

DESCRIPTION

IN VIVO ACETYLCHOLINE PRODUCTION-PROMOTING DEVICE

TECHNICAL FIELD

5 [0001]

The present invention relates to a device for promoting production of acetylcholine *in vivo*.

BACKGROUND ART

[0002]

10 Acetylcholine is the acetic acid ester of choline. Acetylcholine is a substance which is released from the ends of parasympathetic nerves and motor nerves in response to stimuli, and acts as an excitatory transmitter (neurotransmitter) at synaptic sites and end plates. Acetylcholine has physiological actions such as blood-pressure reduction, cardiac depression (heart-rate reduction), intestinal
15 contraction, and skeletal muscle contraction. Acetylcholine bound to an acetylcholine receptor is hydrolyzed by acetylcholine esterase or choline esterase into choline and acetic acid, causing relaxation of a muscle.

[0003]

Use of a choline esterase inhibitor, which increases the acetylcholine
20 concentration in the body, for prevention, therapy, or amelioration of senile dementia, cerebrovascular dementia, attention deficit hyperactivity disorder, glaucoma, myasthenia gravis, or migraine is known (Patent Document 1). Moreover, since acetylcholine has an action to create a relaxed state by stimulation of parasympathetic nerves and to reduce blood pressure and heart rate, acetylcholine is considered to be
25 useful also for blood pressure reduction, resolution of insomnia, and even for suppression of an increase in the blood glucose level due to induction of abnormal glucose tolerance during sympathetic hyperactivity.

[0004]

As described above, choline esterase inhibitors that increase the acetylcholine concentration *in vivo* have been well studied, and various types of compounds are known as the inhibitors. However, there is no known device that promotes
5 production of acetylcholine *in vivo*.

PRIOR ART DOCUMENT

[Patent Document]

[0005]

[Patent Document 1] WO 03/082820

10 SUMMARY OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0006]

An object of the present invention is to provide a device that promotes production of acetylcholine *in vivo*.

15 MEANS FOR SOLVING THE PROBLEMS

[0007]

As a result of intensive study, the present inventors discovered that the production of acetylcholine *in vivo* is promoted by repeating the process of blocking an artery of a limb to cause ischemia and then canceling the blocking of the artery
20 after a predetermined period of time to allow reperfusion. Therefore, a device capable of repeating the cycle of ischemia-reperfusion of a limb artery can promote production of acetylcholine *in vivo*.

[0008]

That is, the present invention provides an *in vivo* acetylcholine production-
25 promoting device comprising: an artery-compressing member which contacts any site on a limb and compresses an artery/arteries located at the site when the artery-compressing member is pressurized; and pressurizing means connected to the artery-

compressing member, which, during use, pressurizes the artery-compressing member for compressing the artery/arteries to cause ischemia and then releases the pressure to allow reperfusion of blood through the artery/arteries. The present invention also provides a method for promoting *in vivo* acetylcholine production, comprising

5 repeating, one or more times, a cycle in which an artery/arteries of an animal is compressed to cause ischemia and reperfusion of blood through the artery/arteries is then allowed.

EFFECT OF THE INVENTION

[0009]

10 By applying the device of the present invention to an animal and repeating the limb artery ischemia-reperfusion, production of acetylcholine can be promoted, and the concentration of acetylcholine in the body can be increased. Therefore, application of the device of the present invention enables achievement of prevention, therapy, or amelioration of senile dementia, cerebrovascular dementia, attention

15 deficit hyperactivity disorder, glaucoma, myasthenia gravis, or migraine; blood pressure reduction; resolution of insomnia; creation of a relaxed state; suppression of an increase in the blood glucose level due to abnormal glucose tolerance; and the like. In particular, in terms of the blood glucose level, the device of the present invention can decrease the blood glucose level or suppress an increase in the blood glucose

20 level, as is evident by the later-described animal experiment using mice.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010]

Fig. 1A to Fig. 1C are diagrams illustrating the results of experiments on deficiency of the choline acetyltransferase (ChAT) gene, which were carried out in

25 the Example 1 below. Deficiency of the ChAT gene affects intercellular communication via β -catenin and Cx43. (A) ChAT knockdown could be confirmed in human tubular epithelial cells, HEK 293 cells, after transient infection with an

miR RNAi expression vector specific to ChAT. A decrease in the immunoreactivity of ChAT (red) in the transfected cells, which are surrounded by broken lines indicating GFP signals (green), was shown.

(B) In ChAT KO (knockdown) cells prepared by stably transfecting the HEK 293 cells, the immunoreactivity of β -catenin observed among cells was lower than in LacZ KO cells used as a control, and the ChAT KO cells were easily dispersed by stirring. In the ChAT KO cells, the ACh immunoreactivity signal found in the cell membrane was lower.

(C) Immunocytochemistry and Western blotting analysis showed that, in ChAT KO HL-1 cells, which are mouse cardiac muscle-derived HL-1 cells prepared by stable transfection with an miR RNAi expression vector specific to ChAT, expression of Cx43 is lower than in LacZ KO HL-1 cells used as a control. In a method for evaluating the intercellular transport using LY dye, transfer of LY dye was more suppressed in linearly arranged ChAT KO HL-1 cells as compared to the control cells.

Fig. 2A to Fig. 2E are diagrams illustrating the results of experiments carried out in the Example 1 below in relation to suppression of ChAT. Suppression of ChAT in the cells promotes energy metabolism accompanied by consumption of more oxygen and ATP. (A) ChAT KO HEK cells, in which the ACh level is low ($9.3 \pm 3.5\%$; comparison with the control), showed an increased MTT activity ($384.8 \pm 20.8\%$, $P < 0.01$; comparison with the control), and also showed higher consumption of oxygen ($-239.4 \pm 26.4\%$, $P < 0.01$; comparison with the control).

(B) The amount of ATP contained in the ChAT KO cells were smaller than in the control (control vs ChAT KO, 765.1 ± 56.9 vs 425.7 ± 18.7 mM/1 g protein, $P < 0.01$)

(C) The number of dead cells observed under low-oxygen conditions chemically induced with 2 mM CoCl_2 was larger in the ChAT KO HEK cells than in

the control (control vs ChAT KO, 100.0 ± 0.35 vs 26.6 ± 2.33 , $P < 0.01$).

(D) Similarly, more ATP was consumed in the ChAT KO HL-1 cells than in the control (control vs ChAT KO, 271.9 ± 28.2 vs 83.1 ± 8.8 mM/1 g protein, $P < 0.01$).

5 (E) BrACh (10 μ M) and hemicholinium-3 (10 μ M), which are ChAT inhibitors, increased the MTT activity in rat cardiac muscle-derived H9c2 cells similarly to the case where ChAT KO HEK cells were used. Hemicholinium-3 also decreased the ATP content in the H9c2 cells.

