AM. Interestingly, the hemodynamic effects of inhaled AM lasted for >45 minutes. A previous study demonstrated that intravenous injection of AM produces a long-lasting vasodilator response because of its long half-life (≈15 minutes).[32] The half-life of plasma AM after inhalation was longer (20 minutes). Thus, inhalation of AM may cause relatively long-lasting pulmonary vasodilator activity in patients with idiopathic pulmonary arterial hypertension. In the present study, plasma cAMP level increased after AM inhalation, suggesting that the hemodynamic effects of AM may be mediated by activation of cAMP.

Earlier studies have shown that peak $\dot{V}o_2$ during exercise is markedly lower in patients with idiopathic pulmonary arterial hypertension than in healthy subjects.[33,34] Peak $\dot{V}o_2$ is determined primarily by the maximal cardiac output during exercise and the potential for O_2 extraction by the exercising muscle.[35] Thus, the decreased peak $\dot{V}o_2$ may reflect insufficient oxygen delivery to the body during exercise, at least in part because of an inadequate increase in cardiac output under conditions of severe pulmonary hypertension. In the present study, inhalation of AM significantly increased peak $\dot{V}o_2$ in patients with pulmonary hypertension. AM also increased the $\Delta\dot{V}o_2/\Delta W$ ratio, which indicates oxygen transport per unit workload to the exercising legs. These results suggest that inhalation of AM improves exercise capacity in patients with idiopathic pulmonary arterial hypertension. It is possible that an increase in cardiac output during exercise may contribute to increases in peak $\dot{V}o_2$ and the $\Delta\dot{V}o_2/\Delta W$ ratio.

The major limitation of this pilot trial relates to the lack of a randomized, placebo-controlled group in acute hemodynamic studies, which was as result not only of invasive assessment of hemodynamics but also of the limited number of patients available. Nevertheless, cardiopulmonary exercise testing was performed in a double-blind, randomized, crossover design. Thus, it is unlikely that the hemodynamic effects of inhaled AM are attributable to the placebo effect.

Inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. An experimental study demonstrated that repeated inhalation of AM (for 30 minutes, 4 times a day) inhibited monocrotaline-induced pulmonary hypertension and markedly improved survival in rats.[36] Recently, pulmonary delivery of a dry-powder insulin has been shown to improve glycemic control without adverse pulmonary effects.[37] Although further studies are necessary to maximize the efficiency and reproducibility of pulmonary AM delivery, combining AM inhalation therapy with other modalities that have a different mode of action may have beneficial effects in patients with idiopathic pulmonary arterial hypertension.

Conclusions

These preliminary results suggest that inhalation of AM may have beneficial effects on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

Acknowledgments

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Adrenomedullin Gene Transfer Induces Therapeutic Angiogenesis in a Rabbit Model of Chronic Hind Limb Ischemia

Benefits of a Novel Nonviral Vector, Gelatin

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Background—Earlier studies have shown that adrenomedullin (AM), a potent vasodilator peptide, has a variety of cardiovascular effects. However, whether AM has angiogenic potential remains unknown. This study investigated whether AM gene transfer induces therapeutic angiogenesis in chronic hind limb ischemia.

Methods and Results—Ischemia was induced in the hind limb of 21 Japanese White rabbits. Positively charged biodegradable gelatin was used to produce ionically linked DNA-gelatin complexes that could delay DNA degradation. Human AM DNA (naked AM group), AM DNA-gelatin complex (AM-gelatin group), or gelatin alone (control group) was injected into the ischemic thigh muscles. Four weeks after gene transfer, significant improvements in collateral formation and hind limb perfusion were observed in the naked AM group and AM-gelatin group compared with the control group (calf blood pressure ratio: 0.60±0.02, 0.72±0.03, 0.42±0.06, respectively). Interestingly, hind limb perfusion and capillary density of ischemic muscles were highest in the AM-gelatin group, which revealed the highest content of AM in the muscles among the three groups. As a result, necrosis of lower hind limb and thigh muscles was minimal in the AM-gelatin group.

Conclusions—AM gene transfer induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia. Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of peripheral vascular diseases. **(*Circulation*. 2004;109:526-531.)**

Key Words: peripheral vascular disease ■ angiogenesis ■ gene therapy ■ ischemia

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.[1] AM and its receptor are expressed mainly in vascular endothelial cells and vascular smooth muscle cells.[2-4] AM not only induces vasorelaxation but also regulates growth and death of these vascular cells.[5-10] These findings suggest that AM plays an important role in maintaining vascular homeostasis in an autocrine and/or paracrine manner.

A recent study has shown that vascular abnormalities are present in homozygous AM knockout mice, suggesting that AM is indispensable for vascular morphogenesis.[11-13] More recently, AM has been shown to activate the PI3K/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation.[7,14] These results raise the possibility that AM plays a role in modulating vasculogenesis and angiogenesis. However, whether AM induces therapeutic angiogenesis remains unknown.

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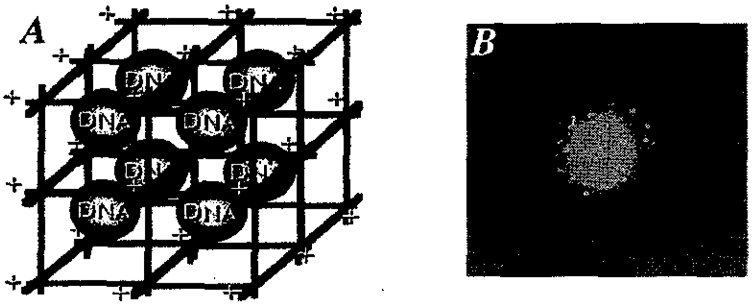

Figure 1. A, Schema of DNA-gelatin complex. Biodegradable gelatin can hold negatively charged plasmid DNA in its positively charged lattice structure. B, RITC-labeled AM DNA particles were incorporated into gelatin.

We prepared biodegradable gelatin that could hold negatively charged protein or plasmid DNA in its positively charged lattice structure.[15,16] Biodegradable gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation.[15] Similarly, ionically linked DNA-gelatin complexes can delay gene degradation.[16] These findings raise the possibility that gelatin may serve as a nonviral vector for gene therapy.

Thus, the purposes of this study were (1) to investigate whether AM gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia and (2) to examine whether the use of biodegradable gelatin as a vector augments AM expression and thereby enhances the therapeutic effects of AM gene transfer.

Methods

Animal Model

All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. Twenty-one male Japanese White rabbits (body weight, 2.9±0.1 kg; Japan Animal Co, Osaka, Japan) were used for physiological and morphological assessment. In addition, 30 rabbits were used for radioimmunoassay, immunohistochemical examination, and Western blot analysis. After anesthetization with pentobarbital sodium (30 to 35 mg/kg), a longitudinal incision was made in the left thigh, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Hind limb ischemia was induced by ligation of the distal left external iliac artery and complete resection of the left femoral artery, as described previously.[17]

Construction of Plasmid DNA

To construct the expression vector for human AM, the *EcoRI/XhoI* fragment of the full-length human AM cDNA was ligated into the EcoRI/XhoI fragment of the pcDNA1.1-CMV expression plasmid (Invitrogen). To verify that the pcDNA1.1-CMV vector encoding AM cDNA produces a biologically active AM protein, the expression vector was transfected into 293 cells, and AM activity in the transfected cells was measured by high-performance liquid chromatography and radioimmunoassay. The pcDNA1.1-CMV vector encoding β-galactosidase (LacZ) cDNA was used as a control DNA.

Preparation of AM DNA-Gelatin Complex

Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of approximately 30 μm, water content of 95%, and an isoelectric point (pI) of 9 after swelling in water.[15,16] Gelatin can hold negatively charged protein or plasmid DNA in its positively charged lattice structure (Figure 1A). Dried gelatin (4 mg, pI 9) was added to human AM DNA solution (500 μg/100 μL in phosphate-buffered saline, pH 7.4). After mixture of DNA and gelatin, DNA-gelatin complexes were incubated at 37°C for 2 hours.

To visualize incorporation of DNA into gelatin. AM plasmid DNA was labeled with rhodamine B isothiocyanate (RITC), as reported previously.[16] In brief, the coupling reaction of RITC to plasmid DNA was carried out by mixing the two substances in 0.2 mol/L sodium carbonate-buffered solution (pH 9.7), followed by gel filtration with a PD 10 column (Amersham-Pharmacia). RITC-labeled AM DNA was incorporated into positively charged gelatin (Figure 1B).

Study Protocol

Ten days after the induction of hind limb ischemia (day 10), AM DNA (naked AM group, n=7), AM DNA-gelatin complex (AM-gelatin group, n=7), or gelatin alone (control group, n=7) was administered intramuscularly into 3 different sites in the ischemic adductor muscle and 2 different sites in the semimembranous muscle. In addition, Lac Z DNA-gelatin complex served as a control DNA (Lac Z-gelatin group, n=5). The amount of plasmid was 500 μg (1 mL) and that of gelatin was 4 mg. Morphological and angiographic analyses and measurements of calf blood pressure and laser Doppler flow were performed 4 weeks after gene transfer (day 38). After completion of these measurements, the adductor, semimembranous, and gastrocnemius muscles were weighed in each hind limb.[18] The muscle weight ratio was calculated for each muscle as follows: muscle weight ratio=muscle weight in ischemic hind limb/muscle weight in nonischemic hind limb. Specimens of the adductor muscle of the ischemic hind limb were obtained for histological examination.

Measurement of Calf Blood Pressure

Calf blood pressure was measured on days 10 and 38 in both hind limbs with a Doppler flowmeter (Hayashi Denki Co, Ltd) and a 25-mm-wide cuff. The pulse of the posterior tibial artery was identified with the use of a Doppler probe, and the systolic blood pressure in both hind limbs was determined by standard techniques. The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic hind limb to that of the normal hind limb.[17]

Laser Doppler Blood Perfusion Analysis

Blood flow of the ischemic hind limb was measured with the use of a laser Doppler blood perfusion image system (moorLDI, Moor Instruments) on day 38.

