Remote ischemic preconditioning protects human neural stem cells from oxidative stress

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Abstract

In previous clinical trials, we showed that remote ischemic preconditioning (rIPC) reduced myocardial damage in children undergoing treatment for congenital heart defects and postoperative renal failure in patients undergoing abdominal aortic aneurysm surgery. In rabbit experiments, pre-treatment with plasma and plasma dialysate (obtained using 15-kDa cut-off dialysis membrane) from donor rabbits subjected to rIPC similarly protected against cardiac infarction. However, the protective substances containing in rIPC plasma have been unknown.

In the present study, we showed that rIPC plasma exerted anti-apoptotic and anti-oxidative effects on human neural stem cells under oxygen glucose deprivation (OGD) that mimics brain ischemia. Additionally, we applied the sample to the liquid chromatography integrated with mass spectrometry to identify candidate key molecules in the rIPC plasma and determine its role in protecting neural stem cells from OGD-induced cell death. Thioredoxin increased significantly after rIPC compared to pre-IPC. Pretreatment with thioredoxin, the antioxidant protein, markedly protected human neural stem cells from OGD-induced cell death. The effect of thioredoxin on brain ischemia in animals should be further evaluated. However, the present study first evaluated the effect of rIPC in the ischemic cellular model.

Introduction

Stroke is one of the leading causes of mortality and severe long-term morbidity worldwide (1, 2). It is estimated that one in six (16.67%) individuals will experience at least one stroke in their lifetime (3, 4). Stroke survivors mostly have consequent permanent neurological deficits (2). Therefore, new therapeutic developments for brain ischemic injury are imperative.

Remote ischemic preconditioning (rIPC) is a well-recognized phenomenon by which a short repeated period of ischemia and reperfusion in a distal organ protects against subsequent ischemia in another vital organ(5-7). Hind limb remote preconditioning is one of the most frequent remote preconditioning methods used to protect against heart and brain ischemia (8-10). The effectiveness of this method has also been confirmed in several studies using cardiac arrest or global ischemia models(10-12). In particular, this method was reported to inhibit hippocampal neuronal apoptosis induced by cerebral ischemia-reperfusion in global ischemia in rats(13). Studies have revealed that this limb remote preconditioning accomplished long-term protective effects against stroke and might block brain injury (14).

In a previous randomized control trial, we showed that rIPC is effective in protecting children undergoing treatment for congenital heart defects(15). Subsequent studies also showed that rIPC reduced myocardial damage and postoperative renal failure in patients undergoing abdominal aortic aneurysm surgery (16), and reduced troponin T release in adults undergoing surgical coronary revascularization (17). However, the mechanism underlying remote preconditioning remains unclear.

In the present study, we determined whether the rIPC plasma can exert protective and anti-apoptotic effects on human neural stem cells (NSCs) under oxygen glucose deprivation (OGD) that mimics ischemia. Additionally, we applied the sample to the liquid chromatography-mass spectrometry (LC-MS/MS) to identify candidate molecules in the rIPC plasma and determine its role in protecting neural stem cells from OGD-induced cell death.

Materials and methods

Blood sample collection and remote ischemic preconditioning

Blood samples were collected before and after rIPC, as previously described (18, 19), in three healthy adult male volunteers who were taking no medications and were with no previous medical history (mean age, 35.0 yrs). Briefly, transient ischemia was induced in the brachium using a blood pressure cuff at 200 mmHg for four cycles of 5-min inflation and 5-min deflation. After a 5-min rest, 30 ml of venous blood was collected from the contralateral arm(8, 19). Samples were collected in standard sterile tubes with EDTA anticoagulant and transferred immediately on ice to prevent protein degradation. Plasma was isolated from whole blood through centrifugation (3000×g for 15 min) at 4℃.

