TABLE I. Reduction in the Coefficient of Variation Using Our Modified, Improved Protocol for Determining CD34+ Cell Levels in Peripheral Blood

	ProCount	Stem-Kit	Improved Protocol
Actual CD34+ cell counts Range	16 ± 1 10–31	34 ± 4 16–58	174 ± 18 88–404
CD34+ cells in leukocytes (%) Range	0.024 ± 0.003 0.012–0.046	0.021 ± 0.001 0.011–0.032	0.019 ± 0.002 0.014–0.038
Circulating CD34+ cells (cells/μL)	0.82 ± 0.05	0.81 ± 0.06	0.88 ± 0.06
Cumulative intra-assay coefficient of variation (%)	30.3	25.7	7.4

ments using the standard method (Table I). The supernatant that was removed during the procedure was also analyzed and was found not to contain either cells or internal control particles.

Improvement in the Cumulative Intra-Assay Coefficient of Variation

In mobilized peripheral blood, the coefficients of variation have been reported to be about 8% and 4% using ProCount and Stem-Kit, respectively, on the basis of the manufacturers' published information. However, in non-mobilized peripheral blood of patients with cardiovascular disease, the mean percentage of CD34+ cells in the leukocyte fraction was less than 10%, compared with mobilized blood, and the calculated cumulative intra-assay coefficients of variation were 30.3% and 25.7%, as evaluated by ProCount and Stem-Kit, respectively. Our method increased the absolute number of CD34+ cells by about 5-fold during the same measurement period, and it resulted in a reduced (7.4%) cumulative intra-assay coefficient of variation (Table I).

Discussion

Although our modified method for quantifying CD34+ cells in blood was similar to established methods for the calculation of mean circulating CD34+ cell counts, our method substantially improved reproducibility of the measurement.

The coefficient of variation of CD34+ cell counts is inversely proportional to the square root of the number of CD34+ cells detected in the sample. A minimum of 100 CD34+ cells is required to ensure a coefficient of variation in the range of 10%.[8] Our modified protocol yielded more than 100 CD34+ cells in each count (confirmed by duplicate counting), and the coefficient of variation was reduced to 7%. Simply increasing the sample volume or lengthening the time for measurement of cell numbers does not necessarily improve reproducibility of the counts.[8] Our results indicate that absolute numbers of circulating CD34+ cells in peripheral blood of patients who have low levels of such cells can now be quantified precisely using a modification of the ISHAGE protocol. This easy method enables precise measurement of the CD34+ cell population of stem cells in peripheral blood and can be broadly applied to screening patients for cardiovascular risk.

References

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964-7.
2. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA, et al. Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. Blood 2001;97:3075-85.
3. Taguchi A, Ohtani M, Soma T, Watanabe M, Kinosita N. Therapeutic angiogenesis by autologous bone-marrow transplantation in a general hospital setting. Eur J Vasc Endovasc Surg 2003;25:276-8.
4. Hamano K, Nishida M, Hirata K, Mikamo A, Li TS, Harada M, et al. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. Jpn Circ J 2001;65:845-7.
5. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003;348:593-600.
6. Taguchi A, Matsuyama T, Moriwaki H, Hayashi T, Hayashida K, Nagatsuka K, et al. Circulating CD34-positive cells provide an index of cerebrovascular function. Circulation 2004;109:2972-5.
7. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother 1996;5:213-26.
8. Sutherland DR, Keeney M, Gratama JW. Enumeration of CD34+ hematopoietic stem and progenitor cells. In: Robinson JP, Darzynkiewicz Z, Dobrucki J, Hyun WC, Nolan JP, Orfao A, Rabinovitch PS, editors. Current protocols in cytometry. New York: John Wiley and Sons, Inc.; 2003. Unit 6.4, p. 1-23.

manufacturers' protocols. (These protocols are based on International Society of Hematotherapy and Graft Engineering (ISHAGE) Guidelines[7] and are frequently used for quantification of CD34+ cells that have mobilized into peripheral blood.) Next, to increase the reproducibility of CD34+ cell counts, the Stem-Kit protocol was modified as follows: the blood sample volume, antibodies, and lysing solution were doubled. After adding 30 µL of internal control particles (stem count: Beckman Coulter), samples were centrifuged for 5 min at 450 G, and 3,860 µL of supernatant was removed carefully with a pipette. Samples were analyzed by Coulter CYTOMICS™ FC500 & XL-system II software (Beckman Coulter) for 6 min each (Fig. 1).

Results

Increase of CD34+ Cell Counts

The mean percentage of CD34+ cells in the leukocyte fraction obtained from mobilized peripheral blood has been reported to be about 0.2% to 0.5%.[7] First, we used the ProCount and Stem-Kit protocols to count circulating CD34+ cells that had been obtained from patients with cardiovascular disease (Table I). The mean percentages of CD34+ cells in the leukocyte fraction were 0.024% ± 0.003% (range, 0.012%–0.046%) for ProCount and 0.021% ± 0.001% (range, 0.011%–0.032%) for Stem-Kit. The actual CD34+ cell counts per analysis were only 16 ± 1 (range, 10–31) for ProCount and 34 ± 4 (range, 16–58) for Stem-Kit. Because the absolute cell count is a major factor in the reproducibility of the measurement,[8] our approach was to modify the original protocol in order to obtain higher numbers of actual CD34+ cells per count. However, simply increasing sample volume or measurement time does not improve reproducibility, because of these factors: adhesion of internal control particles to cells in the patient sample, and precipitation and aggregation of cells in the sample. Our approach, as outlined under Methods, was to seek a method that produces higher cell counts while maintaining short measurement times. Through the use of our method, the mean CD34+ cell count increased to a level of 174 ± 18 (range, 88–404) per analysis, and the mean percentage of CD34+ cells in the leukocyte fraction was 0.019 ± 0.002 (range, 0.014%–0.038%), which was consistent with measure-

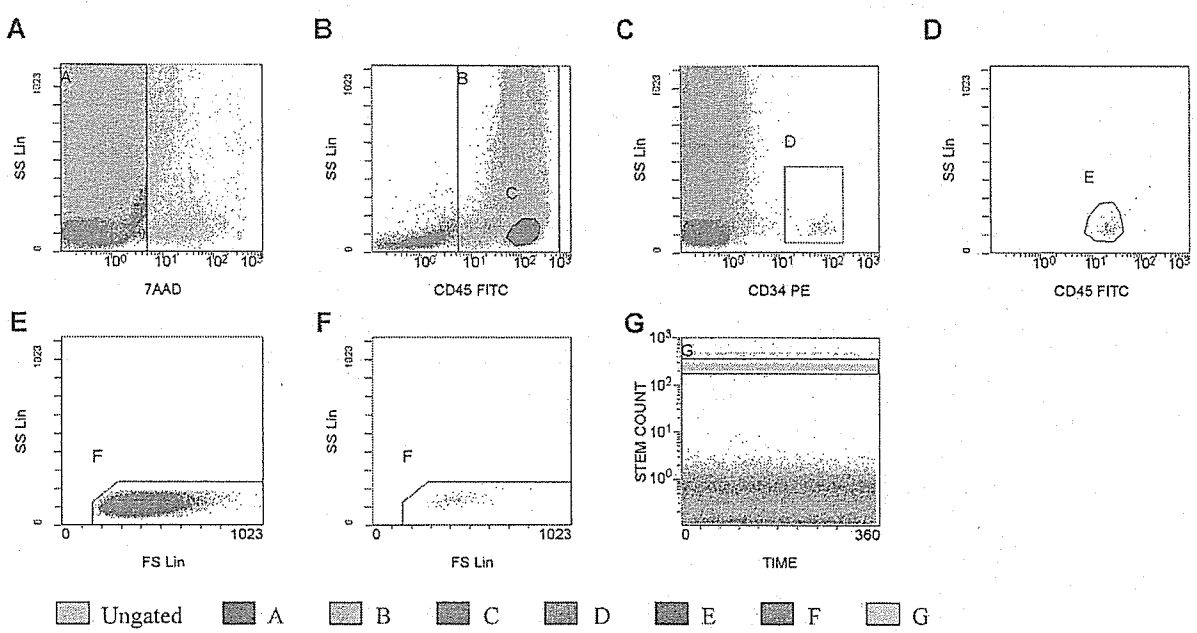

Fig. 1 *Quantification of circulating CD34+ cells by fluorescence-activated cell sorter analysis using our modified, improved protocol.* ***A)*** *All events: 7-aminoactinomycin-D viability dye-positive cells (dead cells) were excluded from region A.* ***B)*** *Events from region A: All CD45+ cells (leukocytes) were included in region B. Region C was adjusted to include only lymphocytes (bright CD45, low side-scatter).* ***C)*** *Events from regions A and B: Region D was adjusted to include CD34+ hematopoietic progenitor cells (HPC).* ***D*** *Events from regions A, B, and D: Region E was adjusted to include cells forming a cluster with characteristic CD34+ HPC (low side-scatter and low-to-intermediate CD45 staining). Brightly stained events were excluded from region E.* ***E)*** *Events from regions A and C: Region F was adjusted to include lymph/blast cells, excluding platelet aggregates if present.* ***F)*** *Events from A, B, D, and E: Lymph/blast region F identified a cluster of events that met all the fluorescence and light-scattering criteria of ISHAGE Guidelines for CD34+ HPC.* ***G)*** *All events: Region G was adjusted to enclose the internal control.*

7AAD = 7-aminoactinomycin-D; CD34 PE = cluster of differentiation 34 phycoerythrin; CD45 FITC = cluster of differentiation 45 fluorescein isothiocyanate; FS Lin = forward-scatter linear scale; ISHAGE = International Society of Hematotherapy and Graft Engineering; SS Lin = side-scatter linear scale

Biphasic Action of Inducible Nitric Oxide Synthase in a Hindlimb Ischemia Model

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Summary We investigated the influence of inducible nitric oxide synthase (iNOS) on acute ischemic injury and chronic angiogenesis. In a hindlimb ischemia model, NO produced by endothelial NO synthase (eNOS) reduces ischemic injury and promotes angiogenesis. However, the effect of the large amounts of NO generated by induced iNOS is unclear. Experimental groups of mice were as follows: (1) wild-type group (Wild), (2) iNOS-knockout group (iNOS-KO), and (3) aminoguanidine-treated wild-type group (Wild + AG), which received aminoguanidine from day 0 to day 3 after ischemia. Acute ischemic injury was evaluated by measuring the plasma CK value and ischemic score. Chronic angiogenesis was evaluated by microangiography and with a non-contact type Doppler blood flowmeter on day 3. Compared with the Wild group (251 ± 34.7 IU/l), the CK value was significantly elevated in the iNOS-KO (497 ± 126.7 IU/l) and Wild + AG (587.2 ± 128.7 IU/l) groups. The ischemic score was significantly increased in the iNOS-KO (92%) and Wild ± AG (66.6%) groups compared with the Wild group (23%). Blood flow was significantly increased in the iNOS-KO group (58.7 ± 15.3%) compared with the Wild (38.1 ± 15.9%) and Wild ± AG (43.5 ± 9.8%) groups in the chronic stage. Microangiography revealed a significantly increased number of blood vessels in the iNOS-KO (0.29 ± 0.02) group compared with the Wild (0.12 ± 0.01) and Wild + AG (0.15 ± 0.02) groups. Our findings indicate that NO generated by iNOS has a biphasic action, reducing acute ischemic injury and inhibiting angiogenesis in the chronic stage.

Key Words: angiogenesis, ischemia, nitric oxide synthase

Introduction

The incidence of refractory peripheral arterial disease is increasing rapidly in developed countries [*1*]. When peripheral arterial disease becomes severe, not only is the quality of life of patients impaired, but also their prognosis is poor [*2*]. Consequently new therapies, including angiogenic treatment with vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and fibroblast growth factor-4 (FGF-4) gene transduction or bone marrow cells, have been developed, with some success [*3-8*].

NO is produced by NO synthase and has multiple bioactivities, including vasodilating, anti-platelet-aggregating

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and anti-microbial activities [*9*]. Among the three NO synthase isoforms, neuronal NO synthase (nNOS) is found in the central nerve system, and iNOS is induced in smooth-muscle cells and inflammatory cells in various diseases, such as endotoxemia or ischemia, while eNOS is found in vascular endothelial cells [*10*].

NO generally has a cytoprotective action on hindlimb ischemia [*11–14*]. During ischemic injury, eNOS is upregulated and iNOS is induced. It is reasonable that NO produced by eNOS reduces acute ischemic injury and induces angiogenesis, as it has been shown to have a vasodilatory action [*15–17*]. Induced iNOS produces large amounts of NO [*18*], but the effect of NO generated by iNOS in hindlimb ischemia remains unclear.