Angiographic Analysis

Development of collateral arteries was evaluated by angiography on days 0 and 38. A 4F catheter was placed in the left internal iliac artery through the common carotid artery, and 3 mL contrast medium (Iopamiron 300, SCHERING) was injected with an automated angiography injector at a rate of 2.5 mL/s. Quantitative angiographic analysis of collateral vessel development in the ischemic hind limb was performed with the use of a 5-mm^2 grid overlay, as described previously.[17] The angiographic score was calculated for each film as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections in the ischemic medial thigh. The angiographic score was determined by 2 blinded observers.

Morphological and Histological Examination

The degree of lower hind limb necrosis and thigh muscle necrosis was macroscopically evaluated on graded morphological scales (grade 1 to 3) for peripheral tissue damage and muscle necrosis area of the adductor, semimembranous, and medial large muscles. Capillary density of the ischemic hind limb was evaluated by alkaline phosphatase staining, as reported previously.[17] A total of 10 different fields from three different sections were randomly selected, and the number of capillaries was counted under a ×40 objective. Capillary density was expressed as the mean number of capillaries per square millimeter. The number of myofibers in each field was also examined and the capillary/muscle fiber ratio calculated.

Radioimmunoassay for Human AM

Human AM production was examined 1, 2, and 4 weeks after gene transfer in the naked AM group, AM-gelatin group, and control group (n=5 each). The muscles were harvested for radioimmunoassay and immunohistochemical examination. Immunoreactive human AM level in rabbit muscles was determined by immunoradiometric assay with the use of a specific kit (Shionogi Co, Ltd).[19] Tissue content of vascular endothelial growth factor (VEGF) was examined by ELISA kit (R&D systems).

Immunohistochemistry for Human AM, Ki67 Antigen, and Phosphorylated Akt

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded 4-μm sections of ischemic thigh muscles 7 days after gene transfer. To elucidate AM expression after gene therapy, immunohistochemistry for human AM was performed with the use of a monoclonal antibody recognizing AM-(12–25) (1:100), as reported previously.[20] To evaluate the proliferative potential of AM, tissue sections were stained for Ki67, a marker for cell proliferation, with the use of monoclonal anti-Ki67 antibody (1:100) (DAKO). AM has recently been shown to promote proliferation of vascular endothelial cells at least in part through the PI3k/Akt pathway.[21] Thus, immunohistochemistry for phosphorylated Akt was performed with mouse monoclonal anti-phosphorylated Akt antibody (1:100) (Cell Signaling Technology).

Western Blot Analysis

To identify Akt phosphorylation in ischemic muscles after AM gene transfer, Western blotting was performed with the use of a commercially available kit (PhosphoPlus Akt [Ser473] Antibody Kit, Cell Signaling Technology). Ischemic muscles in the 3 groups were obtained 7 days after AM gene transfer. These samples were homogenized on ice in 0.1% Tween 20 homogenization buffer with a protease inhibitor (Complete, Roche). After centrifugation for 20 minutes at 4°C, the supernatant was used for Western blot analysis. The 50 μg of protein was transferred into sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose membrane through the use of a wet blotting system. After blocking for 60 minutes, the membranes were incubated with primary antibodies (1:500) at 4°C overnight. The membranes were then incubated with secondary antibodies, which were conjugated with horseradish peroxidase (Cell Signaling Technology), at a final dilution of 1:2000. Signals were detected through the use of LumiGLO chemiluminescence reagents (Cell Signaling Technology).

Statistical Analysis

All results are expressed as mean±SEM. Statistical significance was evaluated by 1-way ANOVA followed by Fisher's analysis, Scheffe's *F* analysis, or Kruskal-Wallis test. A value of $P<0.05$ was considered statistically significant.

Results

Physiological and Morphological Assessment

Complete resection of the left femoral artery resulted in a similar decrease in calf blood pressure ratio among the 3 groups before the initiation of therapy (day 10) (Figure 2A). However, the calf blood pressure ratio on day 38 was highest in the AM-gelatin groups, followed by the naked AM group and subsequently the control group. The laser Doppler flow in hind limb was highest in the AM-gelatin group, followed by the naked AM group and the control group (Figure 2B). The calf blood pressure ratio and laser Doppler flow 4 weeks after gene transfer did not significantly differ between the control group and Lac Z-gelatin group. Lower hind limb necrosis was minimal in the AM-gelatin group, followed by the naked AM group and the control group (Figure 2C). Thigh muscle necrosis was also minimal in the AM-gelatin group. Similarly, the muscle weight ratio (ischemic/normal) on day 38 was highest in the AM-gelatin group (Table). Neither mean arterial pressure nor heart rate significantly differed among the 3 groups.

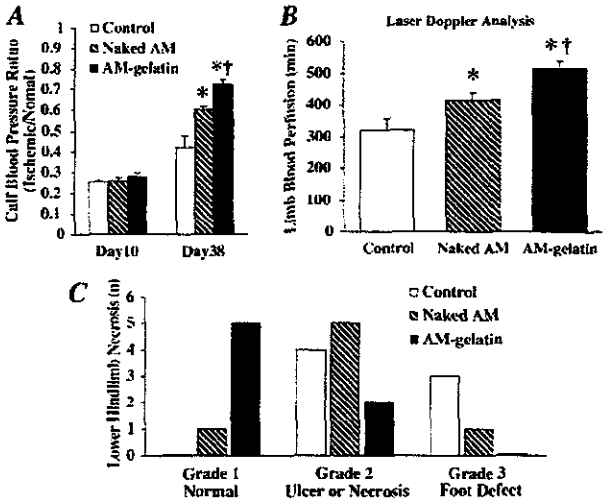

Figure 2. A, Calf blood pressure ratio (ischemic/normal hind limb) before (on day 10) and after (on day 38) gene transfer. B, Measurement of laser Doppler flow on day 38. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. C, Number of cases of each grade of lower hind limb necrosis on day 38. Lower hind limb necrosis was minimal in the AM-gelatin group. Number of necrosis or foot defect is statistically significant among the 3 groups ($P<0.05$ by Kruskal-Wallis test).

Angiographic Analysis

Angiograms 4 weeks after gene transfer (day 38) showed the development of collateral arteries in the naked AM and

Physiological Characteristics

	Control	Naked AM	AM-Gelatin
No. of rabbits	7	7	7
Body weight, kg	2.46±0.06	2.65±0.10	3.16±0.09
MAP, mm Hg	112±3	114±3	116±2
HR, beats/min	269±12	253±5	262±7
Muscle weight ratio	0.71±0.03	0.84±0.02*	0.95±0.02*†

MAP indicates mean arterial pressure; HR, heart rate; and muscle weight ratio, ratio of muscle weight in ischemic hind limb to that in nonischemic hind limb. Data are mean SEM.

*$P<0.01$ vs control group; †$P<0.05$ vs naked AM group.

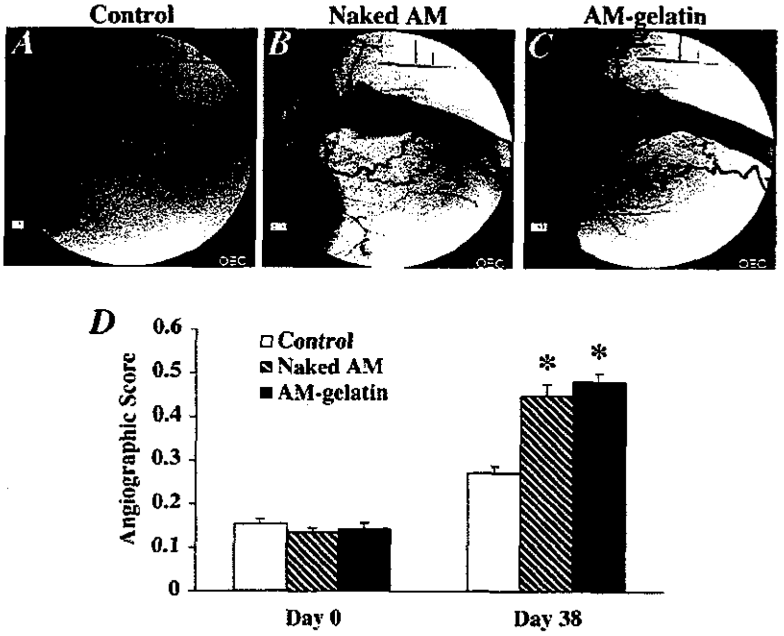

Figure 3. Representative angiograms of control group (A), naked AM group (B), and AM-gelatin group (C) on day 38. Collateral arteries were well developed in the naked AM and AM-gelatin groups. D, Angiographic score on days 0 and 38 in each group. Angiographic score on day 38 was significantly higher in the naked AM and AM-gelatin groups than in the control group. Data are mean±SEM. *$P<0.001$ versus control group.

AM-gelatin groups compared with that in the control group (Figure 3, A through C). Quantitative analysis of collateral vessels demonstrated that the angiographic score in both the naked AM and AM-gelatin groups was significantly higher than that in the control group (Figure 3D). Angiographic score did not significantly differ between the control group and Lac Z-gelatin group.

To examine the development of collateral vessels in an earlier stage, other rabbits (n=4 each) were examined 2 weeks after gene transfer (day 24). Angiograms showed significant collateral development in the naked AM and AM-gelatin groups compared with that in the control group.

Histological Examination

Alkaline phosphatase staining of ischemic hind limb muscle showed marked augmentation of neovascularization in both the naked AM and AM-gelatin groups compared with the control group (Figure 4, A through C). Quantitative analysis demonstrated that capillary density of the ischemic adductor muscle was highest in the AM-gelatin group (Figure 4D). Analysis of the capillary/muscle fiber ratio yielded similar results. Seven days after gene transfer, intense immunostaining for Ki67 was observed in vascular endothelial cells of the naked AM and the AM-gelatin groups (Figure 4, E through G).

