho-CREB labeled smc were calculated.

Data analysis All data were presented as $\bar{x} \pm s$, and the paired t test or one-way anova was used for quantitative data analysis. EC_{50} was calculated according to the Lineweaver-Burk equation.

RESULTS

Ach-induced hypotension In control rats the administration of Ach through femoral arteries (ia) produced dose-dependence but evanescent decrease in MAP, which were completely abolished by pre-injection of atropine 5 mg/kg, reflexing that tachycardia did not occurred in aneasthetized condition until Ach 8 mg/kg was applied. In chronic morphine treated rats the dose-related hypotension induced by Ach was obviously attenuated, and iv naloxone 2 mg/kg decreased MAP temporarily and evoked withdrawal signs including body shakes, limb's twitching, teeth chatting, and defaecation, while Ach-induced hypotension was still blunted (Tab 1).

Responsiveness to NE and Ach in isolated mesenteric vascular beds In mesenteric vascular beds from control rats, the EC_{50} of NE-induced pressor effect was (1.31 ± 0.20) μmol/L ($n = 6$), and acute administration of morphine 40 μmol/L or naloxone 25 μmol/L did not affect this value. However, in the preparations from chronic morphine treated rats the value was equal to (2.06 ± 0.38) μmol/L ($n = 8$, $P < 0.01$ *vs* control) as morphine 20 μmol/L was contained in Kreb's. Moreover, the pressor effect of NE was completely failed when morphine 40 μmol/L was applied for vascular beds of chronic morphine treatment rats. Conversely, After withdrew morphine from Kreb's and naloxone 25 μmol/L was applied for these preparations, the EC_{50} of NE was restored to (1.14 ± 0.21) μmol/L ($n = 8$, $P > 0.05$ *vs* control).

In mesenteric vascular beds from control rats, additions of Ach had no effect on basal perfusion pressure, but very effectively reversed NE-induced increase of perfusion pressure, for Ach 1.1 μmol/L was enough to abolish the pressor effects of NE 0.7 μmol/L from (13.6 ± 0.8) to (10.6 ± 0.4) kPa ($n = 6$, $P < 0.01$). Because of the failure of the preparations in response to NE in the presence of morphine 40 μmol/L, the antagonist interaction between NE and Ach on vascular

Tab 1. Attenuated hypotensive effect of ia Ach in rats undergoing chronic morphine treatment (CMP) and naloxone-precipitated withdrawal syndrome. MAP: mean arterial blood pressure. $\bar{x} \pm s$. $^cP < 0.01$ *vs* Basal. $^eP < 0.05$ *vs* Control.

Pretreatment	n	MAP (kPa) after ia Ach				Withdrawal signs
		0 (Basal)	2	4	8/μg·kg^{-1}	
Control	9	16.0±1.9	7.4±1.5^c	5.7±1.2^c	3.6±0.9^c	0
Atropine	7	15.2±2.8	15.2±2.8	15.4±2.8	16.0±2.7	0
CMT	9	16.2±2.4	8.7±2.0^c	7.9±1.8^{ce}	7.2±1.5^{ce}	4
CMT + Naloxone	9	16.0±2.3	8.9±1.8^c	8.6±2.2^{ce}	7.1±2.3^{ce}	58

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
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beds from morphine-dependent rats was beyond measurement. But following naloxone 25 μmol/L was added into Kreb's and the vascular pressor response was recovered, application of Ach could still reverse the effect of NE (Fig 1).