Fig. 3 is a diagram illustrating the results of experiments on ChAT
 10 knockdown (KO) HL-1 cells, which were carried out in the Example 1 below. ChAT knockdown (KO) HL-1 cells had higher sensitivity to norepinephrine (NE)-induced cell death. The survival rate of ChAT KO cells, as evaluated by comparison with that of control cells, showed a decrease depending on the dose of NE, in contrast to the control cells, which showed a survival rate of almost 100%
 15 after treatment with 2 mM NE. When 2 mM NE was used, the ChAT KO cells produced more reactive oxygen species, and exhibited a higher level of red fluorescence from APF than the control. In accordance with these results, the ChAT KO cells showed higher expression of active caspase-3 under the reference conditions (Hour 0), and the use of NE further increased the caspase-3 activity in the
 20 ChAT KO cells.

Fig. 4 is a diagram illustrating the results of experiments on ischemia-reperfusion of a mouse hind limb, which were carried out in the Example 1 below. The hind limb ischemia-reperfusion increases the expression of ChAT and CHT1 not only in the manipulated hind limb, but also in the heart. By carrying out ischemia-reperfusion of the left hind limb, the expression of ChAT and CHT1 proteins
 25 increased within 16 hours relative to the right hind limb (R). The increases in the expression of the proteins in the skeletal muscle was accompanied by similar

increases with time in the expression of both ChAT and CHT1 in the heart

Fig. 5 is a diagram illustrating the results of experiments on the relationship between the ischemia-reperfusion (IR) treatment and the ChAT activity, which were carried out in the Example 1 below. The ischemia-reperfusion (IR) treatment provides better protection of the heart, via the ChAT activity, against myocardial infarction under ischemia-reperfusion load on the whole heart. Compared to the control without IR (0.61 ± 0.03), the IR-treated group showed better protection of the heart and a smaller infarct area as observed by TTC staining (0.26 ± 0.03 , $P < 0.01$). By pretreatment using hemicholinium-3, the cardioprotective effect by IR was completely lost (0.56 ± 0.03 , $P < 0.05$; comparison with IR).

Fig. 6A to Fig. 6D are diagrams illustrating the results of experiments on the relationship between hind limb ischemia-reperfusion and the cardiac ATP level, which were carried out in the Example 1 below. The remote effect of hind limb ischemia-reperfusion on cardioprotection contributes to activation of the cardiac nonneuronal cholinergic system, which maintains the cardiac ATP level. (A) The cardiac ATP level slowly increased during 16 hours after IR ($256.4 \pm 13.1\%$, 4h, $P < 0.01$; comparison with IR).

(B) The increase in the cardiac ATP level caused by IR (11.5 ± 0.9 mM/1 g protein (IR) vs 5.9 ± 0.5 mM /1 g protein (control), $P < 0.01$) was suppressed by pretreatment using hemicholinium (HC)-3 (6.4 ± 1.0 mM/1 g protein (HC-3), $P < 0.05$; comparison with IR).

(C) Under these conditions, changes in the cardiac ACh level followed these tendencies, and, similarly to ATP level, the ACh level was increased by IR (2.78 ± 0.17 nmol/1 g protein (IR) vs 2.32 ± 0.12 nmol/1 g protein (control), $P < 0.05$), and decreased by HC-3 (2.14 ± 0.07 (HC-3), $P < 0.05$; comparison with IR).

(D) eNOS KO mice did not show up-regulation of the cardiac ACh synthesis by IR (NS, not significant).

Fig. 7 is a schematic diagram illustrating the constitution of a concrete example of the device of the present invention.

Fig. 8 is a diagram illustrating the pressurization-release cycle programmed for the device shown in Fig. 7.

5 Fig. 9 is a diagram illustrating changes in the heart rate with time observed after repeating 3 times an ischemia-reperfusion cycle by application of the device shown in Fig. 7 to a plurality of subjects. The diagram also illustrates, as a control, changes in the heart rate with time observed for subjects who were not subjected to the ischemia-reperfusion cycle.

10 Fig. 10 is a diagram illustrating changes in the heart rate with time observed after carrying out one cycle of ischemia-reperfusion by application of the device shown in Fig. 7 to a plurality of subjects. The diagram also illustrates, as a control, changes in the heart rate with time observed for subjects who were not subjected to the ischemia-reperfusion cycle.

15 Fig. 11 is a diagram illustrating the results of the Example 3 below, that is, the results obtained by feeding mice with high-fat diet for 11 days while performing hind limb IR (3 minutes of ischemia and 2 minutes of reperfusion, 3 cycles) every day, and comparing the increase in BS (blood glucose level) with that of IR-untreated mice.

 Fig. 12 is a diagram illustrating the results of the Example 4 below, that is, the
20 results obtained by feeding mice with high-fat diet while performing IR (3 minutes of ischemia and 2 minutes of reperfusion, 3 cycles) every other day (Day 0, Day 2, and Day 4), and comparing the BS (blood glucose level) with that of IR-untreated mice.

 Fig. 13 is a diagram illustrating the results of the Example 5 below, that is, the
 results obtained by feeding mice with high-fat diet for 11 days while performing IR
25 (2 minutes of ischemia and 2 minutes of reperfusion, 3 cycles) every day, unlike Example 4, and comparing the BS (blood glucose level).

 Fig. 14 is a diagram illustrating the results of the Example 6 below, that is, the

results obtained by feeding mice with high-fat diet for 7 to 11 days while performing IR (2 minutes of ischemia and 2 minutes of reperfusion, 3 cycles) every day, and comparing the changes in the body weight.

MODE FOR CARRYING OUT THE INVENTION

5 [0011]

As concretely described in Example 1 based on animal experiments, the present inventors discovered that the production of acetylcholine *in vivo* is promoted by repeating the process of blocking an artery of a limb to cause ischemia and then canceling the blocking of the artery after a predetermined period of time to allow
10 reperfusion (it should be noted, however, that, since direct measurement of cardiac ACh is impossible in human subjects, an effect to reduce the heart rate was regarded as parasympathetic activation for the heart, that is, promoted secretion of ACh from nerve endings and increased intramyocardial production of ACh caused thereby). The present invention is based on this new discovery. That is, the device of the
15 present invention carries out ischemia and reperfusion of an artery in a limb.

[0012]

The *in vivo* acetylcholine production-promoting device of the present invention comprises an artery-compressing member which contacts any site on a limb and compresses an artery/arteries located at the site when the artery-compressing
20 member is pressurized. The “any site on a limb” herein may be a site on either an arm or leg. In cases of an arm, the site may be on either a brachial region or cubital region, and, in cases of a leg, the site may be on either a femoral region or knee region.