AM Expression and Akt Phosphorylation After Gene Transfer

Seven days after gene transfer, modest immunostaining for human AM was observed in the naked AM group, whereas AM immunoreactivity was intense surrounding the gelatin in the AM-gelatin group (Figure 5, A through C). Tissue content of human AM was significantly increased both in the naked AM and the AM-gelatin groups 7 days after gene transfer (Figure 5D). The AM level in the AM-gelatin group was significantly higher that in the naked AM group. Two weeks after gene transfer, AM overexpression was observed only in the AM-gelatin group. The expression of endogenous VEGF and its receptors (Flt-1 and Flk-1) did not differ among the 3 groups (data not shown). Western blot analysis revealed that phosphorylated Akt in ischemic muscles was increased in both the naked AM and AM-gelatin groups 7 days after gene transfer (Figure 5E). Intense immunostaining for phosphory-

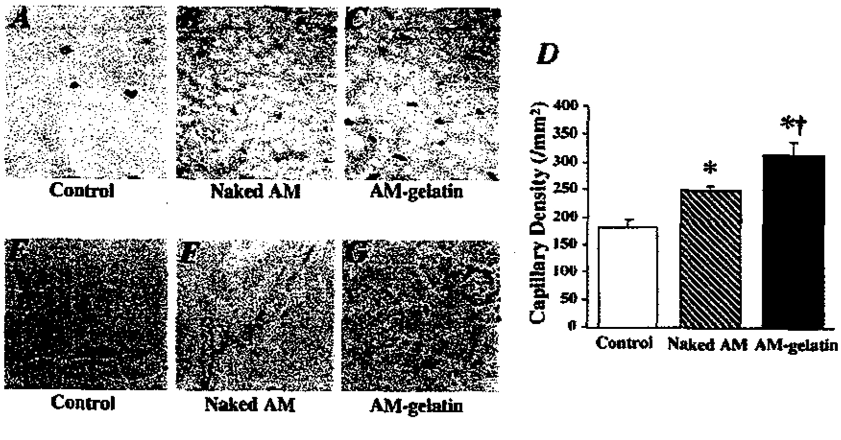

Figure 4. A through C, Representative examples of alkaline phosphatase staining in ischemic hind limb muscles. Magnification ×200. D, Quantitative analysis of capillary density in ischemic hind limb muscles. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. E through G, Immunohistochemical analysis of Ki67 antigen, a marker for cell proliferation. Magnification ×400.

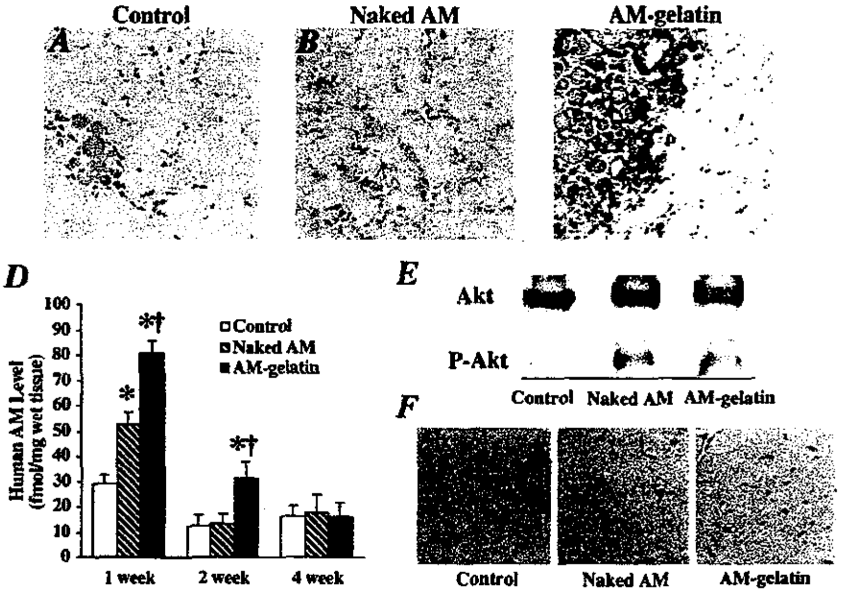

Figure 5. A through C, Immunohistochemistry for human AM 7 days after gene transfer. Intense immunostaining was observed surrounding gelatin in the AM-gelatin group. Magnification ×200. D, Time course of AM production in ischemic muscles after gene transfer. Data are mean±SEM. *$P<0.01$ vs control group; †$P<0.01$ vs naked AM group. E, Western blot analysis for Akt phosphorylation in muscles. F, Immunohistochemical staining for phosphorylated Akt 7 days after gene transfer. Phosphorylated Akt was distributed at least in endothelial cells. Magnification ×400.

lated Akt was observed at least in endothelial cells of the Naked AM and the AM-gelatin groups (Figure 5F).

Discussion

We demonstrated that (1) AM gene transfer induced hemodynamic and angiographic improvements in association with an increase in capillary density in a rabbit model of chronic hind limb ischemia. We also demonstrated that (2) administration of AM DNA-gelatin complexes markedly augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer.

AM has a variety of effects on the vasculature that include vasodilation,[1,5-7] inhibition of endothelial cell apoptosis,[8,9] and regulation of smooth muscle cell proliferation.[10] However, whether AM has angiogenic potential has remained unknown. In the present study, intramuscular administration of naked AM DNA augmented AM production in skeletal muscles, as indicated by increased tissue content and significant immunostaining of AM. As a result, AM gene transfer increased hind limb perfusion and ameliorated lower hind limb and thigh muscle necrosis in a rabbit model of hind limb ischemia. AM gene transfer may protect the ischemic hind limb partly by improving the blood flow in the ischemic hind limb because AM is originally identified as a potent vasodilating peptide.[1] Nevertheless, angiographic collateral development and high capillary density were observed in ischemic muscles after AM gene transfer. Ki67, a marker for cell proliferation, was detected in endothelial cells of microvessels after AM gene transfer. These results suggest that AM overproduction resulting from gene transfer may induce angiogenesis in a rabbit model of hind limb ischemia. Recent studies using AM gene knockout mice have shown that AM is essential for development of the vasculature during embryogenesis.[11-13] These studies support our results that AM may be an angiogenic factor. VEGF is known to induce angiogenesis and to regulate endothelial cell survival through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[22] Thus, the PI3K/Akt pathway is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation.[14] A recent study has reported that AM promotes proliferation and migration of human umbilical vein endothelial cells at least in part through the PI3K/Akt pathway.[21] The present study demonstrated that phosphorylated Akt is increased at least in endothelial cells after AM gene transfer. AM gene transfer did not influence endogenous VEGF and its receptors. Taken together, it is interesting to speculate that AM may directly induce angiogenesis through the PI3K/Akt pathway.

In the present study, we used positively charged biodegradable gelatin as a nonviral vector. We have shown that basic fibroblast growth factor (bFGF) is ionically linked with gelatin, which enhances the angiogenic effects of bFGF by delaying protein degradation.[15] Thus, biodegradable gelatin has been used as a carrier of protein. However, little information is available regarding the therapeutic potential of gelatin as a nonviral vector for gene transfer. In the present study, we demonstrated that RITC-labeled AM DNA was incorporated into positively charged gelatin. In addition, intramuscular administration of AM DNA-gelatin complexes strongly enhanced AM production compared with that of naked AM DNA. These results suggest that biodegradable gelatin may serve as a vector for gene transfer. In fact, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hind limb ischemia than naked AM DNA, as evidenced by significant increases in histological capillary density, calf blood pressure ratio, laser Doppler flow, and muscle weight ratio and a decrease in necrosis of lower hind limb and thigh muscles. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. The angiogenic effects of AM-gelatin complexes were comparable to those of bFGF-gelatin complexes (data not shown). AM DNA-gelatin complexes were distributed mainly in connective tissues. We have recently demonstrated that gelatin-DNA complex is readily phagocytosed by macrophages, monocytes, endothelial progenitor cells, and so on, resulting in gene expression within these phagocytes.[23,24] These findings raise the possibility that AM secreted from these cells acts on muscles in a paracrine fashion. Unlike AM production in the naked AM group, AM overexpression in the AM-gelatin group lasted for longer than 2 weeks. Thus, it is interesting to speculate that delaying gene degradation by gelatin may be responsible for the highly efficient gene transfer.

Currently, a highly efficient and safe gene delivery system is needed for gene therapy in humans. The present study demonstrated that the use of gelatin, which is considered to be less biohazardous than viral vectors, enhanced the angiogenic potential of AM DNA. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular diseases. However, the initial success of gelatin-mediated AM gene therapy reported here should be confirmed by long-term experiments, and extensive toxicity studies in animals are needed before clinical trials.

Study Limitation

First, histological capillary density, calf blood pressure ratio, and laser Doppler flow were significantly higher in the AM-gelatin group than in the naked AM group. However, the angiographic score did not significantly differ between the two. This discrepancy raises the possibility that conventional angiography may have insufficient resolution to fully visualize the angiogenic microvessels. Second, human AM level was slightly elevated in the control group. This implies that the anti-human AM antibody used in this radioimmunoassay had some cross-reactivity with endogenous rabbit AM. Nevertheless, human AM level in the muscles was highest in the AM-gelatin group within 2 weeks after gene transfer. These results suggest that AM DNA-gelatin complexes induces potent and long-lasting AM production.

Conclusions

Intramuscular administration of AM DNA induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia. Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of peripheral vascular diseases.