Preparation of human neural stem cells

Human HB1.F3 (F3) neural stem cells (NSCs) were generated from human fetal telencephalon and were immortalized by transfection with a retroviral vector encoding the v-myc oncogene, as described previously (20). We confirmed that the F3 NSC cell line had self-regenerative and multipotent capacity (20). F3 cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg glucose/L (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Oxygen-glucose deprivation (OGD)

NSCs were plated on 35-mm dishes (1.0×10^6 cells per dish). The cultures were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2 for 24h. After incubation, the medium was aspirated and cells were washed twice with 1X phosphate-buffered saline (PBS). Cultures were then added to glucose-free DMEM and incubated at 37°C under anaerobic conditions of 94% N_2, 5% CO_2, 1% O_2 for the duration (6, 12, or 24 h) of OGD.

Plasma dialysate and fraction via reverse-phase chromatography

To prepare dialysate, approximately 16 ml of plasma (control and rIPC) was transferred to dialysis tubing with a 12–14 kDa cut-off cellulose membrane (Spectra/Por, Rancho Dominguez, CA), and dialyzed against a modified glucose-free Krebs-Henseleit (200-250 ml) for 24 h at 4°C. To fractionate the hydrophobic protein factors, the dialysate was subject to reverse-phase chromatography based on a C_{18} matrix (Sep-pak; Waters, Milford, MA). Briefly, the C_{18} matrix in the column was first washed with 50 ml of 80% acetonitrile, and then equilibrated with 50 ml of Krebs-Henseleit solution. The dialysate was loaded on to the C_{18} column and gravity fed. The resultant flow-through material was collected and treated prior to evaluation for neuro-protective activity. The column was washed further with 50 ml of Krebs-Henseleit solution prior to elution of the bound material with 20 ml of 80% acetonitrile. The C_{18} column eluate was diluted in 20 ml of water and lyophilized. Following freeze-drying, the dried powder was diluted to 1 ml of double distilled water before use.

TUNEL assay

Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assays were performed on NSCs to evaluate OGD-mediated apoptosis. After exposure to OGD for 24 h, the cells were fixed in 4% paraformaldehyde. The TUNEL assay was performed using an *in situ* cell death detection kit (MEBSTAIN Apoptosis TUNEL Kit direct; MEDICAL & BIOLOGICAL LABORATORIES, Nagoya, Japan) according to manufacturer's instructions. Nuclei were visualized by staining with 496-diamidino-2-phenylindole (DAPI) in mounting medium. Four

microscopic fields were randomly selected and evaluated by calculating the ratio of TUNEL-positive cells to the total number of cells in three independent experiments.

Mitochondrial membrane potential and reactive oxygen species (ROS) assay

To visualize mitochondrial membrane potential, NSCs were incubated at room temperature for 30 min. Cells were then washed with PBS, cover-slipped, and observed using a laser confocal microscope. Mitochondrial ROS levels were measured by staining cells with RedoxSensor Red CC-1 (Molecular Probes, Eugene, OR) for 10 min at 37°C in a 5% CO_2 incubator. Cells were then washed with PBS and observed using a laser confocal microscope. Fluorescence intensity of the stained cells was then analyzed using a fluorescence microscope (Olympus IX71, Olympus, Tokyo, Japan). Six fields of view were analyzed for each sample. Duplicates of three independent experiments were analyzed for each treatment group. In addition, cellular ROS levels were measured in cells that had been treated with 2′-7′-dichlorofluorescin diacetate (DCFDA, 20 μM final solution concentration) for 1 h after 6 h of OGD using a DCFDA-Cellular Reactive Oxygen Species Detection Assay Kit (Abcam plc, Cambridge, UK) according to the manufacturer's protocol. The mean fluorescence intensity of 1×10^6 cells was determined using BD FACS Canto™ II flow cytometer (BD, Franklin Lakes, NJ, USA).

Liquid chromatography-mass spectrometry (LC-MS/MS) analysis

Freeze-dried samples obtained from rIPC plasmas were denatured by gentle agitation and incubation in 90% 100 mM ammonium bicarbonate containing 10% acetonitrile at 37°C for 60 min. The resulting solution was immediately combined with 100 mM of iodoacetamide at room temperature for 30 min in the dark. These modified proteins were further subjected to tryptic digestion by the addition of porcine trypsin (2 μL of 200 ng/uL in 50 mM acetic acid solution) and incubated at 37°C for 16 h. The peptide mixture was then dissolved in a 0.1% trifluoroacetic acid (TFA)/2% acetonitrile/distilled water solution, and analyzed using an LC-MS system with LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) in a fully automated manner.

