Cellular Energetics in the Preconditioned State

PROTECTIVE ROLE FOR PHOSPHOTRANSFER REACTIONS CAPTURED BY 18O-ASSISTED 31P NMR

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Cell survival is critically dependent on the preservation of cellular bioenergetics. However, the metabolic mechanisms that confer resistance to injury are poorly understood. Phosphotransfer reactions integrate ATP-consuming with ATP-producing processes and could thereby contribute to the generation of a protective phenotype. Here, we used ischemic preconditioning to induce a stress-tolerant state and 18O-assisted 31P nuclear magnetic resonance spectroscopy to capture intracellular phosphotransfer dynamics. Preconditioning of isolated perfused hearts triggered a redistribution in phosphotransfer flux with significant increase in creatine kinase and glycolytic rates. High energy phosphoryl fluxes through creatine kinase, adenylate kinase, and glycolysis in preconditioned hearts correlated tightly with post-ischemic functional recovery. This was associated with enhanced metabolite exchange between subcellular compartments, manifested by augmented transfer of inorganic phosphate from cellular ATPases to mitochondrial ATP synthase. Preconditioning-induced energetic remodeling protected cellular ATP synthesis and ATP consumption, improving contractile performance following ischemia-reperfusion insult. Thus, the plasticity of phosphotransfer networks contributes to the effective functioning of the cellular energetic system, providing a mechanism for increased tolerance to injury.

Cells with high energy turnover are particularly vulnerable to insults induced by deprivation of oxygen and metabolic substrates (1–3). Recently, endogenous defense mechanisms have been discovered that can “precondition” cells to withstand metabolic stress (3, 4). Preconditioning underlying cytoprotection has implicated multiple metabolic, signal transduction, and electrical events (3–10). However, the actual mechanisms responsible for preservation of cellular energetic systems and, ultimately, functional recovery remain poorly understood. In the heart, coupling of energetics with contractile function is facilitated through phosphotransfer relays, catalyzed by creatine kinase, adenylate kinase, and glycolysis (11–15). Poor contractile performance in the failing myocardium is associated with deficits in phosphotransfer-dependent metabolic signaling (16–19). Furthermore, disruption in phosphotransfer enzymes compromises the ability of heart muscle to respond to metabolic stress (20–23). Alterations in cellular energy metabolism triggered by ischemic preconditioning, including a characteristic creatine phosphate overshoot, indicates that this protective process targets phosphotransfer reactions (24–27). However, direct evidence demonstrating the protective role of phosphotransfer networks in the preconditioned state is still lacking.

Here, we demonstrate that ischemic preconditioning of heart muscle induces remodeling in cellular energy transduction, transfer, and utilization processes, thereby promoting preservation of energy metabolism. Post-ischemic contractile recovery was tightly associated with preconditioning-induced adjustment in metabolic flux through creatine kinase, adenylate kinase, and glycolytic systems. Thus, preconditioning shifts intracellular phosphotransfer networks into a stress-tolerant mode rendering heart muscle more resistant to metabolic injury.

EXPERIMENTAL PROCEDURES

Heart Perfusion—Excised hearts from heparinized (500 units intraperitoneally) and anesthetized (75 mg/kg intraperitoneal pentobarbitonal) male Harlan Sprague-Dawley rats (250–300 g) were perfused on a Langendorff apparatus with a 95% O2/5% CO2 saturated Krebs-Henseleit (K-H) solution (mmol/liter: 118 NaCl, 5.3 KCl, 2.0 CaCl2, 1.2 MgSO4, 11.0 glucose, 0.5 EDTA; 37 °C) at a perfusion pressure of 70 mm Hg. Left ventricular developed pressure, left ventricular end-diastolic pressure (LVEDP), rate-pressure product, and heart rate were derived from the left ventricular pressure signal continuously monitored using a fluid-filled balloon-tipped pressure transducer (Harvard Apparatus).

Experimental Procedures—Control hearts were perfused for 45 min. Preconditioned hearts were perfused for 25 min and then subjected to two “conditioning” cycles, 5 min of ischemia plus 5 min of reperfusion each. Control post-ischemic hearts were perfused for 45 min and then subjected to 45 min of zero-flow normothermic ischemia followed by a 30 min-long reperfusion. Preconditioned post-ischemic hearts were preconditioned as described above and then subjected to 45 min of ischemia and 30 min reperfusion. Hearts in each of the four groups were labeled with 18O at the end of respective protocols.