[0013]

25 As the artery-compressing member, a cuff may be preferably used. A cuff is a belt-shaped bag to be wound around a limb, and swells when air is supplied, resulting in compression of an artery/arteries in the limb by tightening. A cuff is

ordinarily used for blood-pressure measurement, and well known. Also in the present invention, a well-known cuff may be used. A cuff per se is well known and commercially available, and a commercially available product may be used.

[0014]

5 The artery-compressing member is not limited to a cuff, and the constitution of the member is not limited as long as the member is capable of causing ischemia by compression of an artery/arteries. For example, a belt-shaped or ribbon-shaped member may be wound around a limb, and the limb may be directly tightened by a motor without sending air; or a ring-shaped member may be constituted by two rigid
10 members, and these members may be slided using a motor to decrease the diameter of the ring-shaped member, to thereby tighten the limb inserted in the ring-shaped member.

[0015]

 The device of the present invention also comprises pressurizing means
15 connected to the artery-compressing member, which, during use, pressurizes the artery-compressing member for compressing the artery/arteries to cause ischemia and then releases the pressure to allow reperfusion of blood through the artery/arteries. The pressurizing means may be manually driven and stopped. Alternatively, the pressurizing means may be automatically driven and stopped, which is convenient
20 and preferred since the user can do another operation, watch TV, or even sleep. In cases where the pressurizing means is automatically driven and stopped, the pressurizing means is preferably programmed to repeat not less than 1 time, preferably 2 to 10 times, more preferably 2 to 5 times, a cycle in which pressurization is carried out for 1 to 15 minutes and release of the pressure is then carried out.

25 [0016]

 In cases where the artery-compressing member is a cuff, preferred examples of the pressurizing means include those comprising: a pump; an air pipe which

connects the pump to the cuff, and sends air from the pump to the cuff; and a valve provided in the air pipe; and constituted such that the pump is operated to pressurize the cuff, and the valve is opened to release the pressure. Preferably, in such cases, the valve is an electrically-operated valve, and a power source for the electrically-operated valve and a power source for the pump are connected to the electrically-operated valve and the pump, respectively, via a programmable relay, which programmable relay is programmed to repeat, one or more times, a cycle composed of pressurization by driving of the pump and release of the pressure thereafter by opening the electrically-operated valve. More preferably, the device further comprises a pressure switch connected to the air pipe and the programmable relay, which pressure switch detects the pressure in the air pipe and sends a signal indicating whether the detected pressure is less than a predetermined pressure or not to the programmable relay, wherein, in cases where the pressure is less than the predetermined pressure, electric power is supplied from the power source for the pump to the pump to drive the pump and to thereby pressurize the cuff, while in cases where the pressure is not less than the predetermined pressure, no electric power is supplied from the power source for the pump to the pump, and the pump is therefore not driven. In cases of such a device, the programmable relay is preferably programmed to repeat, 2 to 10 times, an ischemia-reperfusion cycle composed of 1 minute to 15 minutes of pressurization and 1 minute to 3 hours of pressure release. The programmable relay is especially preferably programmed to repeat, 2 to 5 times, a cycle composed of 1 minute to 15 minutes of pressurization and 1 minute to 15 minutes of pressure release. In order to avoid damaging of limb tissues as much as possible, the pressure is preferably as low as possible as long as the pressure is enough for achievement of the ischemia (that is, enough for blocking the blood flow through the artery/arteries). Accordingly, the pressure is preferably 5 to 10 mmHg higher than the maximum blood pressure of the user.

[0017]

A preferred specific example of the device of the present invention is described below by way of a drawing.

[0018]

5 Fig. 7 is a schematic view illustrating the constitution of a specific example of the device of the present invention. This specific example is described below. In the specific example, various values such as the voltage of a power source are included. These values are merely preferred examples of values, and other values may also be employed as long as those values do not cause a problem.

10 [0019]

 In Fig. 7, 1 represents a power switch connected to a household alternating-current power source (AC 100 V); 2 represents a direct-current power source which converts 100 V alternating current (AC 100 V) to 24 V direct current (DC 24 V) for supplying DC 24 V; 3 represents a direct-current power source which converts AC
15 100 V to DC 6 V for supplying DC 6 V; 4 represents a pressure switch; 5 represents a programmable relay; 6 represents a pump; 7 represents an electrically-operated valve; 8 represents an air pipe that sends air from the pump 6 to a cuff 9; and 9 represents the cuff. These parts are well known, and commercially available products may be used. The period of each ischemia-reperfusion cycle, as well as the number of
20 cycles, may be changed as appropriate.

[0020]

 When the power switch 1 is closed, AC 100 V is supplied to the power source 2 and the power source 3. This enables the power source 2 to generate DC 24 V, and the power source 3 to generate DC 6V. Since the power source 2 is directly
25 connected (without passing through an open/close switch) to the pressure switch 4 and the programmable relay 5, closure of the power switch 1 allows supply of DC 24 V to the pressure switch 4 and the programmable relay 5, resulting in start-up of the

device. Since the power source 3 is connected to the pump 6 and the valve 7 via the programmable relay 5, DC 6 V is not supplied to the pump 6 and valve 7 even when the power switch 1 is closed, unless the relay switch in the programmable relay 5 is closed. The pressure switch 4, pump 6, valve 7, and cuff 9 are connected to each other via the air pipe 8. The pump 6, when DC 6 V is supplied thereto, draws air from outside the pump, and sends the air into the pipe 8 to pressurize the cuff 9. Since the valve 7 opens at 0 V and closes at DC 6V, the inside of the pipe 8 and the cuff 9 has a pressure equivalent to the atmospheric pressure at 0 V, and is isolated from the atmosphere at DC 6 V. The pressure switch 4 detects the pressure inside the cuff 9 via the pipe 8, and sends different signals to the programmable relay depending on whether the pressure is less than or not less than an arbitrarily set pressure ranging from atmospheric pressure+50 to 250 mmHg. Upon receiving the signal from the pressure switch 4 indicating a pressure lower than the set pressure, the reprogrammable relay 5 supplies DC 6 V to the pump 6 and the valve 7, resulting in pressurization of the inside of the pipe 8 and the cuff 9. Upon receiving the signal indicating a pressure of not less than the set pressure, only the supply of DC 6V to the pump 6 is stopped (while the supply of DC 6V to the valve 7 is continued). Accordingly, the internal pressure of the cuff 9 is maintained at the pressure arbitrarily set with the pressure switch 4.