Acknowledgments

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Simultaneous monitoring of acetylcholine and catecholamine release in the in vivo rat adrenal medulla

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Abstract

To simultaneously monitor acetylcholine release from pre-ganglionic adrenal sympathetic nerve endings and catecholamine release from post-ganglionic adrenal chromaffin cells in the in vivo state, we applied microdialysis technique to anesthetized rats. Dialysis probe was implanted in the left adrenal medulla and perfused with Ringer's solution containing neostigmine (a cholinesterase inhibitor). After transection of splanchnic nerves, we electrically stimulated splanchnic nerves or locally administered acetylcholine through dialysis probes for 2 min and investigated dialysate acetylcholine, choline, norepinephrine and epinephrine responses. Acetylcholine was not detected in dialysate before nerve stimulation, but substantial acetylcholine was detected by nerve stimulation. In contrast, choline was detected in dialysate before stimulation, and dialysate choline concentration did not change with repetitive nerve stimulation. The estimated interstitial acetylcholine levels and dialysate catecholamine responses were almost identical between exogenous acetylcholine (10 μM) and nerve stimulation (2 Hz). Dialysate acetylcholine, norepinephrine and epinephrine responses were correlated with the frequencies of electrical nerve stimulation, and dialysate norepinephrine and epinephrine responses were quantitatively correlated with dialysate acetylcholine responses. Neither hexamethonium (a nicotinic receptor antagonist) nor atropine (a muscarinic receptor antagonist) affected the dialysate acetylcholine response to nerve stimulation. Microdialysis technique made it possible to simultaneously assess activities of pre-ganglionic adrenal sympathetic nerves and post-ganglionic adrenal chromaffin cells in the in vivo state and provided quantitative information about input–output relationship in the adrenal medulla.

Keywords: Anesthetized rats; Microdialysis; Choline; Norepinephrine; Epinephrine

1. Introduction

Although acetylcholine is one of major neurotransmitters in the peripheral autonomic nervous system as well as central nervous system (Collier, 1977; Fibiger, 1991; Calabresi et al., 2000), it has been difficult to measure endogenous acetylcholine in the in vivo state since acetylcholine released from nerve endings is rapidly degraded by tissue acetylcholinesterase (Taylor and Brown, 1998). Recently, microdialysis technique with improved measurement has made it possible to monitor low levels of acetylcholine in the in vivo central nervous system. In the peripheral autonomic nervous system, we have measured acetylcholine release from post-ganglionic parasympathetic nerve endings using microdialysis technique (Akiyama et al., 1994; Akiyama and Yamazaki, 2000, 2001; Kawada et al., 2001). Little information is, however, available on acetylcholine release from pre-ganglionic autonomic nerve endings in the in vivo state. The assessment of pre-ganglionic autonomic nerve activities is important for understanding the autonomic ganglionic transmission under physiological and pathophysiological conditions.

Adrenal medulla is one candidate suitable for investigating acetylcholine release from pre-ganglionic autonomic nerve endings (Holman et al., 1994). Compared to autonomic ganglia, adrenal gland is solid and suited to microdialysis probe implantation. Furthermore, microdialysis technique in the adrenal medulla provides a distinct advantage to monitor catecholamine release from adrenal medulla following acetylcholine release. Thus, we consider it possible to simultaneously assess pre- and post-ganglionic sympathetic nerve activities by monitoring acetylcholine and catecholamine release in the adrenal medulla.

In the present study, we applied the microdialysis technique to the adrenal medulla of anesthetized rats and tested the suitability of microdialysis technique to simultaneously

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monitor acetylcholine and catecholamine release from adrenal medulla.

2. Materials and methods

2.1. Animal preparation

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult male Wistar rats weighing 390–460 g were anesthetized with pentobarbital sodium (50–55 mg/kg i.p.). The rats were ventilated with a constant-volume respirator using room air mixed with oxygen. The left femoral artery and vein were cannulated for monitoring arterial blood pressure and administration of anesthetic, respectively. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (15–25 mg/(kg h) i.v.). Electrocardiogram was monitored for recording heart rate. A thermostatic heating pad was used to keep the esophageal temperature within a range of 37–38 °C. With the animal in the lateral position, the left adrenal gland and left splanchnic nerve were exposed by a subcostal flank incision, and the left splanchnic nerve was transected. In protocols requiring nerve stimulation, shielded bipolar stainless steel electrodes were applied to the distal end of the nerve, which was then stimulated with a digital stimulator (SEN-7203, Nihon Kohden, Japan) with a rectangular pulse (10 V and 1 ms in duration).

2.2. Dialysis technique

The materials of the dialysis probe were the same as those used in our previous dialysis experiments (Akiyama et al., 2003). Briefly, each end of the dialysis fiber (0.31 mm o.d., and 0.20 mm i.d.; PAN-1200 50,000 mol. wt. cutoff, Asahi Chemical, Japan) was inserted into the polyethylene tube (25 cm length, 0.50 mm o.d., and 0.20 mm i.d.; SP-8) and glued. The length of the dialysis fiber exposed was 3 mm. At perfusion speed of 10 μl/min, in vitro recovery rates of acetylcholine, choline, norepinephrine, and epinephrine were (%): 3.08 ± 0.04, 2.93 ± 0.10, 2.09 ± 0.03, and 2.16 ± 0.03, respectively (number of dialysis probes: 3).

The dialysis probe was implanted in the medulla of the left adrenal gland and perfused with Ringer's solution containing the cholinesterase inhibitor, neostigmine (10 μM) at a speed of 10 μl/min using a microinjection pump (CMA/100, Carnegie Medicin, Sweden). Ringer's solution consisted of (in mM) 147.0 NaCl, 4.0 KCl, 2.25 $CaCl_2$. All pharmacological agents tested were locally administered by perfusion through the dialysis probe after being dissolved in Ringer's solution. One sampling period was 2 min (one sample volume = 20 μl). We started the protocols followed by a stabilization period of 3–4 h. Catecholamine release was evoked by 2 min-local administration of acetylcholine or 2 min-electrical stimulation of left splanchnic nerves. In protocols requiring repeated nerve stimulation, electrical stimulation was performed at 30 min-intervals. Taking the dead space volume into account, we continuously collected three dialysate samples per pharmacological or electrical stimulation: one before, one during, and one after stimulation. We subtracted the dialysate acetylcholine, norepinephrine, or epinephrine contents in control from those during stimulation, and expressed these values as indices of dialysate acetylcholine, norepinephrine or epinephrine response to stimulation.

Half of the dialysate sample was injected into high-performance liquid chromatography for the measurement of acetylcholine and choline (Akiyama et al., 1994), and the remaining half was injected into another high-performance liquid chromatography for the measurement of norepinephrine and epinephrine (Akiyama et al., 1991).

2.3. Experimental protocols

2.3.1. Protocol 1

We repeated stimulations of splanchnic nerves at 2 and 4 Hz twice and examined dialysate acetylcholine, choline and catecholamine responses to nerve stimulation and their reproducibility in five rats.

2.3.2. Protocol 2

To compare the estimated interstitial acetylcholine levels between administration of acetylcholine and nerve stimulation, we locally administered acetylcholine (10 μM) in five rats and stimulated splanchnic nerves at 2 Hz in five other rats. The concentration of exogenous acetylcholine was determined to obtain a similar dialysate catecholamine response to nerve stimulation at 2 Hz.

2.3.3. Protocol 3

We raised stepwise the frequency of nerve stimulation from 2 to 4, 10, 20 Hz and examined dialysate acetylcholine and catecholamine responses in five rats. In addition, to examine the input–output relationship in the adrenal medulla, we analyzed the relationship between dialysate acetylcholine and catecholamine responses of five rats.

2.3.4. Protocol 4

We examined the effects of cholinergic receptor antagonists on dialysate acetylcholine and catecholamine responses. Nerve stimulations at 2 and 4 Hz were performed before and after 30 min-local administration of cholinergic receptor antagonists. We tested the nicotinic receptor antagonist, hexamethonium bromide (1 mM) in five rats or the muscarinic receptor antagonist, atropine sulfate (10 μM) in five other rats.

2.4. Statistical methods

To examine the effect of nerve stimulation and pharmacological agents, we analyzed heart rate and mean arterial pressure, and dialysate acetylcholine, choline, norepinephrine and epinephrine responses, using one- or two-way analysis of variance with repeated measures. When statistical significance was detected, the Newman–Keuls test was applied (Winer, 1971). Statistical significance was defined as $P < 0.05$. Values are presented as mean ± S.E.

3. Results

The experiments were carried in anesthetized rats and had been performed in the presence of neostigmine. Local administration of pharmacological agents did not influence heart rate or mean arterial pressure in any of the protocols. In protocol 3 ($n = 5$), nerve stimulation at 2 Hz decreased heart rate from 420 ± 8 to 397 ± 8 beats/min ($P < 0.05$) and increased mean arterial pressure from 125 ± 4 to 136 ± 3 mmHg ($P < 0.05$). Heart rate and mean arterial pressure recovered after cessation of stimulation. Nerve stimulation at 4, 10 and 20 Hz decreased heart rate to 396±9, 393±7 and 392 ± 9 beats/min, respectively, and increased mean arterial pressure to 134±3, 141±3, and 142±3 mmHg, respectively. In the other protocols, nerve stimulation at 2 or 4 Hz evoked the same responses of heart rate and mean arterial pressure.

3.1. Dialysate acetylcholine and catecholamine

3.1.1. Protocol 1

As shown in the *upper panel* of Fig. 1 ($n = 5$), acetylcholine was not detected in dialysate before nerve stimulation, but substantial acetylcholine was detected in dialysate by nerve stimulation. In contrast, choline, norepinephrine, and epinephrine were detected in dialysate before stimulation. Dialysate choline concentration did not change with repetitive nerve stimulation. Dialysate norepinephrine and epinephrine concentrations increased with nerve stimulation. Stimulation at the same frequency elicited almost identical responses on repetition.

3.1.2. Protocol 2

Using in vitro recovery rate of acetylcholine (3.08%), the estimated interstitial acetylcholine levels were 308 nM in acetylcholine infusion (10 μM, $n = 5$) and 276 ± 15 nM in nerve stimulation (2 Hz, $n = 5$; Fig. 1, *lower panel*). There was no statistical difference in the estimated interstitial acetylcholine levels and dialysate catecholamine responses between the two groups.