18O Phosphoryl Labeling—Heart labeling with 18O displays exponential kinetics with saturation occurring after 2 min. 18O labeling was performed for 30 s within the initial linear phase using a K-H buffer with 30% of 18O H2O (Isotec). Hearts were freeze-clamped, pulverized under liquid N2, and extracted in a solution containing 0.6 M HClO4 and 1 mM EDTA (11, 22). Protein content was determined with a DC Protein Assay kit (Bio-Rad). Extracts were neutralized with 2 M KHCO3 and used to determine 31P labeling by 31P NMR spectroscopy. Metabolite levels were determined by 31P and 1H NMR spectroscopy. The percent-

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‡ The abbreviations used are: LVEDP, left ventricular end-diastolic pressure; CrP, creatine phosphate; G6P, glucose-6-phosphate.
ages of recovery of 86 and 92% and of hydrolysis of 3 and 1% were measured for creatine phosphate and ATP, respectively.

**NMR Spectroscopy**—Extracts were processed for NMR spectroscopy as described (22). Samples were pre-cleaned for 1 h with Chelex 100 resin (Sigma) supplemented with internal NMR standards for \(^{31}\)P and \(^{1}H\) NMR spectroscopy. 1250 mmol of methylene, 0.5 mol of 3-trimethylsilyl tetradeutero sodium propionate) and concentrated by vacuum centrifugation (Savant) to a volume of 0.3 ml. Concentrated extracts were filtered (centrifuge filter; 0.22 µm, Milipore) and supplemented with 0.3 ml of \(D_2O\) (Isotec). Samples were cleaned additionally with the Chelex resin by rotation at 4 °C for 12 h. Extensive chelation was required to remove divalent cations, which otherwise reduce spectral resolution and render samples vulnerable to degradation.

High-resolution \(^{31}\)P and \(^{1}H\) NMR spectra were acquired, respectively, at 202.5 and 500 MHz on a Bruker 11 T spectrometer (Avance) in 5-mm tubes at ambient temperature. For \(^{31}\)P spectra, signal accumulation (i.e. 9000 scans) was run without relaxation delay (acquisition time: 1.61 s) using a pulse width of 10 µs (53° angle). Proton decoupling (WALTZ-16 decoupling at 3 kHz radiofrequency; pulse width of 506 µs for \(^{1}H\)) was applied during signal acquisition. The effect of NO (nuclear Overhauser enhancement) on signal intensities was corrected based on factors derived from recordings on typical samples with and without decoupling. The effect of rapid pulsing on signal intensity was corrected using attenuation factors calculated from \(T_1\) relaxation times of each signal, where times \(T_1\) were determined in typical samples by the inversion-recovery technique. Free induction decays were Fourier-transformed after zero filling to 32 K and filtering with a line-broadening factor of 0.3 Hz. Phase and base line were automatically corrected, and peak integrals were determined with a built-in integration routine (XwinNmr 2.5 software, Bruker). For \(^{1}H\) spectra, 64 scans were accumulated under fully relaxed conditions (12.8 s relaxation delay) with a pulse width of 9 µs (90° angle). Free induction decays were zero-filled to 32 K and Fourier-transformed without filtering. Phase and base line were manually corrected, and peak integrals were determined with a built-in integration routine.

**Calculation of Phosphotransfer Rates**—\(^{18}\)O phosphoryl labeling allows measurement of synthesis, transfer, and consumption of high energy phosphoryl-carrying molecules (22, 28). \(^{18}\)O-based state was considered significant when metabolite incorporations were performed by fitting data using linear or sigmoid functions with three parameters. The fidelity of fits was assessed based on \(R^2\) value with variance calculated by the least square method. Statistical significance was calculated for linear fits, and \(p<0.05\) considered significant.

