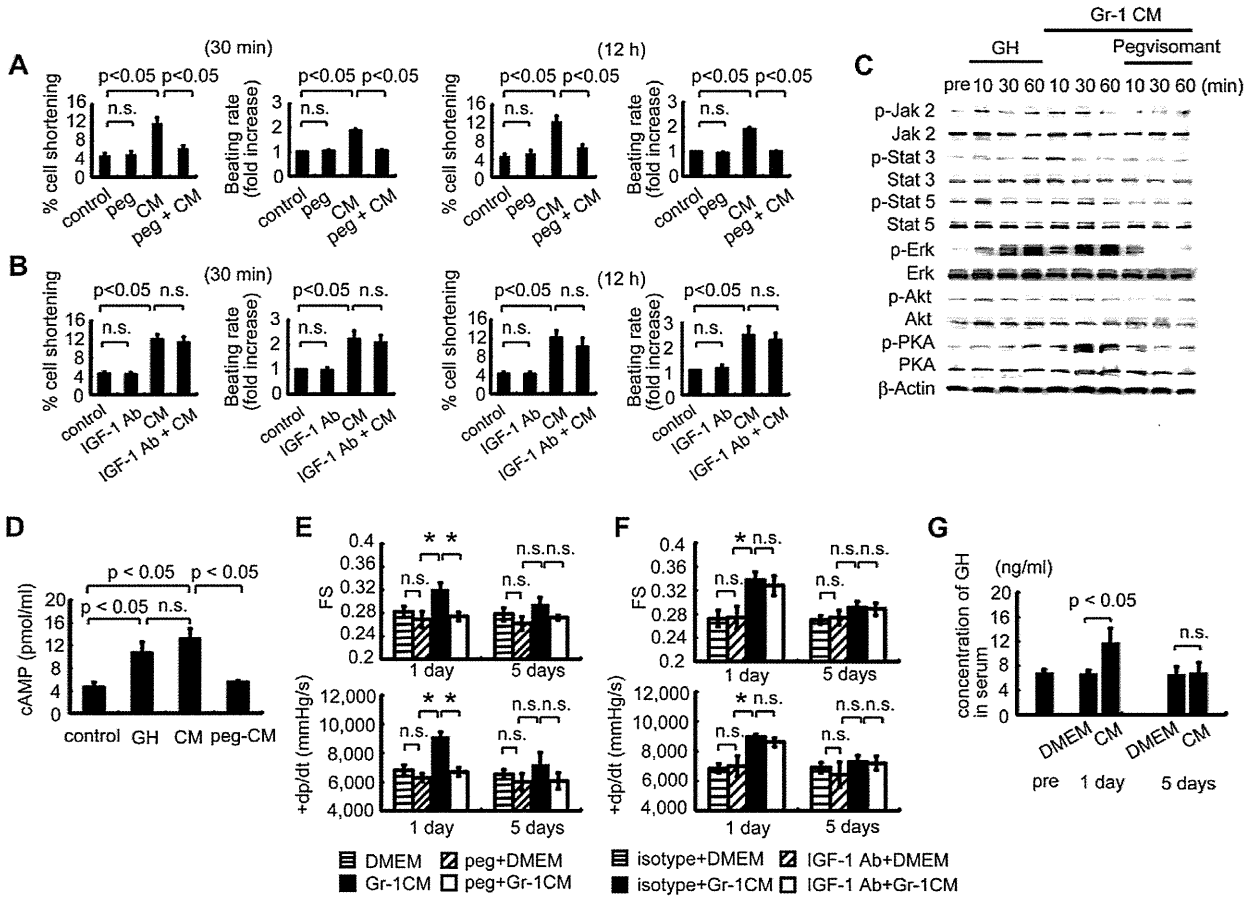

Figure 5. GH mediates the cardioprotective effects of Gr-1(+) cell-derived CM. (A) Pegvisomant (PEG) treatment inhibited the Gr-1(+) cell CM-mediated improvements cardiomyocyte cell shortening and beating rate at 30 min and at 12 h after treatment ($n=27$ cells per group). Left graphs, cell shortening; right graphs, beating rate. (B) Anti-IGF-1 antibody failed to affect the Gr-1(+) cell CM-mediated improvements in cell shortening or beating rate at 30 min or at 12 h after treatment ($n=23$ cells per group). Left graphs, cell shortening; right graphs, beating rate. (C) GH and CM from Gr-1(+) cells phosphorylated Akt, Erk, Jak2, Stat3/5 and PKA in cardiomyocytes ($n=3$), which was inhibited by pegvisomant ($n=3$). (D) GH (500 pg/ml) and CM from Gr-1(+) cells increased the cAMP concentration in cardiomyocytes ($n=5$), which was inhibited by pegvisomant ($n=5$). (E, F) Cardiac function analysis by echocardiography (upper graphs, $n=8$) and catheterization (lower graphs, $n=8$). Pegvisomant (E), but not anti-IGF-1 antibody (F), inhibited the improvements in FS and +dp/dt elicited by the infusion of CM from Gr-1(+) cells. *$p<0.05$ ($n=8$). (G) Serum GH concentrations in DOX mice treated with CM from Gr-1(+) cells ($n=4$ per group). The infusion of CM from Gr-1(+) cells from wild-type mice increased the serum GH concentration at 1 d, but not at 5 d. Data are means ± s.e.m.
doi:10.1371/journal.pone.0027901.g005

cells *in vitro*. These findings suggest that activin A, which is upregulated in heart failure, inhibits GH expression in various tissues/cells, including BMMNC. Treatment with anti-activin A antibody restored GH levels in Gr-1(+) cells and serum of EGFRdn mice and improved cardiac function, suggesting that normalizing the GH levels by inhibiting activin A is a novel therapeutic strategy for heart failure. Since many humoral factors such as AngII and TNFα are upregulated in heart failure and increased activin A expression by activating NFκB, the molecules that modulate NFκB activation might be also therapeutic targets to restore GH levels. On the other hand, anti-activin A treatment also increased expression levels of GH mRNA in the pituitary (N.F. K.M., unpublished data), suggesting that upregulation of activin A in heart failure might inhibit the expression of GH not only in Gr-1(+) cells but also in the pituitary, and that anti-activin A treatment might improve cardiac function of DCM mice in part by restoring GH expression in the pituitary.

The effects of GH on heart failure have been examined in many animal experiments and clinical trials [35]. A recent meta-analysis revealed that GH treatment improved several clinical parameters including left ventricular end-diastolic dimension, ejection fraction and New York Heart Association functional class [36]. Conversely, non-response to GH treatment for heart failure has been ascribed to GH resistance [37]. In patients with cardiac cachexia, GH levels were reported to be enhanced when compared with non-cachectic patients and normal subjects [38]. In this study, GH levels in heart failure mice and patients were significantly lower than those in healthy control subjects. Moreover, GH derived from Gr-1(+) cells improved cardiac function of heart failure animals, suggesting that our models were in a non-cachectic state and non-cachectic patients of heart failure might be suitable for GH treatment. Because of only temporary improvements in cardiac function (Figure 2A), bone marrow cell infusion might not be an appropriate treatment for heart failure, however inhibition

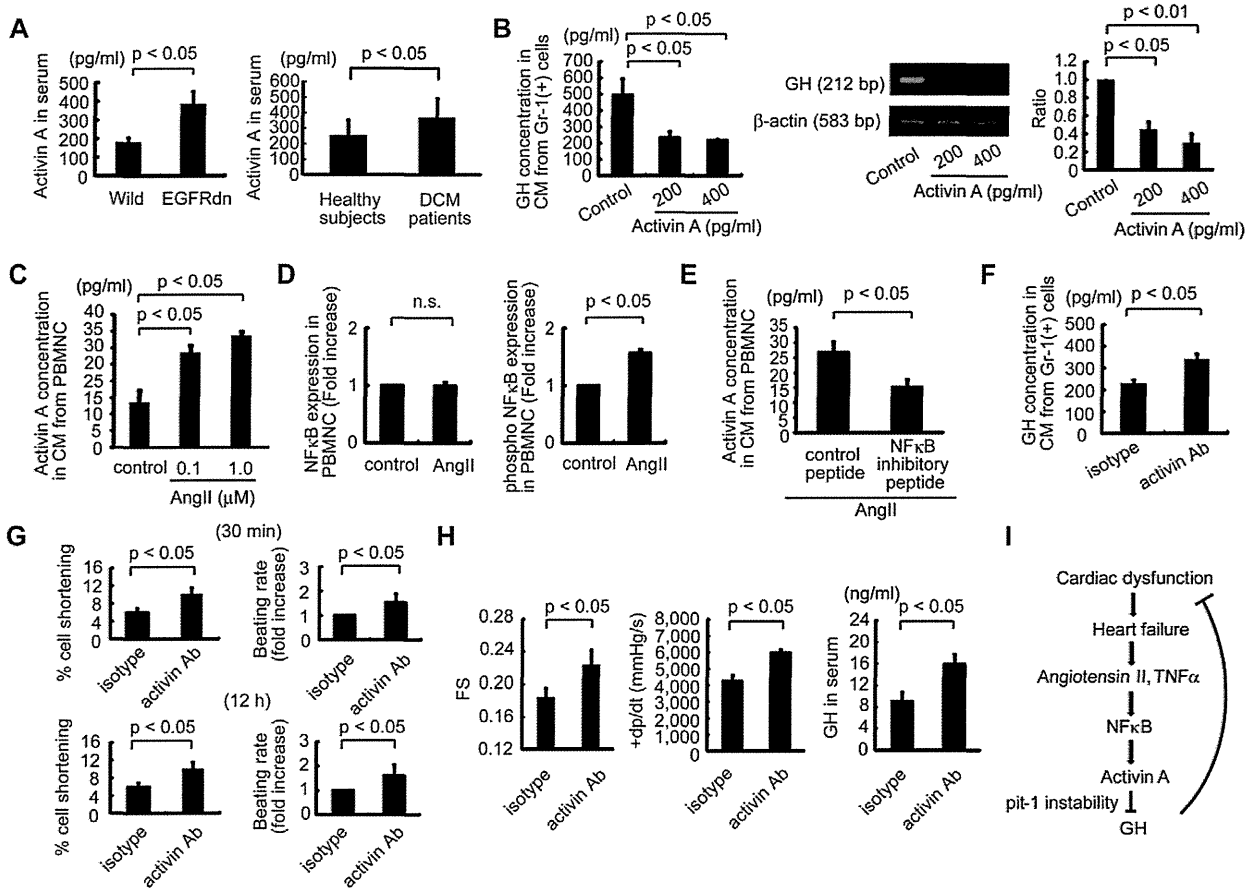

Figure 6. Regulatory mechanisms of GH in heart failure. (A) The serum activin A concentration was higher in EGFRdn mice (left, $n=5$) and in DCM patients (right, $n=10$) than in wild-type mice ($n=5$) and healthy subjects ($n=11$). (B) Activin A downregulated GH mRNA expression in Gr-1(+) cells and GH protein levels in Gr-1(+) cell CM. Left graph, GH protein concentration; middle photographs, representative semi-quantitative RT-PCR images; right graph, GH mRNA expression ($n=3$). (C, D) AngII upregulated activin A secretion (C, $n=4$) and phosphorylated NFκB expression (D, $n=5$) in wild-type PBMNC. (D) Left graph, total NFκB; right graph, phosphorylated NFκB. (E) Inhibition of NFκB [50 μM; NFκB p65 (Ser276) inhibitory peptide] suppressed AngII (10 μM)-mediated upregulation of activin A in CM derived from wild-type PBMNC ($n=5$). Isotype peptide was used as control. (F) The GH concentration in CM from EGFRdn Gr-1(+) cells ($n=5$) was significantly increased by treatment with an anti-activin A antibody ($n=5$). (G) Effects of anti-activin A antibody treatment on cell shortening and the beating rate of cardiomyocytes induced by CM from Gr-1(+) cells isolated from EGFRdn mice ($n=18$ cells per group). (H) Treatment with the anti-activin A antibody improved the cardiac function of EGFRdn mice. Left graph, echocardiography ($n=7$). Middle graph, miller catheter results ($n=7$). Right graph, serum GH concentration in EGFRdn mice after antibody treatment ($n=7$). Data are means ± s.e.m. (I) Proposed mechanism underlying impaired GH expression by activin A in heart failure.
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of activin A and enhancement of GH levels might offer novel therapeutic strategies for heart failure.