In this experiment, we examined the contributions of iNOS to the acute phase of ischemic injury and to angiogenesis in the chronic phase of ischemia in a mouse hindlimb ischemia model, using iNOS knockout mice and wild-type mice treated with aminoguanidine (a selective inhibitor of inducible nitric oxide synthese in macrophages)[*19–21*] in the acute phase.

Materials and Methods

Mice

All mice used in experiments were male, 2 to 3 months of age, weighing 18 to 26 g each. Wild-type (Wild) 129 SvEv mice were purchased from CLEA, Japan. iNOS –/– mice, with a mixed C57BL/6J × 129 SvEv genetic background, were obtained from Merck & Co, Inc.. INOS +/+ mice were obtained by crossing 129 SvEv mice with C57BL/6J mice twice. INOS –/– and iNOS +/+ strains have similar genetic backgrounds of 75% C57BL/6J and 25% 129/SvEv [*22*]. For the pharmacologically iNOS-inhibited group, Wild mice were given aminoguanidine (AG; Sigma, 50 mg/kg, i.p., KI value of 55 micro M and a $K_{inact\ max}$ value of 0.09 min^{-1}) [*23*] 24 hr before operation and daily for 3 days postoperatively [*24, 25*]. The animals were maintained in a pathogen-free barrier facility with a 12-hour light/dark cycle and had free access to food and water. Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and hindlimb ischemia was created by ligation of the left common iliac artery and external iliac artery and resection of the femoral artery [*26, 27*]. Mice were killed 7 days (acute phase) or 14 to 21 days (chronic phase) after surgery [*28*]. The study was approved by the Animal Care Committee of Tokai University.

Evaluation of acute ischemic injury

Serum CK value—To estimate skeletal muscle injury, CK release was estimated in the effluent collected from the infraorbitalis vein on day 3. Plasma was obtained through centrifugation of the whole blood for 10 minutes at 12000 g at 4°C. Plasma was collected and CK was assayed by SRL Co..

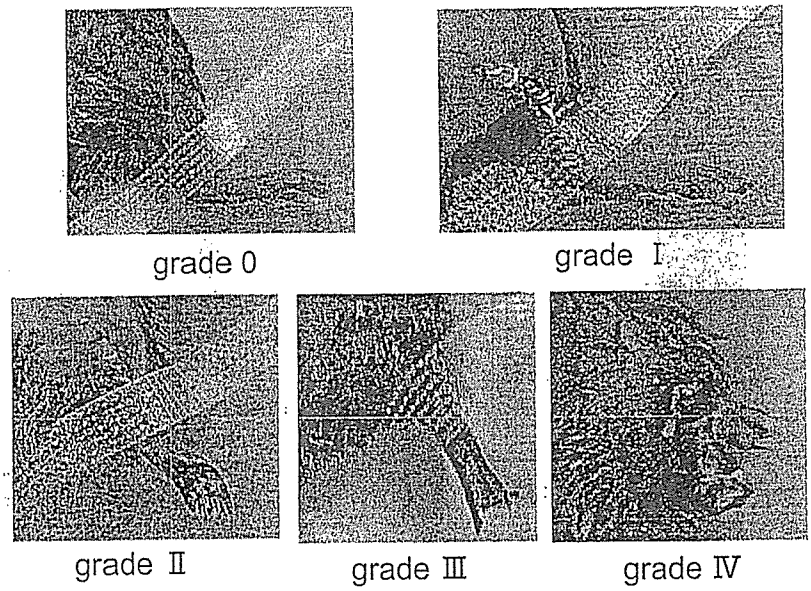
Ischemic score—On day 7, the degree of ischemic insult in the limb was macroscopically evaluated by using graded morphological scales for tissue necrosis (grade 0 to IV): grade 0: absence of necrosis: grade I, necrosis only of toes; grade II, necrosis extending to dorsum of a foot; grade III, necrosis extending to crus; grade IV, necrosis extending to a thigh or complete necrosis (Fig. 1).

Evaluation of chronic angiogenesis

Non-contact type laser Doppler measurement—We employed laser Doppler flowmetry (LDF), a non-nvasive technique for measuring tissue blood flow [*16, 29*], using a FLO-N1 device (OMEGAWAVE, Japan), which delivers light generated by a semiconductor laser diode operating at a wavelength of 780 nm, with a maximum accessible power of 3 mW. Briefly, the skin was removed so that only deep muscle blood flow would be measured, and the probe (ST-N probe, OMEGAWAVE, Japan) was placed on 4 points of the femoral muscles. Blood flow was expressed as ml/min/100 g. The contralateral hindlimb served as an internal control.

Sequential microangiography in in vivo—A PE-10 (10-gauge polyethylene) catheter was placed in the right common carotid artery of a mouse fixed on a board (1.0 mm thick) in the standing position under general anesthesia. Sequential images of the hind limb were obtained by the injection of non-ionic contrast material (1 ml/s for 2 s, Iopamidol, Nihon Schering, Tokyo, Japan) via the arterial catheter [*30*] on day 0 and day 14. Monochromatic synchrotron radiation with an energy level of 33.3 keV was obtained with a silicon crystal from beamlines NE5 and BL-14 at the High Energy Accelerator Research Organization, Tsukuba, Japan. To improve contrast resolution, subtraction images were created in the computer from the digital images obtained immediately before and during contrast material injection [*31*]. Angiogenesis was evaluated in terms of vessel density and assigned an angiographic score [*28, 32, 33*]. The ischemic signal in the acute phase is a critical factor inducing angiogenesis [*34*], and angiogenesis increases in proportion to the degree of ischemia [*35, 36*]. Therefore, angiogenesis should be compared among groups with comparable severity of acute ischemic injury. For this reason, we compared results among groups using only animals with grade I ischemic score (refer to Figure 3).

FITC gel angiography—To visualize microvessel networks, the FITC-gelatin conjugate fluorescence injection method (dialyzed FITC, 30 mg/mL conjugated gelatin solution) was employed [*37*]. Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and a PE-10 (10-gauge polyethylene) catheter was placed in the right common carotid artery. The FITC gelatin solution (20 ml) was injected into the catheter



grade	necrotic area
0	none
I	only toes
II	extending to dorsum pedis
III	extending to crus
IV	extending to thigh or complete necrosis

Fig. 1. The grading of necrosis in ischemic hindlimb. The ischemic limb was macroscopically evaluated by using a graded morphological scale for tissue necrosis area (grade 0 to IV).

(1 ml/min) and the right common carotid vein was cut. After complete perfusion, the left leg were resected and immediately fixed in ice-cold graded paraformaldehyde (4%). A confocal laser scanning microscopy (CLSM) system (LSM-410, Carl Zeiss, Jena, Germany), equipped with a 488-nm argon laser (for FITC), was employed on thick sections (1–2 mm) to visualize microvessel networks in detail [*38*]. After computer-assisted 3-D imaging of microvessel networks by the CLSM system, the images were stored on hard disk memory or a magnetic optical disk, EDM-230C (Sony, Tokyo, Japan) and were printed with a digital Pictrostat 400 (Fuji Film Co/Ltd., Tokyo, Japan).

Statistical Analysis

Data are presented as mean values ± SD. Differences were assessed by using one-way ANOVA with Tukey's post test.

Results

Acute ischemic injury

Serum CK value—Firstly, we measured serum CK value to evaluate the acute ischemic injury in the three experimental groups. In the control (Wild) group, the serum CK value was 251 ± 34.7 IU/l. The serum CK values in the Wild + AG group (587.2 ± 128.7 IU/l) and iNOS-KO group (497 ± 126.7 IU/l) were significantly higher than that in the Wild group (Figure 2).

Ischemic score on day 7—The ischemic scores in the iNOS-KO group (92%) and the Wild + AG group (66.6%) were significantly higher than that in the Wild group (23%) (Figure 3). Percentages of grade I in the Wild, iNOS-KO and Wild + AG groups were 23%, 25% and 33%, respectively.

Chronic angiogenesis

Laser Doppler (non contact type) measurement—The

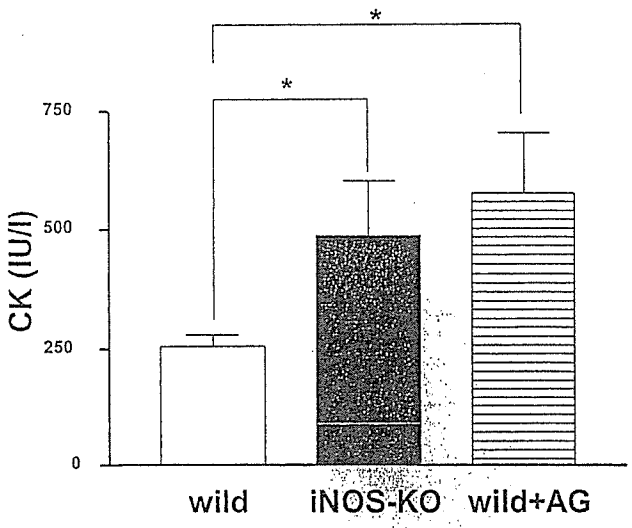


Fig. 2. Serum CK value in acute hindlimb ischemia. Open bar, control group; closed bar, iNOS-knockout (iNOS-KO) group; hatched bar, aminoguanidine-treated group (Wild + AG). The CK values in the iNOS-KO group and Wild + AG group were significantly higher than that in the control (Wild) group (*p<0.05).

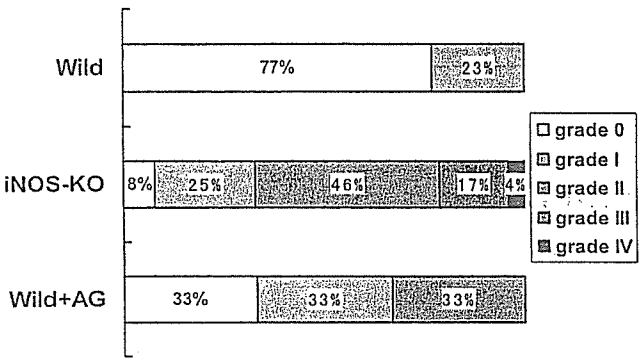


Fig. 3. Ischemic score in acute hindlimb ischemia. Open column, grade 0; dotted column, grade I; vertically lined column, grade II; hatched column, grade III; cross lined column, grade IV.

blood flow at the ischemic lesion was significantly reduced in all three groups on day 3 after surgery. However, at post-operative day 14, it was significantly higher in the iNOS-KO group (58.7 ± 8.7%) than in the Wild group (38.1 ± 5.2%) or the Wild + AG group (43.5 ± 6.4%) (Figure 4).

Sequential microangiography in in vivo—No vessels were apparent in hind limb angiography on day 0, and fine vessels were barely visible on day 14 in the Wild group (Figure 5 A and B). In contrast, many vessels were supplying the hind-limb on the injured side on day 14 in the iNOS-KO group. The angiogenic score on day 14 was significantly increased in the iNOS-KO group (0.29 ± 0.02) compared with that in

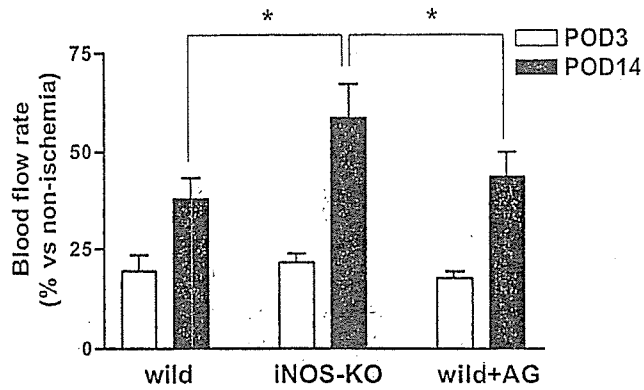


Fig. 4. Blood flow rate in non-contact laser-Doppler flowmetry. Open bar, blood flow ratio on day 3. Closed bar, blood flow ratio on day 14. The blood flow ratio on day 14 in the iNOS-KO group was significantly higher than in the Wild group or Wild + AG group (*p<0.05).

the Wild group or the Wild + AG group (0.12 ± 0.01 and 0.15 ± 0.02, respectively) (p<0.05) (Figure 5).

FITC angiography—The presence of fine vascular networks in the iNOS-KO group (Fig. 6B) implies that marked angiogenesis had occurred. There was a distinct difference in induction of vascular networks between the iNOS-KO group and the Wild and Wild + AG groups (Figure 6 A,B,C).