Briefly, peptide separation was performed with a Paradigm MS4 LC instrument (Bruker Michrom, Auburn, CA, USA) containing a MAGIC C18 capillary LC column (0.2-mm id, 50-mm length, 3-μm particle size, and 200-Å pore size; Bruker Michrom). The mobile phase consisted of formic acid, acetonitrile, and water at a volume ratio of 0.1:2:98 for mobile phase A, and 0.1:90:10 for mobile phase B. The flow rate was reduced from 100 μL/min to approximately 1 μL/min using a flow splitter. Ten microliters of the peptide solution, containing 1 μg peptide, was applied using an HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) onto a Peptide CapTrap column (0.5-mm id, 2.0-mm length, 0.5 μL

bed volume; Bruker Michrom), and equilibrated with a solution of TFA, acetonitrile, and water at a volume ratio of 0.1:2:98. The peptides concentrated and purified on the trap column were injected onto the C18 capillary LC column by valve switching. The peptides were continuously eluted at a rate of 1 μL/min on a gradient mode: The initial ratio of 5% of mobile phase B was increased linearly to 40% B over 70 min, followed by an increase to 95% B over the next 5 min. After washing with a non-gradient flow at 95% B, the column was equilibrated again with the solvent of 5% B for the next separation. The total analysis lasted 90 min. Forionization of the protonated peptides, the LC effluent was interfaced with an nano electrospray ionization (nESI) source in a positive ion mode, on a Finnigan LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific). The nESI source via coupling to a spray emitter (150-μm OD/20-μm ID; OmniSeparo-TJ, Hyogo, Japan) directly connected with the outlet of the LC column. The ESI voltage was 2.0 kV and the transfer capillary of the LTQ inlet was heated at 200°C. No sheath gas was supplied. The other parameters on the ion separation and detection were optimized according to an Autotune function on the mass spectrometer instrument. For MS/MS, protonated peptides in gas phase were sequentially analyzed using a data-dependent scanning mode. A full scan at an m/z range of 450 to 2,000 and subsequent product ion scans of the three most intense precursor ions were analyzed. The data acquisition was made in a Centroid mode for both scans. The product ion scan was performed under conditions including an intensity threshold of 1×10^3, 30% normalized collision energy, 2.0 Da isolation m/z width, and dynamic exclusion for 30 sec. The ESI-MS/MS operation and data acquisition were carried out on an Xcalibur Revision 1.4 SR1 system controller (Thermo Fisher Scientific). All LC-MS data were analyzed against the SwissProt 55.6 Homo sapiens database (20,009 entries) using Mascot (version_2.3.02, Matrix Science, London, UK) (21). Peptide and fragment mass tolerance were 2.0 Da and 0.8 Da (22-24), respectively. For variable peptide modifications, methionine oxidation, and [+charge parameter (eg. N-formilation)] were taken into account. A p-value of less than 0.05 was considered statistically significant and the ions score cut-off was 0.05. All results were obtained from triplicate LC-MS/MS runs for each sample with all peptide hits included (25, 26). Unique peptides and proteins were selected in a low-molecular-mass (10–15 kDa), which transient limb ischemia may release as hydrophobic circulating factors (8).

Treatment of neural stem cells with plasma dialysates or thioredoxin

After NSCs (1.0×10^6 cells per dish) were cultured for 24 h, 20 μL of freeze-dried extract from plasma dialysates of pre-rIPC and post-rIPC, or thioredoxin (final concentration of 10 μg/ml, Sigma-Aldrich Japan G.K) were added directly to the culture medium for 10 min prior to OGD. The concentration of thioredoxin was physiologically reasonable according to the manufacturer's instruction. The medium was removed and cells were washed with PBS. Cultures were then placed in 2 ml of glucose-free DMEM and incubated for 24 h under OGD.