$[Ca^{2+}]_i$ changes in cultured aec and smc

Control smc acutely exposed to morphine 0.5 mmol/L exhibited different responses, including $[Ca^{2+}]_i$ elevation from (101 ± 19) to (140 ± 24) nmol/L ($P < 0.05$, $n = 10$), no change in 28 cells, and biophasic $[Ca^{2+}]_i$ fluctuations in other 12 smc. Acute administration of naloxone 0.1 or 0.2 mmol/L had no direct effect on $[Ca^{2+}]_i$ but abolished acute effect of morphine on $[Ca^{2+}]_i$. However, in smc preincubated by morphine 0.1 and 0.5 mmol/L for 48 h, addition of naloxone 0.2 mmol/L resulted in abrupt $[Ca^{2+}]_i$ increases from (97 ± 20) to (167 ± 29) nmol/L ($P < 0.01$, $n = 9$), and from (100 ± 22) to (227 ± 42) nmol/L ($P < 0.01$, $n = 10$), respectively, the ratio between $[Ca^{2+}]_i$ increase and total tested cells was 19/30.

In control aec, neither morphine nor naloxone produce recordable $[Ca^{2+}]_i$ changes. In contrast, one third morphine (0.5 mmol/L)-preincubated aec exhibited $[Ca^{2+}]_i$ decrease from (146 ± 34) to (78 ± 24) nmol/L ($n = 10$, $P < 0.01$) after challenged by naloxone.

Immunocytochemistry for phospho-CREB

The percentage of positive phospho-CREB-labeled control smc was (3.4 ± 0.8) % ($n = 5$, Fig 2A), so was it in smc incubated with naloxone 0.2 mmol/L for 30 min (data not showed). In smc preincubated with forskolin 20 μmol/L for 48 h, the ratio of phospho-CREB-labeled cells was (19.4 ± 5.8) % ($n = 5$, $P < 0.01$ *vs* control). Addition of naloxone 0.2 mmol/L following incubation with morphine 0.1 or 0.5 mmol/L resulted in obvious increases of positive phospho-CREB-labeled smc with the respective percentage of (10.2 ± 3.9) % and (19.6 ± 4.7) % ($n = 5$, $P < 0.01$ *vs* control, Fig 2C and D), while incubation with morphine 0.5 mmol/L for 48 h only slightly increased the positively-labeled smc to (7.7 ± 3.2) % ($n = 6$, $P < 0.05$, Fig 2B).