Discussion

In this experiment, ischemic injury was severe, but angiogenesis was markedly greater in the iNOS-KO group than in the Wild + AG or Wild group. The results indicate that NO generated by iNOS inhibited acute ischemic injury, but reduced angiogenesis in the chronic stage of ischemia.

Of the three NO synthese isoforms, nNOS is mainly localized in the central nervous system, postsynaptic density (PSD), and muscular sarcolemma (muscle fiber myelin) and participates in neural transmission [*39-41*]. iNOS is usually not expressed, but is induced in vascular smooth muscle cells and macrophages *via* cytokine stimulation during sepsis or ischemia with or without reperfusion, and produces large quantities of NO [*42*]. eNOS is mainly localized in vascular endothelial cells and produces NO continuously in response to shear stress, playing important roles in platelet aggregation and vasodilation [*12, 17*]. So, it appears reasonable that NO inhibits acute ischemic injury. In contrast, many studies have shown that NO production by iNOS aggravates injury in the hindlimb ischemia model. Nevertheless, we found that iNOS reduced ischemic injury in the acute stage in the present experiment. A key difference between our study and the others is the presence or absence of reperfusion following the ischemic period. We have

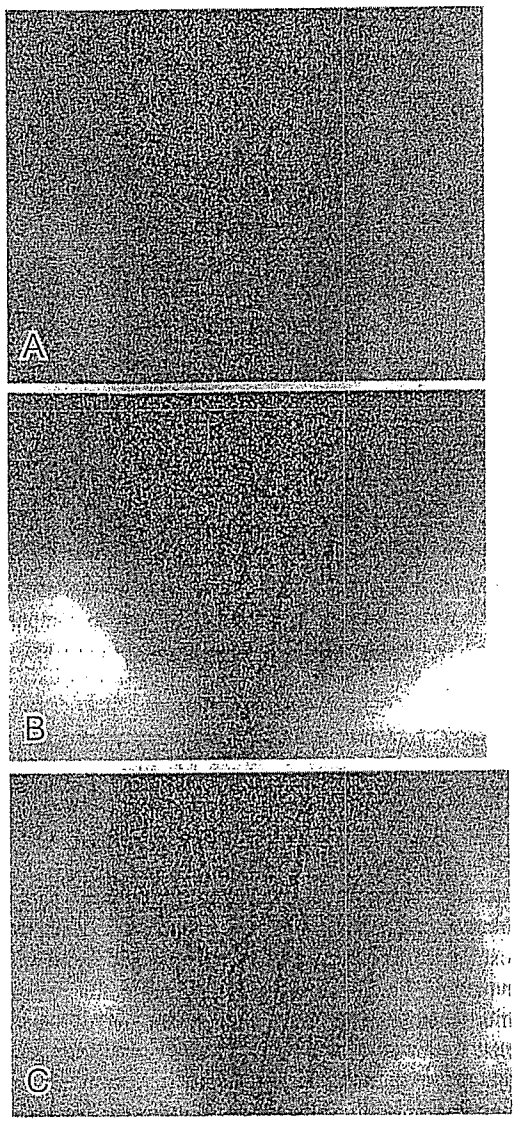


Fig. 5. Representative microangiograms. A : angiogram taken on day 0 in an animal of the Wild group ; B : angiogram taken on day 14 in an animal of the Wild group ; C : angiogram taken on day 14 in an animal of the iNOS-KO group.

already reported that superoxide ($O_2^{\cdot-}$) is produced in ischemic tissue at the time of reperfusion, and reacts with NO to form peroxynitrite [*43*]. Peroxynitrite is a potent oxidant that directly oxidizes sulfhydryl groups at a 1000-fold greater rate than hydrogen peroxide. It inhibits the function of various enzymes, including components of the mitochondrial electron transport chain. In our experiment, we examined ischemia without reperfusion, so that $O_2^{\cdot-}$ (and hence peroxynitrite) would not be produced, and only NO was present.

A second difference from previous experiments is that we used mice treated with iNOS inhibitor in the acute stage, as well as iNOS knockout mice, to examine the effect of iNOS [*44*]. It is noteworthy that one study in which iNOS knockout mice were used and reperfusion was not performed (similar to our protocol) found that injury was severe and angiogenesis in the chronic stage was augmented [*45*]. This is consistent with our results, and indicates that reperfusion plays a critical role in the outcome [*46*].

As the ischemic signal in the acute phase is a critical factor inducing angiogenesis [*34*], and angiogenesis increases in proportion to the degree of ischemia [*35, 36*], angiogenesis has to be compared among groups with comparable severity of acute ischemic injury. We therefore selected animals with grade I ischemic score in all cases for comparison among groups. Aminoguanidine was administered for only three days in the Wild + AG group in order to allow iNOS to function in the chronic stage. At corresponding levels of acute ischemia, angiogenesis in the chronic stage was obviously enhanced in the iNOS-KO group in comparison with the Wild + AG group, i.e., angiogenesis in the chronic stage was inhibited by the function of iNOS.

Many reports indicate that iNOS enhances angiogenesis in various neoplastic disease models [*44, 47–49*]. However, factors secreted by the cancer cells may play important roles in these models. It is important to note that our results showing a biphasic action of iNOS depended on the use of both an acutely iNOS inhibitor-treated wild-type group and an iNOS knockout group in an ischemic model. The mechanism underlying the inhibitory action of NO appears to be down-regulation of the VEGF receptor [*50*]. Possible compensatory roles of eNOS and nNOS in iNOS knockout mice have been ruled out by a previous study, in which their expression was shown to remain unchanged [*51*].

In summary, we have shown that iNOS reduces acute ischemia, but inhibits angiogenesis in a hindlimb ischemia model. Thus we suggest to use iNOS inducer or agents to increase NO production such as arginine with acute phase and supplement iNOS inhibitor in chronic stage. However these remained to be examined prior to clinical trial.

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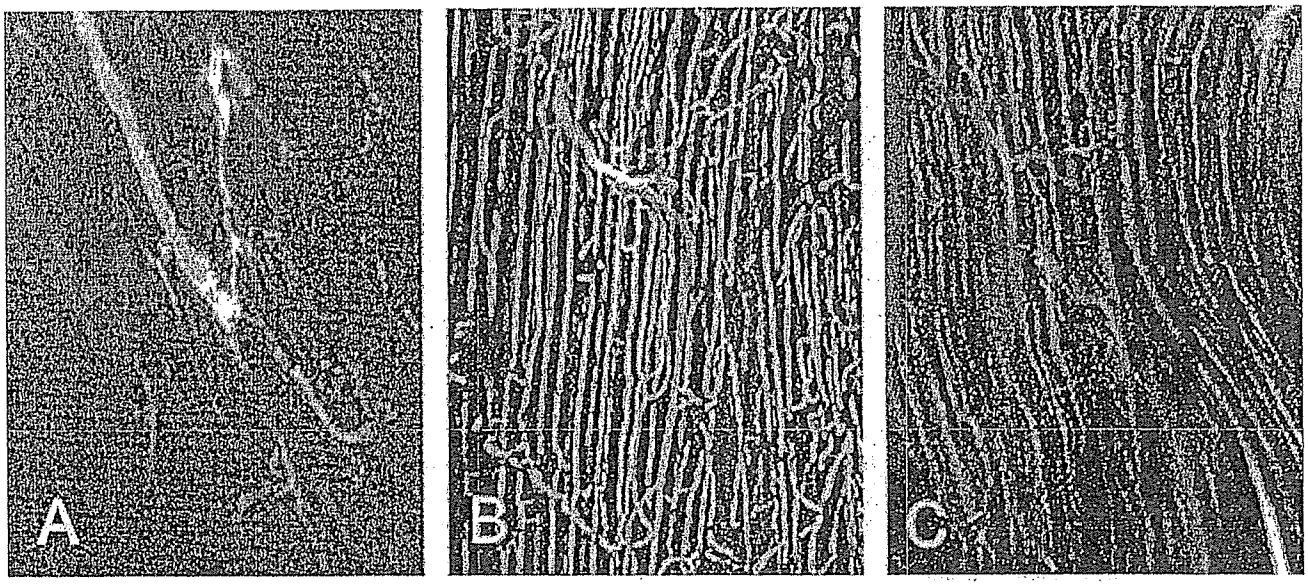

Fig. 6. FITC angiography. FITC angiograms were evaluated on day 14. A: in an animal of the Wild group; B: in an animal of the iNOS-KO group; C: in an animal of the Wild + AG group. Clear angiogenesis was visualized in the iNOS-KO group compared with the control group and aminoguanidine-treated group.

References

[1] Rosamond, W.D., Chambless, L.E., Folsom, A.R., Cooper, L.S., Conwill, D.E., Clegg, L., Wang, C.H., and Heiss, G.: Trends in the incidence of myocardial infarction and in mortality due to coronary heart disease, 1987 to 1994. *N. Engl. J. Med.*, **339**, 861-867, 1998.

[2] Mukherjee, D., Bhatt, D.L., Roe, M.T., Patel, V., and Ellis, S.G.: Direct myocardial revascularization and angiogenesis-How many patients might be eligible? *Am. J. Cardiology.*, **84**, 598-600, 1999.

[3] Isner, J.M. and Asahara, T.: Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. *J. Clin. Invest.*, **103**, 1231-1236, 1999.

[4] Carmeliet, P., Ng, Y.S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V.V., Stalmans, I., Mattot, V., Perriard, J.C., Dewerchin, M., Flameng, W., Nagy, A., Lupu, F., Moons, L., Collen, D., Amore, P.A.D., and Shima, D.T.: Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms $VEGF_{164}$ and $VEGF_{188}$. *Nature. Med.*, **5**, 495-502, 1999.

[5] Jayasnkar V., Woo J., Bish L.T., Pirolli T.J., Chatterjee S., Berry M.F., Burdick J., Gardner T.J., and Sweeney H.L.: Gene transfer of hepatocyte growth factor attenuates postinfarction heart failure. *Circulation*, **108**[suppl II], II-230-II-236, 2003.

[6] Taniyama, Y., Morishita, R., Hiraoka, K., Aoki, M., Nakagami, H., Yamasaki, K., Matsumoto, K., Nakamura, T., Kaneda, Y., and Ogihara,T.: Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat diabetic hind limb ischemia model. Molecular mechanisms of delayed angiogenesis in diabetes. *Circulation*, **104**, 2344-2350, 2001.

[7] Grines, C.L., Watkins, M.W., Helmer, G., Penny, W., Brinker, J., Marmur, J.D., West, A., Rade, J.J., Marrott, P., Hammond, H.K. and Engler, R.L.: Angiogenic gene therapy (AGENT) trial in patients with stable angina pectoris. *Circulation*, **105**, 1291-1297, 2002.

[8] Tateishi-Yuyama, E., Matsubara, H., Murohara, T., Ikeda, U., Shintani, S., Masaki, H., Amano, K., Kishimoto, Y., Yoshimoto, K., Akashi, H., Shimada, K., Iwasaka, T., and Imaizumi, T.: Therapeutic angiogenesis for patients with limb ischemia by autologous transplantation of bone-marrow cells: a pilot study and a randomized controlled trial. *Lancet*, **360**, 427-435, 2002.

[9] Randomski, M.W., Vallance, P., Whitley, G., Foxwell, N., and Moncada, S.: Platelet adhesion to human vascular endothelium is modulated by constitutive and cytokine induced nitric oxide. *Cardiovasc. Res.*, **27**, 1380-1382, 1993.

[10] Förstermann, U., Closs, E.I., Pollock, J.S., Nakane, M., Schwarz, P., Gath, I., and Kleinert, H.: Nitric oxide synthase isozymes characterization, molecular cloning, and functions. *Hypertension*, **23**, 1121-1131, 1994.

[11] Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H.J., Maggi, C.A., Geppetti, P., and Ledda, F.: Nitric Oxide Mediates Angiogenesis *in vivo* and endothelial cell growth and migration *in vitro* promoted by substance P. *J. Clin. Invest.*, **94**, 2036-2044, 1994.

[12] Papapetropoulos, A., Desai, K.M., Rudic, R.D., Mayer, B., Zhang, R., Ruiz-Torres, M.P., García-Cardeña, G., Madri, J.A., and Sessa, W.C.: Nitric oxide synthase inhibitors attenuate transforming-growth-factor-β_1-stimulated capillary organization *in vitro*. *Am. J. pathol.*, **150**, 1835-1844, 1997.