3.1.3. Protocol 3

When the frequency of nerve stimulation was increased from 2 to 20 Hz, dialysate acetylcholine, norepinephrine and epinephrine responses were enhanced ($n = 5$; Fig. 2, *upper panel*). We plotted the relationship between dialysate catecholamine response (ordinate) and dialysate acetylcholine response (abscissa) of five rats (Fig. 2, *lower panel*). Dialysate norepinephrine and epinephrine responses correlated with dialysate acetylcholine responses.

3.1.4. Protocol 4

At both 2 and 4 Hz of nerve stimulation, hexamethonium suppressed dialysate norepinephrine and epinephrine responses, but did not affect acetylcholine response ($n = 5$; Fig. 3, *upper panel*). Atropine suppressed epinephrine response at both 2 and 4 Hz of nerve stimulation, but did not affect norepinephrine and acetylcholine responses ($n = 5$; Fig. 3, *lower panel*).

4. Discussion

By now, simultaneous monitoring of adrenal acetylcholine and catecholamine release has been limited to only a few studies using perfused adrenal gland. Collier et al. (1984) measured endogenous acetylcholine and catecholamine effluxes from perfused cat adrenal gland. O'Farrell et al. (1997) preloaded bovine adrenal glands with [^{3}H]-choline and measured the subsequent efflux of [^{3}H]-labelled compound as an index of acetylcholine release and catecholamine efflux. In the present in vivo study, dialysate acetylcholine and catecholamine responses served as indices of acetylcholine release from splanchnic nerve endings and catecholamine release from adrenal medulla, respectively. This simultaneous monitoring implies quantitative measurement of pre- and post-ganglionic neurotransmitter release at the adrenal medulla.

4.1. Source of dialysate acetylcholine

The stimulation of splanchnic nerve induced acetylcholine release from pre-ganglionic nerve endings and increased dialysate acetylcholine concentration. It has been demonstrated that adrenal gland receives parasympathetic efferent and afferent innervation (Coupland et al., 1989; Niijima, 1992; Parker et al., 1993). Branches of parasympathetic efferent nerves conduct through celiac nerves, celiac ganglion and splanchnic nerves to adrenal nerves (Niijima, 1992). In the present study, we electrically stimulated the portion just distal to the sympathetic chain and proximal to the celiac ganglion. This portion does not contain branches of parasympathetic efferent nerves. After transection of splanchnic nerves, basal dialysate acetylcholine was less than the detection limit of high performance liquid chromatography (10 fmol), and substantial acetylcholine was detected in dialysate during the stimulation of this portion. Thus, most of the detected acetylcholine in dialysate derives from pre-ganglionic sympathetic nerve endings.

4.2. Interstitial choline levels in the adrenal medulla

Under physiological conditions, there is enough acetylcholinesterase activity in splanchnic nerve endings, chromaffin cells, and interstitial cells (Coupland, 1965; Palkama, 1967; Lewis and Shute, 1969; Somogyi et al., 1975). Released acetylcholine is degraded to choline and acetate by

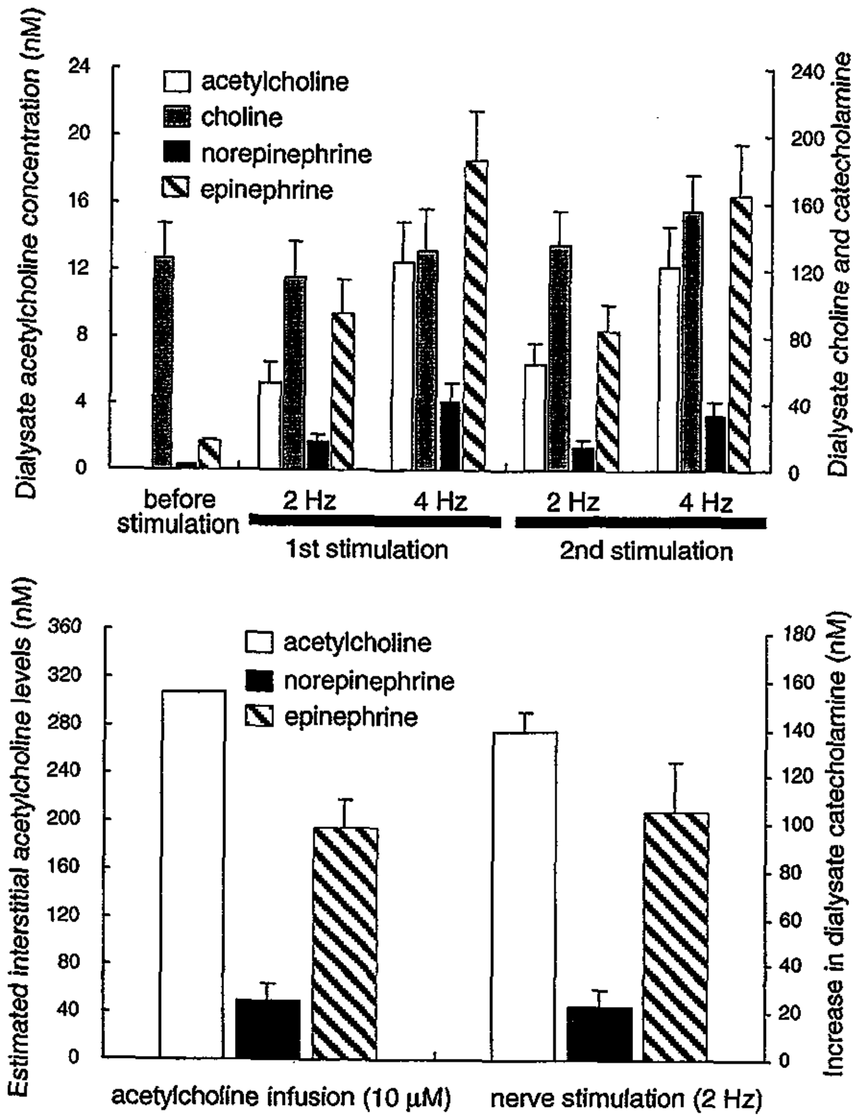

Fig. 1. (*Upper panel*) Acetylcholine was not detected in dialysate before nerve stimulation, but substantial acetylcholine was detected in dialysate by nerve stimulation (5 ± 1 nM at 2 Hz and 12 ± 2 nM at 4 Hz). Dialysate choline concentration did not change with nerve stimulation. Dialysate norepinephrine and epinephrine concentrations increased with nerve stimulation (17 ± 5 and 94 ± 20 nM at 2 Hz, respectively, and 41 ± 12 and 185 ± 29 nM at 4 Hz, respectively). Stimulation at the same frequency elicited almost identical responses on repetition, $n = 5$. Values are mean ± S.E. (*Lower panel*) There was no statistical difference in the estimated interstitial acetylcholine levels and dialysate catecholamine responses between acetylcholine infusion (10 μM, $n = 5$) and nerve stimulation (2 Hz, $n = 5$). Values are mean ± S.E.

acetylcholinesterase. Interstitial choline is carried into the nerve endings through neuronal transporters and used as a precursor for synthesis of acetylcholine (Taylor and Brown, 1998). In in vitro perfused experiments, continuous administration of choline sustains the synthesis and release of acetylcholine from nerve endings. In the present study, the concentration of dialysate choline was more than 10 times that of dialysate acetylcholine during nerve stimulation, and repetitive acetylcholine release did not induce a decrease in dialysate choline concentration. Moreover, nerve stimulation elicited almost identical responses of dialysate acetylcholine on repetition. These results indicate that repetitive acetylcholine release did not decrease interstitial choline levels and did not affect release of acetylcholine. Thus, under in vivo conditions, adrenal interstitial choline levels may be sufficiently high to sustain acetylcholine synthesis in the pre-ganglionic nerve endings.

4.3. Catecholamine release induced by endogenous and exogenous acetylcholine

Either exogenous or endogenous acetylcholine evokes catecholamine release by activating cholinergic receptors on the surface of chromaffin cells (Douglas, 1975). The interstitial acetylcholine levels serves as an index of input into chromaffin cells. We examined whether the estimated interstitial acetylcholine levels were identical between exogenous acetylcholine and nerve stimulation when