[0021]

The programmable relay 5 is also capable of stopping the supply of DC 6 V to the pump 6 and the valve 7 at once to make the internal pressure of the pipe 8 and the cuff 9 equivalent to the atmospheric pressure. The programmable relay 5 is capable of repeating these actions, that is, the action to maintain the internal pressure of the cuff 9 at the pressure set with the pressure switch 4 and the action to make the internal pressure of the cuff 9 equivalent to the atmospheric pressure, for an arbitrary period in an arbitrary pattern.

[0022]

In the programmable relay 5 of the device of this specific example, the control program shown in Fig. 8 is stored. During the ischemia, the internal pressure of the cuff 9 becomes the pressure set with the pressure switch 4, and the cuff tightens a brachial region or femoral region of the subject to cause ischemia. When the pressure is released, the internal pressure of the cuff 9 becomes almost equivalent to the atmospheric pressure, to allow perfusion.

[0023]

During use, according to the program, the power switch 1 is closed to cause ischemia for an arbitrary period of 1 to 15 minutes, and perfusion is then allowed for an arbitrary period of 1 to 15 minutes. This cycle is repeated an arbitrary number of times of 0 to 5, and perfusion is then allowed for an arbitrary period of 0 to 3 hours. The above actions are repeated an arbitrary number of times of 0 to 5.

EXAMPLES

[0024]

The present invention is described below more concretely by way of Examples. However, the present invention is not limited to the Examples below.

[0025]

First, an animal experiment for demonstrating that repeating a ischemia-reperfusion cycle promotes *in vivo* production of acetylcholine (Ach) is described as Example 1.

[0026]

Example 1

Methods

1. Knockdown of ChAT gene

Knockdown of ChAT gene was performed using BLOCK-iT™ Pol II miR RNAi Expression Vector, pcDNA™ 6.2-GW/ EmGFP-miR (Invitrogen Corporation,

Carlsbad, CA, USA). According to the manufacturer's protocol, commercially recommended and pre-designed RNAi sequences specific for human or mouse ChAT were used to prepare for the double-stranded oligos, which were subcloned into the expression vector. As a negative control, Lac Z specific RNAi sequences were

5 subcloned to develop a negative control expression vector, which was also recommended commercially. To confirm that the targeted gene expression of ChAT was suppressed, HEK293 cells, transiently transfected using Effectene transfection reagent (QIAGEN), were examined by immunocytochemical studies. Stably

transfectants of ChAT knockdown expression vectors were developed with proper

10 selective antibiotics in HEK293 cells, ChAT KO HEK cells, as well as in HL-1 cells, ChAT KO HL-1 cells, which was derived from murine atrial myocardium and the transfected cells were easily detected due to the GFP expression.

[0027]

2. Immunocytochemical studies

15 Immunocytochemical analysis was performed using first antibodies, as follows; a goat anti-ChAT polyclonal antibody (MILLIPORE, Billerica, MA, USA) with 1:200 dilution, a rabbit anti--catenin polyclonal antibody (Cell Signaling TECHNOLOGY, Inc., Danvers, MA, USA) with 1:200 dilution, rat anti-ACh polyclonal antibody (MILLIPORE) with 1:500 dilution, and rabbit anti-Cx43

20 polyclonal antibody (ZYMED Laboratories Inc., South San Francisco, CA, USA). After overnight incubation at 4 °C, followed by PBS washing, each proper secondary antibody conjugated with immunofluorescence was applied and samples were examined by laser confocal microscopy (OLYMPUS, Tokyo, Japan).

[0028]

25 3. Western blot analysis

As already reported in the previous studies of the present inventors, Western blot analysis was performed [2, 3, 7, 10, 11]. First antibodies applied in this study

were a goat anti-ChAT polyclonal antibody (MILLIPORE) with 1:500 dilution, a rabbit anti-CHT1 polyclonal antibody (Antagene, Inc., Limonest, France) with 1:500 dilution, a rabbit anti-cleaved caspase-3 monoclonal antibody (Cell Signaling TECHNOLOGY, Inc.) with 1:500 dilution. After reaction with a proper HRP-conjugated secondary antibody, followed by washing with 1TBST, signals were detected using Luminata™ Forte Western HRP Substrate (MILLIPORE).

[0029]

4. LY dye migration assay

To assess effects of the cardiac non-neuronal cholinergic system on cardiomyocytes-derived cells, we obtained permanently ChAT knockdown HL-1 cells, ChAT KO HL-1 cells. These cells were inoculated onto glass bottom dishes, which were processed for cells to be aligned linearly with about 60 μ m in width (Cytograph, Dai Nippon Printing Co., Ltd., Tokyo, Japan). The each band formed by linearized ChAT KO HL-1 cells was patterned in parallel a 200 μ m apart from each other. The linearized cells were scratched perpendicularly by a 27 gauge needle after removal of culture medium and 1 % of Lucifer Yellow dye was applied onto the center of the scratched region. One minute later the cells were washed by PBS three times, fixed with 4 % paraformaldehyde for 10 min, and then the distance of the dye transferred from the scratched region was evaluated by immunofluorescent microscope.

[0030]

5. Cell culture

HEK293 cells and H9c2 cells, which were spontaneously immortalized ventricular myoblasts from rat embryos, were cultured in DMEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% FBS and antibiotics. Especially HEK293 cells were cultured on type I collagen (Cellmatrix, Nitta Gelatin Inc., Osaka, Japan)-coated culture dishes. HL-1 cells, derived from

atrial cardiac muscle, were cultured in Claycomb medium (SAFC Biosciences, Inc., enexa, KS, USA) supplemented with 10% FBS, 4 mM L-glutamine, 0.1 mM norepinephrine and 3 ML ascorbic acid on the culture dishes coated with 0.02% gelatin and 25 g/mL fibronectin.

5 [0031]

6. Measurement of ACh concentration in cells

As already reported in our recent study, the cellular or whole heart ACh concentration was determined by HPLC [11]. To prepare cell lysates, cells scraped from 10-cm culture dish were washed by PBS and suspended in 1 mL of PBS
 10 containning 0.1 mM physostigmine and 2×10^{-8} M IPHC as an internal control. And then, these samples were freeze-thawed three times. The heart excised from a mouse was adequately minced and homogenated in the presence of 1 mL homogenate solution including 0.1 M perchloric acid, 0.1 mM physostigmine and 2×10^{-8} M IPHC. After centrifugation at 0 °C with 2×10^4 G for 15 min, the supernatant with pH
 15 adjusted by 1 M potassium bicarbonate was filtered using Ultrafree MC column (MILLIPORE), and the eluted solution was used for quantification of an ACh concentration.