[13] Ziche, M., Morbidelli, L., Choudhuri, R., Zhang, H.T., Donnini, S., Granger, H.J., and Bicknell, R.: Nitric oxide

synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J. Clin. Invest.*, 99, 2625-2634, 1997.

[14] Papapetropoulos, A., García-Cardeña, G., Madri, J.A., and Sessa, W.C.: Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest.*, **100**, 3131-3139, 1997.

[15] Park, K.M., Byun, J.Y., Kramers, C., Kim, J.I., Huang, P.L., and Bonventre, J.V.: Inducible nitoric-oxide synthase is an important contributor to prolonged protective effects of ischemic preconditioning in the mouse kidney. *J. Biol. Chem.*, 278, 27256-27266, 2003.

[16] Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J.F., Fishman, M.C., Huang, P.L., and Isner, J.M.: Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J. Clin. Invest.*, **101**, 2567-2578, 1998.

[17] Brevetti, L.S., Chang, D.S., Tang, G.L., Sarkar, R., and Messina, L.M.: Overexpression of endothelial nitric oxide synthase increases skeletal muscle blood flow and oxygenation in severe rat hind limb ischemia. *J. Vasc. Surg.*, 38, 820-826, 2003.

[18] Wildhirt, S.M., Suzuki, H., Horstman, D., Weismüller, S., Dudek, R.R., Akiyama, K., and Reichart, B.: Selective modulation of inducible nitric oxide synthase isozyme in myocardial infarction. *Circulation*, **96**, 1616-1623, 1997.

[19] Misko, T.P., Moore, W.M., Kasten, T.P., Nickols, G.A., Corbett, J.A., Tilton, R.G., McDaniel, M.L., Williamson, J.R., and Currie, M.G.: Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur. J. Pharmacol.*, **233**, 119-125, 1993.

[20] Joly, G.A., Ayres, M., Chelly, F., and Kilboum, R.G.: Effects of NG-methyl-L-arginine, NG-nitro-L-arginine, and aminoguanidine on constitutive and inducible nitric oxide synthase in rat aorta. *Biochem. Biophys. Res. Commun.*, **199**, 147-154, 1994.

[21] Cross, A.H., Misko T.P., Lin, R.F., Hickey, W.F., Trotter, J.L., and Tilton, R.G.: Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice. *J. Clin. Invest.*, **93**, 2684-2690, 1994.

[22] Niu, X.L., Yang, X., Hoshiai, K., Tanaka, K., Swamura, S., Koga, Y., and Nakazawa, H.: Inducible nitric oxide synthase deficiency dose not affect the susceptibility of mice to atherosclerosis but increases collagen content in lesions. *Circulation*, **103**, 1115-1120, 2001.

[23] Wolff, D.J., Gauld, D.S., Neulander, M.J., and Southan, G.: Inactivation of nitric oxide synthase by substituted aminoguanidines and aminoisothioureas. *J. Pharmacol. Exp. Ther.*, **283**, 265-273, 1997.

[24] Wildhirt, S.M., Schulze, C., Conrad, N., Kornberg, A., Horstman, D., and Reichart, B.: Aminoguanidine inhibits inducible NOS and reverses cardiac dysfunction late after ischemia and reperfusion-implications for iNOS-mediated myocardial stunning. *Thorac. Cardiovasc. Surg.*, **47**, 137-143, 1999.

[25] Tamarat, R., Silvestre, J.S., Huijberts, M., Benessiano, J., Ebrahimian, T.G., Duriez, M., Wautier, M.P., Wautier, J.L., and Lévy, B.I.: Blockade of advanced glycation end-product formation restores ischemia-induced angiogenesis in diabetic mice. *Proc. Natl. Acad. Sci. U. S. A.*, **100**, 8555-8560, 2003.

[26] Couffinhal, T., Silver, M., Zheng, L.P., Kearney, M., Witzenbichler, B., and Isner, J.M.: Mouse model of angiogenesis. *Am. J. Pathol.*, **152**, 1667-1679, 1998.

[27] Kasahara, H., Tanaka, E., Fukuyama, N., Sato, E., Sakamoto, H., Tabata, Y., Ando, K., Iseki, H., Shinozaki, Y., Kimura, K., Kuwabara, E., Koide, S., Nakazawa, H., and Mori, H.: Biodegradable gelatin hydrogel potentiates the angiogenic effect of fibroblast growth factor 4 plasmid in rabbit hindlimb ischemia. *J. Am. Coll. Cardiol.*, **41**, 1056-1062, 2003.

[28] Tanaka, E., Hattan, N., Ando, K., Ueno, H., Sugio, Y., Mohammed, M.U., Voltchikhina, S.A., and Mori H.: Amelioration of microvascular myocardial ischemia by gene transfer of vascular endothelial growth factor in rabbits. *J. Thorac. Cardiovasc. Surg.*, **120**, 720-728, 2000.

[29] Lindén, M., Sirsjö, A., Lindbom, L., Nilsson, G., and Gidlöf, A.: Laser-Doppler perfusion imaging of microvascular blood flow in rabbit tenuissimus muscle. *Am. J. Physiol. Heart Circ. Physiol.*, **269**, H1496-H1500, 1995.

[30] Kuwabara, E., Furuyama, F., Ito, K., Tanaka, E., Hattan, N., Fujikura, H., Kimura, K., Goto, T., Hayashi, T., Taira, H., Shinozaki, Y., Umetani, K., Hyodo, K., Tanioka, K., Mochizuki, R., Kawai, T., Koide, S., and Mori, H.: Inhomogeneous vasodilatory responses of rat tail arteries to heat stress: evaluation by synchrotron radiation microangiography. *Jpn. J. Physiol.*, **52**, 403-408, 2002.

[31] Sekka, T., Volchikhina, S.A., Tanaka, A., Hasegawa, M., Tanaka, Y., Ohtani, Y., Tajima, T., Makuuchi, H., Tanaka, E., Iwata, Y., Sato, S., Hyodo, K., Ando, M., Umetani, K., Kubota, M., Tanioka, K., and Mori, H.: Visualization, quantification and therapeutic evaluation of angiogenic vessels in cancer by synchrotron microangiography. *J. Synchrotron Rad.*, 7, 361-367, 2000.

[32] Takeshita, S., Zheng, L.P., Brogi, E., Kearney, M., Pu, L.G., Bunting, S., Ferrara, N., Symes, J.F., and Isner, J.M.: Therapeutic angiogenesis: a single intra-arterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J. Clin. Invest.*, **93**, 662-670, 1994.

[33] Takeshita, S., Isshiki, T., Ochiai, M., Eto, K., Mori, H., Tanaka, E., Umetani, K. and Sato, T.: Endothelium-dependent relaxation of collateral microvessels after intramuscular gene transfer of vascular endothelial growth factor in a rat model of hindlimb ischemia. *Circulation*, 98, 1261-1263, 1998.

[34] Chung, N.A.Y., Lydakis, C., Belgore, F., Blann, A.D., and Lip, G.Y.H.: Angiogenesis in myocardial infarction An acute or chronic process? *Eur. Heart J.*, **23**, 1604-1608, 2002.

[35] Gavin, J.B., Maxwell, L., and Edgar, S.G.: Microvascular Involvement in Cardiac Pathology. *J. Mol. Cell. Cardiol.*, 30, 2531-2540, 1998.

[36] Sennlaub, F., Courtois, Y., and Goureau, O. Inducible nitric

oxide synthase mediates the change from retinal to vitreal neovascularization in ischemic retinopathy. *J. Clin. Invest.*, **107**, 717-725 2001.

[37] Itoh, J., Kawai, K., Serizawa, A., Yasumura, K., Ogawa, K., and Osamura, R.Y.: A new approach to three-dimensional reconstructed imaging of hormone-secreting cells and their microvessel environments in rat pituitary glands by confocal laser scanning microscopy. *J. Histochem. Cytochem.*, **48**, 569-577, 2000.

[38] Itoh, J., Yasumasa, K., Takeshita, T., Ishikawa, H., Kobayashi, H., Ogawa, K., Kawai, K., Serizawa, A., and Osamura, R.Y.: Three-dimensional imaging of tumor angiogenesis. *Analyt. Quant. Cytol. Histol.*, **22**, 85-90, 2000.

[39] Kobzik, L., Reid, M.B., Bredt, D.S., and Stamler, J.S.: Nitric oxide in skeletal muscle. *Nature*, **372**, 546-548 1994.

[40] Brenman, J.E., Christopherson, K.S., Craven, S.E., McGee, A.W., and Bredt, D.S.: Cloning and Characterization of Postsynaptic Density 93, a Nitric Oxide Synthase Interacting Protein. *J. Neurosci.*, **16**, 7407-7415, 1996.

[41] Dreyer, J., Schleicher, M., Tappe, A., Schilling, K., Kuner, T., Kusumawidijaja, G., Müller-Esterl, W., Oess, S., and Kuner, R.: Nitric Oxide Synthase (NOS)-Interacting Protein Interacts with Neuronal NOS and Regulates Its Distribution and Activity. *J. Neurosci.*, **24**, 10454-10465, 2004.

[42] Sharshar, T., Gray, F., Geoffroy, L.G., Hopkinson, N.S., Ross, E., Dorandeu, A., Orlikowski, D., Raphael, J.C., Gajdos, P., and Annane, D.: Apoptosis of neurons in cardiovascular autonomic centres triggered by inducible nitric oxide synthase after death from septic shock. *Lancet*, **362**, 1799-1805 2003.

[43] Liang, F., Gao, E., Tao, L., Liu, H., Ou, Y., Christopher, T.A., Lopez, B.L., and Ma, X.L.: Critical timing of L-arginine treatment in post-ischemic myocardial apoptosis—role of NOS isoforms. *Cardiovasc. Res.*, **62**, 568-577, 2004.

[44] Kane, A.J., Barker, J.E., Mitchell, G.M., Theile, D.R.B., Romero, R., Messina, A., Wagh, M., Fraulin, F.O.G., Morrison, W.A., and Stewart, A.G.: Inducible nitric oxide synthase (iNOS) activity promotes ischemic skin flap survival. *Br. J. Pharmacol.*, **132**, 1631-1638, 2001.

[45] Cuzzocrea, S., Chatterjee, P.K., Mazzon, E., Dugo, L., De Sarro, A., Van de Loo, F.A., Caputi, A.P., and Thiemermann, C.: Role of induced nitric oxide in the initiation of the inflammatory response after postischemic injury. *Shock*, 18, 169-176, 2002.

[46] Bolli, R.: Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research. *J. Mol. Cell. Cardiol.*, 33, 1897-918, 2001.

[47] Kisley, L.R., Barrett, B.S., Bauer, A.K., Dwyer-Nield, L.D., Barthel, B., Meyer, A.M., Thompson, D.C., and Malkinson, A.M.: Genetic ablation of inducible nitric oxide synthase decreases mouse lung Tumorigenesis. *Cancer. Res.*, **62**, 6850-6856 2002.

[48] Deininger, M.H., Wybranietz, W.A., Graepler, F.T., Lauer, U.M., Meyermann, R., and Schluesener, H.J.: Endothelial endostatin release is induced by general cell stress and modulated by the nitric oxide/cGMP pathway. *F.A.S.E.B. J.*, **17**, 1267-1276 2003.

[49] Cianchi, F., Cortesini, C., Fantappiè, O., Messerini, L., Sardi, I., Lasagna, N., Perna, F., Fabbroni, V., Felice, A.D., Perigli, G., Mazzanti, R., and Masini, E.: Cyclooxygenase-2 activation mediates the proangiogenic effect of nitric oxide in colorectal cancer. *Clin. Cancer. Res.*, **10**, 2694-2704, 2004.

[50] Fukumura, D., Gohongi, T., Kadambi, A., Izumi, Y., Ang, J., Yun, C.O., Buerk, D.G., Huang, P.L., and Jain, R.K.: Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. *Proc. Natl. Acad. Sci. U. S. A.*, **98**, 2604-2609, 2001.

[51] Qi, W., Chen, L.E., Zhang, L., Eu, J.P., Seaber, A.V., and Urbaniak, J.R.: Reperfusion injury in skeletal muscle is reduced in inducible nitric oxide synthase knockout mice. *J. Appl. Physiol.*, **97**, 1323-1328, 2004.