We used EGFRdn for DCM model mice in this study. It has been reported that cardiac-specific mutant of *ErbB2*, a member of the EGFR/erbB family, shows a severe dilated cardiomyopathy in mice [39]. In the clinical setting, trastuzumab, an anti-cancer agent, is humanized monoclonal antibody that targets the extracellular domain of the human epidermal growth factor receptor 2 and the use of trastuzumab demonstrated an unexpectedly high incidence of both asymptomatic and symptomatic cardiomyopathy. EGFRdn is a compatible DCM model mouse, resembling the cardiotoxic effects observed in patients treated with trastuzumab [40], [41].

There is a limitation in this study. We examined the surface area of neonatal rat cardiomyocytes after the treatment with CM from Gr-1(+) cells or BMMNC as an index for cardiac hypertrophy. However, the surface area not only depends on cell volume, but also on the degree of adhesion and spreading on the culture dishes.

Materials and Methods

Ethics Statement. The ethical committee of Tokyo Women's Medical University reviewed and approved the study protocol (approval ID: 1795). The study was conducted in accordance with the Declaration of Helsinki. We obtained informed consent from the all patients and the all healthy subjects by written before inclusion in this study.

Animals. Wild-type mice (C57BL/6) were purchased from Japan SLC. Adult GFP transgenic mice (C57BL/6) were a kind gift from Dr. M. Okabe (Osaka University). Cardiac-specific dominant-negative STAT3 mice were a kind gift from Dr. K. Yamauchi-Takihara (Osaka University). Neonatal Wistar rats (0–1 d old) were purchased from Saitama Experimental Animals Supply. All protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University and Chiba University. The approval IDs for the animal experiments were 11–34 in Tokyo Women's Medical University

and A21–178 in Chiba University. Doxorubicin (10 mg/kg body weight) was intraperitoneally injected into wild-type male mice (C57BL/6) once-weekly at weeks 7 and 8 after birth. After both Doxorubicin injections, the mice were reared for a further 2 weeks, and the surviving mice were used for experiments. Myocardial infarction models were prepared using wild-type male mice (C57BL/6) as previously described [11]. Serum and Gr-1(+) cells were isolated 4 weeks after inducing myocardial infarction (11 weeks of age).

Generation of EGFRdn mice. The C-terminal 533 amino acids [42] were deleted from the full-length human *EGFR* cDNA (a gift from Professor T. Kadowaki, The University of Tokyo) by introducing a stop codon (TGA) after the R677 codon by site-directed mutagenesis. The truncated *EGFR* (*EGFRdn*) cDNA was then subcloned into the *αMHC* promoter-containing expression vector (a gift from Professor J. Robbins, Cincinnati Children's Hospital). The 8.2-kb DNA fragment was microinjected as a transgene into pronuclei of eggs from BDF1 mice. The eggs were then transferred into the oviducts of pseudopregnant ICR mice. The transgenic founders were identified by Southern blot and PCR analysis. Line 2–5 and Line 9–12 were established and maintained by breeding to C57BL/6 mice. Line 9–12 was selected for further analysis on the basis of a higher level of transgene expression.

BMMNC infusion and CM injection. BMMNC (2.0×10^7) isolated from a male wild-type mouse and suspended in 200 μl of PBS or an equal volume of PBS as a control were injected into the tail veins of anesthetized (4% inhaled isoflurane) 8-week-old male EGFRdn mice and 11-week-old male DOX and OMI mice. CM (200 μl) from Gr-1(+) cells from male wild-type mice or isovolume serum-depleted DMEM were infused into the tail veins of anesthetized 8-week-old male EGFRdn mice and 11-week-old male DOX mice under anesthesia. Anti-mouse insulin-like growth factor-1 (IGF-1) (0.1 μg/g body weight) or anti-goat immunoglobulin G (IgG) (0.1 μg/g body weight) antibodies were intraperitoneally injected into 11-week-old male DOX mice 2 h before CM infusion. Anti-activin A (20 μg) or anti-mouse IgG (20 μg) antibodies were intraperitoneally injected at 48-h intervals into male EGFRdn mice from 10 to 12 weeks of age. Pegvisomant (10 mg/kg body weight) or vehicle (control) were intraperitoneally injected into 8-week-old male DOX mice 30 min before CM infusion.

Evaluation of cell shortening and the beating rate of cardiomyocytes. After 12 h starvation with 500 μl serum-depleted DMEM in 12-well dishes, rat cardiomyocytes were cultured with 500 μl of CM or serum-depleted DMEM. At specific times, the cultured cardiomyocytes were video recorded for 10 sec, and the percentage of cell shortening was analyzed using ImageExpress version 5.5 (Nippon Roper). To measure the percentage of cell shortening, two regions of interest were fixed by the software, which analyzed the beating distance of a single cardiomyocyte, and divided the distance by the length between the regions of interest. The number of beats of single cardiomyocyte was counted for 10 sec to determine the beating rate. For antibody treatment *in vitro*, the starved cardiomyocytes were pretreated with anti-IGF-1 (10 μg/ml) or anti-goat IgG (10 μg/ml) antibodies for 2 h before adding CM. For pegvisomant treatment *in vitro*, the cardiomyocytes were pretreated with pegvisomant (12.5 μg/ml) for 30 min before adding CM.

Echocardiography and catheterization. Transthoracic echocardiographic analysis and catheterization analysis were performed as previously described [11]_ENREF_9. Briefly, the +dp/dt in the left ventricle was measured using a catheter, which was introduced retrogradely *via* the carotid artery.

Cell isolation. Neonatal rat cardiomyocytes were isolated and separately collected as described previously [43]. Cardiomyocytes were plated at a density of 1×10^5 cells/cm^2 on six-, 12- and 24-well dishes (BD Falcon) coated with 1% gelatin and cultured in DMEM supplemented with 10% FBS. Adult cardiomyocytes were prepared as previously described [44]. BMMNC and PBMNC were isolated from 8-week-old male C57BL/6, male GFP mice, and male EGFRdn mice by density gradient centrifugation with Histopaque-1083, as previously described [45]. PBMNC were also isolated from human subjects, as previously described [46].

Sorting of harvested BMMNC into sub-populations and collection of CM. After BMMNC were harvested from male wild-type mice, the cells were sorted into Gr-1(+) cells, B220(+) cells, TER(+) cells, and lineage-negative populations using a Magnetic Cell Sorting system (Miltenyi Biotec), as previously described [47]. To collect the CM, the individual sub-populations were seeded onto 24-well dishes with 200 μl of serum-depleted DMEM. After incubation for 24 h in serum-depleted DMEM, the supernatant (CM) was collected, and any cells were removed by filtering through a 0.45-μm filter (BD Falcon).

Phase-contrast live imaging. Live images of beating cardiomyocytes were taken using a Leica inverted microscope (Leica) equipped with a phase-contrast objective and a CCD camera (Leica).

Flow cytometry. The percentage of cells expressing each cell surface antigen was analyzed using a FACSCalibur (Becton Dickinson Immunocytometry Systems) and Cell Quest Pro version 5.2 software.

RNA extraction and DNA microarray analysis. Total RNA was extracted from 12-week-old male wild-type ($n=4$) and age-matched male EGFRdn mice ($n=4$) using a RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). cRNA preparation, fragmentation, hybridization, and scanning of a GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix) were performed according to the manufacturer's protocol. cRNA was labeled using a Two-cycle Eukaryotic Target Labeling assay with a GeneChip Expression 3′ amplification two-cycle labeling and control reagents kit (Affymetrix). Briefly, cDNA was generated from total RNA (100 ng) using SuperScript II (Invitrogen) and a T7-oligo(dT) promoter primer (Affymetrix). After second-strand cDNA synthesis, cDNA was converted to cRNA by an *in vitro* transcription reaction (MEGAscript T7 kit, Ambion). The cRNA was then purified using a Sample Cleanup Module (Affymetrix), and the yield was monitored with a spectrophotometer. The second cycle of cDNA synthesis was performed, followed by the same cleanup as above and a second *in vitro* transcription reaction cycle with biotin-labeled ribonucleotides and T7 RNA polymerase. The labeled cRNA was purified, using a Sample Cleanup Module and denatured at 94°C before hybridization. The samples were hybridized to GeneChip® Mouse Genome 430 2.0 Arrays at 45°C for 16 h with rotation at 60 rpm. The arrays were then washed, stained with phycoerythrin–streptavidin (Molecular Probes), washed, and scanned with a GeneChip Scanner 3000 7G (Affymetrix). The data were analyzed with GeneSpring version 7.3.1 software (Agilent Technologies).

Reverse transcriptase-PCR. RNA extraction and RT-PCR were performed as previously described [11]. Real-time PCR amplification was performed using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems) with QuantiTect SYBR Green PCR Master Mix (Qiagen). The PCR protocol comprised an initial denaturation step (94°C, 15 sec) followed by 60 cycles of amplification and quantification (55°C for 30 sec and 72°C for 35 sec) and a melting curve program (60–95°C). The

relative mRNA expression level was calculated using the standard curve of GAPDH. All samples were independently analyzed at least three times for each gene. Semi-quantitative RT-PCR of GH was performed using 0.4 μg of total RNA and followed by 40 cycles of the above conditions. The primer sequences were QT00311654 (Qiagen) for GH in real-time PCR, 5′-TCCTGTGGACAGATCACTGC-3′ and 5′-AATGTAGGCACGCTCGAACT-3′ for GH in semi-quantitative PCR, QT00309099 (Qiagen) for GAPDH, and 5′-GGACCTGGCTGGCCGGGACC-3′ and 5′-GCGGTGCACGATGGAGGGGC-3′ for β-actin. For semi-quantitative RT-PCR, the PCR products were size-fractionated by 2% agarose gel electrophoresis.

Northern blot analysis. For northern blot analysis, total RNA (20 μg) was extracted from hearts using TRIzol Reagent (Invitrogen) and hybridized with a cDNA probe for *EGFRdn*. 18S rRNA ethidium bromide staining was used to quantify RNA loading.

Analysis of phosphorylated ErbB receptor expression. Four-week-old mice were anesthetized by intraperitoneal injection of urethane (2 mg/g body weight) followed by intravenous injection of HB-EGF (0.5 μg/g body weight, R&D Systems), NRG-1β (0.5 μg/g body weight, R&D Systems), or vehicle *via* the inferior vena cava. After 5 min, the hearts were immediately excised and homogenized in a buffer containing 50 mmol/l HEPES (pH 7.5), 137 mmol/l NaCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10 mmol/l Na-pyrophosphate, 2 mmol/l EDTA, 1% NP-40, 10% glycerol, 2 mmol/l Na_3VO_4, 10 mmol/l NaF, and protease inhibitor cocktail (Complete Mini, Roche Applied Science). To analyze the tyrosine phosphorylation of ErbB receptors, equivalent amounts of proteins were subjected to immunoprecipitation with the specific antibodies, fractionated by 6% SDS-PAGE, and immunoblotted with the mouse monoclonal anti-phosphotyrosine antibody 4G10 (Millipore). Horseradish peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare) was used as the secondary antibody, and the bound antibodies were detected using an ECL detection kit (GE Healthcare).

ELISA. Serum and CM concentrations of cAMP, GH and activin A were measured by ELISA (cAMP and activin A, R&D Systems; GH, LINCO Research). To prepare cell lysates for cAMP analysis, cardiomyocytes were seeded (4×10^5 cells/cm) onto six-well dishes coated with 1% gelatin and cultured in DMEM supplemented with 10% FBS. After 5 d, the cells were washed three times with PBS and the medium was changed to serum-depleted DMEM. After incubation for 12 h in the serum-depleted medium, the cells were washed three times with PBS and the medium was replaced with 1 ml of serum-depleted DMEM with CM (1 ml), 2 ml of serum-depleted DMEM with 500 pg/ml GH, 2 ml of serum-depleted DMEM with 12.5 μg/ml pegvisomant, or 1 ml of serum-depleted DMEM plus 1 ml of CM and 12.5 μg/ml pegvisomant. Thirty minutes later, the cardiomyocytes were resuspended in lysis buffer in six-well dish.

To examine the expression of NFκB and phosphorylated NFκB in PBMNC, PBMNC isolated from wild-type male mice were cultured with AngII or TNFα. Thirty minutes later, PBMNC were resuspended in lysis buffer and the expression of NFκB and phosphorylated NFκB were examined using sandwich ELISA kits (Cell Signaling). Some cells were also treated with 50 μM NFκB p65 (Ser276) inhibitory peptide to inhibit NFκB activity.

Western blot analysis. Whole-cell lysates (30–50 μg) were resolved by SDS-PAGE. The separated proteins were transferred to a PVDF membrane (GE Healthcare) and incubated with the primary antibody, followed by an anti-IgG-horseradish peroxidase-conjugated secondary antibody. Proteins were detected using an ECL-Plus kit (GE Healthcare).