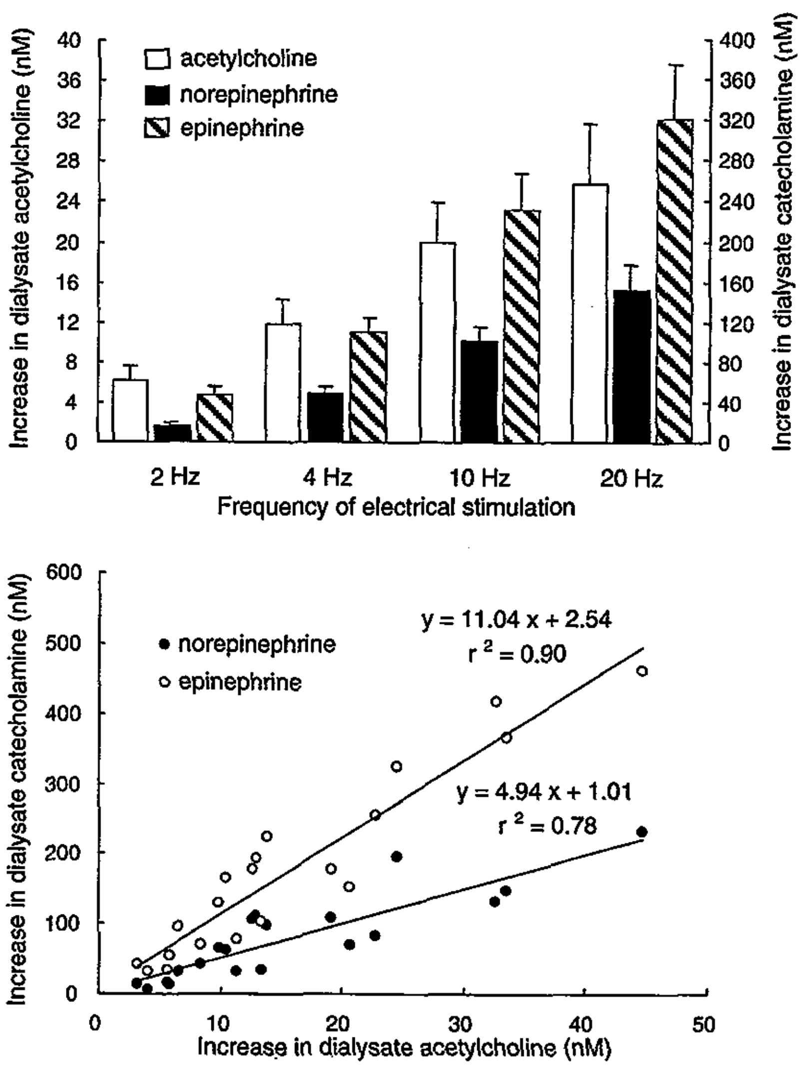

Fig. 2. (*Upper panel*) Dialysate acetylcholine response was enhanced from 6 ± 1 to 26 ± 6 nM when the frequency of nerve stimulation was increased from 2 to 20 Hz. Similarly, dialysate norepinephrine and epinephrine responses were enhanced from 16 ± 4 to 152 ± 26 nM and 48 ± 9 to 321 ± 54 nM, respectively, $n = 5$. Values are mean ± S.E. (*Lower panel*) The relationship between dialysate catecholamine response (*ordinate*) and dialysate acetylcholine response (*abscissa*) of five rats. Dialysate norepinephrine and epinephrine responses correlated with dialysate acetylcholine responses. These relations were expressed by regression equations with correlation coefficients of $y = 4.94x + 1.01$, $r^2 = 0.78$, and $y = 11.04x + 2.54$, $r^2 = 0.90$, respectively.

dialysate catecholamine responses were equal. Actually the estimated interstitial acetylcholine levels during nerve stimulation (2 Hz) were identical with those during acetylcholine infusion (10 μM). These data indicate that inputs into chromaffin cells were almost identical between the two stimulations. It could be inferred from this finding that dialysate acetylcholine concentration reflects acetylcholine levels at the surface of chromaffin cells and serves as an index of cholinergic transmission in the adrenal medulla.

4.4. Relationship of acetylcholine and catecholamine release

Dialysate norepinephrine and epinephrine responses were correlated with the frequency of splanchnic nerve stimulation. This norepinephrine and epinephrine release occurred as a consequence of acetylcholine release by splanchnic nerve stimulation. We found a linear relation between dialysate acetylcholine response and dialysate catecholamine responses. This indicates that the input–output relationship in the adrenal medulla is linear over the range of frequency from 2 to 20 Hz. Dialysate acetylcholine response of 1 nM evoked dialysate norepinephrine response of about 5 nM and dialysate epinephrine response of about 11 nM. This relation between dialysate acetylcholine and catecholamine responses could provide quantitative information about the input–output relationship in the adrenal medulla.

4.5. Effects of cholinergic receptor antagonists

It has been suggested that acetylcholine release from pre-ganglionic nerve endings is modulated by pre-synaptic

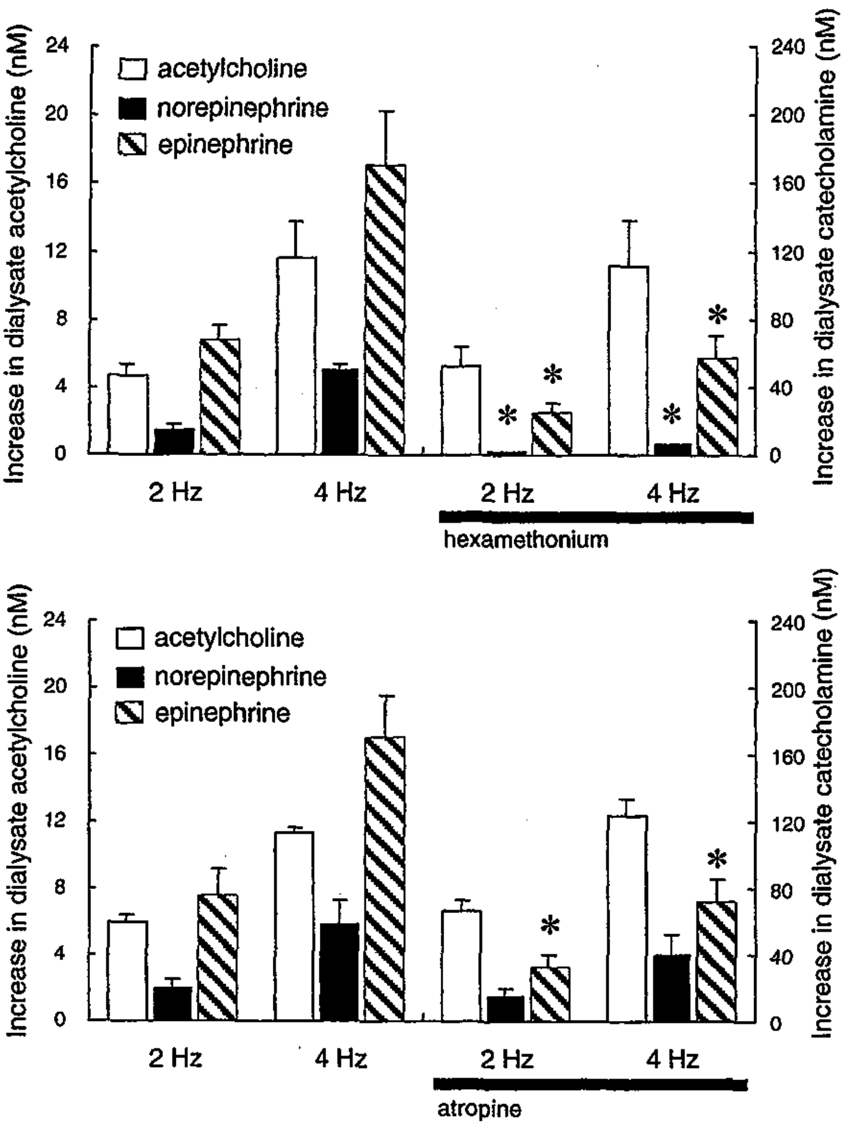

Fig. 3. (*Upper panel*) At both 2 and 4 Hz of nerve stimulation, hexamethonium suppressed dialysate norepinephrine and epinephrine responses, but did not affect acetylcholine response, $n = 5$. Values are mean ± S.E. $^{*}P < 0.05$ vs. concurrent dialysate norepinephrine or epinephrine response before administration of hexamethonium. (*Lower panel*) At both 2 and 4 Hz of nerve stimulation, atropine suppressed epinephrine response, but did not affect norepinephrine and acetylcholine responses, $n = 5$. Values are mean ± S.E. $^{*}P < 0.05$ vs. concurrent dialysate norepinephrine or epinephrine response before administration of atropine.

cholinergic autoreceptors (Dujic et al., 1990; Myers and Undem, 1996; Barbara et al., 1998). Neostigmine might induce the activation of pre-synaptic cholinergic receptors by increasing the acetylcholine levels in synaptic regions (Brehm et al., 1992) and suppress acetylcholine release by activating pre-synaptic autoreceptors. In the present study, neither hexamethonium nor atropine affected dialysate acetylcholine response to nerve stimulation at either 2 or 4 Hz. Thus, autoinhibition of acetylcholine release can be considered insignificant in our experimental condition, and dialysate acetylcholine response reflects pre-ganglionic nerve activities. In contrast, hexamethonium suppressed norepinephrine and epinephrine releases by nerve stimulation whereas atropine suppressed only epinephrine release. The muscarinic agonist, muscarine or pilocarpine preferentially enhanced epinephrine release (Douglas and Poisner, 1965; Wakade and Wakade, 1983). These results suggest that both nicotinic and muscarinic receptors exist on the surface of epinephrine-storing cells, while, on the surface of norepinephrine-storing cells, nicotinic receptors are primarily present.

4.6. Methodological limitations

We locally administered neostigmine to adrenal medulla through dialysis probe. Cholinesterase inhibitor was necessary to detect acetylcholine even during splanchnic nerve stimulation because released acetylcholine is rapidly degraded by acetylcholinesterase before reaching the dialysis fiber. In the same preparation, local administration of neostigmine enhanced the dialysate catecholamine response to nerve stimulation by about three-fold, but did not influence the responses of heart rate and mean arterial pressure (Akiyama et al., 2003). Total catecholamine release from adrenal gland might not change by the local administration of neostigmine. In the present study, dialysate catecholamine response was correlated with the frequency of splanchnic nerve stimulation. Thus, in the presence of neostigmine, absolute value of dialysate catecholamine response is exaggerated, but could reflect relative changes in catecholamine release from adrenal gland.

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Short communication

In vivo assessment of catechol *O*-methyltransferase activity in rabbit skeletal muscle

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Abstract

With the use of microdialysis technique in the anesthetized rabbit, we examined the catechol *O*-methyltransferase (COMT) activity at the skeletal muscle interstitium. We implanted a dialysis probe into the adductor muscle, and monitored dialysate catecholamines and their metabolites with chromatogram-electrochemical detection. Administration of COMT inhibitor (entacapone) decreased dialysate 3-methoxy 4-hydroxyphenylglycol (MHPG) levels. Local administration of dihydroxyphenylglycol induced increases in dialysate MHPG levels. These increases in dialysate MHPG levels were suppressed by the addition of entacapone. The concentration of MHPG in the skeletal muscle dialysate corresponded to the COMT activity in the skeletal muscle. Furthermore, local administration of norepinephrine or epinephrine increased normetanephrine or metanephrine levels in dialysate but not MHPG levels. Skeletal muscle microdialysis with local administration of catecholamine offers a new method for in vivo assessment of regional COMT activity.