THYROID
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Triiodothyronine Acutely Increases Blood Flow in the Ventricles and Kidneys of Anesthesized Rabbits

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Thyroid hormone (triiodothyronine [T_3]) has various nongenomic effects, including alterations in glucose and fatty acid metabolism, augmentation of intracellular Ca^{2+}, enhancement of myocardial contractility, and vascular dilatation. However, its effect on regional blood flow remains to be established. We have measured the effect of T_3 on blood flow in major organs of anesthetized rabbits *in vivo* using the microsphere method. Under artificial respiration, nonradioactive microspheres (5×10^5) labeled with barium were injected to measure blood flow at control level. Then, T_3 (50 μg/kg per milliliter) was administered and microspheres labeled with iodine (5×10^5) were injected. The atria, ventricles, kidneys, and right upper limb were excised and their contents of microspheres were evaluated. Blood flow in the ventricles was significantly increased by T_3 (2.9 $\pm$ 0.3 versus 3.4 $\pm$ 0.3 mL/min per gram, vehicle versus T_3). Similarly, blood flow in the kidneys was significantly higher after T_3 injection (4.3 $\pm$ 0.5 versus 5.1 $\pm$ 0.5 mL/min per, vehicle versus T_3). The blood flow in the atria and skeletal muscles remained unchanged. These results indicate that the vasodilatory response to T_3 is not uniform and occurs preferentially in major organs such as cardiac ventricles and kidneys; this may be relevant to the T_3-induced improvement of cardiac function.

Introduction

THYROID HORMONE (triiodothyronine [T_3]) exerts its effect by binding the specific receptor in the nucleus, and activating or repressing transcription of target genes. In addition to this genomic action, T_3 has nongenomic effects that occur very quickly upon T_3 exposure (1,2) and are insensitive to inhibitors of protein synthesis. Nongenomic effects include alterations in glucose (3) and fatty acid metabolism (4), increase in mitochondrial oxygen consumption (5), augmentation of intracellular Ca^{2+} (6–9), enhancement of myocardial contractility (10) and dilatation of vasculature (11–13). These effects have been demonstrated in cells or tissue *in vitro*, as well as in perfused heart preparations or *in vivo*. Of particular importance are the improvement of cardiac function in conscious dogs with pacing-induced cardiomyopathy (14) and the beneficial outcome in patients undergoing cardiopulmonary bypass (15). These results suggest that acute administration of T_3 may become a therapeutic option for patients with impaired ventricular function. A recent study (16) performed in volunteers provided further support for its use in the clinical situation, because T_3 administration acutely induced a decrease in systemic vascular resistance and an increase in cardiac output. This indicates that T_3 can produce acute arterial dilation in healthy euthyroid humans. However, further basic studies are required before clinical use, because important information remains to be obtained. One such point is whether or not the decrease in systemic vascular resistance actually increases regional blood flow. It is also important to examine whether the vasodilatory response to T_3 administration occurs uniformly in all organs.

The purpose of this study was to examine whether the vasodilatory effect of T_3 differs among various organs. To test this, we measured regional blood flow in major organs using the microsphere method *in vivo*.

Materials and Methods

Animals

Animals were managed in accordance with guiding principles for the care and use of animals proposed by the Physiological Society of Japan and the *American Journal of Physiology*.

Japanese White rabbits weighing 3.1 $\pm$ 0.1 kg were anesthetized with pentobarbital sodium (20 mg/kg intravenously), intubated, and ventilated with room air mixed with oxygen (0.5 L/min). Four catheters were placed: one in the left ventricle for microsphere injection, two in the femoral arteries for blood pressure measurement and blood sample

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withdrawal, and one in the femoral vein for vehicle or T_3 injection. The blood pressure and electrocardiogram were continuously recorded throughout the experiment.

Experimental protocol

After stabilization of the hemodynamics, 1 mL of the vehicle of T_3 was administered (over 1 minute) and the first injection of 5×10^5 microspheres labeled with barium (Sekisui Plastic Co. Ltd., Japan) was made 5 minutes after the vehicle injection. Then T_3 (50 μg/kg per milliliter; 144.3–177 μg/mL) was administered over 1 minute and the second injection of 5×10^5 microspheres labeled with iodine was made 5 minutes after T_3 injection. In an additional four rabbits microspheres were injected 5 minutes and 30 minutes after T_3 injection to observe time course of T_3 effect. At the end of the experiment, the rabbits were sacrificed with an overdose of pentobarbital sodium, and the heart, kidneys, and skeletal muscles of the right upper limb were removed for blood flow measurements.

Measurements of regional blood flow

Regional blood flow was determined by the nonradioactive microsphere technique, as previously reported (17). Briefly, heavy metal microspheres (barium and iodine 15 μm optical density [OD], 5×10^5) suspended in 1 mL of normal saline containing 0.05% Tween 80 were infused into the left ventricle for 1 minute, followed by flushing with 0.5 mL of saline for 30 seconds. Withdrawal of reference blood from the femoral artery was continued for 30 seconds after the saline flush was completed. Each of the tissues and the reference blood samples was dissolved completely in 2 N KOH solution in a vial. The vials were centrifuged and the microspheres were aspirated and transferred to the surface of filter paper under vacuum. Heavy metals on the paper were quantitated by x-ray fluorescence spectrometry (PW1480, Phillips, Almelo, The Netherlands) (17). We used microspheres labeled with barium (Ba) in the first injection and iodine (I) in the second injection. In preliminary experiments we confirmed that this number of microspheres (5×10^5) did not impair the microcirculation, and provided sufficient sensitivity of evaluation in this size of rabbit.

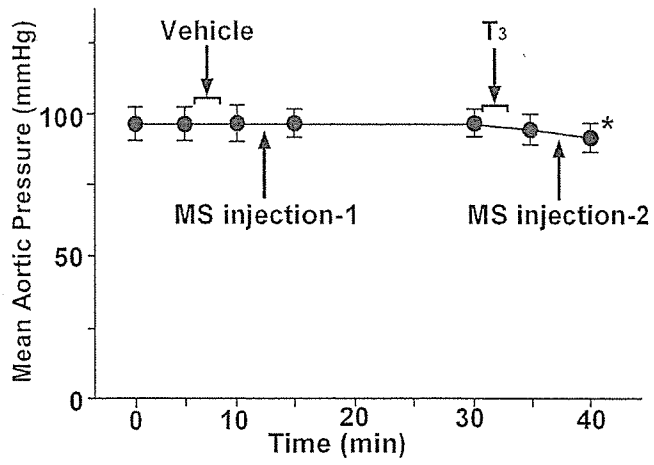

FIG. 1. Time course of blood pressure in four rabbits. Arrows indicate timing of each injection. MS injection 1, barium microspheres injection (5×10^5); MS injection 2, iodine microspheres injection (5×10^5); vehicle, vehicle of triiodothyronine (T_3) solution injection (1 mL); T_3, T_3 injection (50 μg/kg, 1 mL). *Indicates significant difference between blood pressure prior to T_3 injection and that 10 minutes after T_3 injection.

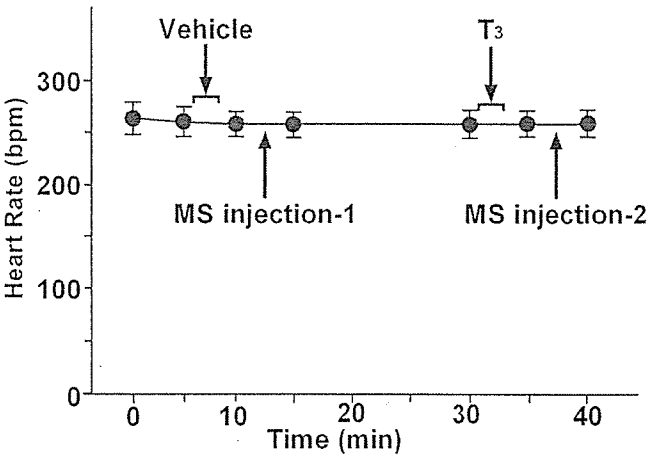

FIG. 2. Time course of heart rate. MS injection 1, barium microspheres injection (5×10^5); MS injection 2, iodine microspheres injection (5×10^5); vehicle, vehicle of triiodothyronine (T_3) solution injection (1 mL); T_3, T_3 injection (50 μg/kg, 1 mL). *Indicates significant difference between blood pressure prior to T_3 injection and that 10 minutes after T_3 injection.

The stock solution of T_3 (Sigma, St. Louis, MO) was prepared fresh each day and serially diluted to the necessary concentrations. The free T_3 level in plasma was measured with a commercial electrochemiluminescence assay (ECLIA; Roche Molecular Biochemicals, Mannheim, Germany).

Statistics

All results are presented as means ± standard error (SE). A paired t test was used to compare the data before and after T_3 injection. A p value of less than 0.05 was considered significant.

Results

The basal plasma free T_3 level was 3.23 ± 0.28 (range, 2.4–3.63) pg/mL, and free T_3 increased to over 32.6 pg/mL at 20 minutes after T_3 injection. As T_3 exceeded the upper quantitation limit of the test (32.6 μg/mL), peak values could not be determined

Figure 1 shows the time course of mean blood pressure during the experiment. Vehicle injection did not affect the blood pressure (96.5 ± 5.9 and 97.0 ± 5.9 mm Hg before and after, respectively). Blood pressure prior to T_3 injection was 97.0 ± 4.3 mm Hg, and was slightly decreased at 5 minutes after the injection (94.5 ± 5.4 mm Hg). Blood pressure at 10 minutes after the injection was 91.2 ± 5.5 mm Hg (signifi-

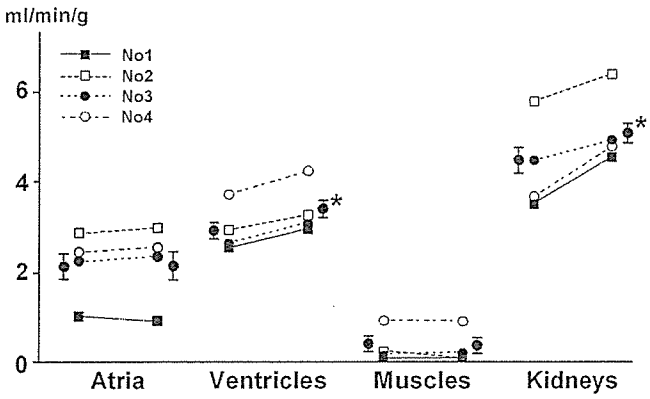

FIG. 3. Regional blood flow during vehicle and triiodothyronine (T_3) injections. Different symbols are used to indicate individual rabbits. *Indicates significant difference between vehicle and T_3 injections. ($p < 0.05$)

cantly lower than that prior to T_3 injection). Heart rate remained unaltered (Fig. 2). Figure 3 shows the changes in regional blood flow in four organs. T_3 injection increased blood flow to the ventricles and kidneys (ventricles: 2.9 ± 0.3 versus 3.4 ± 0.3 and kidneys: 4.3 ± 0.5 versus 5.1 ± 0.5 mL/min/g, before versus after T_3 injection), but the flows in the atria and skeletal muscles remained unchanged (atria: 2.1 ± 0.4 versus 2.2 ± 0.4 and muscles: 0.3 ± 0.2 versus 0.3 ± 0.3 mL/min/g, before versus after). In additional experiments to evaluate the time course of T_3 effect, barium was injected 5 minutes and iodine was injected 30 minutes after T_3 injection. Blood flows remained unchanged: 2.1 ± 0.2 versus 2.1 ± 0.3 in atria, 3.0 ± 0.3 versus 3.3 ± 0.2 in ventricle, 0.1 ± 0.2 versus 0.2 ± 0.2 in muscles, and 5.0 ± 0.3 versus 5.1 ± 0.4 in kidneys, indicating that the effect of T_3 lasted at least for 30 minutes.

Discussion

This is the first study to evaluate regional blood flows immediately after T_3 administration, and the results show that vasodilatory response to T_3 is diverse in various organs. Blood flow in the ventricles and kidneys increased, but that in atria and skeletal muscles remained unaffected. As the cardiovascular system is one of the major targets of genomic as well as nongenomic T_3 action (1,2), and the ventricles and kidneys are important for the cardiovascular system, the significant increase of blood flow in these organs appears to provide a basis for the T_3-induced improvement of cardiac function. No increase in blood flow to skeletal muscles appears to contradict to previous studies in which relaxation was observed in isolated skeletal muscle artery (13) and in cultured smooth muscle cells (11). However, experimental conditions differ markedly since both studies were performed in *in vitro*. Particularly dilatation of the artery by T_3 was evaluated under the preconstricted condition by thromboxane, in which vasodilatory effect appears to be easily demonstrable.

The mechanism of the increase in blood flow by T_3 is dilatation of relevant arteries because systemic blood pressure decreased. In addition, an increase in cardiac output through the positive inotropic effect of T_3 may have contributed, because a rapid increase in cardiac output or in contractility following T_3 administration has been demonstrated *in vitro* (6,7,10,18), *ex vivo* (4,19), and *in vivo* (20,21).