Immunohistology. The hearts were fixed with 4% paraformaldehyde and embedded in paraffin, or fixed in 10% neutralized formalin and embedded in Tissue-Tek OCT cryo-embedding compound (Sakura Finetek). The specimens were sectioned (5 μm thick), and stained with hematoxylin/eosin or Masson trichrome.

Evaluation of cardiac hypertrophy. To evaluate the mean diameter of LV cardiomyocytes, the shortest diameter of each cardiomyocyte was measured in nucleated transverse sections stained with hematoxylin-eosin. Thirty cardiomyocytes in each LV were measured using an ocular micrometer disc with a linear scale at a magnification of 400×, and the average cardiomyocyte diameter was calculated for each specimen. Four hearts were measured in each group. The cell surface area of isolated neonatal and adult cardiomyocytes was measured by planimetry in 50 randomly selected cells per specimen.

Immunofluorescence staining. Immunostaining was performed as previously described [45]. Images were taken using a fluorescent microscopy (Leica) with LAS AF software (Leica).

Human subjects. We enrolled 10 subjects who were outpatients of Department of Cardiology of Tokyo Women's Medical University Hospital. We obtained 10 ml of whole blood from each patient. Half of the blood sample was used to measure the serum activin A concentration and the remaining blood was used to measure GH in CM after PBMNC isolation. All patients were receiving medical therapies and exhibited New York Heart Association class II symptoms. We also enrolled 11 healthy age- and body mass index-matched volunteers. Characteristics of the patients and healthy subjects are summarized in Table S1.

Statistics. Data are presented as means ± s.e.m. We examined differences between groups by Student's *t* test or analysis of variance followed by Bonferroni's correction to compare means. A value of $P<0.05$ was considered to be significant.

Supporting Information

Figure S1 Overexpression of EGFRdn inhibited the functional activation of endogenous ErbB receptors in a dominant-negative manner. (A) Northern blot analysis for the transgene expression in hearts from wild-type and two different founder lines of EGFRdn mice (L2–5 and L9–12). (B) Tyrosine phosphorylation of ErbB receptors in hearts from wild-type and EGFRdn mice (L9–12) at 5 min after injection of HB-EGF. In wild-type mice, intravenous injection of HB-EGF enhanced cardiac tyrosine phosphorylation of EGFR, ErbB2 and ErbB4, which was abrogated in EGFRdn hearts. HB-EGF, heparin-binding EGF-like growth factor. (C) Tyrosine phosphorylation of ErbB receptors in hearts from wild-type and EGFRdn mice (L9–12) at 5 min after the injection of NRG-1β. NRG-1β induced tyrosine phosphorylation of ErbB2 and ErbB4 in wild-type hearts, but not in EGFRdn hearts. NRG-1, neuregulin-1.
(TIF)

Figure S2 Echocardiographic analysis of DOX mice. (A) Representative M-mode images of wild-type and DOX mice. (B) Left ventricular diastolic and systolic dimensions, and FS of 11-week-old DOX mice ($n=36$) and age-matched wild-type mice ($n=10$). LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension. Data are means ± s.e.m.
(TIF)

Figure S3 Analysis of cardiac hypertrophy. (A) The shortest diameter of each cardiomyocyte ($n=30$ per group). Lower photographs, H&E-stained tissue sections. Scale bar, 75 μm. (B) Surface area of isolated adult cardiomyocytes ($n=50$ per group).

Lower photographs, representative images. Scale bar, 75 μm. Data are means ± s.e.m.
(TIF)

Figure S4 Flow cytometric analysis. The left and right panels show the expression of each cell surface marker before and after magnetic sorting (MACS), respectively.
(TIF)

Figure S5 Cardiac hypertrophy *in vitro*. Upper graph, cell surface area of neonatal rat cardiomyocytes ($n = 50$); lower photographs, representative images of the cells. Cardiomyocytes were stained with sarcomeric α-actinin (red). Nuclei were stained with Hoechst 33258 (blue). Scale bars, 75 μm. Data are means ± s.e.m.
(TIF)

Figure S6 Comparison of GH concentration. GH concentration in CM from Gr-1(+) cells isolated from old myocardial infarction (OMI) mice and DOX mice ($n = 5$). Data are means ± s.e.m.
(TIF)

Figure S7 BMMNC improve the cardiac function of OMI mice via the GH receptor. (A) At 4 weeks after coronary ligation, BMMNC were infused via the tail vein. Pegvisomant (10 mg/kg body weight) or vehicle (control) was intraperitoneally injected into OMI mice 30 min before infusing BMMNC. BMMNC infusion improved FS and +dp/dt at 3 d after infusion and these improvements were inhibited by pegvisomant (n = 5). (B) Masson trichrome staining. Panels show representative images. Scale bars: 1 mm. Data are means ± s.e.m.
(TIF)

Figure S8 Direct effects of GH in the CM from Gr-1(+) cells on cardiomyocytes. CM from Gr-1(+) cells from wild-type mice was infused into DOX-treated wild-type mice (wild-DOX) or DOX-treated cardiac-specific STAT3dn mice (STAT3dn-DOX). Gr-1(+) cell-derived CM improved FS (left) and +dp/dt (right) in wild-DOX mice ($n = 5$) at 1 d after infusion, but not in STAT3dn-DOX ($n = 5$). Data are means ± s.e.m.
(TIF)

Figure S9 Serum activin A concentrations ($n = 5$). Data are means ± s.e.m.
(TIF)

Figure S10 TNFα increases the secretion of activin A from PBMNC *via* NFκB. (A) Activin A levels in CM from PBMNC were upregulated by treatment with TNFα ($n = 5$). (B) TNFα (50 ng/ml) activated NFκB in PBMNC ($n = 5$). Left, total NFκB; right, phosphorylated NFκB. (C) TNFα (50 ng/ml) -mediated upregulation of activin A in PBMNC was inhibited by treatment with the NFκB inhibitory peptide ($n = 5$). Isotype peptide was used as control. Data are means ± s.e.m.
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Table S1 Characteristics of human subjects.
(PDF)

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Author Contributions

Conceived and designed the experiments: KM IK. Performed the experiments: NF KM HA AH TN TT AS KMM. Analyzed the data: NF KM HA. Contributed reagents/materials/analysis tools: TS TO. Wrote the paper: NF KM NH IK.

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Review

Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures

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Sanada S, Komuro I, Kitakaze M. Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures. *Am J Physiol Heart Circ Physiol* 301: H1723–H1741, 2011. First published August 19, 2011; doi:10.1152/ajpheart.00553.2011.—Heart diseases due to myocardial ischemia, such as myocardial infarction or ischemic heart failure, are major causes of death in developed countries, and their number is unfortunately still growing. Preliminary exploration into the pathophysiology of ischemia-reperfusion injury, together with the accumulation of clinical evidence, led to the discovery of ischemic preconditioning, which has been the main hypothesis for over three decades for how ischemia-reperfusion injury can be attenuated. The subcellular pathophysiological mechanism of ischemia-reperfusion injury and preconditioning-induced cardioprotection is not well understood, but extensive research into components, including autacoids, ion channels, receptors, subcellular signaling cascades, and mitochondrial modulators, as well as strategies for modulating these components, has made evolutional progress. Owing to the accumulation of both basic and clinical evidence, the idea of ischemic postconditioning with a cardioprotective potential has been discovered and established, making it possible to apply this knowledge in the clinical setting after ischemia-reperfusion insult. Another a great outcome has been the launch of translational studies that apply basic findings for manipulating ischemia-reperfusion injury into practical clinical treatments against ischemic heart diseases. In this review, we discuss the current findings regarding the fundamental pathophysiological mechanisms of ischemia-reperfusion injury, the associated protective mechanisms of ischemic pre- and postconditioning, and the potential seeds for molecular, pharmacological, or mechanical treatments against ischemia-reperfusion injury, as well as subsequent adverse outcomes by modulation of subcellular signaling mechanisms (especially mitochondrial function). We also review emerging translational clinical trials and the subsistent clinical comorbidities that need to be overcome to make these trials applicable in clinical medicine.

calcium overload; reactive oxygen species; mitochondria; transition pore; comorbidities; clinical trials

WHAT DO WE KNOW ABOUT ischemia-reperfusion injury? Because the morbidity and mortality due to ischemic heart diseases have come to the fore in developed countries and are still increasing, it is critically important, both scientifically and socially, to know how cardioprotection is achieved in ischemic myocardium. In fact, in the clinical setting, the application of coronary thrombolysis or immediate percutaneous coronary intervention for faster recanalization has been shown to dramatically improve the outcomes of patients with acute or chronic myocardial ischemia due to impaired coronary blood supply. This finding is founded on the premise that a shorter period of index ischemia causes less damage (212).

However, even if the ischemic period is short or limited, the functional recovery of a reperfused heart is often less successful than expected due to "reperfusion injury" (188), and we still do not have a definitive intervention to eliminate reperfusion-induced myocardial damage. Therefore, it is important to fully understand the mechanisms of ischemia-reperfusion injuries and to consider cardioprotective strategies.

Dynamic Sequence of Ischemia-Reperfusion Injury

ATP depletion as the original hypothesis. To maintain cellular homeostasis, the intracellular use of both ATP and high-energy phosphates is critically important. Cellular energy metabolism depends on acetyl-CoA, which is generated through aerobic/anaerobic glycolysis or β-oxidation of free fatty acids and is then metabolized through the tricarboxylic acid cycle, which supplies ATP. Cardiomyocytes are rich in mitochondria because the highest continuous amount of ATP is consumed within the myocardium, and this demand for ATP can only be met by aerobic metabolism. When hearts are exposed to ischemia, coronary arterioles and resistant vessels significantly

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dilate to increase coronary blood flow up to three to five times above basal levels and to supply as much oxygen as possible and maintain aerobic needs. However, because of the lack of anaerobic metabolic pathways, the absence of a supply of oxygen leads to the depletion of intramyocardial ATP in a short period of time, making the myocardium very susceptible to ischemia.

Initial studies on ischemic myocardium revealed that contractile arrest generally occurs within several minutes of index ischemia, followed by cellular bulging and rupture of intracellular microstructures starting 15 to 30 min later (4). This discovery led to the ATP depletion hypothesis as a central cause of cell death because a 90% decrease in ATP results in irreversible structural changes in the myocardium (4). Therefore, supplementation of ATP in the myocardium was initially proposed as an effective therapy for the prevention of ischemic myocardial death.

However, a complete depletion of ATP within the myocardium takes ~40–60 min, whereas the intracellular inorganic phosphate level promptly increases just after the onset of ischemia (120), suggesting the rapid exhaustion of intracellular high-energy phosphate. Therefore, it was then thought that a change in the intracellular pH was a trigger for this lethal cascade.

Decreasing pH and counterbalancing Ca^{2+} accumulation. Anaerobic glycolysis prevails in ischemic myocardium, causing a rapid decrease in the intracellular pH and deactivation of troponin C sensitivity to phosphofructokinase and Ca^{2+}. Within several minutes, this decrease results in contractile arrest and cellular bulging. To buffer this decrease in pH, excessive H^+ is excreted by accelerated Na^+/H^+ exchange, which in turn causes substantial Na^+ inflow (152). Meanwhile, intracellular ATP depletion gradually inactivates ATPases, such as the Na^+/K^+ ATPase, ATP-dependent Ca^{2+} reuptake, and active Ca^{2+} excretion, resulting in Ca^{2+} overload. These results have also been confirmed in vivo (121). These changes are also accompanied by a subsequent activation of intracellular proteases, such as calpain, which causes a fragile cellular structure, hypercontracture, which leads to contraction band necrosis, and the initiation of apoptotic cascades (Fig. 1) and mitochondrial transition pore opening, which will be discussed later. Each of these factors can occur within minutes, but they proceed gradually because a low intracellular pH slows or inhibits all of them (76). This idea agrees with the observation that reoxygenation within 5 min avoids irreversible cellular damage, whereas index ischemia for more than 15 min gradually affects intracellular structures (123).