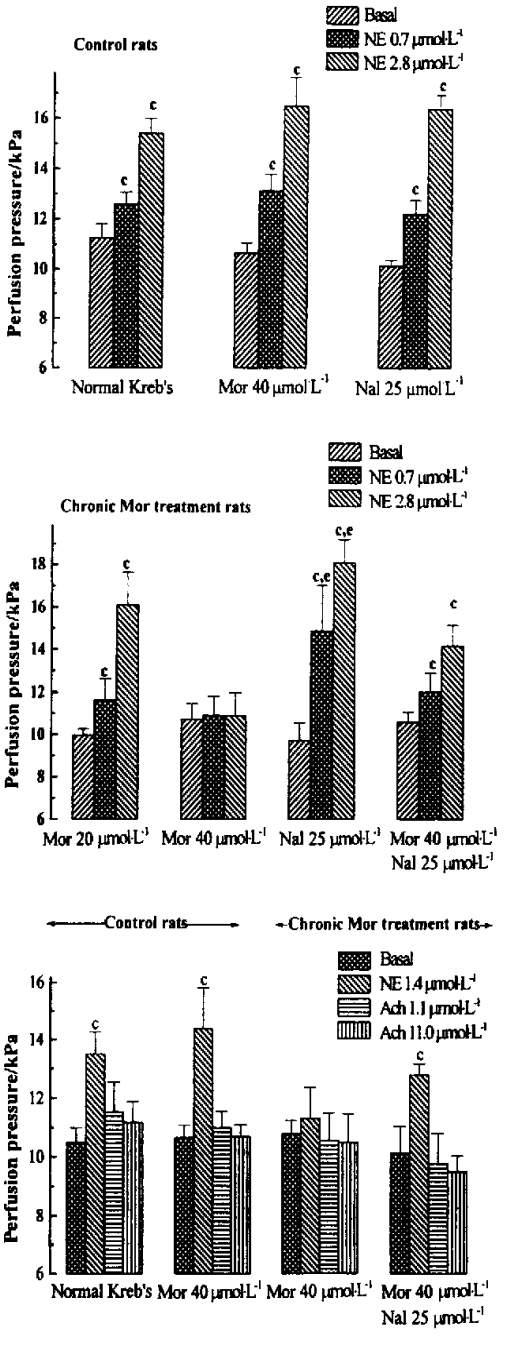

Fig 1. The responsiveness of isolated mensenteric vascular beds to norepinephrine (NE) and acetylcholine (Ach). The drugs contained in perfusion medium are noted below the columns, and the treatments for rats after which mesenteric vascular beds were isolated are noted above the columns. Mor: morphine, Nal: naloxone. $\bar{x} \pm s$. $^cP < 0.01$ *vs* Basal. $^eP < 0.05$ *vs* the relative value in groups without naloxone.

DISCUSSION

The present study demonstrated that chronic morphine treatment in rats not only blunted the hypotension of Ach *in vivo* but also increased the EC_{50} of the pressor effects of NE and even completely inhibited it in mesen-

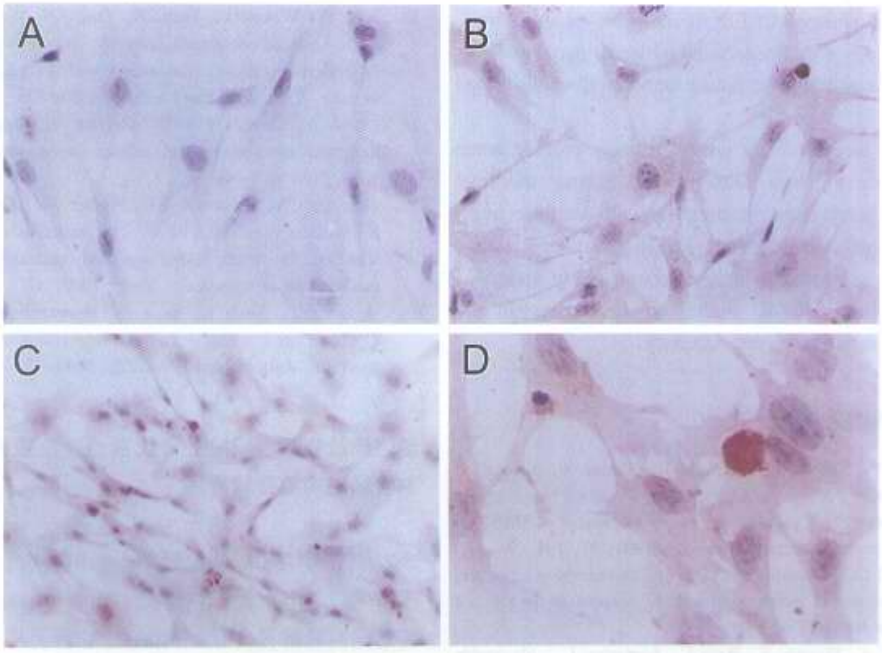

Fig 2. Immunoreactivity to phospho-CREB in cultured bovine vascular smooth muscle cells that underwent different drug pretreatment as follow: A) Control, × 200. B) Exposed to morphine for 48 h, × 200. C) and D): Adding naloxone to smc preincubated with morphine, × 100 and × 400, respectively.

teric vascular beds during perfusion with morphine 20 or 40 μmol/L, suggesting the peripheral vascular functions are abnormally adjusted during morphine tolerance and dependence. On the other hand, the administration of naloxone following chronic morphine treatment potently reversed the responsive defect in mesenteric vascular beds and increased EC_{50} of NE, providing a new plausible explaination to abnormal blood flow and defective vascular function during opiate abstinence[1,2].

The question may be arisen about whether the present vascular defective reaction to NE possibly resulted from non-specific effects of high concentration of morphine so as to doubt the inner relationship between opiate dependence and the abnormal vascular function. One answer comes from the present results in that the control vascular beds did not show similar defevtive reaction to NE during the same concentration of morphine was applied, indicating only acute morphine exposure is not enough to induced the change of vascular responsiveness. In fact, with opiate tolerance and dependence being developed, the dose of opiate for abuse had to be increased surprisingly. Therefore, it is comprehensible that the vascular functional abnormality associates with high concentration of morphine in bodies.