Studies that have shown acute beneficial effects of T_3 were performed in patients with impaired cardiac function or undergoing cardiopulmonary bypass (15,22), both of which conditions are known to decrease T_3 levels (the low T_3 syndrome). Several animal studies have found that T_3 administration did not cause any hemodynamic changes under basal conditions or in a normal thyroid state, but significant effects were seen in hearts subjected to ischemic injury or ischemia-reperfusion injury, or in a hypothyroid condition (14,20). Thus a noteworthy feature of this study is that the effect was observed in the euthyroid state.

A membrane-associated receptor of T_3 is necessary for T_3 to exert its acute effect, and its existence seems very likely, by analogy with the recent discovery of membrane receptors for progestins and estrogens, which has clarified the mechanism underlying the nongenomic actions of steroid hormone (23,24). In the presence of such membrane-associated T_3 receptors, acute effects should be manifested even under physiologic and euthyroid conditions. The recent novel discovery by Scanlan et al. (25) provided evidence that metabolite of T_3 act through G-protein–coupled receptor.

Heart rate remained unaltered by T_3 administration. This may appear peculiar, because tachycardia is one of the most common manifestations in hyperthyroidism. However, it is in agreement with a previous study in which heart rate was not changed by T_3 in an isolated perfused heart (19), even though a marked increase in left ventricular developed pressure was observed. A review (11) also commented on unchanged heart rate despite a significant decrease in peripheral vascular resistance after T_3 administration.

In conclusion, T_3-induced acute vasodilatation is accompanied by an increase in blood flow in the ventricles and kidneys, and this may be one of the mechanisms underlying the T_3-induced improvement of cardiac function.

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References

1. Davis PJ, Davis FB 2002 Nongenomic actions of thyroid hormone on the heart. Thyroid 12:459–466.
2. Bassett JH, Harvey BC, Williams GR 2003 Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. Mol Cell Endocrin 213:1-11.
3. Segal J 1989 A rapid, extranuclear effect of 3,5,3′-triiodothyronine on sugar uptake by several tissues in the rat in vivo. Evidence for a physiological role for the thyroid hormone action at the level of the plasma membrane. Endocrinology 124:2755–2764.
4. Liu Q, Clanachan AS, Lopaschuk GD 1998 Acute effects of triiodothyronine on glucose and fatty acid metabolism during reperfusion of ischemic rat hearts. Am J Physiol 275:E392–399.
5. Wrutniak-Cabello C, Casas F, Cabello G 2001 Thyroid hormone action in mitochondria. J Mol Endocrinol 26:67–77.
6. Segal J 1990 Calcium is the first messenger for the action of thyroid hormone at the level of the plasma membrane: first

evidence for an acute effect of thyroid hormone on calcium uptake in the heart. Endocrinology **126**:2693–2702.
7. Davis PJ, Davis FB 1993 Acute cellular actions of thyroid hormone and myocardial function. Ann Thorac Surg **56**:S16–23.
8. Gotzsche LB 1994 L-triiodothyronine acutely increases Ca^{2+} uptake in the isolated, perfused rat heart. Changes in L-type Ca^{2+} channels and beta-receptors during short- and long-term hyper- and hypothyroidism. Eur J Endocrinol **130**: 171–179.
9. Segal J, Masalha S, Schwalb H, Merin G, Borman JB, Uretzky G 1996 Acute effect of thyroid hormone in the rat heart: Role of calcium. J Endocrinol **149**:73–80.
10. Wang YG, Dedkova EN, Fiening JP, Ojamaa K, Blatter LA, Lipsius SL 2003 Acute exposure to thyroid hormone increases Na^+ current and intracellular Ca^{2+} in cat atrial myocytes. J Physiol **546**:491–499.
11. Ojamaa K, Balkman C, Klein IL 1993 Acute effects of triiodothyronine on arterial smooth muscle cells. Ann Thorac Surg **56**:S61–66.
12. Yoneda K, Takasu N, Higa S, Oshiro C, Oshiro Y, Shimabukuro M, et al. 1998 Direct effects of thyroid hormones on rat coronary artery: Nongenomic effects of triiodothyronine and thyroxine. Thyroid 8:609–613.
13. Park KW, Dai HB, Ojamaa K, Lowenstein E, Klein I, Sellke FW 1997 The direct vasomotor effect of thyroid hormones on rat skeletal muscle resistance arteries. Anesth Analg **85**:734–738.
14. Jamall IN, Pagel PS, Hettrick DA, Lowe D, Kersten JR, Tessmer JP, et al. 1997 Positive inotropic and lusitropic effects of triiodothyronine in conscious dogs with pacing-induced cardiomyopathy. Anesthesiology **87**:102–109.
15. Vavouranakis I, Sanoudos G, Manios A, Kalogeropoulou K, Sitaras K, Kokkinos C 1994 Triiodothyronine administration in coronary artery bypass surgery: Effect on hemodynamics. J Cardiovasc Surg **35**:383–389.
16. Schmidt BM, Martin N, Georgens AC, Tillmann HC, Feuring M, Christ M, Wehling M 2002 Nongenomic cardiovascular effects of triiodothyronine in euthyroid male volunteers. J Clin Endocrinol Metab **87**:1681–1686.
17. Mri H, Haruyama S, Shinozaki Y, Okino H, Iida A, Takanashi R, et al. 1992 New nonradioactive microspheres and more sensitive X-ray fluorescence to measure regional blood flow. Am J Physiol **263**:H1946–9.
18. Dudley SC Jr, Baumgarten CM 1993 Bursting of cardiac sodium channels after acute exposure to 3,5,3′-triiodo-L-thyronine. Circ Res **73**:301–313.
19. Tielens ET, Forder JR, Chatham JC, Marrelli SP, Ladenson PW 1996 Acute L-triiodothyronine administration potentiates inotropic responses to beta-adrenergic stimulation in the isolated perfused rat heart. Cardiovasc Res **32**:306–310.
20. Dyke CM, Yeh T Jr, Lehman JD, Abd-Elfattah A, Ding M, Wechsler AS, Salter DR 1991 Triiodothyronine-enhanced left ventricular function after ischemic injury. Ann Thorac Surg **52**:14–19.
21. Gotzsche LB 1994 Acute increase in cardiac performance after triiodothyronine: Blunted response in amiodarone-treated pigs. J Cardiovasc Pharmacol **23**:141–148.
22. Novitzky D, Cooper DK, Barton CI, Greer A, Chaffin J, Grim J, Zuhdi N 1989 Triiodothyronine as an inotropic agent after open heart surgery. J Thorac Cardiovasc Surg **98**:972–977.
23. Li L, Haynes MP, Bender JR 2003 Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. Proc Natl Acad Sci USA **100**:4807–4812.
24. Zhu Y, Bond J, Thomas P 2003 Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci USA **100**:2237–2242.
25. Scanlan TS, Suchland KL, Hart ME, Chiellini G, Huang Y, Kruzich PJ, Frascarelli S, Crossley DA, Bunzow JR, Ronca-Testoni S, Lin ET, Hatton D, Zucchi R, Grandy DK 2004 3-Iodothyronamine is an endogenous and rapid-acting derivative of thyroid hormone. Nat Med **10**:638–642

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Effect of sustained limb ischemia on norepinephrine release from skeletal muscle sympathetic nerve endings

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Abstract

Acute ischemia has been reported to impair sympathetic outflow distal to the ischemic area in various organs, whereas relatively little is known about this phenomenon in skeletal muscle. We examined how acute ischemia affects norepinephrine (NE) release at skeletal muscle sympathetic nerve endings. We implanted a dialysis probe into the adductor muscle in anesthetized rabbits and measured dialysate NE levels as an index of skeletal muscle interstitial NE levels. Regional ischemia was introduced by microsphere injection and ligation of the common iliac artery. The time courses of dialysate NE levels were examined during prolonged ischemia. Ischemia induced a decrease in the dialysate NE level (from 19 ± 4 to 2.0 ± 0 pg/ml, mean ± S.E.), and then a progressive increase in the dialysate NE level. The increment in the dialysate NE level was examined with local administration of desipramine (DMI, a membrane NE transport inhibitor), ω-conotoxin GVIA (CTX, an N-type Ca^{2+} channel blocker), or TMB-8 (an intracellular Ca^{2+} antagonist). At 4 h ischemia, the increment in the dialysate NE level (vehicle group, 143 ± 30 pg/ml) was suppressed by TMB-8 (25 ± 5 pg/ml) but not by DMI (128 ± 10 pg/ml) or CTX (122 ± 18 pg/ml). At 6 h ischemia, the increment in the dialysate NE level was not suppressed by the pretreatment. Ischemia induced biphasic responses in the skeletal muscle. Initial reduction of NE release may be mediated by an impairment of axonal conduction and/or NE release function, while in the later phase, the skeletal muscle ischemia-induced NE release was partly attributable to exocytosis via intracellular Ca^{2+} overload rather than opening of calcium channels or carrier mediated outward transport of NE.

Keywords: Catecholamine; Interstitial space; Microdialysis; Rabbit; Striate muscle

1. Introduction

Acute ischemia has been reported to be associated with impairment of the sympathetic tract (Schömig et al., 1984; Toyohara et al., 1986; Fujii et al., 2003). A well-known example is myocardial ischemia associated with impairment of the regional cardiac sympathetic nerve endings (Schömig et al., 1984; Ciuffo et al., 1985). Outward norepinephrine (NE) transport through uptake$_1$ carrier has been proposed as one of the main mechanisms responsible for ischemia-induced NE efflux from sympathetic nerve endings (Schömig et al., 1984; Akiyama and Yamazaki, 2001). However, little is known about the sympathetic impairment evoked by skeletal muscle ischemia. Histochemical and electrophysiological studies (Barker and Saito, 1981; Hill et al., 1996) have identified sympathetic innervation in skeletal muscle, which exerted actions on the regulation of regional blood flow and glucose metabolism (Thompson and Mohrman, 1983; Fagius and Berne, 1994). During and after exercise, muscle sympathetic nerve activity has been reported to be modulated by ischemia-induced metaboreceptor stimulation (Cornett et al., 2000; Cui et al., 2001). Furthermore, skeletal muscle may be exposed to prolonged severe ischemia (Welsh and Lindinger, 1993). Severe skeletal muscle ischemia occurs with trauma, vascular diseases, and compartment syndrome. It is so far unknown whether severe muscle ischemia induces excessive NE release from muscle sympathetic nerve endings.

In view of energy metabolism, cardiac ischemia is characterized by rapid deterioration of cardiac function, which has been linked to a fall in intracellular pH, increased levels of inorganic phosphate and reduction in free energy changes of ATP-hydrolysis (Mair, 1999). In contrast to cardiac muscle,

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energy requirements in skeletal muscle are dependent on exercise and are reduced in the resting state since only resting tone is maintained (Idström et al., 1990; Lindsay et al., 1990). Typically, prolonged skeletal muscle ischemia imposes a metabolic stress that results in a depletion of glycogen, high-energy phosphagen, and adenine nucleotides (Welsh and Lindinger, 1993). Thus, a differential time course of energy metabolism occurs in the skeletal muscle and cardiac myocardium. No studies have systematically characterized the impairment of sympathetic nerves in the skeletal muscle ischemia.

Recently, we reported that microdialysis technique with high-performance liquid chromatography is a sensitive and versatile method for monitoring interstitial NE concentrations in myocardial ischemic regions (Akiyama et al., 1991, 1993). Moreover, we applied microdialysis technique to skeletal muscle and have reported that skeletal muscle dialysate NE serves as an index of muscle sympathetic nerve activity (Tokunaga et al., 2003a). Using this method, we investigated how acute skeletal muscle ischemia affects NE release from skeletal muscle sympathetic nerve endings and the mechanism of skeletal muscle ischemia-induced NE release with regional pharmacological intervention.

2. Methods

2.1. Animal model

The investigation conformed 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). Forty-two male Japanese white rabbits weighing 2.2–3.8 kg were used for the model of skeletal muscle ischemia. The animals were anesthetized with pentobarbital sodium (30–35 mg/kg) and ventilated with room air mixed with oxygen. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). Body temperature was maintained with a heated pad and lamp. An electrocardiogram, heart rate (HR), and mean arterial blood pressure (MAP) were simultaneously monitored with a data recorder. 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.