Rapid normalization of pH and overload of Ca^{2+} and reactive oxygen species upon reperfusion. Prompt reperfusion or reoxygenation will bring about a rapid restoration of substrates essential for the generation of ATP, such as glucose or free fatty acids, an instantaneous increase in the oxygen supply, and prompt normalization of the extracellular pH by pericellular washout. Indeed, all of these factors are crucial for the prevention of further ischemic cellular injury and for restoration of cellular homeostasis; however, they can also concurrently cause reperfusion injury (188). Rapid normalization of the extracellular pH will instantly create an extreme H^+ gradient across the plasma membrane that triggers a robust Na^+/H^+ exchange and a massive Na^+ influx. This unphysiological gradient can instantly trigger the passive, inverted action of the surface Na^+/Ca^{2+} exchanger, called "reverse mode," which absorbs Na^+ accumulation via excretion but, in turn, allows intracellular Ca^{2+} overload (179) (Fig. 2). Meanwhile, rapid normalization of intracellular pH disinhibits a low pH-derived inhibition of the Ca^{2+}-dependent protease calpain, hypercontracture, and the mitochondrial permeability transition all at once and promptly accelerates myocardial damage in the early stages of reperfusion (76). This also occurs when, upon reoxygenation, ectopic xanthine oxidase is activated by Ca^{2+}-sensitive proteases (89) and the intramitochondrial respiratory chain. This activation causes a sudden recovery of aerobic metabolism and results in an overload of reactive oxygen species (ROS), mainly superoxide. Physiologically, superoxide becomes hydrogen peroxide via superoxide dismutase (SOD) (53), which is then inactivated by catalase and becomes H_2O and O_2. However, robust ROS generation beyond this catalytic process generates excessive hydroxyl radicals, which are very unstable but have a high potential to damage cellular structures, enzymes, or channel proteins on the cellular membrane (200). These events, together with activation of inflammatory cascades and facilitation of bioactive autacoids, such as cytokines or catecholamines, make cells more susceptible to death (Fig. 3) or myocardial contractile

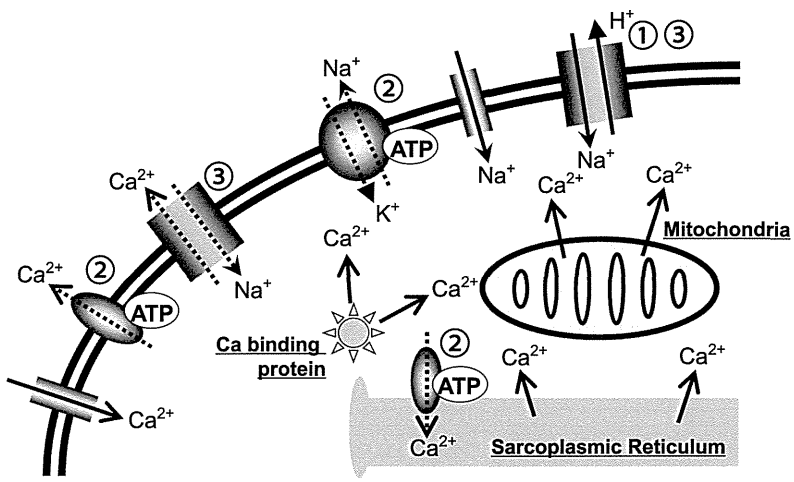

Fig. 1. Ion exchanges during ischemia: *1*) excretion of H^+ due to pH lowering, *2*) deactivation due to loss of ATP, and *3*) reduction of Na^+/Ca^{2+} exchange due to lowered extracellular pH and intracellular accumulation of Na^+.

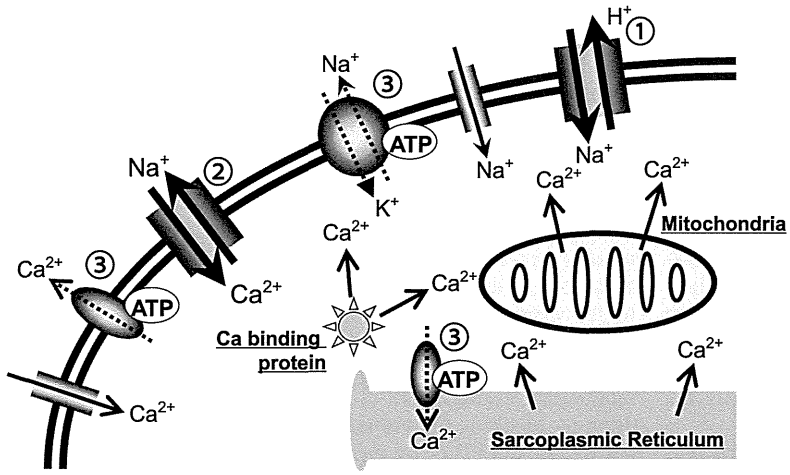

Fig. 2. Ion exchanges at reperfusion: *1*) robust excretion of H^+ due to prompt recovery of extracellular pH, *2*) "reverse mode" excretion of accumulated Na^+ and Ca^{2+} influx in turn, and *3*) reexcretion of Ca^{2+} followed by recovery of ATP synthesis.

dysfunction (183) immediately after the onset of reperfusion. Furthermore, impaired intracellular Ca^{2+} and ROS regulation can propagate to adjacent cells through gap junctions to further spread injury (159). Finally, after 30–60 min of reperfusion, a gradual recovery of Ca^{2+} excretion and ATP-dependent Ca^{2+} reuptake in sarcoplasmic reticulum takes place, and the cells return to homeostasis. This ischemia-reperfusion process makes the intracellular Ca^{2+} concentration dual peaked (120), with one peak occurring at 15–60 min after the onset of index ischemia and the other peak occurring within 30 min of reperfusion.

Various modes of death and their regulation under ischemia-reperfusion. During the above sequence of events, Ca^{2+} overload and excessive ROS generation can trigger multiple modes of cell death, such as necrosis and apoptosis (168). Myocardial necrotic changes generally include cellular swelling as an initial change, followed by rupture of cellular membranes, degradation of intracellular proteins or structures induced by Ca^{2+}-dependent proteases such as calpain (89), Ca^{2+}-induced hypercontracture [which induces mechanical rupture of muscle fibers (159)], and direct cleavage of DNA by free radicals originating from excessive ROS. Necrotic changes often require focal recruitment of inflammatory cells for subsequent scavenging activity.

Apoptosis is usually triggered by intracellular Ca^{2+} overload, which induces the processing of procaspase-8 into active caspase-8 and the activation of Bax, which lead to the release of the apoptosis-inducing factor, Smac, and cytochrome-*c* from mitochondria. Apoptosis-inducing factor translocates into the nucleus and facilitates nonspecific DNA fragmentation. Smac inactivates X chromosome-linked inhibitor of apoptosis protein, which inhibits caspase-3, and cytochrome-*c* forms an apoptosome complex with procaspase-9 and apoptotic protease-activating factor-1, which activates caspase-9. Together, these cascades ultimately contribute to irreversible cellular dysfunction. Ca^{2+} accumulation (57) and ROS induction (175) are also crucial for opening the pore that enables trafficking of nonspecific, small molecules across mitochondrial membranes. Subsequently, H^+ influx into mitochondria and a decrease in the mitochondrial membrane potential result in mitochondrial

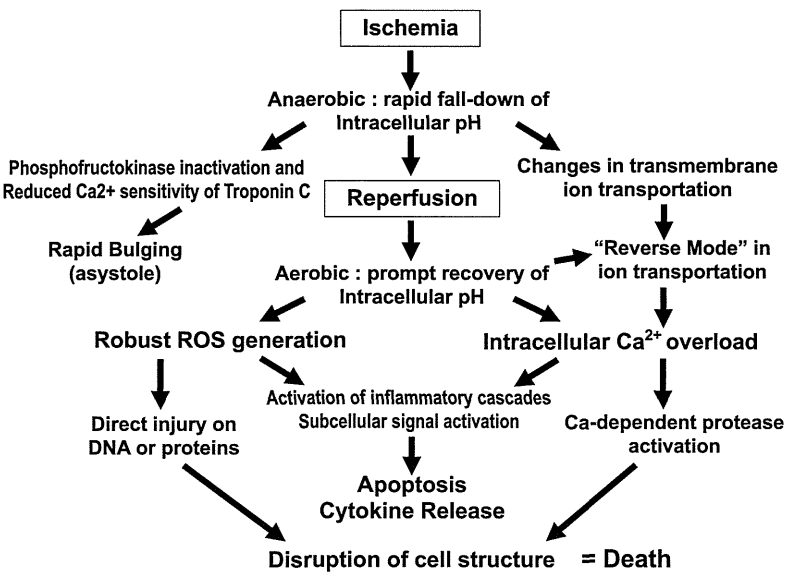

Fig. 3. Putative cascades of cell death due to ischemia-reperfusion injury. ROS, reactive ocygen species.

swelling and apoptotic changes (66). Histological findings of apoptosis include nuclear chromatin condensation, making cells nonfunctional, followed by cellular shrinking and fragmentation, usually independently of the inflammatory reaction, and subsequent phagocytotic clearance (7).

Necrosis prevails within the ischemic myocardium and its adjacent regions, whereas apoptosis predominantly occurs in the ischemic border and in nonischemic regions (202), reflecting the differences in the focal magnitude of anoxia, ROS accumulation, neurohormonal activation, and mechanical stress. Recent reports propose that necrotic cell death prevails when the intramitochondrial Ca^{2+} level becomes extremely low or completely lost (14). Similarly, the intracellular ATP level might also serve as a "molecular switch," with high levels resulting in apoptosis and low levels resulting in necrosis (105).

Adaptation and therapeutic targets for ischemia-reperfusion injury. Because the prompt recovery of intracellular pH and reoxygenation enhances Ca^{2+} overload or ROS generation and promotes reperfusion injury as described above in *Rapid normalization of pH and overload of Ca^{2+} and reactive oxygen species upon reperfusion* and *Various modes of death and their regulation under ischemia-reperfusion*, it has long been proposed that the transient acidosis during early reperfusion (97) as well as acidic or staged reperfusion procedures (67) buffer the Ca^{2+} or ROS overload upon reperfusion. They can lead to a smaller infarct size or preservation of myocardial function (96, 97) by inhibiting Na^+/Ca^{2+} exchange and Ca^{2+} overload (91) or by directly disrupting proapoptotic signals (229), all of which might be important therapeutic targets of cardioprotection. Accordingly, some of the membrane ion channels, such as the Na^+/H^+ exchanger (228), the Na^+/K^+ ATPase (59), and the Ca^{2+}-activated K^+ channel (185), are possible candidates for cardioprotection due to preconditioning because of their ability to directly inhibit intracellular Ca^{2+} accumulation.

Another key therapeutic target might be excessive ROS production or prolonged catecholamine release. When myocardial ischemia occurs, presynaptic vesicles release norepinephrine via the accumulation of Na^+ and subsequent activation of reverse uptake-1 (180), which facilitates norepinephrine release. Norepinephrine activates both α-adrenoceptors that cause vasoconstriction and β-adrenoceptors that increase myocardial oxygen consumption. Both of these may make the cells more susceptive to ischemic damage. Indeed, many reports show that the blockade of the β-adrenoceptor will protect the myocardium from prolonged ischemia-reperfusion. However, possible pharmacological mechanisms other than hemodynamic benefits are under discussion (9, 180, 238).

On the other hand, there are a number of adaptive responses to the various kinds of stresses induced by ischemia-reperfusion, i.e., Ca^{2+} overload, ROS accumulation, neurohormonal stimuli, focal mechanical strain, and endoplasmic reticulum stress from excessive production of unfolded or misfolded proteins. Autophagy, the lysosomal bulk digestion pathway of intracellular structural proteins, contributes to physiological turnover as well as pathological removal of intracellular proteins as a housekeeping system. The process of autophagy begins by recruiting Atg-5, -12, and -16 to the intracellular lipid bilayer. These proteins are then polymerized by LC-3 to form the autophagosome. The autophagosome sequesters proteins targeted for destruction and then fuses with the protease-rich lysosome, resulting in digestion of its contents. Mitochondria also have an adaptive system involving dynamic fusion and "fission" with each other (222). A shift toward fusion favors the generation of interconnected mitochondria that form large networks and are beneficial in metabolically active cells against the dissipation of energy. Alternatively, a shift toward fission produces numerous mitochondrial fragments as morphologically and functionally distinct small spheres or short rods with an increased distribution and surface area, which is usually beneficial in quiescent cells (222). Therefore, the facilitation of autophagy (69) and mitochondrial fusion (141) might also protect against ischemia-reperfusion injury by maintaining intracellular homeostasis. In addition, the blockade of cell-to-cell connections, such as gap junctions, by modulating connexin-43 (136) could also protect the myocardium by blocking the propagation of cellular damage to neighboring cells (159) or by modulating mitochondrial ATP-sensitive K^+ (K_{ATP}) channel opening (164).