[0032]

7. MTT activity measuremen

20 MTT activity was measured using Cell Counting Kit-8 (DOJINDO LABORATORIES, Kumamoto, Japan) according to a manufacturer's protocol. This activity was already known to be dependent on cell viability, i.e., the number of intact cells, however, it was reported that the activity was determined not only by the cell number but also by the cell metabolism [10, 12]. Specifically MTT activities of cells
 25 with malfunctioned mitochondria showed a trend of activity reduction even with a comparable cell numbers.

[0033]

8. Measurement of cellular oxygen consumption

Oxygen contents in culture medium was determined by Fibox 3 (PreSense Precision Sensing, Regensburg, Germany), as shown in our previous study [11]. A specific oxygen-sensitive sensor was attached at the bottom of a culture dish, and cells were incubated in the culture dish. An Oxygen content was sequentially measured, recorded and compared to that at time 0 h.

[0034]

9. Measurement of cellular ATP content

Cellular and heart ATP levels were determined using XL-ATP kit (APRO Life Science Institute, Tokushima, Japan) according to the protocol. An ATP level from lysates was corrected by the protein concentration.

[0035]

10. Assessment of cellular reactive oxygen species production

Using an indicator for reactive oxygen species aminophenyl fluorescein (APF, SEKISUI MEDICAL CO., LTD., Tokyo, Japan), the production level from ChATKO HL-1 cells or control HL-1 cells was evaluated in the presence of each concentration of norepinephrine (0.5, 1.0, 2.0 mM). APF was added to be a final concentration of 10 μ M into culture medium. Cells were incubated with APF for 1 h and observed by laser confocal microscopy.

[0036]

11. Global ischemia model of murine heart with Langendorff apparatus

After anesthesia with intraperitoneal injection of Nembutal, a male C57BL/6J was suffered from left hindlimb ischemia (5 min)-reperfusion (3 min) repeated 3 times, performed by not a surgical but physical procedure. The right and left quadriceps femoris muscles were excised for the assessment of ChAT and CHT1 protein expressions 16 h after reperfusion. Furthermore, after reperfusion 4 h, 8 h or 16 h, the heart was excised for evaluation of protein expression, and the other heart

was connected to Langendorff apparatus and perfused with filtered Krebs–Henseleit buffer at 90 mmHg, equilibrated with 5% carbon dioxide and 95% oxygen. After stabilization, the Langendorff-perfused heart was subjected to 30 min global ischemia by stopping the perfusion of Krebs–Henseleit buffer, followed by 60 min reperfusion. And then, the heart was immersed into 1% TTC stain solution for 10 min at 37 °C, the stained heart was horizontally sliced in the mid-body and infarcted area was compared. Infarcted area ratio was determined by division of infarcted area by the whole area using NIH image software.

[0037]

10 12. Statistical analysis

Data were expressed as mean $\pm$ standard error. A non-parametric test for comparison of between two groups was performed with a Mann-Whitney U-test. Nonparametric multiple-comparison tests among three groups were performed with Kruskal-Wallis test followed by Fisher's PLSD tests. Differences were considered significantly at $P < 0.05$.

[0038]

Results

1. A non-neuronal cholinergic system regulates cell-cell communication.

To reveal the specific roles of the non-neuronal cholinergic system *in vitro*, ChAT gene was knocked down by a ChAT specific miRNA expression vector, and after confirming the effects thereof, permanent ChAT KO cells were prepared using HEK293 cells and HL-1 cells, which were derived from murine atrial myocardium. Knockdown of ChAT gene was immunocytochemically confirmed in transiently transfected HEK293 cells (Figure 1A). The transfected cells were confirmed by the GFP expression (green in Figure 1A). ChAT immunoreactivities, as observed in non-transfectants (red), were completely attenuated in ChAT KO cells.

[0039]

Permanent ChAT KO cells proliferated without growth retardation comparably with control HEK293 cells stably transfected with a LacZ gene knockdown vector (control cells). However, reinoculation of ChAT KO cells, following the agitation-induced detachment, unmasked a characteristic of the cells, i.e., failure of efficient cell-cell communication, compared with control cells (Figure 1B). After agitation, ChAT KO cells were easily scattered with single cells, however, control cells sustained the cell-cell interaction, forming aggregates. In control cells, ACh-positive signals were detected on the cytoplasmic membrane, however, the signals were attenuated in ChAT KO cells. Likewise, further weakened immunoreactive β -catenin signals were detected between ChAT KO cells, great contrast to the more intense and strong signals between control cells.

[0040]

ChAT KO HL-1 cells were also prepared, as were control cells with LacZ gene knockdown (control cells). ChAT KO HL-1 cells suppressed Cx43 protein expression, confirmed by Western blot and immunocytochemical analysis (Figure 1C). Cx43 positive signals, clearly detected between control cells, were remarkably attenuated in ChAT KO HL-1 cells, suggesting that cell-cell communication was disturbed. Dye transfer assay also revealed that LY dye was not efficiently transferred between ChATKO HL-1 cells. In contrast, the dye applied to control cells was remarkably transferred with a long distance, suggesting that non-neuronal cholinergic system regulated a gap-junction function.

[0041]

Taken together, these data suggest that non-neuronal cholinergic system plays a role in cell-cell communication.

[0042]

2. A non-neuronal cholinergic system plays a role in sparing energy metabolism. To investigate further roles of this system, ChAT KO HEK cells, with

potency to synthesize less than 10% of the ACh production level by control cells (control vs. ChAT KO: 100 ± 8.7 vs. 9.3 ± 3.5 %, $P < 0.01$), were evaluated in terms of cellular energy metabolism. Even with comparable cell numbers between each other, MTT activities of ChAT KO HEK cells were more elevated than those of control cells (384.8 ± 20.8 % vs. control, $P < 0.01$). Consistent with this, ChAT KO HEK cells consumed more oxygen than control (-239.4 ± 26.4 % vs. control, $P < 0.01$) (Figure 2A). As a result, the ATP level of ChAT KO HEK cells was significantly decreased (control vs. ChAT KO: 765.1 ± 56.9 vs. 425.7 ± 18.7 mM/g protein, $P < 0.01$) (Figure 2B). These results showing reciprocal changes between MTT activities as well as oxygen consumption and ATP contents suggest that the nonneuronal cholinergic system negatively regulates cellular energy metabolism, and this system sustains a cellular ATP content. Consequently, in the case of interference of oxygen utilization with a chemical hypoxic mimetic cobalt chloride (2 mM), ChAT KO HEK cells were more susceptible to cell death, compared with control cells, which were intact even with the dose (control vs. ChAT KO: 100.0 ± 0.35 vs. 26.6 ± 2.33 %, $P < 0.01$) (Figure 2C).