**RESULTS**

**Preconditioning-induced Stress-tolerant State**—Vigorous myocardial performance was abruptly lost at the onset of ischemia, and could be only marginally recovered upon reperfusion indicating poor tolerance of heart muscle to ischemia-reperfusion injury. Conditioning with two periods of brief (5-min) ischemia, prior to a prolonged ischemia-reperfusion insult increased cardiac tolerance to post-ischemic injury. In control hearts, work performance expressed as the rate pressure product and lusitropic properties reflected by the LVEDP were 35,000 ± 1,500 mm Hg/s and 7.7 ± 0.3 mm Hg, respectively (\(n=7\)). Preconditioning per se did not significantly disturb heart function, with rate pressure product and LVEDP maintained at 32,000 ± 1,800 mm Hg/s and 9.4 ± 0.2 mm Hg, respectively (\(n=7\)). However, preconditioning did provide significant protection against subsequent prolonged (45-min long) ischemia. On average, rate pressure product and LVEDP following 30 min of reperfusion were 6,100 ± 2,200 mm Hg/s and 91 ± 5 mm Hg in nonconditioned (\(n=7\)) versus 17,600 ± 4,300 mm Hg/s and 53 ± 7 mm Hg in preconditioned (\(n=7\)) post-ischemic hearts, respectively (\(p<0.05\)). Thus, preconditioning induces a stress-tolerant state characterized by improved performance of the post-ischemic myocardium.

**Remodeling of Intracellular Phosphotransfer in the Preconditioned State**—Muscle performance critically depends on nucleotide homeostasis, optimal high energy phosphoryl transfer, and efficient ATP utilization (35, 36). Here, the dynamics of phosphotransfer reactions were quantified by capturing the rates of \(^{18}\)O incorporation in high energy phosphoryls using \(^{18}\)O-NMR spectroscopy (37). The rates of \(^{18}\)O incorporation in high energy phosphoryls using \(^{18}\)O-assisted \(^{31}\)P nuclear magnetic resonance spectroscopy (\(^{18}\)O/\(^{31}\)P NMR). Creatine kinase phosphotransfer rate was monitored in control and preconditioned myocardium through the appearance of phosphoryl species of CrP containing \(^{18}\)O, \(^{18}\)O, \(^{18}\)O, and \(^{18}\)O (Fig. 1A). In control hearts, net creatine kinase phosphoryl transfer was vigorous at 330 ± 20 mmol-mg protein\(^{-1}\) min\(^{-1}\) (\(n=7\); Fig. 1B), in line with the major energetic role of this enzyme in the heart (11, 16). Following ischemia-reperfusion, creatine kinase flux dropped over 4-fold to 74 ± 10 mmol-mg protein\(^{-1}\) min\(^{-1}\) (\(n=7\), \(p<0.05\); Fig. 1B), indicating deficient phosphotransfer in the post-ischemic heart.
heart. In conditioned hearts, creatine kinase phosphotransfer flux was 409 ± 5 nmol/mg protein min⁻¹ (n = 7, p < 0.05; Fig. 1B) before ischemia, conditioning the myocardium for subsequent metabolic stress. Accordingly, creatine kinase flux upon reperfusion was better preserved in the preconditioned heart at 180 ± 30 nmol/mg protein min⁻¹ (n = 7), a value significantly higher than that of nonconditioned hearts (Fig. 1A and B; p < 0.05). Consistent with alterations in creatine kinase flux, the levels of CrP were higher in preconditioned hearts before and after ischemia (Fig. 1C). Specifically, before ischemia, CrP was 30.4 ± 1.6 versus 39.1 ± 0.7 nmol/mg protein⁻¹ (p < 0.05), and following ischemia-reperfusion CrP was 9.0 ± 0.8 versus 18.8 ± 2.5 nmol/mg protein⁻¹ (p < 0.05) in nonconditioned (n = 7) and pre-conditioned (n = 7) hearts, respectively (Fig. 1C). Preconditioning also reduced the loss of total creatine following ischemia-reperfusion (52 ± 3 versus 45 ± 2 nmol/mg protein⁻¹, p < 0.05; n = 7). In general, the efficiency of cellular phosphotransfer appeared improved by preconditioning, as indicated by the increase in the CrP/Pi ratio (Fig. 1D). Before ischemia-reperfusion injury, the CrP/Pi ratio was 1.09 ± 0.07 and 1.67 ± 0.07 in control and preconditioning hearts, respectively (p < 0.05). Following ischemia-reperfusion, the CrP/Pi ratio was dramatically reduced to 0.15 ± 0.02 in nonconditioned but was partially preserved, at 0.37 ± 0.06, in preconditioned hearts (p < 0.05; n = 7). Thus, preconditioning shifts the creatine kinase phosphotransfer system into a new, more efficient steady state.