2.2. Dialysis technique and NE measurements

With the dialysis technique, dialysate NE levels were measured as an index of skeletal muscle interstitial NE levels. 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 cut-off, 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). The dialysis probe was perfused with Ringer solution using a microinjection pump (CMA 102, Carnegie Medicin, Stockholm, Sweden). Similar to previous studies (Tokunaga et al., 2003a, 2003b), we chose a perfusion speed of 10 μl/min for skeletal muscle. Sampling periods were set at 15 min for skeletal muscle. Dialysate NE levels were measured by high-performance liquid chromatography with electrochemical detection (ECD-300, Eicom, Kyoto, Japan) after removing interfering compounds in the dialysate by an alumina procedure (Anton and Sayer, 1962; Akiyama et al., 1991). Dialysate dihydroxyphenylglycol (DHPG) levels were measured by separate high-performance liquid chromatography with electrochemical detection (Akiyama and Yamazaki, 2001).

2.3. Experimental protocols

Acute skeletal muscle ischemia was induced by injection of non-radioactive iodine-labeled microspheres (15 μm in diameter, 3×10^7/kg, Sekisui Plastic, Osaka, Japan) through the left common iliac artery, as previously described (Tanaka et al., 2000). After the injection of microspheres, the common iliac artery was ligated.

2.3.1. Protocol 1: time courses of dialysate NE levels during acute ischemia

To examine the time courses of dialysate NE levels during acute skeletal muscle ischemia, we measured dialysate NE levels over 60-min periods of skeletal muscle ischemia ($n = 6$). We collected four consecutive 15-min dialysate samples. Furthermore, we measured dialysate NE samples over a period of 6 h of skeletal muscle ischemia with 2 h interval in separate rabbits. To examine intraneuronal NE kinetics in the skeletal muscle, the measurement of dialysate DHPG level was added during 6 h of skeletal muscle ischemia ($n = 6$).

2.3.2. Protocol 2: involvement of NE uptake$_1$ transport, Ca^{2+} channels and cytosol Ca^{2+} in dialysate NE levels during acute ischemia

To examine the mechanism underlying the increment of NE release during the prolonged ischemia, dialysate NE levels were measured with regional pharmacological intervention. Neurotransmitter release from sympathetic nerve endings can be caused by a variety of different mechanisms (Schömig et al., 1987; Kawada et al., 2000; Akiyama and Yamazaki, 2001). In the present studies, we examined the roles of membrane NE transport, N-type Ca^{2+} channels and cytosol Ca^{2+} in the time courses of dialysate NE levels during prolonged ischemia. To examine the involvement of membrane NE transport in the ischemia-induced NE release, we locally administered an uptake$_1$ carrier blocker, desipramine (100 μM) through a dialysis probe and observed the responses of dialysate NE (Akiyama and Yamazaki, 2001) ($n = 6$). The same protocol was performed with addition of a voltage-dependent N-type Ca^{2+} channel blocker, ω-conotoxin GVIA (10 μM) ($n = 6$) or intracellular Ca^{2+} antagonist, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8, 1 mM) ($n = 6$) through a dialysis probe. From data on protocol 1, we observed increases in dialysate NE levels after 2 h of skeletal muscle ischemia. Therefore, the time course of dialysate NE for skeletal muscle ischemia was examined over a period of 6 h with a 2 h-interval ($n = 6$). The effectiveness of ω-conotoxin GVIA (10 μM) ($n = 6$) or TMB-8 (1 mM) ($n = 6$) was tested before the experiment in separate rabbits. We administered high potassium (KCl, 100 mM) locally through the dialysis probe, and the dialysate NE response was obtained in the presence and absence of ω-conotoxin GVIA or TMB-8. High-K increased dialysate NE from 11.7 ± 2.8 to 84.7 ± 20.8 pg/ml ($n = 6$). This KCl-induced increment in dialysate NE was attenuated by the addition of ω-conotoxin GVIA or TMB-8 (Fig. 1).

2.3.3. Protocol 3: time courses of dialysate lactate levels during the hind limb ischemia

To confirm whether this perturbation induces tissue ischemia, we examined the time course of dialysate lactate levels as an index of tissue ischemia. The dialysate lactate levels were measured by kinetic enzymatic analysis with CMA 600 (Carnegie Medicin). In the skeletal muscle ischemia, four consecutive 15-

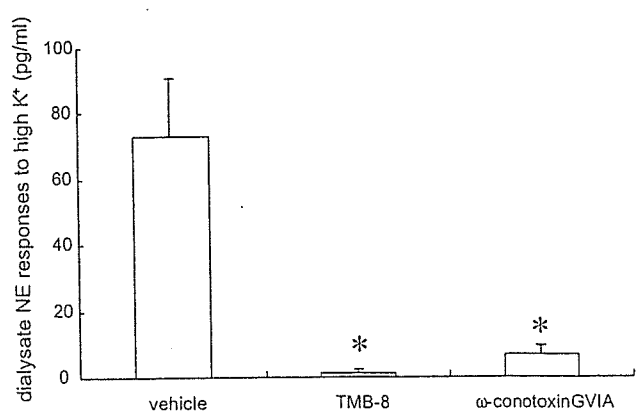

Fig. 1. Effects of pharmacological intervention on dialysate norepinephrine (NE) responses to high K^+ (KCl, 100 mM). Both TMB-8 (1 mM) and ω-conotoxin (10 μM) suppressed dialysate responses to high K^+. Values are means ± S.E. ($n = 6$).

min dialysate samples were collected during the initial 60-min and subsequently three consecutive samples were collected over a period of 6 h with a 2 h-interval ($n = 6$).

2.4. Statistical analysis

All data are presented as mean ± S.E. values. Hemodynamic and dialysate data responses to acute ischemia were statistically analyzed by analysis of variance with repeated measures. When a statistically significant effect of ischemia was detected as a whole, the Dunnett's test was applied to determine which mean values differed significantly from the control level. When a statistically significant effect of the treatment was detected, Newman–Keuls test was applied to determine which treatment differed significantly from the vehicle.

3. Results

Table 1 summarizes changes in HR and MAP. MAP and HR increased during 6 h-hind limb ischemia. Changes in MAP at 2 h and HR at 6 h-hind limb ischemia were significant.

3.1. Time courses of dialysate NE levels during short and prolonged ischemia

Skeletal muscle dialysate NE levels decreased from 19 ± 4 pg/ml at control to 9 ± 4 pg/ml at 30 min of ischemia and reached 2 ± 0 pg/ml at 60 min of ischemia (Fig. 2). The decrease in dialysate NE level was maintained after 2 h of ischemia. Then skeletal muscle dialysate NE levels markedly increased to 143 ± 30 pg/ml at 4 h of ischemia. The dialysate NE levels continued to increase progressively and reached 289 ± 45 pg/ml at 6 h of ischemia. Skeletal muscle dialysate DHPG levels decreased from 38 ± 2 pg/ml at control to 5 ± 1 pg/ml at 2 h of ischemia and reached 7 ± 1 pg/ml at 6 h of ischemia.

3.2. Involvement of NE uptake$_1$ transport, Ca^{2+} channels and cytosol Ca^{2+} in dialysate NE levels during prolonged ischemia

Dialysate NE increases at 4 and 6 h-skeletal muscle ischemia were not suppressed by treatment with desipramine (Fig. 3). Dialysate NE increases at 4 and 6 h-skeletal muscle ischemia were not suppressed by treatment with ω-conotoxin GVIA. Treatment with TMB-8 significantly suppressed the dialysate NE increase at 4 h-skeletal muscle ischemia. But at 6 h-skeletal muscle ischemia, there was no significant difference in dialysate NE levels among treatments.

Table 1
Changes in heart rate (HR) and mean arterial pressure (MAP) in 6 h-hindlimb ischemia

	Control	2 h	4 h	6 h
HR (beats/min)	283 ± 10	292 ± 4	293 ± 8	302 ± 8*
MAP (mmHg)	104 ± 6	114 ± 3*	111 ± 4	108 ± 4

Values are means ± S.E. from six rabbits. Data were obtained during control, after 2, 4, and 6 h of hind limb ischemia.
* $P < 0.05$ vs. control.

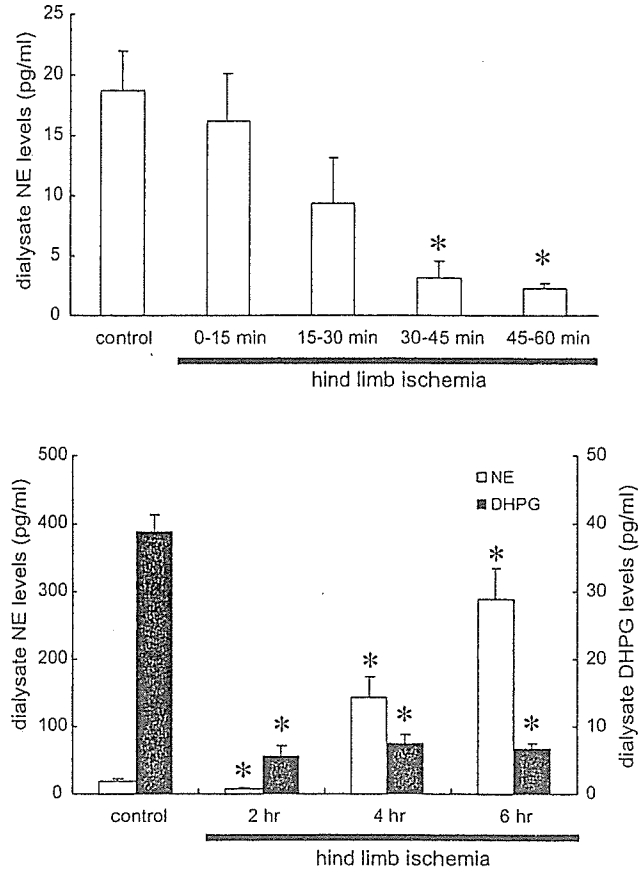

Fig. 2. (Upper panel) Time course of dialysate norepinephrine (NE) levels during 60 min-hind limb ischemia. Values are means ± S.E. ($n = 6$). *$P < 0.05$ vs. control value. (Lower panel) Time courses of dialysate NE and dihydroxyphenylglycol (DHPG) levels during 6 h-hind limb ischemia. Values are means ± S.E. ($n = 6$). *$P < 0.05$ vs. control value.

3.3. Time course of dialysate lactate levels during hind limb ischemia

Skeletal muscle dialysate lactate levels increased from 0.6 ± 0.07 nmol/l at control to 1.73 ± 0.17 nmol/l at 45–60 min

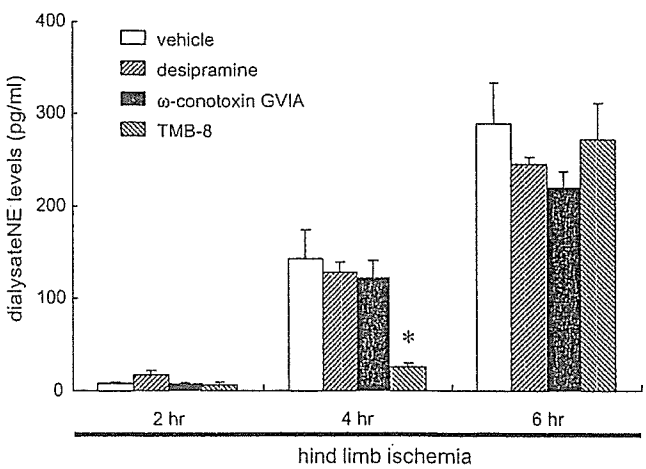

Fig. 3. Effects of pharmacological intervention on dialysate norepinephrine (NE) levels evoked by 6 h-hind limb ischemia. Desipramine (100 μM), ω-conotoxin (10 μM), or TMB-8 (1mM) was locally administered through the probe. Values are means ± S.E. ($n = 6$). *$P < 0.05$ vs. concurrent value of vehicle group.

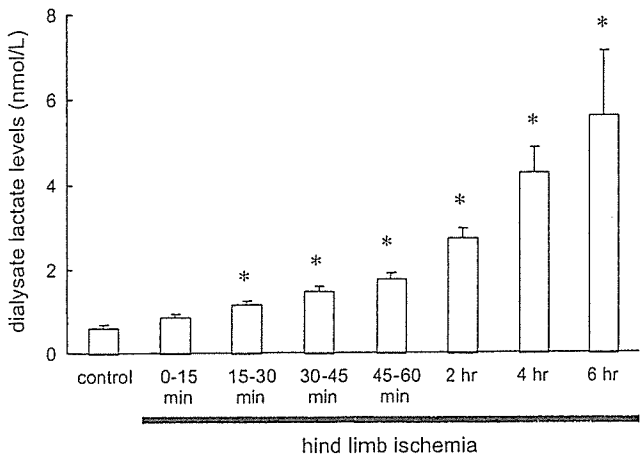

Fig. 4. Time course of dialysate lactate levels during 6 h-hind limb ischemia. Values are means ± S.E. ($n = 6$). $^{*}P < 0.05$ vs. control value.

of ischemia (Fig. 4). These step-wise increases were continued for 6 h of the hind limb ischemia.