[0043]

In HL-1 cells, likewise, the non-neuronal cholinergic system plays an important role in regulating cell energy metabolism. The ATP contents in ChAT KO HL-1 cells were more reduced than control (control vs. ChAT KO: 271.9 ± 28.2 vs. 83.1 ± 8.8 mM/g protein, $P < 0.01$) (Figure 2D). And this result was also followed by H9c2 cells derived from rat myocardium. Two ChAT inhibitors, bromoacetylcholine (BrACh) and hemicholinium-3, were used to inhibit ChAT activities instead of ChAT gene deletion. H9c2 cells did not show any morphological changes when 10 μ M of each inhibitor was used. Each inhibitor increased a MTT activity in H9c2 cells, and hemicholinium-3 reciprocally decreased the ATP level (control vs. ChAT KO: 92.0 ± 1.0 vs. 61.0 ± 1.1 mM/g protein, $P < 0.05$) (Figure 2E), as ChAT gene

deletion did. These data also suggest that the non-neuronal cholinergic system negatively regulates energy metabolism and suppresses oxygen consumption to save cellular ATP.

[0044]

5 3. The Non-neuronal cholinergic system protects cells from norepinephrine toxicity. ChAT KO HL-1 cells were treated with norepinephrine (NE) (Figure 3). In this experiment, we used the cell viability ratio between NE-treated ChAT KO HL-1 cells and control cells as an indicator of cell viability. Therefore, the ratio of 100% indicated that both cells are equally intact even in the presence of NE. Nevertheless,
10 as clearly demonstrated in Figure 3, NE dose-dependently decreased cell viability of ChAT KO HL-1 cells (vs. control: 73.6 ± 1.4 % at 0.5 mM of NE, $P < 0.01$; 62.0 ± 1.0 % at 1.0 mM of NE, $P < 0.01$). Two mM of NE induced more cell death of ChAT KO HL-1 cells (vs. control: 29.4 ± 2.4 %, $P < 0.01$), accompanied with enhancement of caspase-3 activity compared with control cells. In addition, ChAT
15 KO HL-1 cells produced more reactive oxygen species during this cell death by NE, assessed by a ROS indicator APF. Such increased production of ROS was supposed to be partly contributed to cell death. These results clearly indicate that the non-neuronal cholinergic system in cardiomyocytes is essential to protect the cells from NE toxicity.

20 [0045]

Taken together with these *in vitro* data, the non-neuronal cholinergic system is responsible for sustaining the cell-cell communication, negatively regulating cellular energy metabolism and protecting cells from cell death.

[0046]

25 4. In vivo acceleration of the non-neuronal cholinergic system in skeletal muscle
To investigate a regulatory mechanism of this system *in vivo*, we performed murine transient hindlimb ischemia-reperfusion (IR) by manually attenuating the left

femoral artery flow with an intermittent fashion, 3 cycles of ischemia (5 min) with reperfusion (3 min), similar protocol to preconditioning (Figure 4). After 16 h since IR, the subjected left quadriceps femoris muscle remarkably increased protein expression of ChAT and CHT1, compared with contralateral one. This suggests that IR per se works as a trigger to activate the skeletal non-neuronal cholinergic system. [0047]

Of note, during this procedure the system was also activated in the heart, suggesting that IR activates the non-neuronal cardiac cholinergic system remotely. The protein expression of ChAT was gradually increased within 24h. The time course of CHT1 protein expression also followed the similar pattern with that of ChAT. These data suggest that IR upregulates the non-neuronal cholinergic system not only in a subjected organ, but also in the remote organ, e.g., the heart. [0048]

5. Hindlimb ischemia-reperfusion *in vivo* salvages the whole heart from global ischemia through the cardiac non-neuronal cholinergic system.

To confirm that IR protects the heart from global ischemia, the heart, excised 16 h after IR to be perfused through Langendorff apparatus, was subjected to the global ischemia-reperfusion, i.e., 30 min ischemia and 60 min reperfusion (Figure 5). As a consequence of reperfusion injury, an infarcted area ratio in control mice, which did not receive IR, was 0.61 ± 0.03 . In contrast, the ratio in the heart excised from mice with IR was significantly reduced to 0.26 ± 0.03 (vs. control, $P < 0.01$), confirming a remote protective effect of IR on the heart in mice. This phenomenon was well known as a late phase of preconditioning, however, pretreatment of IR-treated mice with a ChAT inhibitor hemicholinium-3 before IR blunted this myocardial infarction sparing effect exerted by IR and the ratio returned to the comparable level with the control 0.56 ± 0.03 (vs. IR, $P < 0.05$). These results suggest that the remote effect of cardiac protection was mediated by upregulation of ChAT in

the heart, because hemicholinium-3 canceled this effect.

[0049]

6. The mechanisms underlying the cardioprotection by the remote effect

The hearts were isolated after IR at 4 h, 8 h and 16 h, respectively and ATP
 5 contents in hearts were measured. It was revealed that the heart at 16 h contained a
 higher level of ATP content (256.4 ± 13.1 % vs. IR 4h, $P < 0.01$), suggesting that the
 IR causes elevation of the ATP level in the heart (Figure 6A). To investigate whether
 an increased ATP level in the heart was attributed to the upregulation of the cardiac
 non-neuronal cholinergic system, hemicholinium-3 was administered before IR. As
 10 shown in Figure 4 and 6C, IR upregulated ChAT protein expression in the heart and
 increased the cardiac ACh (IR: 2.78 ± 0.17 nmol/g protein vs. control: 2.32 ± 0.12
 nmol/g protein, $P < 0.05$) and ATP levels (IR: 11.5 ± 0.9 mM /g protein vs. control:
 5.9 ± 0.5 mM /g protein, $P < 0.01$). However, hemicholinium-3, a ChAT inhibitor,
 surprisingly attenuated an increase in the cardiac ATP levels (HC-3: 6.4 ± 1.0 mM /g
 15 protein vs. IR, $P < 0.05$) to a control level (Figure 6B). This suggests that the cardiac
 non-neuronal cholinergic system is definitely involved in maintaining an ATP level
 in the heart. Measuring an ACh level in the heart from mice subjected to IR also
 supported this issue. Pretreatment with hemicholinium-3 completely suppressed the
 elevation of cardiac ACh level (HC-3: 2.14 ± 0.07 vs. IR, $P < 0.05$) (Figure 6C).

20 [0050]

In eNOS (vascular endothelial nitric oxide synthase) KO mice, IR-induced
 upregulation of ACh synthesis in the heart was abolished. Even 16 h after IR, the
 cardiac ACh level, which was supposed to increase in wild-type mice, was
 comparable with that before IR. This suggests that NO was partly responsible for
 25 upregulation of a non-neuronal cardiac cholinergic system induced by the remote
 effect of IR.