Cells were then stained with TUNEL, RedoxSensor, and DCFDA.

Real-Time RT-PCR of the thioredoxin-ROS axis

Total RNA was isolated using TRIzol (Thermo Fisher Scientific Inc., Waltham, MA, USA) and Phasemaker™ (Thermo Fisher Scientific Inc.). Reverse transcription (RT) from RNA to cDNA was performed using a ReverTra Ace® qPCR RT Kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. A real-time polymerase chain reaction (PCR) was performed on the cDNA solution (10% of the volume) using THUNDERBIRD® SYBR® qPCR Mix (Toyobo Co., Ltd.) and a LightCycler® 480 Instrument II (Roche Diagnostics Corporation, Indianapolis, IN, USA). The following primers were obtained from Hokkaido System Science Co., Ltd. (Hokkaido, Japan): thioredoxin-interacting protein (TXNIP), 5'-GATCACCGATTGGAGAGCCC-3' and 5'-TGCAGGGATCCACCTCAGTA-3' and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-GCATCTTCTTTTGCGTCGCC-3' and 5'-CCCAATACGACCAAATCCGTTG-3'. GAPDH was used as an internal control. RT-PCR was performed with the following thermal cycling conditions: 10 s of denaturation at 95°C, 10 s of primer annealing at 60°C, and 10 s of synthesis at 72°C. The PCR products were determined using a melting curve analysis (heating from 65°C to 95°C) with 10 s of cooling at 50°C. Each experiment was repeated three times, and the relative levels of expression of TXNIP mRNA were calculated using the $2^{-\Delta\Delta C}{}_{T}$ method.

Results

Oxygen-Glucose Deprivation induces apoptosis in neural stem cells by oxidative stress

Human NSCs were subjected to OGD for 6, 12, or 24 h and then stained with TUNEL and RedoxSensor. TUNEL- and RedoxSensor-positive cells were best observed 24 h after OGD (data not shown). The number of positive cells was counted in four independent fields. The ratio was expressed as a percentage of positive cells among all cells. OGD markedly increased the ratio of both TUNEL and RedoxSensor-positive cells up to approximately 10-20%, respectively (Figs 1 and 2). The results indicated that OGD activated apoptosis in neural cells by inducing oxidative stress, reflecting the mechanism of cerebral ischemia (27).

Extract of rIPC plasma dialysates through C18 column protects neural stem cells from apoptosis by oxidative stress

Approximately 16 ml of plasma (pre-rIPC and post-rIPC) was placed in a dialysis tube with a 12–14-kDa cut-off cellulose membrane, and dialysed against a modified glucose-free Krebs-Henseleit buffer for 24 h at 4°C. To fractionate the hydrophobic protein factors, the dialysate was subject to reverse-phase chromatography based on a C_{18} matrix. The

flow-through material was collected and treated prior to evaluation for neuroprotective activity. The C_{18} column eluate was diluted in 20 ml of water and freeze-dried. Following freeze-drying, the dried powder was diluted in 1 ml of double distilled water before use. The neural stem cells were then pretreated with the resulting dialysate solution from either the pre-rIPC or post-rIPC, and were subject to OGD. OGD induced apoptosis in approximately 21% and 16% of neural stem cells pretreated with buffer and pre-rIPC respectively. In contrast, the solution of freeze-dried extract from post-rIPC plasma dialysis protected NSCs from apoptosis significantly (Fig 1B). Similarly, extracts of post-rIPC plasma dialysates prevented oxidative stress in OGD-induced cells (Fig 1C).