Discovery of Preconditioning

Before the concept of ischemic preconditioning arose, cardiologists observed that patients with severe, unstable angina or acute myocardial infarction and who had experienced at least one episode of prodromal angina often exhibited less chest pain, less variation in the ST segment of ECGs, less cardiac dysfunction, or even smaller myocardial infarct size. This was despite a paradoxical increase in the total period of time suffered from ischemia, and they called this the "cardiac warm-up phenomenon" (80). In 1986, Murry et al. (134) confirmed "preconditioning with ischemia" using an in vivo canine model and defined it as a phenomenon where brief periods of ischemia accompanied by reperfusion just before sustained ischemia exert *1*) a delay in ATP depletion, *2*) a reduction in oxygen consumption, *3*) a retention of intracellular structure, and *4*) a delay or reduction of cellular necrosis due to ATP expiration, finally resulting in delayed progression or reduction of infarct size, despite an increase in the total ischemic period. This is now recognized as a narrow definition of ischemic preconditioning. The concept of cardioprotection due to preconditioning currently prevails and has been expanded on to include not only acute irreversible injuries, such as necrosis and apoptosis, but also chronic disorders, such as myocardial hibernation or remodeling, although it appears to be irrelevant to acute myocardial contractile dysfunction, such as stunning (98).

Critical Dual Time Window: Early and Late Phases

The original report on preconditioning (134) also mentioned that the strength of protection by ischemic preconditioning critically depends on the duration from the end of preconditioning ischemia to the onset of index ischemia. Later, the existence of dual periods for this duration was reported (123). The first period is more than several seconds and <3 h and the second one, associated with the increased expression of cardioprotective heat shock proteins (HSPs) (123), is 24–72 h. These are now widely recognized as "early and late phase" preconditioning. The two phases appear to involve different types of reactions; the former involves reactions that are completed in a short period of time, such as activation of ion channels, phosphorylation/activation of existing enzymes, or

rapid turnover/translocation of substances, whereas the latter involves more time-consuming reactions, such as genomic modulation and expression of channel proteins, receptor proteins, enzymes, molecular chaperon proteins, or immunotransmitters.

Although these two phases differ in timing, they share some common triggers, mediators, and effectors. Finally, myocardial injury at the time of reperfusion might be a major target of both types of preconditioning as well as postconditioning; this will be discussed later (64).

Factors Underlying the Mechanisms of Preconditioning

G protein-coupled receptor agonists: adenosine, α- and β-adrenoceptor activation, and others. Downey and colleagues (109) opened the door to the investigation on mechanisms of preconditioning with a report showing that protection by ischemic preconditioning was abolished by the inhibition of the adenosine receptor before sustained ischemia in vivo. This suggested that adenosine was a trigger of ischemic preconditioning. However, they later reported a conflicting result in vitro that preischemic treatment with either adenosine or selective adenosine-A_1 receptor agonist, 2-chloro-N^6-cyclopentyladenosine, exerted minimal protection (49), implying that adenosine is not a candidate for pharmacological preconditioning. This inconsistency was highlighted by later reports from other groups showing successful pharmacological preconditioning with selective adenosine-A_1 receptor agonists, 2-chloro-N^6-cyclopentyladenosine or R-phenylisopropyladenosine, in early phase (130, 233) and late phase (91) infarct limitations in vivo, strongly suggesting the inclusion of adenosine receptor activation in ischemic preconditioning.

Later reports proposed α (68, 214)- and β (193, 209)-adrenoceptor activation as a putative trigger of ischemic preconditioning, based on the findings that receptor blockade abrogated ischemic preconditioning-induced cardioprotection. However, there were contradictory findings. Downey and colleagues reported that neither specific catecholamine receptor antagonists (207) nor depletion of intramyocardial catecholamine storage and release (8) blocked ischemic preconditioning, whereas exogenous catecholamine or adrenoceptor agonists did precondition the heart (207, 214). Vatner and colleagues also reported a similar observation that cardiac denervation did not block early phase but did blunt late-phase ischemic preconditioning via α_1-adrenoceptor signaling (102). In search of effective receptor subtypes, Tsuchida et al. showed the direct involvement of α_{1b}-adrenoceptor in ischemic preconditioning (214), but studies using transgenic mice support cardioprotection by α_{1a} rather than by α_{1b} (160). On the other hand, the β_2-adrenoceptor is reported to confer cardioprotection in ischemic preconditioning (209), whereas preischemic β_1-activation could be an alternative (193).

Indeed, reasons for such contradictions between the receptor blockade of ischemic preconditioning and pharmacological preconditioning with receptor agonists, or the inconsistency of positive or negative cardioprotection among the reports, are not fully understood. One reason for these differences may be due to the experimental models used; however, we should consider other reasons.

First, a feasible explanation is that there are multiple, parallel mechanisms induced by preconditioning ischemia, all of which can exert cardioprotection by themselves or in harmony. Accordingly, adenosine-dependent and α_1-adrenoceptor mechanisms might reflect this relationship (12, 214).

Second, pharmacological interventions do not necessarily mimic ischemic preconditioning accurately because of the half-life of mimetic agents; the majority of the agents might act for a longer period of time than the brief period of time required for preconditioning ischemia. Because the increased endogenous adenosine production induced by ischemic preconditioning confers cardioprotection in both preconditioning and reperfusion periods (95) and because adenosine-A_1 receptor activation around the onset of reperfusion also protects the myocardium (111), the preischemic administration of agents might still be effective beyond the sustained ischemic period.

Third, a critical time window for the involvement of the respective receptor activation is considered. For example, sustained transgenic activation of α_{1b}-adrenoceptor did not elicit cardioprotection (160), whereas temporal blockade during the preconditioning period protected the myocardium (68, 214). Also, β_1-adrenoceptor activation is reported to be beneficial during the preconditioning period but deleterious after reperfusion (193).

Finally, some reports do not support the contribution of adenosine in ischemic preconditioning-induced cardioprotection, because they failed to show that preconditioning-induced cardioprotection resulted in an increase in the intramyocardial level of adenosine. One possible reason for this failure might be due to how the level of adenosine was measured and the extremely short biological half-life of adenosine. We have also experienced this inconsistency when collecting samples without the prompt usage of a stop solution containing EDTA, adenosine deaminase inhibitors, and dipyridamole. Therefore, this may be due to the prompt intracellular uptake or ultrarapid degradation of adenosine under physiological conditions. Accordingly, this idea might be further supported by the observation that the loss of cardioprotection by the addition of exogenous adenosine was restored by extending its biological half-life by either a coadministration with dipyridamole to delay clearance through intracellular uptake (11) or a sustained targeting release using a liposomal envelope (201).

Further pharmacological analyses revealed the importance of other G protein-coupled receptor (GPCR) members, opioid receptors (22, 181), and bradykinin-B_2 receptors (52, 101) in pharmacological and ischemic preconditioning-induced cardioprotection in both the early (181) and late (22) phases of ischemic preconditioning. For example, the contribution of each opioid receptor (δ-, κ-, and μ-subtypes) has been reported in direct (107), remote, (144) or secondary protection of cardiac myocytes by acting on the central nervous system (106). However, the protection in the clinical setting still remains controversial (206, 219). As is summarized in Fig. 4, *top left*, various endogenous as well as exogenous stimulants cause their respective cardioprotective effects via the activation of unique or combined GPCR subtypes.

Downstream of GPCR: PKC, PKA cascades, and more. GPCRs couple to G proteins, consisting of a Gα- and a Gβγ-subunit, and the overall properties of each GPCR upon activation are generally defined by Gα-subtypes, such as G_s, G_i, and G_q. G_s and G_i positively and negatively modulate, respectively, cAMP production, whereas G_q activates phospholipase C and leads to PKC activation (Fig. 4).

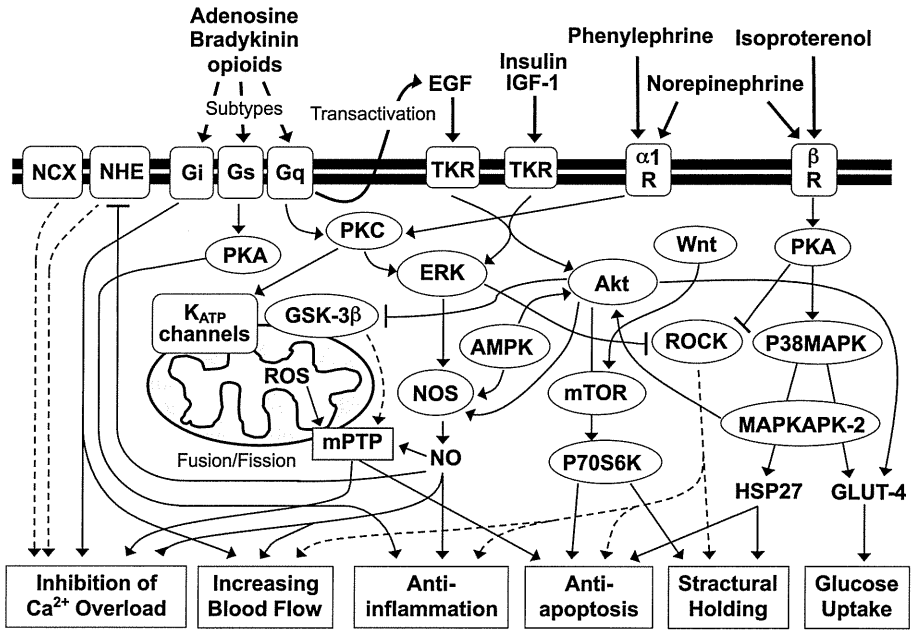

Fig. 4. Putative major cascades of cardioprotection and phenotypes of protection, afforded by preconditioning and postconditioning, either by brief ischemia(s) or by pharmacological agents. NCX, Na^+/Ca^{2+} exchanger; NHE, Na^+/H^+ exchanger; TKR, tyrosine kinase receptor, R, receptor; EGF, epidermal growth factor; IGF, insulin-like growth factor; mTOR, mammalian target of rapamycin; ROCK, Rho-associated protein kinase; K_{ATP}, ATP-sensitive K^+ channel; AMPK, 5′-AMP-activated kinase; NOS, nitric oxide (NO) synthase; MAPKAPK-2, MAPK-activated protein kinase 2; mPTP, mitochondrial permeability transition pore; HSP27, heat shock protein 27; GLUT-4, glucose transpoter 4. Dashed arrows indicate signal transduction through upstream inhibition.

Many reports have shown that ischemic preconditioning activates PKC (93, 237) and that PKC blockade abrogates infarct limitation induced by ischemic preconditioning (194, 237). In addition, repeated ischemic preconditioning instantly increases intramyocardial cAMP levels; moreover, either transient β-adrenoceptor stimulation (110) or intramyocardial pharmacological cAMP accumulation (167) mimics the cardioprotective behavior of preconditioning, and PKA inhibition significantly and substantially reduced the preconditioning-induced cardioprotection (167). Among PKC subtypes, PKC-δ inhibition or PKC-ϵ activation have been reported to be relevant to preconditioning-induced cardioprotection (186) in human myocardium, whereas we have previously reported the importance of Ca^{2+}-dependent, classic PKC in animal models (236).

As a possible downstream effector, we have previously reported that PKC directly activates ecto-5′-nucleotidase, located on the cell membrane (92), suggesting that adenosine could benefit the myocardium as not only a trigger but also a mediator of ischemic preconditioning (94). This has been supported by reports from other groups (95, 196). Recent reports also revealed that adenosine is an endogenous bioactive substance with multiple cardiovascular effects, including a negative inotropic effect, a negative contractile effect, and promotion of coronary blood flow and anti-platelet activity (90), as well as inhibition of apoptosis (158) and enhancement of autophagy (233). However, the role of elevated intramyocardial cAMP levels and PKA activation in cardioprotection of ischemic preconditioning has been reported (110), but they were both shown to be independent of PKC (117, 170, 176). Instead, mitochondrial ion channel (176) or p38 MAPK activation (170) was reported as a putative downstream mechanism in vivo. The reason for transient activation of p38 MAPK by PKA can be explained by PKA phosphorylation of the catalytic site of protein tyrosine phosphatase and inhibition of the dephosphorylation of p38 MAPK, leading to physiological augmentation of p38 MAPK activity (178). We also reported that Rho kinase plays an important role downstream of PKA during sustained ischemia to confer cardioprotection (167). Other reports support the involvement of SOD induction (48).