[0051]

Example 2

The device of the present invention concretely described with reference to Fig. 7 and Fig. 8 was prepared. Using this device, the right femoral region of human was subjected to 5 minutes of ischemia and 3 minutes of reperfusion. This ischemia-reperfusion cycle was repeated once or 3 times. The changes in the heart rate with time observed in these cases are shown in Fig. 9 and Fig. 10.

[0052]

In Fig. 9 and Fig. 10, “trained” represents human individuals subjected to the ischemia-reperfusion using the device of the present invention, and “untrained” represents human individuals who have not been subjected to the ischemia-reperfusion (control). As shown in Fig. 9 and Fig. 10, a decrease in the heart rate (which indicates an increase in ACh in the heart and central activation of the parasympathetic nervous system) was found from immediately after the ischemia-reperfusion cycle, and this continued for about 16 hours. Continuation of this effect could be found for about 24 hours, and, after that, the heart rate became normal again. When the ischemia-reperfusion was carried out again, the heart rate decreased (data not shown). This suggests systemic activation of the parasympathetic nervous system and activation of the acetylcholine synthetic system in the heart.

[0053]

Example 3

Mice were fed with high-fat diet for 11 days while hind limb IR (3 minutes of ischemia and 2 minutes of reperfusion, 3 cycles) was performed every day, and the BS (blood glucose level) was compared with that of IR-untreated mice. The results are shown in Fig. 11.

[0054]

As shown in Fig. 11, the IR-untreated mice showed an increase in the blood glucose level, but an action to decrease the blood glucose level was found in the IR-

treated mice.

[0055]

Example 4

Mice were fed with high-fat diet while IR (3 minutes of ischemia and 2
5 minutes of reperfusion, 3 cycles) was performed every other day (Day 0, Day 2, and
Day 4), and the BS (blood glucose level) was compared with that of IR-untreated
mice. The results are shown in Fig. 12.

[0056]

As shown in Fig. 12, the IR-untreated mice showed an increase in the blood
10 glucose level, but an action to decrease the blood glucose level was found in the IR-
treated mice. This action to decrease the blood glucose level was found to be
evidently effective even in the cases where the IR was carried out every other day.

[0057]

Example 5

15 Mice were fed with high-fat diet for 11 days while IR (2 minutes of ischemia
and 2 minutes of reperfusion, 3 cycles) was performed every day, and the BS (blood
glucose level) was compared with that of IR-untreated mice. The results are shown
in Fig. 13.

[0058]

20 As shown in Fig. 13, an action to decrease the blood glucose level was found
in the IR-treated mice. This action to decrease the blood glucose level continued for
11 days even in the cases where the period of ischemia was set to 2 minutes in order
to reduce invasiveness.

[0059]

25 Example 6

Mice were fed with high-fat diet for 7 to 11 days while IR was performed
every day. The IR was carried out by repeating, 3 times, 2 minutes to 3 minutes of

ischemia and 2 minutes of reperfusion. The body weight was measured during the experiment. The results are shown in Fig. 14.

[0060]

As shown in Fig. 14, the pc (IR) group did not show a significant increase in the body weight as compared to the control group. The daily intake was not different between these groups. On the other hand, in cases where the IR was carried out every other day, no effect to decrease the body weight was found. However, as shown in Fig. 12, the action to decrease blood glucose could still be found.

[0061]

REFERENCE LIST

1. Kakinuma Y, Ando M, Kuwabara M, Katare RG, Okudela K, Kobayashi M, Sato T. Acetylcholine from vagal stimulation protects cardiomyocytes against ischemia and hypoxia involving additive non-hypoxic induction of HIF-1alpha. FEBS Lett. 2005;579:2111-2118.
2. Ando M, Katare RG, Kakinuma Y, Zhang D, Yamasaki F, Muramoto K, Sato T. Efferent vagal nerve stimulation protects heart against ischemia-induced arrhythmias by preserving connexin43 protein. Circulation. 2005;112:164-170.
3. Zhang Y, Kakinuma Y, Ando M, Katare RG, Yamasaki F, Sugiura T, Sato T. Acetylcholine inhibits the hypoxia-induced reduction of connexin43 protein in rat cardiomyocytes. J Pharmacol Sci. 2006;101:214-222.
4. Katare RG, Ando M, Kakinuma Y, Arikawa M, Handa T, Yamasaki F, Sato T. Vagal nerve stimulation prevents reperfusion injury through inhibition of opening of mitochondrial permeability transition pore independent of the bradycardiac effect. J Thorac Cardiovasc Surg. 2009;137:223-231.
5. Handa T, Katare RG, Kakinuma Y, Arikawa M, Ando M, Sasaguri S, Yamasaki F,

- Sato T. Anti-Alzheimer's drug, donepezil, markedly improves long-term survival after chronic heart failure in mice. *J Card Fail.* 2009;15:805-811.
6. Katare RG, Ando M, Kakinuma Y, Arikawa M, Yamasaki F, Sato T. Differential regulation of TNF receptors by vagal nerve stimulation protects heart against acute ischemic injury. *J Mol Cell Cardiol.* 2010;49:234-244.
7. Kakinuma Y, Furihata M, Akiyama T, Arikawa M, Handa T, Katare RG, Sato T. Donepezil, an acetylcholinesterase inhibitor against Alzheimer's dementia, promotes angiogenesis in an ischemic hindlimb model. *J Mol Cell Cardiol.* 2010;48:680-693.
8. Arikawa M, Katare RG, Kakinuma Y, Handa T, Ando M, Yamasaki F, Sato T. Progression of ventricular dysfunction after myocardial infarction is prevented by anti-Alzheimer's disease drug, donepezil. *Circulation* 116: II_291.
9. Okazaki Y, Zheng C, Li M, Sugimachi M. Effect of the cholinesterase inhibitor donepezil on 19 cardiac remodeling and autonomic balance in rats with heart failure. *J Physiol Sci.* 2010;60:67-74.
10. Kakinuma Y, Katare RG, Arikawa M, Muramoto K, Yamasaki F, Sato T. A HIF-1 α -related gene involved in cell protection from hypoxia by suppression of mitochondrial function. *FEBS Lett.* 2008;582:332-340.
11. Kakinuma Y, Akiyama T, Sato T. Cholinoceptive and cholinergic properties of cardiomyocytes involving an amplification mechanism for vagal efferent effects in sparsely innervated ventricular myocardium. *FEBS J.* 2009;276:5111-5125.
12. Rapoport N, Marin AP, Timoshin AA. Effect of a polymeric surfactant on electron transport in HL-60 cells. *Arch Biochem Biophys.* 2000;384:100-108.
13. Kirkpatrick CJ, Bittinger F, Nozadze K, Wessler I. Expression and function of the non-neuronal cholinergic system in endothelial cells. *Life Sci.* 2003;72:2111-2116.
14. Kawashima K, Fujii T. Expression of non-neuronal acetylcholine in lymphocytes