Keywords: Catecholamine; Catechol *O*-methyltransferase; Entacapone; Microdialysis; Skeletal muscle

Catechol *O*-methyltransferase (COMT) exerts a critical action on the inactivation of catecholamrines and catecholestrogens (Boulton and Eisenhofer, 1998). COMT enzyme exists in almost all mammalian tissues and organs (Karhunen et al., 1994; Männistö and Kaakkola, 1999). The wide distribution of COMT in different tissues suggests an important physiological role for COMT activity. In vitro COMT activity has been widely assessed in various tissues (Männistö and Kaakkola, 1999; Tsunoda et al., 2002), while in vivo COMT activity has been assessed only in erythrocyte (Toumainen et al., 1996). To determine whether COMT activity is involved in cardiovascular regulation, we need information about in vivo COMT activity in organs and tissues.

A sophisticated technique using radiotracers has been employed for spillover of organ specific metabolite formed by COMT activity (3-methoxy 4-hydroxyphenylglycol, MHPG) (Lambert et al., 1995). This study suggested that majority of MHPG in plasma was derived from skeletal muscle, with the exception of central nervous system. Dispersed organs, such as skeletal muscle, have a thin and diffuse sympathetic innervation, but skeletal muscle is one candidate suitable for investigating regional MHPG production (Tokunaga et al., 2003a,b). This organ is suited to microdialysis probe implantation. Recently we have developed the skeletal muscle microdialysis for the monitoring of catecholamines and their metabolites. At the skeletal muscle, the small amounts of dialysate norepinephrine and its metabolites could be determined by microdialysis with electrochemical detection.

In the present study, we examined whether COMT blocker affected regional norepinephrine kinetics at the skeletal muscle interstitial spaces. With the use of dialysis technique, the dialysate was sampled from the skeletal muscle, and dialysate catecholamines and their metabolites levels were measured with liquid chromatography. Further, the study was designed to examine regional *O*-methylation products evoked by local administration of catecholamine and determine whether these data provide information about in vivo regional COMT activity.

Male Japanese white rabbits weighing 2.6–3.1 kg each were anesthetized with pentobarbital sodium (30–35 mg/kg,

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i.v.). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). After tracheotomy, the animals were ventilated with room air mixed with oxygen. Body temperature was maintained with a heated pad and lamp. All protocols were performed in accordance with the American Physiological Society guidelines for the use of animals. After a longitudinal skin incision was made in the left groin, the dialysis probes were implanted in the left adductor muscle with a fine guiding needle.

For skeletal muscle dialysis, we designed a transverse dialysis probe. The dialysis fiber (13 mm length, 0.31 mm O.D. and 0.2 mm I.D.; PAN-1200, 50,000 molecular mass cutoff, Asahi Chemical, Tokyo, Japan) was glued at both ends into a polyethylene tube (25 cm length, 0.5 mm O.D. and 0.2 mm I.D.) (Akiyama et al., 1991; Tokunaga et al., 2003a,b). The dialysis probe was perfused with Ringer solution at a speed of 10 μl/min using a microinjection pump (CMA 102, Carnegie Medicin, Stockholm, Sweden). Dialysate catecholamines and their metabolite concentrations were measured by high-performance liquid chromatography with electrochemical detection (Takauchi et al., 1997; Tokunaga et al., 2003a,b; Yamazaki et al., 1995).

Basal dialysate norepinephrine, dihydroxyphenylglycol (DHPG) and MHPG levels were presented in Table 1. Entacapone (COMT blocker) was intraperitoneally administered (10 mg/kg) (Illi et al., 1995; Scheinin et al., 1998). Administration of entacapone decreased the MHPG level of dialysate but increased the DHPG levels of dialysate. The dialysate norepinephrine levels were not affected by entacapone. These changes were preserved 2 h after administration of entacapone.

To examine regional COMT activity, we measured the formation of MHPG evoked by local administration of exogenous DHPG via dialysis probe. We determined doses of DHPG based on the dialysate DHPG concentration in the previous experiments (Akiyama and Yamazaki, 2001). Local administration of DHPG (25, 250 ng/ml) dose-dependently increased the MHPG levels of dialysate (Fig. 1). These increases in the MHPG levels were prevented by pretreatment with entacapone.

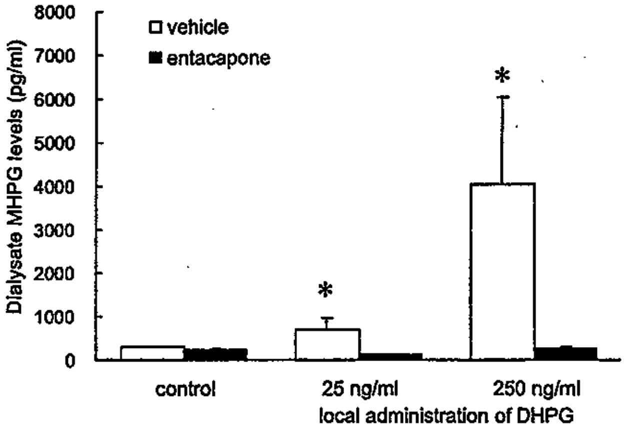

Fig. 1. Effects of exogenous dihydroxyphenylglycol (DHPG) infusion on the 3-methoxy 4-hydroxyphenylglycol (MHPG) production. Local administration of DHPG dose-dependently increased the MHPG levels of dialysate. These increases in the MHPG levels were prevented by pretreatment with entacapone. Values are means±SE (n=5). *P<0.05 vs. control.

In this study, exogenous DHPG dose-dependently increased the MHPG levels of dialysate. Exogenous DHPG via the dialysis probe easily traversed the cell membrane and reached skeletal muscle (Goldstein et al., 1998). In contrast, entacapone significantly decreased the MHPG levels of dialysate. These data suggest that regional COMT activity corresponds to the production of dialysate MHPG levels. Furthermore, entacapone induced a decrease in the dialysate MHPG level accompanied by an increase in the dialysate DHPG but not norepinephrine level. Therefore we consider that regional DHPG is one possible substrate for MHPG production, and that the concentration of MHPG or MHPG/DHPG ratio in the skeletal muscle dialysate might correspond to the COMT activity in the skeletal muscle.

Table 1
Basal dialysate NE, DHPG, and MHPG levels in rabbit skeletal muscle

	Before entacapone	After entacapone
NE (pg/ml)	8±1	10±1
DHPG (pg/ml)	27±4	53±11*
MHPG (pg/ml)	198±12	147±18*

NE, norepinephrine; DHPG, dihydroxyphenylglycol; MHPG, 3-methoxy 4-hydroxyphenylglycol. Values are means±SE. n=5.

* P<0.05 vs. values before entacapone.

Earlier studies suggested species and organ differences in extraneuronal uptake and COMT activity (Scheinin et al., 1998; Tsunoda et al., 2002). Extraneuronal norepinephrine uptake and COMT activity were well examined in rabbit heart with the findings suggesting that rabbit heart hardly metabolizes isoprenaline to methoxyprenaline (Lindmar and Löffelholz, 1974). Thus rabbit heart seems to have a very poorly developed extraneuronal system, including weak COMT activity, for the uptake and metabolism of catecholamines (Trendelenburg, 1978). On the other hand, rabbit aortic strips have a high capacity for COMT activity (Levin, 1974). From these and previous data (Tokunaga et al., 2003a,b), the ratio of MHPG/DHPG in myocardium and skeletal muscle were 1.0±0.2 and 7.9±1.3, respectively. Rabbit skeletal muscle seems to have a well-developed COMT activity. In the skeletal muscle sympathetic innervation was not dense, and the DHPG levels were less than that of heart (Tokunaga et al., 2003a,b). Therefore, other compounds or plasma DHPG might be involved in the regional formation of MHPG in the skeletal muscle.

MHPG is produced by extraneuronal *O*-methylation of DHPG formed intraneuronally from norepinephrine or by the extraneuronal combination of COMT and monoamine oxidase (MAO) on norepinephrine and epinephrine (Akiyama and Yamazaki, 2001; Eisenhofer et al., 1988). Therefore, MHPG is mainly yielded from DHPG, norepinephrine or epinephrine at the skeletal muscle. Furthermore,

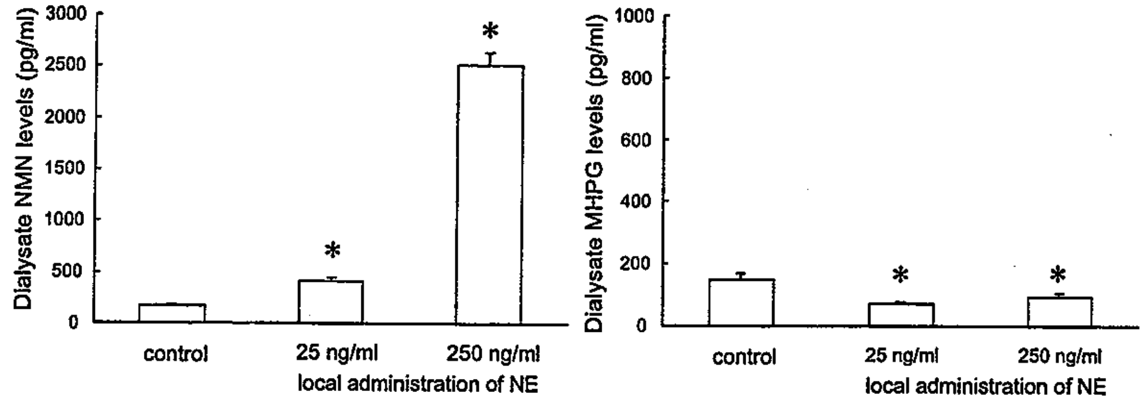

Fig. 2. Effects of exogenous norepinephrine (NE) infusion on the 3-methoxy 4-hydroxyphenylglycol (MHPG) and normetanephrine (NMN) production. Local administration of NE dose-dependently increased the NMN levels of dialysate but not MHPG levels. Values are means±SE ($n=5$). *$P<0.05$ vs. control.