Taken together, while adenosine or α_{1b}-adrenoceptor stimulation could activate PKC (68) and while β_2-adrenoceptor or some unknown mechanism could activate PKA and cAMP response element-binding protein (119) following ischemic preconditioning, both mechanisms might independently but synergistically mediate preconditioning in response to various stimuli, including brief periods of ischemia (168). This might include a switch in second messenger signaling; cardioprotection that was induced by PKA activation through β_2-adrenoceptor stimulation was observed together with a switch of the second messenger from G_s to G_i (209). Also, α_{1b}-adrenoceptor stimulation resulted in PKC activation that was mediated by G_i, not G_q (68). Furthermore, a recent report (87) regarding heat-induced preconditioning intriguingly proposes p38 MAPK activation as a common cardioprotective mechanism in the PKC and PKA pathways.

p38 MAPK, ERK, and phosphatidylinositol 3-kinase/Akt cascades. The first report supporting the role of p38 MAPK activation during indexed ischemia in preconditioning-induced cardioprotection (221) was followed by opposing reports, in vivo (113) and in vitro (115), suggesting that p38 MAPK activation during prolonged ischemia could promote ischemic damage. In fact, continuous hypoxia causes biphasic activation of p38 MAPK in rat neonatal cardiomyocytes (115), with a transient peak within 30 min, followed by continuous activation after 4 h, leading to the hypothesis that p38 MAPK activation might have diverse effects; transient activation is protective, whereas continuous activation is harmful. We observed in vivo (171) that brief preconditioning ischemia causes transient but robust activation of p38 MAPK, followed by inactivation of p38 MAPK during sustained ischemia. The inhibition of p38 MAPK during the preconditioning period substantially blunted infarct limitation by ischemic preconditioning, implying a major role for p38 MAPK activation before

index ischemia in preconditioning-induced cardioprotection. However, Marber and colleagues (177) have shown in vitro a p38 MAPK subtype specificity in cardioprotection and that ischemic preconditioning reversed p38 MAPKα activation during index ischemia, whereas p38 MAPKβ was inhibited during index ischemia in the presence and absence of ischemic preconditioning. They have also shown that TGF-β-activated kinase-1-binding protein-1 might be important in this subtype-specific action of p38 MAPKα (205). While we also documented partial cardioprotection by nonspecific pharmacological p38 MAPK inhibition during index ischemia in the above study (171), the critical role of MAPKs during sustained ischemia in preconditioning-induced cardioprotection remains obscure (17).

As downstream effectors, p38 MAPK and phosphatidylinositol 3-kinase (PI3K) induce expression, membrane transportation, and activation of glucose transporter-4, which facilitates glucose uptake (191), partly regulated by 5′-AMP-activated kinase (138). Furthermore, MAPK-activated protein kinase-2 and HSP27 are also activated in the preconditioned myocardium at the onset of sustained ischemia (171). HSP27 binds to the z-bands of myofibrils and prevents ischemic myofilament degradation and the interaction of apoptotic protease-activating factor-1 with procaspase-9 by binding to cytochrome-*c* and reducing apoptotic changes (24). Interestingly, MAPK-activated protein kinase-2 also potentially activates Akt, another possible antagonist of apoptotic signals (157).

By contrast, the pharmacological and ischemic preconditioning-induced activation of ERK, another component of MAPKs, also plays an important role in cardioprotection. Initial findings showed that the inhibition of ERK during preconditioning and after reperfusion blunted the infarct limitation of ischemic preconditioning (196). Later reports indicated limited or enhanced MAPK contribution to cardioprotection during sustained ischemia (17) or after reperfusion (155), respectively, supporting the protective role of postischemic ERK activation in preconditioning-induced cardioprotection. Intriguingly, a counteracting effect of Rho-kinase activation at reperfusion is raised as a putative mechanism downstream of ERK (241). We have also observed this in vivo downstream of preischemic p38 MAPK activation (167). The cross talk between p38 MAPK and ERK in preconditioning-induced cardioprotection might be an important issue for further analysis.

PI3K and Akt, which are activated by ischemic preconditioning, are denoted as "reperfusion injury salvage kinase (RISK) pathways" (63), a set of signals together with ERK that confer cardioprotection against ischemia-reperfusion. They are also reported to induce nitric oxide (NO) production upon both ischemic preconditioning (211) and reperfusion (21); additionally, they are reported to protect the myocardium. We will discuss this later.

Finally, the cascades shown above are summarized in Fig. 4, *right*.

NO and cGMP pathways. NO is reported as a major factor that primarily provides cardioprotection (20). Unlike adenosine, NO can activate guanylate cyclase to use cGMP as a second messenger while exerting similar cardiovascular effects. The sources of NO can be both endogenous and exogenous. Although it still remains somewhat controversial (137), endogenous NO generated by either activated endothelial NO synthase (NOS) or upregulated inducible NOS can confer cardioprotection both immediately and long after the triggering signal, respectively (20). This supports the idea that NO works as both a signal mediator and an effector (90, 109), both immediately after ischemic insult and at later time points.

Recently, numerous reports revealed that the cardioprotective properties of NO are partly induced by vasodilation hemodynamic effects or anti-inflammatory effects (142), which are generally cGMP dependent (33) and similar to adenosine (168). However, they also include direct cGMP-independent effects: the inhibition of GSK-3β (30), which might cause the aforementioned cardioprotection as well as the inhibition of mitochondrial permeability transition pore (mPTP) (143) and the opening of the mitochondrial K_{ATP} channel, which are both dependent on (25) and are independent of (30) cGMP-mediated signaling. It is likely that the direct effects of NO largely target mitochondria. NO might also protect the myocardium by preventing mitochondrial fission (35, 141), opening mitochondrial K_{ATP} channel, and inhibiting mPTP to maintain energy metabolism against ischemic energy disturbances. We will discuss these issues in detail later.

While NO confers many aspects of cardioprotection as described above, the induction of NO synthesis colocalizes with the site of Ca^{2+} and ROS in action. NO could act as a double-edged sword when simultaneously exposed to excessive oxidative stress, resulting in both the uncoupling and generation of further oxidative/nitrosative stress (142).

K_{ATP} channels. The K_{ATP} channel, usually an octamer [4 inward rectifier K^+ channel (Kir) family and 4 sulfonylurea receptor (SUR) subunits] on the membrane modulated by Mg and ATP (190), was first identified by cardiovascular physiology studies as a relaxing and negative inotropic factor (234).

K_{ATP} channels, which are inward rectifiers (190), can raise a depolarization threshold and reduce the excitation of either vascular smooth muscle or cardiomyocytes, resulting in intracellular Ca^{2+} unloading and reduced metabolic demand (190, 234). However, Inoue et al. (75) also found ATP-sensitive inward rectifier activity on the inner mitochondrial membrane, suggesting the existence of "mitochondrial K_{ATP} channels" in contrast to "sarcolemmal K_{ATP} channels." Cardioprotection derived from both subtypes has been previously reported (172). The concept of the mitochondrial K_{ATP} channel as a final effector of cardioprotection involves the stabilization of the mitochondrial inner membrane and the prevention of membrane uncoupling (57), which provides similar benefits with the inhibition of mPTP due to preconditioning (32). For example, δ-opioid receptor signaling might also contribute to preconditioning with K_{ATP} channels as a putative downstream mechanism, but it is also reported to be involved in GSK-3β inhibition (53) or mitochondrial PTP-induced cardioprotection (32). Accordingly, pharmacological K_{ATP} opening prevents not only ischemic damages but also cardiac remodeling due to chronic nonischemic stimuli (169), suggesting that this may be a putative therapeutic strategy.

GSK-3β and mPTP. In search of the downstream cascades of PI3K, Tong et al. (210) reported on the involvement of GSK-3β inhibition in the cardioprotection of ischemic preconditioning. The emerging importance of this kinase, which is inactivated through phosphorylation by PI3K, is supported by its multiple roles as a critical downstream mediator of ischemic or pharmacological preconditioning that are induced by opioids (53) or mitochondrial K_{ATP} channel openers (32). This kinase

subsequently confers either triggered or immediate cardioprotection and serves as a downstream effector of the Wnt/Frizzled pathway-induced cardioprotection (16). These roles implicate this developmental signal in cardioprotection afforded by preconditioning. It has also been reported to target the mammalian target of rapamycin, one of the major common downstream effectors of the PI3K/Akt pathways (217).

However, mitochondrial protection against ischemia-reperfusion is necessary to ensure cellular respiration and aerobic ATP generation and to inhibit the release of cytotoxic agents such as ROS and proapoptotic factors (such as cytochrome-*c*) from mitochondria upon myocardial stress (57). There were two great breakthroughs in the understanding of mitochondrial protection: the discovery of the mitochondrial K_{ATP} activity as described above in *K_{ATP} channels* and the discovery of mPTP (56, 57).

The mitochondrial outer membrane contains a pore protein called the voltage-dependent anion channel (VDAC) that lets nonspecific small substances of less than ~5 kDa into the intermembrane space and cytosol, whereas the inner membrane is less permeable and may let only molecules such as H_2O, O_2, CO_2, or NH_3 go through. Therefore, the mechanism that induces mitochondrial release of larger molecules, such as cytochrome-*c* under stress, was not well understood. Originally, VDAC was considered to be a crucial component of this canal but was ruled out later because the intrinsic apoptotic pathway was also induced in VDAC knockout mice (13). Currently, Halestrap et al. (56) propose that mPTP is composed of adenine nucleotide translocase, mitochondrial phosphate carrier, and their regulator cyclophilin-D. Ca^{2+} overload (57) or ROS (175) is crucial to open the pore that enables small molecules to pass between the mitochondrial matrix and cytosol, allowing for an H^+ influx into mitochondria. This cancels the membrane potential across the mitochondrial membrane and results in mitochondrial swelling and subsequent apoptotic changes (66).

Cyclosporine A binds to cyclophilin D, located on the mitochondrial inner membrane, and inhibits mPTP opening by causing its dissociation from mPTP and thereby facilitating pore opening induced by Ca^{2+} and ROS (27, 57). Ischemic preconditioning is also reported to prevent, upon ischemia-reperfusion, the mPTP opening downstream of GSK-3β (84) or PKC-ε (15), possibly through the modulation of cyclophilin D (62). Finally, these models conform well to the idea that both necrotic (128) and apoptotic (32, 238) cell death occurs upon ischemia-reperfusion injury, and both are reduced by ischemic preconditioning (70).

Further investigation of the intersectional relationship between the RISK/survival and Wnt/developmental pathways, as well as mPTP (a putative contributing mechanism), in cardioprotection is necessary.

Ca^{2+} and free radicals. Importantly, it is widely recognized that both Ca^{2+} and ROS serve as signaling mediators under physiological conditions, while the dose, time period, and location are critical (43). It is well known that intracellular Ca^{2+} overload or induction of ROS causes cellular damage because of ischemia-reperfusion injury, especially when it is prolonged or intense. Moreover, as described above in *NO and cGMP pathways*, even a typically beneficial signal such as NO could cause deleterious effects when colocalized with excessive oxidative stress (142). However, short or transient exposure to them is reported to be beneficial because of their ability to trigger ischemic preconditioning (139, 198) and postconditioning (29). In preconditioning mechanisms, intracellular Ca^{2+} is required to activate classical PKC, an important component for triggering the preconditioning signal (139). Also, the Ca^{2+}-activated K^+ channel protects the myocardium when it is activated at the time of reperfusion (176, 185). Furthermore, Ca^{2+} is indispensable for myocardial twitching (142) and for the generation of ROS from mitochondria (43) within physiological range. On the other hand, ROS can reciprocally modulate intracellular Ca^{2+} homeostasis (43), and the transient increase in ROS through the increase in NO levels can trigger both early (24, 137) and late (198) phases of ischemic preconditioning. However, the role of ROS as a final effector of cardioprotection is rather controversial, especially in postconditioning, as discussed in the next section.

We finally summarized these mitochondria-oriented protective mechanisms in Fig. 4, *bottom left.*

Cardioprotection Afforded by Postconditioning

Postconditioning, defined as brief periods of ischemia alternating with brief periods of reflow applied at the onset of reperfusion following sustained ischemia (143), has recently been shown to have potential as a novel cardioprotective intervention against ischemia-reperfusion injury (197, 218). Ischemic postconditioning results in cardioprotection similar to preconditioning and provides a variety of crucial clues to cardioprotective mechanisms that can be directly applied in the clinic (28). In contrast to preconditioning, cardioprotection from postconditioning requires the instant sequence starting from triggers, through mediators and leading to effectors. The question as to whether cardioprotection from preconditioning and postconditioning use different mechanisms is currently under discussion (143), together with supportive (230) or negative (242) reports for additional cardioprotection from preconditioning beyond concomitant ischemic postconditioning.