As a very sensitive intracellular second messenger, cytoplasmic free calcium ($[Ca^{2+}]_i$) plays an important role in mediation of cellular function[14]. In this regard, it is necessary to profile the intereaction between the effects of morphine and naloxone on $[Ca^{2+}]_i$ in vascular cells. The present results showed that high concentration of morphine made $[Ca^{2+}]_i$ unsteady in a part of control smc, but smc preincubated by morphine for 48 h did not show obvious different $[Ca^{2+}]_i$ value from control. Much interestingly, in consistent with the outcome that naloxone reversed the responsive defect in morphine-exposed vascular beds, naloxone triggered remarkable $[Ca^{2+}]_i$ elevation only in morphine-exposed smc, indicating $[Ca^{2+}]_i$ signal in vascular smooth muscle cells involves in the intereaction between two drugs during opiate withdrawal syndrome.

As one of constitutive nuclear transcription factor, CREB is phosphorylated at Ser^{133} by cAMP-dependent protein kinase A (PKA)[15]. It is known that intracellular cAMP-generating system involves in morphine tolerance and dependence[16], thus the phosphrylation at Ser^{133} in cultured vascular cells may be sensitive to chronic morphine treatment and naloxone precipitation. The present outcome showed naloxone increased immuno-

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
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reactivity of phospho-CREB in morphine-exposed smc, implicating this cAMP-associated molecule may be affected by naloxone-triggered opiate withdrawal within blood vessels.

Taken together, the present studies provide some new evidence to show naloxone may trigger abnormal vascular responsiveness in company with vascular intracellular signal transduction alterations after chronic morphine treatment and high concentration of morphine exposure, though further studies on the mechanism and therapeutic significance are necessary.

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纳洛酮改变慢性吗啡处理大鼠血管反应性和培养的血管细胞内信号转导[1]

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关键词 吗啡; 纳洛酮; 去甲肾上腺素; 钙; 培养的细胞

目的: 探讨吗啡戒断反应对血管反应性及其细胞内信号转导的影响. **方法**: 大鼠注射递增剂量的吗啡两周后 iv 纳洛酮催瘾, 记录乙酰胆碱(Ach)的降压效应. 用含不同药物的 Kreb's 液灌流大鼠离体肠系膜血管床. AR-CM-MIC 阳离子测定系统检测培养牛胸主动脉内皮细胞(aec)和血管平滑肌细胞(smc)胞浆内游离钙($[Ca^{2+}]_i$). 计算 smc 呈磷酸化 cAMP 反应元件结合蛋白(Phospho-CREB)免疫阳性反应的比例. **结果**: 纳洛酮 iv 2 mg/kg 催瘾后钝化的 Ach 降压效应同催瘾前一致. 以纳洛酮 25 μmol/L 取代灌流液所含吗啡 20 μmol/L 使成瘾大鼠肠系膜血管的去甲肾上腺素(NE)升压效应的 EC_{50}(μmol/L)从 2.06 ± 0.38 降至 1.14 ± 0.21 ($n=8$, $P<0.01$), 而吗啡 40 μmol/L 完全预防 NE 的作用. 即时加入吗啡后对照组血管平滑肌细胞内$[Ca^{2+}]_i$反应不一. 纳洛酮使 2/3 的吗啡预处理组的血管平滑肌细胞内$[Ca^{2+}]_i$显著升高, 呈 Phospho-CREB 免疫阳性反应的比例也因之增加. 部分内皮细胞的$[Ca^{2+}]_i$明显下降. **结论**: 纳洛酮增加慢性吗啡处理大鼠血管的反应性可能与血管平滑肌细胞内钙增加有关, 并伴有 cAMP 反应元件结合蛋白的磷酸化增强.

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