Liquid chromatography-mass spectrometry (LC-MS/MS) analysis

Freeze-dried samples obtained from pre/post-rIPC plasmas were analyzed using Liquid chromatography integrated with mass spectrometry. For MS/MS, protonated peptides in a gas phase were sequentially analyzed by data-dependent scanning mode. A full scan at an m/z range of 450 to 2,000 and subsequent product ion scans of the three most intense precursor ions were analyzed. The data acquisition was made in a Centroid mode for both scans. The product ion scan was performed under conditions including an intensity threshold of 1×10^3, 30% normalized collision energy, 2.0-Da isolation m/z width, and dynamic exclusion for 30 sec. The ESI-MS/MS operation and data acquisition were carried out on an Xcalibur Revision 1.4 SR1 system controller (Thermo Fisher Scientific). A p-value of less than 0.05 was assumed to be statistically significant and the ions score cut-off was 0.05. All results were obtained from triplicate LC–MS/MS runs for each sample with all peptide hits included. Proteins (10–15 kDa) specifically present in post-rIPC plasma were listed in Table 1. Of the proteins listed in Table 1, the potential candidates were thioredoxin, macrophage migration inhibitory factor, and dermcidin. The remaining proteins, cystatin A, histones, and ribosomal proteins, were found in the samples because of mechanical release due to pressure in the brachium. Dermcidin is involved in host skin defenses (28). Macrophage migration inhibitory factor, which inhibits macrophage accumulation in chronic inflammation, has protective effects in long-term diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, and atherosclerosis (29). Because thioredoxin with a molecular weight of 12,015 Da is an antioxidant, it could have acute protective effects against oxidative stress and apoptosis. Thus, we considered thioredoxin the most reasonable candidate hydrophobic-circulating factor that was released following transient limb ischemia (8).

Treatment of neural stem cells with thioredoxin

After NSCs were cultured for 24 h, thioredoxin (final concentration of 10 μg/ml, Sigma-Aldrich Japan G.K) was added directly to the culture medium for 10 min prior to OGD.

Cultures were then placed in 2 ml of glucose-free DMEM and incubated for 24 h under OGD. Cells were then stained with TUNEL and RedoxSensor. OGD itself reduced cell growth, and approximately 14% and 8% of cells were stained with TUNEL and RedoxSensor, respectively. However, pretreatment with thioredoxin prior to OGD prevented NSCs from OGD-induced cell death and oxidative stress up to 2% and 0.5% as measured by TUNEL and RedoxSensor staining, respectively (Fig 2). A well-known function of TXNIP is the covalent binding to thioredoxin, which both inhibits thioredoxin's ability to scavenge ROS and interferes with thioredoxin's binding to other signaling molecules(30). Because the TXNIP gene is dramatically induced by glucose(31), we investigated the transcription of TXNIP after 24 h of OGD. The levels of TXNIP were markedly decreased to 20% of the levels of the nonOGD controls after 24 h of OGD, and pretreatment with thioredoxin resulted in recovery of the levels of TXNIP to approximately 70% (Fig. 3A). We set out to try to detect ROS earlier to investigate whether ROS that were detected by RedoxSensor at 24 h were the cause or result of the TXNIP reduction. Thus, DCFDA flow cytometry was performed after 6 h of OGD. At 6 h, the number of DCFDA-labeled cells was already significantly increased, and the thioredoxin levels had returned the cells to near-normal control levels (Fig. 3B).

Discussion

Stroke accounts for ~9% of deaths worldwide. The majority of strokes are ischemic. A recent study showed that reactive oxygen species (ROS) were increased after ischemic stroke (32). When cells become hypoxic, the production of ROS increases. These metabolic changes interact with the mitochondrial respiratory chain to increase the release of the superoxide anion and hydrogen peroxide(33-36). In this study, neural stem cells were cultured under oxygen-glucose deprivation (OGD) in order to mimic cellular ischemic conditions. We confirmed that OGD not only induced cell death but also exerted oxidative stress as measured by RedoxSensor and DCFDA staining.

A randomized controlled trial demonstrated that rIPC reduced myocardial damage in children undergoing repair of congenital heart defects(15). However, the mechanism involved in remote preconditioning remains unclear. Previously, we demonstrated that infarct size after coronary artery ligation and reperfusion was substantially reduced by rIPC in rabbits. Further, pre-treatment with plasma and plasma dialysate (obtained using 15-kDa cut-off dialysis membrane) from donor rabbits subjected to rIPC similarly protected against cardiac infarction. So far, although we know that in vivo transient limb ischemia releases a low-molecular-mass (<15 kDa) hydrophobic circulating factor(s) which induce(s) potent protection against myocardial ischemia/reperfusion injury, the factor(s) remain(s) unknown. We referred to this unknown factor as Factor X (8).