O-methylation of catechol compounds includes MHPG, normetanephrine and metanephrine. We examined the relation between catecholamines and their metabolites. To compare norepinephrine and epinephrine with DHPG infusion, norepinephrine or epinephrine infusion with similar doses of DHPG was administered. Local administration of norepinephrine increased the normetanephrine levels of dialysate but not the MHPG levels (Fig. 2). Local administration of epinephrine increased the metanephrine levels of dialysate but not the MHPG levels (Fig. 3). Our data suggest that only DHPG is a possible substrate for MHPG production. Local administration of norepinephrine or epinephrine produced normetanephrine or metanephrine but not MHPG. Or rather, norepinephrine or epinephrine caused a decrease in the dialysate MHPG level. These data are consistent with data on the origins of plasma MHPG in rats, which indicated that most MHPG arises from *O*-methylation of the DHPG by intraneuronal deamination of norepinephrine (Eisenhofer et al., 1994).

Our data indicate that COMT exerts an important role on the degradation of cathecholamines in the skeletal muscular interstitium. Muscular catecholamines derive from circulating blood and surrounding sympathetic nerve systems (Tokunaga et al., 2003a,b). Therefore, COMT activity in the skeletal muscle may be related to regional or systemic sympathetic nerve activity. The relationship between regional COMT activity and sympathetic nerve activity remains to be further examined. Muscle sympathetic nerve activity is involved in the regulation of vascular tone and glucose metabolism in the skeletal muscle (Lundvall and Edfeldt, 1994; Spraul et al., 1994). Further studies concerning the physiological role of regional COMT activity on vascular or metabolic control are warranted.

To our knowledge, this is the first report on the in vivo assessment of COMT activity by direct measurement of dialysate MHPG, normetanephrine, and metanephrine obtained from skeletal muscle. Local administration of DHPG increased the MHPG levels of dialysate. These increases in MHPG were prevented by pretreatment with a COMT inhibitor. Therefore we consider that the concentration of MHPG in the skeletal muscle dialysate might correspond to the COMT activity in the skeletal muscle. Measurement of MHPG/DHPG ratio or MHPG formation evoked by DHPG infusion in skeletal muscle may be particularly appropriate for providing information about regional COMT activity. Thus skeletal muscle microdialysis with local administration of catecholamine offers a new method for in vivo assessment of regional COMT activity.

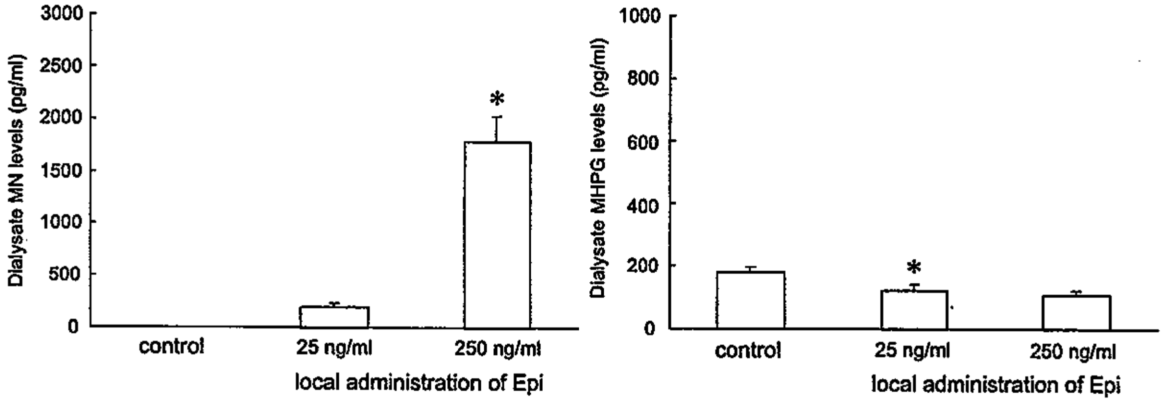

Fig. 3. Effects of exogenous epinephrine (Epi) infusion on the metanephrine (MN) and 3-methoxy 4-hydroxyphenylglycol (MHPG) production. Local administration of Epi increased the MN levels of dialysate but not MHPG levels. Values are means±SE ($n=5$). *$P<0.05$ vs. control.

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Effects of Ca^{2+} channel antagonists on acetylcholine and catecholamine releases in the in vivo rat adrenal medulla

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Akiyama, Tsuyoshi, Toji Yamazaki, Hidezo Mori, and Kenji Sunagawa. Effects of Ca^{2+} channel antagonists on acetylcholine and catecholamine releases in the in vivo rat adrenal medulla. *Am J Physiol Regul Integr Comp Physiol* 287: R161–R166, 2004. First published March 18, 2004; 10.1152/ajpregu.00609.2003.—To elucidate the types of voltage-dependent Ca^{2+} channels controlling ACh and catecholamine releases in the in vivo adrenal medulla, we implanted microdialysis probes in the left adrenal medulla of anesthetized rats and investigated the effects of Ca^{2+} channel antagonists on ACh, norepinephrine, and epinephrine releases induced by nerve stimulation. The dialysis probes were perfused with Ringer solution containing a cholinesterase inhibitor, neostigmine. The left splanchnic nerves were electrically stimulated at 2 and 4 Hz before and after intravenous administration of Ca^{2+} channel antagonists. ω-Conotoxin GVIA (an N-type Ca^{2+} channel antagonist, 10 μg/kg) inhibited ACh release at 2 and 4 Hz by ∼40%, norepinephrine release at 4 Hz by ∼50%, and epinephrine release at 2 and 4 Hz by ∼45%. A fivefold higher dose of ω-conotoxin GVIA (50 μg/kg) did not further inhibit these releases. ω-Conotoxin MVIIC (a P/Q-type Ca^{2+} channel antagonist, 50 μg/kg) inhibited ACh and epinephrine releases at 4 Hz by ∼30%. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) inhibited ACh release at 2 and 4 Hz by ∼70% and norepinephrine and epinephrine releases at 2 and 4 Hz by ∼80%. Nifedipine (an L-type Ca^{2+} channel antagonist, 300 and 900 μg/kg) did not change ACh release at 2 and 4 Hz; however, nifedipine (300 μg/kg) inhibited epinephrine release at 4 Hz by 20%, and nifedipine (900 μg/kg) inhibited norepinephrine and epinephrine releases at 4 Hz by 30%. In conclusion, both N- and P/Q-type Ca^{2+} channels control ACh release on preganglionic splanchnic nerve endings while L-type Ca^{2+} channels do not. L-type Ca^{2+} channels are involved in norepinephrine and epinephrine releases on chromaffin cells.

anesthetized rats; microdialysis; norepinephrine; epinephrine; preganglionic autonomic nerve endings

CA^{2+} INFLUX through the voltage-dependent Ca^{2+} channels induces the release of transmitters from neuronal or secretory cells by initiating exocytosis from vesicles. Voltage-dependent Ca^{2+} channels have been classified into L-, N-, P-, Q-, R-, and T-types (12, 25, 30). To better understand the mechanism controlling the release of transmitters, it is important to determine the type of Ca^{2+} channels involved in the release of the transmitters on neuronal or secretory cells.

In the in vivo adrenal medulla, catecholamine release is controlled by central sympathetic neurons through preganglionic splanchnic nerves. Splanchnic nerve endings make synaptic-like contacts with chromaffin cells (9). ACh released from splanchnic nerve endings consequently evokes catecholamine release from chromaffin cells by activation of cholinergic receptors. Thus, in vivo catecholamine release requires Ca^{2+} influx through the voltage-dependent Ca^{2+} channels at two different sites in the adrenal medulla: splanchnic nerve endings and chromaffin cells. Numerous studies have investigated the nature of Ca^{2+} channels controlling transmitter release from postganglionic autonomic nerve endings (8, 11, 32, 33, 36, 37). Little information is, however, available on the type of Ca^{2+} channels controlling the ACh release from preganglionic autonomic nerve endings including splanchnic nerve endings. Moreover, although the types of Ca^{2+} channels controlling catecholamine release have been investigated using isolated chromaffin cells in various species (5, 6, 13, 16, 21, 23, 24), it remains unknown whether endogenous ACh induces Ca^{2+} influx through the same types of Ca^{2+} channels on chromaffin cells.

We have recently developed a dialysis technique to simultaneously monitor ACh and catecholamine releases in the in vivo adrenal medulla (2). This method makes it possible to characterize Ca^{2+} channels controlling ACh release from splanchnic nerve endings and catecholamine release from adrenal medulla in the in vivo state. In the present study, we applied the microdialysis technique to the adrenal medulla of anesthetized rats and investigated the effects of Ca^{2+} channel antagonists on dialysate ACh and catecholamine responses induced by the electrical stimulation of splanchnic nerves.

MATERIALS AND METHODS

Animal preparation. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult male Wistar rats weighing 380–450 g were anesthetized with pentobarbital sodium (50–55 mg/kg ip). A cervical midline incision was made to expose the trachea, which was then cannulated. The rats were ventilated with a constant-volume respirator using room air mixed with oxygen. The left femoral artery and vein were cannulated for monitoring arterial blood pressure and administration of anesthetic, respectively. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (15–25 $\mathrm{mg \cdot kg^{-1} \cdot h^{-1}}$ iv). Electrocardiogram was monitored for recording heart rate. A thermostatic heating pad was used to keep the esophageal temperature within a range of 37–38°C. With the animal in the lateral position, the left adrenal gland and left splanchnic nerve were exposed by a subcostal flank incision, and the left splanchnic nerve was transected. Shielded bipolar stainless steel electrodes were applied to the distal end of the nerve, which was then stimulated with a digital stimulator (SEN-7203, Nihon Kohden) with a rectangular pulse (10 V and 1 ms in duration).

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