- and its contribution to the regulation of immune function. *Front Biosci.* 2004;9:2063-2085.
15. Lips KS, Volk C, Schmitt BM, Pfeil U, Arndt P, Miska D, Ermert L, Kummer W, Koepsell H. Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. *Am J Respir Cell Mol Biol.* 2005;33:79-88.
16. Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K. Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. *Circulation.* 2004;109:120-124.
17. Schwartz PJ, De Ferrari GM, Sanzo A, Landolina M, Rordorf R, Raineri C, Campana C, Revera M, Ajmone-Marsan N, Tavazzi L, Otero A. Long term vagal stimulation in patients with advanced heart failure: first experience in man. *Eur J Heart Fail* 2008;10:884-891.
18. De Ferrari GM, Schwartz PJ. Vagus nerve stimulation: from pre-clinical to clinical application: challenges and future directions. *Heart Fail Rev.* 2011;16:195-203.
19. Hoover DB, Ganote CE, Ferguson SM, Blakely RD & Parsons RL. Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. *Cardiovasc Res* 2004;62:112-121.
20. Kawano H, Okada R & Yano K. Histological study on the distribution of autonomic nerves in the human heart. *Heart Vessels* 2003;18:32-39.
21. Kuwabara M, Kakinuma Y, Katare RG, Ando M, Yamasaki F, Doi Y, Sato T. Granulocyte colony-stimulating factor activates Wnt signal to sustain gap junction function through recruitment of beta-catenin and cadherin. *FEBS Lett.* 2007;581:4821-4830.
22. Kostin S, Hein S, Bauer EP, Schaper J. Spatiotemporal development and distribution of intercellular junctions in adult rat cardiomyocytes in culture. *Circ Res* 1999;85:154-167.

23. Wu JC, Tsai RY, Chung TH. Role of catenins in the development of gap
junctions in rat cardiomyocytes. *J Cell Biochem* 2003;88: 823-835.
24. Hausenloy DJ, Mwamure PK, Venugopal V, Harris J, Barnard M, Grundy E,
Ashley E, Vichare S, Di Salvo C, Kolvekar S, Hayward M, Keogh B, MacAllister RJ,
5 Yellon DM. Effect of remote ischaemic preconditioning on myocardial injury in
patients undergoing coronary artery bypass graft surgery: a randomised controlled
trial. *Lancet* 2007;370:575-579.
25. Ali ZA, Callaghan CJ, Lim E, Ali AA, Nouraei SA, Akthar AM, Boyle JR, Varty
K, Kharbanda RK, Dutka DP, Gaunt ME. Remote ischemic preconditioning reduces
10 myocardial and renal injury after elective abdominal aortic aneurysm repair: a
randomized controlled trial. *Circulation* 2007;116 (11 Suppl):I98-105.
26. Botker HE, Kharbanda R, Schmidt MR, Bottcher M, Kaltoft AK, Terkelsen CJ,
Munk K, Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH,
Krusell LR, Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sorensen HT,
15 Redington AN, Nielsen TT. Remote ischaemic conditioning before hospital
admission, as a complement to angioplasty, and effect on myocardial salvage in
patients with acute myocardial infarction: a randomised trial. *Lancet* 2010 ;375:727-
734.
27. Hausenloy DJ, Yellon DM. Remote ischaemic preconditioning: underlying
20 mechanisms and clinical application. *Cardiovasc Res* 2008;79:377-386.

DESCRIPTION OF SYMBOLS

[0062]

- | | |
|------|-----------------------------|
| 1 | Power switch |
| 2 | Direct-current power source |
| 25 3 | Direct-current power source |
| 4 | Pressure switch |
| 5 | Programmable relay |

- 6 Pump
- 7 Electrically-operated valve
- 8 Air pipe
- 9 Cuff

CLAIMS

1. An *in vivo* acetylcholine production-promoting device comprising:
 - an artery-compressing member which contacts any site on a limb and compresses an artery/arteries located at the site when the artery-compressing member
 - 5 is pressurized; and
 - pressurizing means connected to the artery-compressing member, which, during use, pressurizes the artery-compressing member for compressing the artery/arteries to cause ischemia and then releases the pressure to allow reperfusion of blood through the artery/arteries.
- 10 2. The device according to claim 1, wherein said pressurizing means is programmed to repeat, one or more times, a cycle in which the pressurization is carried out for 1 to 15 minutes and then the release of the pressure is carried out.
3. The device according to claim 2, wherein said pressurizing means is programmed to repeat said cycle 2 to 10 times.
- 15 4. The device according to any one of claims 1 to 3, wherein said artery-compressing member is a cuff.
5. The device according to claim 4, wherein said pressurizing means comprises:
 - a pump;
 - an air pipe which connects said pump to said cuff, and sends air from said
 - 20 pump to said cuff; and
 - a valve provided in said air pipe;
 - which pump is operated to pressurize said cuff, and which valve is opened to release the pressure.
- 25 6. The device according to claim 5, wherein said valve is an electrically-operated valve, and a power source for said electrically-operated valve and a power source for said pump are connected to said electrically-operated valve and said pump, respectively, via a programmable relay, which programmable relay is programmed to

repeat, one or more times, a cycle composed of pressurization by driving of said pump and release of the pressure thereafter by opening said electrically-operated valve.

7. The device according to claim 6, further comprising a pressure switch
5 connected to said air pipe and said programmable relay, which pressure switch detects the pressure in said air pipe and sends a signal indicating whether the detected pressure is less than a predetermined pressure or not to said programmable relay, wherein, in cases where the pressure is less than the predetermined pressure, electric power is supplied from the power source for said pump to said pump to drive said
10 pump and to thereby pressurize said cuff, while in cases where the pressure is not less than the predetermined pressure, no electric power is supplied from the power source for said pump to said pump, and the pump is not driven.

8. The device according to claim 7, wherein said cycle is composed of 1 minute to 15 minutes of pressurization and 1 minute to 3 hours of pressure release, and
15 repeated 2 to 10 times.

9. The device according to claim 8, wherein the pressurization time is 1 minute to 15 minutes; the pressure release time is 1 minute to 15 minutes; and said cycle is repeated 2 to 5 times.

10. The device according to any one of claims 1 to 9, which is a blood glucose
20 level reducing device or a blood glucose level elevation suppressing device.

11. A method for promoting *in vivo* acetylcholine production, comprising repeating, one or more times, a cycle in which an artery/arteries of an animal is compressed to cause ischemia and reperfusion of blood through said artery/arteries is then allowed.

12. The method according to claim 11, which is a blood glucose level reducing
25 method or a blood glucose level elevation suppressing method.