4. Discussion

Using dialysis techniques in the in vivo rabbit skeletal muscle, we examined interstitial levels of NE in the control and ischemic period, and observed the biphasic response of dialysate NE in ischemic skeletal muscle. Ischemia induced an initial reduction followed by a progressive increment in dialysate NE levels. Here we discuss changes in interstitial NE and possible mechanisms underlying sympathetic nerve impairment.

Within 2 h of acute skeletal muscle ischemia, unlike acute myocardial ischemia, skeletal muscle interstitial NE levels continued to decline progressively, decreasing to one-tenth of control at 60 min of ischemia. A previous study demonstrated that skeletal muscle ischemia modulated the baroreflex control of regional muscle sympathetic activity (Cornett et al., 2000). At 75 min of acute skeletal muscle ischemia, hemodynamic responses to carotid occlusion were preserved while the interstitial NE response to carotid occlusion was blunted in the ischemic region (Tokunaga et al., 2003b). These results indicate that the systemic response to baroreflex remained intact while the skeletal muscle sympathetic response was impaired in ischemic regions. Earlier studies reported that acute limb ischemia reduced the conduction of motor nerves such as sciatic nerve (Fern and Harrison, 1994), and induced axonal degeneration histologically (Makitie and Teravainen, 1977; Nukada and Dyek, 1987). Axonal conduction in the ischemic muscle sympathetic nerve may be impaired as well as in sensory and motor nerves. In addition to diminished axonal conductance, the interstitial NE response to high K^+ but not tyramine was suppressed during the 75 min of acute skeletal muscle ischemia, although NE content at muscle sympathetic nerve endings was preserved during the ischemia (Tokunaga et al., 2003b). This result indicates that exocytotic NE releasing function in muscle sympathetic nerve endings might be suppressed during 75 min of acute skeletal muscle ischemia. Therefore, initial reduction of NE release may be mediated by an impairment of axonal conduction and/or NE releasing function.

After 2 h of acute skeletal muscle ischemia, skeletal muscle interstitial NE levels significantly increased and finally reached 20-fold that of control. This amount of NE release is higher than that evoked by baroreflex or high K^+. This level is similar to that evoked by the Na^+–K^+ ATPase inhibitor, ouabain (Tokunaga et al., 2003a). The amount of NE release evoked by ischemia may be dependent on the density of sympathetic innervation. Dispersed organ systems such as skeletal muscle have a thin and diffuse sympathetic innervation. This is the first report to describe that marked NE release is induced from muscle sympathetic nerve endings in the ischemic region after 2 h of skeletal muscle ischemia. Numerous histological changes of skeletal muscle have been reported after ischemia and reperfusion injury (Patterson and Klenerman, 1979; Turchányi et al., 2005). However, there is no histochemical evidence of the impaired sympathetic nerves in the skeletal muscle ischemia.

In the case of skeletal muscle ischemia, ω-conotoxin GVIA did not suppress NE efflux. N-type Ca^{2+} channels are not involved in this NE efflux. Desipramine did not alter NE efflux during skeletal muscle ischemia. Desipramine inhibits carrier-mediated NE transport in both directions. Considering that desipramine did not alter interstitial NE levels, the amounts of NE release and uptake via normal transport can be surmised to be negligible. Second, the increase in skeletal muscle interstitial NE levels was not associated with an increase in skeletal muscle interstitial DHPG levels, indicating that skeletal ischemia fails to induce axoplasmic NE elevation via alterations in monoamine activity, NE mobilization from stored vesicle, and NE uptake. Further, desipramine did not suppress NE efflux. These results exclude the possibility that marked increases in skeletal muscle interstitial NE could be due to carrier-mediated outward transport of NE for removal of elevated axoplasmic NE concentration. The membrane NE transporter exists in the skeletal muscle sympathetic nerve endings (Cabassi et al., 2001; Tokunaga et al., 2003a), but was not involved in outward transport of NE. Thus, we consider that a ω-conotoxin GVIA insensitive and desipramine-resistant NE release mechanism exists after 2 h of acute skeletal muscle ischemia.

TMB-8 significantly suppressed the marked NE release at 4 h of skeletal muscle ischemia. TMB-8 is well known to inhibit Ca^{2+} release from intracellular Ca^{2+} stores. TMB-8 inhibits caffeine-induced catecholamine release from perfused adrenal gland in the absence of extracellular Ca^{2+} (Yamada et al., 1988). Studies using chromaffin cells, brain slices and synaptosomes have suggested that metabolic inhibition induces intracellular Ca^{2+} overload (Milusheva et al., 1992), and a rise in the intracellular Ca^{2+} causes exocytotic catecholamine release without membrane depolarization (Dry et al., 1991; Du et al., 1997). Moreover, an in vitro study with adrenergic nerves of guinea-pig vas deferens suggested that Ca^{2+} release from intracellular Ca^{2+} stores is to some extent involved in the NE release evoked by elevation of intracellular Na^+ (Katsuragi et al., 1994). Under energy-depleted conditions, Ca^{2+} overload

in synaptosomes of noradrenergic neurons from the brain is an important mechanism for the enhanced release of neurotransmitter, with a reversal of Na^+–Ca^{2+} exchange possibly the key pathway leading to intraneuronal Ca^{2+} overload (Du et al., 1997). We consider that Ca^{2+} release from intracellular Ca^{2+} stores is partly involved in the NE release at 4 h of skeletal muscle ischemia.

At 6 h of skeletal ischemia, increment in dialysate NE level was not suppressed by the pretreatments. This result suggests that another mechanism may be involved in NE release, which is insensitive to desipramine, ω-conotoxin GVIA, and TMB-8. Alternatively, the NE release may occur with development of irreversible membrane damage and can no longer be inhibited by pharmacological interventions. Future work should concentrate on these aspects of NE release during the later period.

4.1. Methodological considerations

The limitation of this experiment is related to the methodology and the duration of the hind limb ischemia. In a variety of these experimental models for organ ischemia, we chose microsphere injection and iliac artery occlusion for the short and prolonged hind limb ischemia model. A preliminary experiment indicated that common iliac artery occlusion did not yield severe ischemia or muscle necrosis in a chronic ischemic model because collateral flow prevents skeletal muscle ischemia. The combination of artery occlusion and injection of microsphere was used for the hind limb ischemic model. In the hind limb ischemia, however, we did not measure skeletal muscle blood flow. To confirm whether this perturbation induced reduction of blood flow and tissue ischemia, we measured dialysate lactate levels in skeletal muscle as an index of tissue ischemia. This perturbation induced increases in dialysate lactate levels. In the present study, dialysate NE responses were examined in prolonged 6 h ischemia. Temporal changes in MAP and HR appeared but sustained significant hemodynamic changes were not observed. This duration was referred to the experiments on the tourniquet application and release time (Sapega et al., 1985; Mitrev et al., 1996). Four to 6 h of ischemic periods has been thought to produce extensive and reversible damage of skeletal muscle. Therefore, data on pharmacological intervention were obtained within 6 h of skeletal muscle ischemia.

Ischemia induced biphasic NE responses in the skeletal muscle. Initial reduction of NE release may be mediated by an impairment of axonal conduction and/or NE releasing function, while in the later phase, the skeletal muscle ischemia-induced NE release was partly attributable to exocytosis via intracellular Ca^{2+} overload rather than opening of calcium channels or carrier mediated outward transport of NE.

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References

Akiyama, T., Yamazaki, T., Ninomiya, I., 1991. In vivo monitoring of myocardial interstitial norepinephrine by dialysis technique. Am. J. Physiol. 261, H1643–H1647.

Akiyama, T., Yamazaki, T., Ninomiya, I., 1993. Differential regional responses of myocardial interstitial noradrenaline levels to coronary occlusion. Cardiovasc. Res. 27, 817–822.

Akiyama, T., Yamazaki, T., 2001. Myocardial interstitial norepinephrine and dihydroxyphenylglycol levels during ischemia and reperfusion. Cardiovasc. Res. 49, 78–85.

Anton, A.H., Sayer, D.F., 1962. A study of the factors affecting the aluminum oxide-trihydroxyindole procedure for the analysis of catecholamine. J. Pharmacol. Exp. Ther. 138, 360–375.

Barker, D., Saito, M., 1981. Autonomic innervation of receptors and muscle fibers in cat skeletal muscle. Proc. Roy. Soc. Lond., B: Biol. Sci. 212, 317–332.

Cabassi, A., Vinci, S., Quartieri, F., Moschini, L., Borghetti, A., 2001. Norepinephrine uptake is impaired in skeletal muscle of hypertensive rats in vivo. Hypertension 37, 698–702.

Ciuffo, A.A., Ouyang, P., Becker, L.C., Levin, L., Weisfeldt, M.L., 1985. Reduction of sympathetic inotropic response after ischemia in dogs. Contributor to stunned myocardium. J. Clin. Invest. 75, 1504–1509.

Cornett, J.A., Herr, M.D., Gray, K.S., Smith, M.B., Yang, Q.X., Sinoway, L.I., 2000. Ischemic exercise and the muscle metaboreflex. J. Appl. Physiol. 89, 1432–1436.

Cui, J., Wilson, T.E., Shibasaki, M., Hodges, N.A., Grandall, C.G., 2001. Baroreflex modulation of muscle sympathetic nerve activity during postgrip muscle ischemia in human. J. Appl. Physiol. 91, 1679–1686.

Dry, K.L., Phillips, J.H., Dart, A.M., 1991. Catecholamine release from bovine adrenal chromaffin cells during anoxia or metabolic inhibition. Circ. Res. 69, 466–474.

Du, X.-J., Bobik, A., Little, P.J., Esler, M.D., Dart, A.M., 1997. Role of Ca^{2+} in metabolic inhibition-induced norepinephrine release in rat brain synaptosomes. Circ. Res. 80, 179–188.

Fagius, J., Berne, C., 1994. Increase in muscle sympathetic activity in humans after food intake. Clin. Sci. (London) 86, 159–167.

Fern, R., Harrison, P.J., 1994. The relationship between ischaemic conduction failure and conduction velocity in cat myelinated axons. Exp. Physiol. 79, 571–581.

Fujii, T., Kurata, H., Takaoka, M., Muraoka, T., Fujisawa, Y., Shokoji, T., Nishiyama, A., Abe, Y., Matsumura, Y., 2003. The role of renal sympathetic nervous system in the pathogenesis of ischemic acute renal failure. Eur. J. Pharmacol. 481, 241–248.

Hill, J.M., Adreani, C.M., Kaufman, M.P., 1996. Muscle reflex stimulates sympathetic postganglionic efferents innervating triceps surae muscle of cats. Am. J. Physiol. 271, H38–H43.

Idström, J.-P., Soussi, B., Elander, A., Bylund-Fellenius, A.-C., 1990. Purine metabolism after in vivo ischemia and reperfusion in rat skeletal muscle. Am. J. Physiol. 258, H1668–H1673.

Katsuragi, T., Ogawa, S., Furukawa, T., 1994. Contribution of intra- and extracellular Ca^{2+} to noradrenaline exocytosis induced by ouabain and monensin from guinea-pig vas deferens. Br. J. Pharmacol. 113, 795–800.

Kawada, T., Yamazaki, T., Akiyama, T., Sato, T., Shishido, T., Inagaki, M., Tetewaki, T., Yanagiya, Y., Sugimachi, M., Sunagawa, K., 2000. Cyanide intoxication induced exocytotic epinephrine release in rabbit myocardium. J. Auton. Nerv. Syst. 80, 137–141.

Lindsay, T.F., Liauw, S., Romaschin, A.D., Walker, P.M., 1990. The effect of ischemia/reperfusion on adenine nucleotide metabolism and xanthine oxidase production in skeletal muscle. J. Vasc. Surg. 12, 8–15.

Mair, J., 1999. Tissue release of cardiac markers: from physiology to clinical applications. Clin. Chem. Lab. Med. 37, 1077–1084.

Makitie, J., Teravainen, H., 1977. Peripheral nerve injury and recovery after temporary ischemia. Acta Neuropathol. (Berl.) 37, 55–63.