Critical conditions for ischemia and reperfusion/reoxygenation in postconditioning. Because the prompt recovery of intracellular pH and reoxygenation upon reperfusion causes Ca^{2+} overload or excessive ROS generation and enhanced reperfusion injury, reduction of the levels of intracellular Ca^{2+} or ROS is one of the critical strategies for preventing reperfusion injuries. The initial successful attempts to reduce these levels were done using acidic (97) or staged (67) reperfusion, which can temper the promptness of pH recovery and reoxygenation upon reperfusion. On the other hand, recent studies have proposed that postconditioning can also elicit cardioprotection; however, it might instead induce the chances of repeated overshooting of intracellular Ca^{2+} and ROS levels at the time of reperfusion when preventive action has to take place. Accordingly, the latest reports have revealed that the benefits of postconditioning require the following conditions to be optimal: the duration of the index ischemia (118), the duration between the onset of reperfusion and the first brief ischemia, the duration and number of ischemias, and the duration of the interspersed reperfusion (143). First, postconditioning reduced the infarct size after index ischemia longer than 45 min in vivo, whereas it adversely impaired injury after index ischemia shorter than 30 min (118), possibly because the

ischemia-reperfusion postconditioning maneuver per se causes some extent of ischemia-induced damage, and the injury made by relatively short-index ischemia was too small to be substantially rescued by ischemic postconditioning. Second, a short time period of <1 min after the onset of reperfusion/reoxygenation before the onset of the brief ischemia postconditioning might protect the myocardium (230), whereas a longer time period invalidates cardioprotection from the following brief ischemia (88, 230). While time periods longer than 1 min fail to exert cardioprotection in small rodent models (88), it is still effective after 3 min in large animal models (189), whereas a 5-min interval seems to be beyond the cardioprotective limit (218) even in the human. Third, the duration and number of ischemic postconditioning procedures that could affect the strength of stimulus might also be critical (143). In vivo, the predominant findings across the species are that at least three cycles of brief occlusion and reperfusion are needed to elicit substantial cardioprotection as measured by reduced myocardial cell death (71, 118, 143), but additional cycles did not seem to provide further protection (88, 230). Also, a 10-s to 1-min period appears to be the optimal duration for an ischemic postconditioning mechanism to limit infarct injury (88, 143), and this tends to be shorter in small animals (26, 230).

Role of brief ischemia in putative mechanisms of postconditioning. Taken together, the postconditioning procedure should start within a few minutes after index ischemia, and periods of ischemia, <1 min, should be repeated at least several times within the total duration of several minutes (230). Because preconditioning ischemia and reperfusion periods do not require such strict time windows as postconditioning, preconditioning and postconditioning transient ischemia might use different mechanisms and conditions: postconditioning brief ischemia at the moment of reperfusion injury activates triggers or mediators that cause "instant" cardioprotection, whereas the brief preconditioning ischemia has additional time before the final effectors are activated, either during index ischemia or upon reperfusion. Therefore, limited signals and events, which are caused by brief ischemia and altered within seconds or a few minutes, could actually confer the same cardioprotection as postconditioning.

Indeed, transient acidosis, which may result from the brief ischemic period, might directly confer cardioprotection by attenuating intracellular Ca^{2+} levels, regional ROS generation (88, 118), and mPTP opening (29), independent of subcellular kinase signaling pathways (135). However, accumulating evidence shows that postconditioning strictly requires a brief ischemic/anoxic phase to activate the RISK pathways (ERK and Akt) (118, 230), produce NO, and open mitochondrial K_{ATP} channels (230), thereby preserving mitochondrial function (143) and preventing apoptotic changes (149). This requirement implies a common cardioprotective mechanism for ischemic preconditioning and postconditioning because postischemic activation of RISK pathways is also important for preconditioning, as we described in *p38 MAPK, ERK, and phosphatidylinositol 3-kinase/Akt.* Prompt activation of the RISK pathways (63) could also subsequently inhibit mediators, such as GSK-3β, prevent mPTP opening, or induce NO synthesis, and most of these are common to ischemic preconditioning (242) and reduce ROS production and Ca^{2+} overload (197).

Role of transient reperfusion in the postconditioning mechanism. Meanwhile, Cohen et al. (29) reported that the restoration of oxygenation is necessary to activate PKC and cardioprotective cascades, while maintaining an acidic myocardial pH for several minutes until RISK cascades can be activated. Other reports also showed that the paradoxical effects of postconditioning might be related to the divergent effects of postconditioning on Akt phosphorylation and ROS production (118). Such mechanisms are recognized as the reason why brief ischemia and reperfusion should be frequently repeated upon reperfusion to obtain the unique protection of postconditioning. Accordingly, cotreatment with the ROS scavenger *N*-(2-mercaptopropionyl)glycine is reported to blunt cardioprotection by ischemic postconditioning (29).

Subsequent cardioprotective mechanisms of postconditioning. Furthermore, Garcia-Dorado and colleagues (76) reported that the activation of the cGMP/PKG pathway is upstream of delayed normalization of intracellular pH upon reperfusion via PKG-dependent inhibition of Na^+/H^+-exchange. This indicates that the pre- and postconditioning mechanisms differ substantially because the Na^+/H^+ exchange inhibition seems distant from cardioprotection that is induced by ischemic preconditioning (55).

In addition, the contribution of some endogenous autacoids, such as adenosine and opioids as well as their receptors (mostly GPCR), on postconditioning-induced cardioprotection is also expected (143). Adenosine is increasingly released or focally accumulated upon reperfusion (90), and it is recognized to cause cardioprotection before index ischemia as a trigger of preconditioning, as well as at reperfusion as an effector of both preconditioning (95) and postconditioning (143). In a rodent model, the nonselective opioid receptor antagonist naloxone, as well as the selective antagonists of specific (δ, κ, or μ) opioid receptors, blunted cardioprotective postconditioning; however, the nonselective agonist morphine exerted pharmacological postconditioning (239). There are other possible mediators of postconditioning-induced cardioprotection such as bradykinin or tyrosine kinase receptors (143), but their mechanisms of action are still under discussion.

Clinical Translational Trials Based on Cardioprotection of Pre- and Postconditioning

It would be extremely beneficial if direct or mimicking procedures of cardioprotection by preconditioning and postconditioning in the clinical fields are successful. Confirmation of a clinical presence of cardioprotection induced by ischemic preconditioning (78) and postconditioning (58) against ischemia-reperfusion injury has warranted recent clinical investigations.

Preconditioning procedures for elective ischemia-reperfusion. Yellon et al. initially found in open-heart surgery that intermittent cross clamping preserved cardiac ATP levels (231) and protected the myocardium (81). Accordingly, unstable angina during the last 48 h before surgery mimicked preconditioning in the early postoperative period (224). Furthermore, ischemic preconditioning significantly reduced lethal postoperative ventricular arrhythmia (225) as well as postoperative atrial fibrillation (226). However, because scheduled cardiac surgeries usually employ cardioplegia and anesthesia, which have the potential to provide cardioprotection (42), the added benefit of

additional treatments seems controversial even after prospective clinical trials (216). Even in the case of elective percutaneous coronary intervention, the preceding use of some drugs such as statins was reported to reduce myocardial injury (132), but the application of its putative downstream mediator by nitroglycerin before ischemic insult failed to exert any significant cardioprotection (82). One of the reasons it may be difficult to identify preischemic pharmacological strategies mimicking "triggers of preconditioning" could be the critical timing and intensity required for procedures in clinical situations.

By contrast, in a procedure called "remote preconditioning," promising results have been obtained in recent trials where cardioprotection was successfully achieved by applying repeated cycles of transient ischemia on distant organ(s), such as limb muscles (23, 220).

Postconditioning mimetics for predictable or unexpected ischemia-reperfusion. Because cardioprotection by both preconditioning and postconditioning mainly focuses on the reduction of ischemia-reperfusion injury and because the accessibility of sudden unexpected ischemia-reperfusion, such as acute coronary syndrome, is usually quite convenient after the onset of ischemic insult, most of the recent translational therapeutic strategies are applied at the time of reperfusion. This protocol is followed because of findings of "final effectors of preconditioning" and "contributors of postconditioning" at reperfusion, which share some common pathways (64). Unfortunately, very few large clinical trials to date have successfully shown sufficient cardioprotection (129). As the initial translational application in the clinical field, the Acute Myocardial Infarction Study of Adenosine (AMISTAD) trial (116a) proposed cardioprotection by adenosine, which is considered to be a final effector of preconditioning at reperfusion. It revealed that patients with acute myocardial infarction who underwent continuous intravenous adenosine infusion together with percutaneous transluminal coronary recanalization had a smaller infarct size and a better functional recovery than those without adenosine infusion, especially in the instances of anterior wall infarction. However, a following AMISTAD-II trial (162), which specifically evaluated anterior wall infarction, found no difference in the primary end point of new congestive heart failure, rehospitalization for CHF, or death from any cause within 6 mo, although the infarct size tended to decrease in a dose-dependent manner. Cohen and Downey (31) addressed an important limitation in the AMISTAD-II study regarding the method for calculating the infarct size and the different measurements of infarction in the placebo group (45 and 27% in AMISTAD-I and -II, respectively). Similarly, the Japan-Working Groups of Acute Myocardial Infarction for the Reduction of Necrotic Damage (J-WIND) trial (89a) successfully found cardioprotection when recombinant human atrial natriuretic peptides (ANP) were administered at reperfusion as adjunctive therapy just after successful percutaneous coronary intervention in acute phase, which reduced myocardial creatine kinase (CK) release and increased left ventricular (LV) ejection fraction at 6–12 mo. In another portion of this study, a hybrid of the K_{ATP} channel opener and the NO donor nicorandil failed to show any infarct limitation in the acute phase, although oral administration during follow-up increased LV ejection fraction. Differences in the results among these studies might be due to differences in the details of the protocols used, but the favorable outcome, especially in chronic phase, is also supported by other clinical trials (79, 89a), as well as the preceding Impact of Nicorandil in Angina (IONA) study (77), demonstrating chronic cardioprotection due to nicorandil by reducing coronary heart disease death, nonfatal myocardial infarction, or unplanned hospital admission for anginal heart attack. Therefore, at least the chronic use of nicorandil as well as the adjunctive use of ANP might be recognized as evidence-based medicine in the clinical field, originated in the knowledge of pre- and postconditioning-induced cardioprotection. On the other hand, following the initial success in protecting the myocardium in both the acute (195) and chronic phase (206) in patients suffering from left anterior descending coronary artery or right coronary artery infarction within 6 h of the onset with four cycles of 60 min ischemia-reperfusion, the direct application of postconditioning brief ischemia-reperfusion is also intensively evaluated. Actually, the salvage impact varies among the studies, probably depending on critical requirements in postconditioning maneuvers, and it seems less protective by the protocols that have fewer cycles or longer intermission (143).

Among recent studies using the cardioprotective signaling of postconditioning, the use of 3-hydroxy-3-methylglutaryl-CoA inhibitors (statins) is reported as a readily available, safe, and hopeful option to date. The immediate use of statins either before the onset of ischemia (72, 215) or around the reperfusion period (173) reduced infarct size regardless of hyperlipidemia. This result was also shown in humans (125); in addition, immediately using statins at these time point also reduced adverse outcomes when used as late as 24 h after reperfusion (182). This evidence strongly suggests that cardioprotection induced by statins goes beyond lipid lowering and involves the signaling cascades of postconditioning, such as Akt activation (173) and oxidative stress reduction (72).

Another emerging target is the use of the mPTP inhibitor cyclosporine A, a noteworthy and quite reasonable pharmacological intervention to date, which exhibited cardioprotection in acute phase (153) by infarct-limitation, measured in terms of CK release and MRI image on *day 5* after infarction, as well as in chronic phase (126) by restoring LV function. The findings of protection by cyclosporine are now expanded in endothelial function upon reperfusion in humans (140) but seem still immature and need further confirmation in large-scale clinical trials to establish evidence-based medicine.

Comorbidities and Cardioprotection: Prevailing Knowledge into Real-World Clinical Medicine

The most important issue is to bridge the results of basic research with clinical medications or therapeutic procedures. It is unfortunately true that there is an inevitable gap in the scientific approaches used in basic science and in clinical medicine. Clinical science largely relies on statistics because of heterogeneity of disease conditions and individuals, such as age, sex, background diseases, and their comorbidities (45).

Hypertension and cardiac hypertrophy. Hypertrophied myocardium is at greater risk of exacerbating myocardial injury after ischemia-reperfusion through the development of rigor contracture during ischemia, resulting in reduced contractile function and increased CK release (45). Also, epidemiologic (165) and experimental studies (17) show that ventricular

tachyarrhythmias are highly associated with hypertensive LV hypertrophy (LVH), probably via abnormalities in ectopic ion channel currents (45) and increased dispersion of action potential duration through reentrant mechanisms induced by increased interstitial fibrosis (221). Accordingly, the preconditioning effect of prodromal angina is reportedly attenuated in acute myocardial infarction patients with hypertensive LVH (203). It also seems to be the case with cardioprotection by ischemic (150) and pharmacological (151) postconditioning, although few studies have been published on this point.