Adenylate kinase phosphotransfer flux was monitored here through the appearance of 18O-labeled phosphoryl species (18O1 and 18O2) in β-ADP (Fig. 2A) and β-ATP (not illustrated). In control hearts, net adenylate kinase phosphotransfer flux was 45.6 ± 6.5 nmol/mg protein min⁻¹ but was significantly reduced to 11.3 ± 30 nmol/mg protein min⁻¹ following ischemia-reperfusion (p < 0.05, n = 7; Fig. 2B). This represents a 4-fold drop in adenylate kinase phosphotransfer, contributing to the overall cellular phosphotransfer deficit. In the preconditioned heart, however, the adenylate kinase phosphotransfer flux was less pronounced and dropped more moderately following ischemia-reperfusion (Fig. 2B). On average, less than a 3-fold reduction (36.4 ± 5.3 versus 13.8 ± 1.0 nmol/mg protein⁻¹ min⁻¹, n = 7) was observed, suggesting that preconditioning limited somewhat the post-ischemic decline in adenylate kinase phosphotransfer.

In addition to its energy-producing role, glycolysis also has the capability of catalyzing spatially directed high energy phosphoryl transfer (15, 37). Here, glycolytic phosphotransfer was monitored by measuring the rate of appearance of 18O-labeled phosphoryl species in G6P (Fig. 3A). Ischemia-reperfusion...
halved hexokinase flux (from 3.8 ± 0.8 to 2.0 ± 0.2 nmol-mg protein⁻¹-min⁻¹, p < 0.05; n = 7; Fig. 3B). Preconditioning significantly promoted hexokinase phosphotransfer, apparently conditioning glycolysis for subsequent ischemic stress (Fig. 3B). Glycolytic phosphotransfer was increased about 2-fold following brief conditioning events (7.6 ± 0.4 nmol-mg protein⁻¹-min⁻¹) and was reduced to a lesser extend following ischemia-reperfusion (4.1 ± 0.6 nmol-mg protein⁻¹-min⁻¹) compared with nonconditioned hearts (p < 0.05, n = 7; Fig. 3, A and B). Before ischemia-reperfusion injury, stimulation of glycolytic phosphotransfer in the preconditioned myocardium was accompanied by increased tissue levels of lactate (2.1 ± 0.1 to 4.1 ± 0.3 nmol-mg protein⁻¹; Fig. 3C). Following ischemia-reperfusion, lactate levels were markedly elevated in the nonconditioned myocardium (16.3 ± 3.3 nmol-mg protein⁻¹). In contrast, preconditioned hearts had much lower lactate levels (5.6 ± 0.8 nmol-mg protein⁻¹, n = 7; p < 0.05) despite higher glycolytic flux (Fig. 3B). This could indicate that preconditioning promotes more efficient lactate conversion along with improved pyruvate oxidation in the post-ischemic myocardium. Along with changes in glycolytic flux and lactate concentration, preconditioning also increased tissue levels of α-glycerophosphate (from 0.20 ± 0.03 in control to 0.73 ± 0.03 nmol-mg protein⁻¹ following preconditioning, p < 0.05; Fig. 3D), indicating an altered myocardial redox state. Following ischemia-reperfusion, increase in α-glycerophosphate levels was less pronounced in preconditioning than nonconditioned hearts (2.5 ± 0.1 versus 3.3 ± 0.1 nmol-mg protein⁻¹, n = 7, p < 0.05; Fig. 3D). Thus, preconditioning-induced redistribution of intracellular phosphotransfer is associated with more efficient utilization of glycolytic substrates and improved redox balance of cellular energetics.

**Phosphotransfer Flux Supports Post-ischemic Recovery**—The significance of phosphotransfer in supporting myocardial recovery was further indicated by the relationship observed between individual phosphotransfer enzyme fluxes and post-ischemic contractile function (Fig. 4). In particular, creatine kinase flux correlated closely with the performance of the myocardium following ischemia-reperfusion. The squared correlation coefficient (R²) was 0.81 (n = 6, p < 0.05) and 0.96 (n = 6, p < 0.05) in control and preconditioned hearts, respectively (Fig. 4A), suggesting coupling of creatine kinase phosphotransfer with muscle performance. Flux through adenylate kinase also correlated well with contractile performance following ischemia-reperfusion. R² was 0.77 (n = 6, p < 0.05) and 0.83 (n = 5, p < 0.05) in control and preconditioned hearts, respectively (Fig. 4B), indicating contribution of adenylate kinase-catalyzed β-phosphate energetics in support of cardiac function. Correlation between hexokinase flux and post-ischemic functional recovery was not significant in nonconditioned hearts (R² = 0.57, n = 6; p = 0.08). However, it became significant in the preconditioned state (R² = 0.68, n = 6, p < 0.05; Fig. 4C), suggesting that glycolytic phosphotransfer was recruited by the conditioning process to augment post-ischemic contractile recovery. Thus, promotion of phosphotransfer reactions by ischemic preconditioning is associated with protection against ischemia-reperfusion injury.