In the present study, we sought to identify Factor X in the OGD-induced neural stem cell system. To our knowledge, the molecular weight of Factor X should be less than 15 kDa. We extracted rIPC plasma dialysates through a C18 column followed by the freeze-drying, and subjected the concentrated solution to LC-MS/MS, resulting in the list of candidate proteins ranging 10–15 kDa (Table 1). Given the fact that OGD-induced cell death was related to oxidative stress, we focused on thioredoxin (12 KDa), even though other candidates may exist.

ROS affects cellular function though redox-based modification of proteins, particularly S-oxidation. Reversible redox-based modification of cysteine thiols regulates the activity of proteins to influence diverse cellular processes such as cell division and differentiation, metabolism, and cell death (37). While the mechanisms need to be defined more precisely, ROS markedly decreased the levels of TXNIP, which suggested that ischemic cells require an energy source to regenerate their ATP stores. Thus, it will be necessary to increase the glucose uptake into the cells by decreasing TXNIP (38). The antioxidant protein thioredoxin represents one of the major defenses against ROS (39). Accordingly, when NSCs were pretreated with thioredoxin prior to OGD, ROS-mediated cell death was significantly inhibited in vitro. The TXNIP-thioredoxin interaction has the potential to affect the regulation of mitochondrial bioenergetics and respiratory function and provide neuroprotection against ischemic injury.

The effect of thioredoxin on brain ischemia in animals should be further evaluated. However, the present study first evaluated the effect of rIPC in the ischemic cellular model, and identified thioredoxin as a possible Factor X that may protect heart and brain from ischemia.

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Figure legends

Figure 1. Extract of rIPC plasma dialysates through C18 column protects neural stem cells from apoptosis by oxidative stress.

The neural stem cells were then pretreated with the resulting dialysate solution from either the pre-rIPC or post-rIPC, and were subject to OGD. OGD induced apoptosis in approximately 21% and 16% of neural stem cells pretreated with buffer and pre-rIPC respectively. In contrast, the solution of freeze-dried extract from post-rIPC plasma dialysis protected NSCs from apoptosis significantly (B). Similarly, extracts of post-rIPC plasma dialysates prevented oxidative stress in OGD-induced cells (C). Scale bar, 200 μm (A), error bar, standard errors in four independent experiments (B, C)

Figure 2. Treatment of neural stem cells with thioredoxin.

Thioredoxin (final concentration of 10 μg/ml) was added directly to the culture medium for 10 min prior to OGD. Cultures were then placed in 2 ml of glucose-free DMEM and incubated for 24 h under OGD. Cells were then stained with TUNEL and RedoxSensor. OGD itself reduced cell growth, and approximately 14% and 8% of cells were stained with TUNEL and RedoxSensor, respectively. However, pretreatment with thioredoxin prior to OGD prevented NSCs from OGD-induced cell death and oxidative stress up to 2% and 0.5% as measured by TUNEL (B) and RedoxSensor (C) staining, respectively. Scale bar, 200 μm (A), error bar, standard errors in four independent experiments (B, C)

Figure 3. The effects of oxygen glucose deprivation (OGD) and thioredoxin on the levels of expression of thioredoxin-interacting protein (TXNIP) and the early detection of oxidative stress

The transcription of TXNIP was examined with quantitative polymerase chain reaction after 24 h of OGD. The levels of TXNIP were markedly decreased to 20% of the nonOGD control levels after 24 h of OGD, and thioredoxin pretreatment resulted in recovery of the TXNIP levels to approximately 70% (A). 2′-7′-dichlorofluorescin diacetate (DCFDA) flow cytometry was performed after 6 h of OGD. At 6 h, the number of DCFDA-labeled cells was already significantly increased, and thioredoxin returned the number of the labeled cells close to normal control levels (B). The error bars represent the standard errors of seven independent experiments (B).