It is important to distinguish between the primary benefits of regression of LVH or the reduction of blood pressure from those of acute treatments during ischemia-reperfusion when considering the effects of cardioprotective interventions. The regression of LVH by lowering blood pressure leads to a reduction in the susceptibility to negative outcomes, especially arrhythmias. In fact, the pharmacological reduction of acute blood pressure with LVH reduced mortality and infarct size to control levels (74), supporting the contribution of perfusion pressure rather than LVH to potentiating irreversible injury in hypertensive animals. Also, classic ischemic preconditioning preserved cardioprotection in the isolated model (40) of rats with pressure-overloaded LVH. On the other hand, there is evidence that normalization of myocardial action potential duration is related to a restoration of transient outward current density after LVH regression (235), and pharmacological cardioprotection by bradykinin was attenuated in the isolated pressure-overloaded hypertrophied hearts (40), even though the isolated effects of either high blood pressure or LVH are still arguable.

Although the influence of acutely applied cardioprotective procedures on reducing injury in established LVH has been immature to date, Rajesh et al. (156) propose the effectiveness of opening K_{ATP} channels in the protection of preconditioning.

Heart failure. The unfavorable situations are quite similar in long-duration hypertension in the status of cardiac remodeling and in heart failure. Studies in isolated hearts of aged rats showed that preconditioning is protective in control hearts collected from normotensive animals, but in hearts collected from age-matched hypertensive animals, it neither enhanced postischemic functional recovery nor attenuated creatine phosphate release during global ischemia-reperfusion (41, 131). This finding highly suggests a reduced efficacy of preconditioning in chronic hypertension or in the presence of aging and hypertension. In rabbits, ischemic preconditioning failed to reduce infarct size in postinfarction remodeled hearts, whereas cardioprotection by pharmacological preconditioning with diazoxide was not affected (127). Accordingly, in right atrial appendages obtained from patients, ischemic preconditioning reduced myocardial injury in the myocardium in the presence of mild LV failure but failed to rescue those with severe LV failure. By contrast, diazoxide treatment resulted in similar protection for all groups (51).

These data are consistent with a defect within the upstream cardioprotective signal transduction pathway of failing hearts that does not interfere with direct activation of the downstream cardioprotective signaling cascade of preconditioning. In this way, an insufficiency in the signal transduction cascade in failing hearts might occur upstream of mitochondrial K_{ATP} channels. Ferdinandy et al. (45) have described an important potential modification of upstream cardioprotective signaling in diseased hearts at the level of adenosine metabolism. These authors found that in patients with heart failure, increased ecto-5′-nucleotidase activity results in an increased serum adenosine level (90) and leads to the loss of cardioprotection by ischemic preconditioning due to tachyphylaxis (60). These observations could potentially explain the failure of the adenosine A_1/A_{2A} receptor agonist, AMP579, to reduce the infarct size in patients with impaired LV function in the AMP579 Delivery for Myocardial Infarction Reduction (ADMIRE) study. In line with this, pharmacological postconditioning with isoflurane reduced infarct size (112) and activated the salvage kinase pathway (44) in the post-myocardial infarction-remodeled heart.

Another possible explanation could be found in mitochondrial malfunction (161), such as decreased electron transport chain activity in the status of cardiac remodeling and the failing heart. Recent studies reveal that mPTP can modulate mitochondrial function in the heart (37) and that ischemic pre- and postconditioning effects might be impaired under mitochondrial insufficiency (62) but that the direct pharmacological restoration of mitochondrial function by inhibiting cyclophilin D might protect against the failing heart (108).

Hyperlipidemia. It was initially reported that protection conferred by classic preconditioning against myocardial stunning and electrophysiological changes was lost when rabbits developed hypercholesterolemia, irrespective of atherosclerosis, which was restored by normalization of serum lipid levels (46) or the administration of statins (215). Other more recent reports (83) have shown that increasing the number of preconditioning cycles can aggravate infarct size in isolated rabbit hearts that are subjected to ischemia-reperfusion after 8 wk of experimental hypercholesterolemia. However, a number of recent studies have shown a limited impact of hypercholesterolemia on the cardioprotective effects of ischemic preconditioning (38). Taken together, it is likely that hyperlipidemia modifies the effect of preconditioning to some extent but that the net result of this effect is critically dependent on the strength of the cardioprotective signals. This also applies to late preconditioning (199) and postconditioning (73). In fact, the same rabbit in the ischemia-reperfusion model was used to show the loss of the infarct limitation in response to late preconditioning (204).

The use of statins has been reported to reduce myocardial injury regardless of hyperlipidemia both before the onset of ischemia (72, 215) and around the reperfusion period (173), suggesting that the cardioprotection induced by statins is not merely the result of a lipid-lowering effect.

Diabetes and hyperglycemia. The reduced protective effect of classic preconditioning in vivo on infarct size, ischemia-reperfusion-induced arrhythmias, and contractile dysfunction in experimental streptozotocin-induced diabetic hearts have been shown in a variety of species including rats, dogs, and sheep (45). Resistance to the protective effect of preconditioning in this experimental model has also been described for late preconditioning (39), isoflurane-induced pharmacological preconditioning (240), and postconditioning (243). Some clinical observations also suggest that patients with diabetes and ischemic heart disease might present a reduced response to preconditioning-like events, such as prodromal angina (78) and brief ischemic events, which can produce infarct limitation and increase survival after coronary angioplasty (104) in normal

hearts. Furthermore, hyperglycemia per se has been shown to be a significant risk factor for mortality in a very large cohort of hospitalized patients with acute myocardial infarction (100), as well as a determinant of infarct size, irrespective of the presence of diabetes (86).

Mitochondrial dysfunction (6) or rather mitochondrial K_{ATP} dysfunction (51) has been proposed to be the mechanism underlying this characteristic behavior of diabetic or hyperglycemic hearts. This might entail an impaired integrity of mitochondrial DNA (124), impaired Akt phosphorylation in response to ischemic preconditioning (213), increased oxidative or nitrosative stress (47) caused by impairment of mitochondrial respiratory capacity (114), as well as enhanced susceptibility to mPTP opening, caspase activation, and apoptosis (223).

In the experimental conditions described above, PKC or p38 MAPK activation were still protective, suggesting that insufficiency in the cardioprotective signaling cascade arises upstream of PKC and p38 MAPK (61). Accordingly, treatment of diabetes with insulin or pioglitazone has been suggested as a way to overcome the negative effects on cardioprotection by activating ERK and Akt, the downstream effectors and central mediators of the RISK pathway (227).

Antidiabetic drugs might have an effect on cardioprotection, beyond their direct effect on diabetes and hyperglycemia. Insulin-secreting drugs such as sulfonylureas and glinides increase insulin secretion by blocking K_{ATP} channel on the pancreatic β-cell (SUR1/Kir6.2). However, in coronary smooth muscle cells (SUR2b/Kir6.1), the K_{ATP} channel modulates coronary blood flow at rest and in hypoxia, and myocardial sarcolemmal K_{ATP} channels (SUR2a/Kir6.2) contribute to the adaptation of the myocardium to stress. Interestingly, the inhibition of cardiovascular K_{ATP} channels by sulfonylureas increases mortality in diabetic patients after coronary angioplasty (50, 104). In particular, despite the absence of structural information, mitochondrial K_{ATP} channels have been suggested to play an important role in cardioprotective mechanisms. This is thought to occur because nonselective K_{ATP} channel blocker glibenclamide and the selective mitochondrial K_{ATP} channel blocker 5-hydroxydeaconate block, at least in part (172), the cardioprotection of classic as well as late preconditioning. This has been shown in several species including humans (45). Interestingly, the selective pancreatic K_{ATP} channel blocker glimepiride does not appear to have any negative effect on cardioprotection, even when clinical data were analyzed (104).

Aging. The morbidity and mortality due to ischemic cardiovascular diseases are significantly higher in the elderly than in young adults (99). It is also generally recognized that aged hearts are resistant to cardioprotection from various kinds of preconditioning procedures (18), although there is still some controversy about the specific effects of classical, late-phase, and pharmacological preconditioning in certain animal models (34, 184).

Besides changes in structural components of the myocardium, such as increased fibrosis (174), the aged myocardium displays functional alteration of the cardiomyocytes (18). Intriguingly, telomere dysfunction even in quiescent cells, such as cardiomyocytes, produces aging-induced impaired mitochondrial biogenesis and function that lead to reduced respiratory capacity (114). This produces insufficient gluconeogenesis, cardiomyopathy, and increased ROS through the p53-PGC axis (166). The aging-induced increase in ROS generation is also the product of NADPH oxidases (116) and increased cardiac monoamine oxidase-A activity (18), as well as of reduced antioxidant capacity (187). In addition, in the aged myocardium the expression of several genes is altered (19). Among these, the decreased expression of IGF-IGF receptor, PKC-ϵ, ERK, Akt, MnSOD, and catalase might not only weaken the impact of the protective effect of preconditioning or postconditioning but also increase susceptibility to ROS. Furthermore, increased inducible NOS and decreased connexin-43 expressions (19) might be considered as an adaptation to continuous stress. Finally, aged myocardium shows a reduced tolerance to ischemic injury (1).

Ischemic preconditioning reduces infarct size and LV remodeling and therefore potentially improves the prognosis of patients with an acute myocardial infarction (78, 232). However, these benefits seem diminished in patients older than 65 yr (2). Ischemic postconditioning failed to reduce infarct size (154), but a longer and more intense postconditioning procedure restored protection (20). In accordance with this, chronic opioid treatment confers cardioprotection in both the young and senescent mouse heart via PKA activation, independently of acquisition of analgesic tolerance (145), whereas protection with acute morphine treatment, which is PKC dependent (146), is lost in aged hearts (147). Therefore, prolonged or stronger preconditioning stimuli might provide a powerful cardioprotection for the aging heart.

Regular exercise, especially endurance exercise, protects against ischemia-reperfusion injury in both young and old animals through the induction of myocardial HSPs and endothelial NOS, and either improves cardiac antioxidant capacity or restores mitochondrial function (10). Aging-induced increases in LV cardiomyocyte apoptosis and subsequent remodeling are improved by exercise, which also normalizes the Bax-to-Bcl-2 ratio in the LV of the aged rat heart (103). In the clinical setting, the protective effect of prodromal angina against subsequent acute myocardial infarction was reported to be preserved in aged patients with a high level of physical activity (3).

Sex difference. Premenopausal women have a reduced risk for cardiovascular disease, but this risk arises after menopause (65). However, a large clinical trial unexpectedly showed that hormone replacement therapy increases cardiovascular events in healthy postmenopausal women (163).

In most animal studies, no sex difference in ischemia-reperfusion injury has been observed, except in the rat model where injury was smaller in female than in male hearts (148). Furthermore, estrogen administration, at least for short duration, has been shown to reduce ischemia-reperfusion injury via acute nongenomic responses that involve the activation of Akt pathway (192). Also, nuclear estrogen receptor activation has been shown to result in an altered expression of a number of cardioprotective genes, such as NOS and HSPs, and a number of genes involved in metabolism, such as lipoprotein lipase, PGD2 synthase, and PGC-1α (133). Recently, the third category of G protein-coupled estrogen receptors has been shown to protect the myocardium regardless of sex (36), allowing us to reconsider sex-induced differences in ischemia-reperfusion injury as well as in preconditioning-induced cardioprotection.

Summary and Future Directions

In this review, we first discussed the original hypotheses and current findings regarding the nature of ischemia-reperfusion injury. Based on both basic and clinical findings, ischemic pre- and postconditioning with a cardioprotective potential has been discovered and established. We summarized here the ongoing investigation on the protective mechanisms of ischemic pre- and postconditioning as well as its potential application for molecular, pharmacological, or mechanical treatments against ischemia-reperfusion injury and subsequent adverse outcomes. Among various factors, Ca^{2+} overload and ROS generation are recognized as the key players of injury, whereas modulation on mitochondrial homeostasis as well as activation of intracellular salvaging kinase signaling (such as RISK pathway) is thought to be the emerging target of therapeutic interventions. We also reviewed major previous and upcoming translational clinical trials upon such basic findings, but we still need to further optimize such trials along with clinical comorbidities to make these trials more applicable and adaptive in clinical medicine.

Although further work is needed to understand the mechanism of cardioprotection and to make it fully applicable in the clinical setting, the connection between the bench and the bedside can be achieved by additional translational studies and established by large-scale clinical trials. We need to facilitate the creation of large clinical trials in variable situations to bring the results obtained by basic research into the real world.

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