**Preconditioning Protects ATP-generating and ATP-consuming Cellular Processes**—The ATP synthesis rate was monitored in the control and preconditioned myocardium through incorporation of 18O into γ-phosphoryls of ATP (Fig. 5A). In controls, ischemia-reperfusion significantly reduced the rate of ATP synthesis, expressed as percentage of γ-ATP phosphoryls replaced by 18O, from 84 ± 1 to 66 ± 4% (p < 0.05, n = 7; Fig. 5B). Preconditioning limited the drop in ATP production, which was 92 ± 2% prior to and 78 ± 3% following ischemia-reperfusion (n = 7), a drop of 14% versus 28% in nonconditioned hearts. ATP production, reflected as 18O labeling of γ-ATP, correlated tightly (p < 0.05) with heart work (Fig. 5C), demonstrating the requirement for sufficient ATP production in support of cardiac contractile function. Indeed, ischemia-reperfusion produced a quite dramatic reduction (>8-fold) in ATP levels from 29.1 ± 2.5 to 4.1 ± 0.3 nmol-mg protein⁻¹ in the nonconditioned hearts (Fig. 5D). In preconditioning hearts, however, the drop in ATP levels was less prominent, from 25.1 ± 1.3 to 7.0 ± 0.7 nmol-mg protein⁻¹, a 3.5-fold reduction (n = 7; Fig. 5D).

The ATP consumption rate, monitored by the appearance of 18O-labeled species in Pᵢ, was also protected by ischemic preconditioning (Fig. 6A). Total myocardial ATPase activity, expressed as percentage of Pᵢ phosphoryls replaced by 18O, was...
Phosphotransfer Networks in Preconditioning

Preconditioning Improves Feedback Metabolic Communication—Cardiac contractile function depends not only on the delivery of high energy phosphoryls but also on removal of end-products of the ATPase reaction (38). Here, we took advantage of the unique feature of $^{18}$O labeling to monitor concomitantly phosphotransfer flux and metabolite exchange between cellular compartments. Specifically, $^{18}$O-labeled P$_i$ produced during ATP hydrolysis at an ATPase site must reach a distinct ATP production site to be incorporated into γ-ATP (Fig. 7A). The efficiency of P$_i$ removal and the exchange rate between cellular ATPases and mitochondrial ATP synthase can, therefore, be monitored as the ratio of $^{18}$O-labeled P$_i$ over $^{18}$O-labeled γ-ATP ($[^{18}\text{O}]P/[^{18}\text{O}]γ$-ATP). Under nonischemic conditions, both in control and preconditioned hearts, the ($[^{18}\text{O}]P$/[^{18}\text{O}]γ$)-ATP ratio was high, on the order of 0.84 ± 0.01 and 0.83 ± 0.01, indicating rapid P$_i$ removal and/or delivery to ATP production sites (Fig. 7A). Ischemia-reperfusion dramatically reduced the metabolically active P$_i$ pool (Fig. 7A), and the ($[^{18}\text{O}]P$/[^{18}\text{O}]γ$)-ATP ratio in nonconditioned hearts to 0.34 ± 0.03 (n = 7). This finding suggests that P$_i$ removal from cellular ATPases as well as communication between ATPases and ATP synthases are markedly compromised (Fig. 7A). Indeed, there was negative correlation between LVEDP, a parameter defining muscle relaxation, and the severity of decline in the ($[^{18}\text{O}]P$/[^{18}\text{O}]γ$)-ATP ratio, reflecting impaired intracellular P$_i$ mobility (Fig. 7B). Preconditioning, however, limited the decline in the ($[^{18}\text{O}]P$/[^{18}\text{O}]γ$)-ATP ratio and maintained it at a value (0.49 ± 0.04, n = 7) significantly higher (p < 0.05) than that recorded in nonconditioned hearts (Fig. 7A). Thus, preconditioning promotes removal of the end-products of ATP hydrolysis following ischemia-reperfusion, thereby improving the cardiac contraction-relaxation cycle.

**DISCUSSION**

Induction of the cytoprotective response is fundamental to cell survival under stress, and yet the mechanisms underlying acquired tolerance to metabolic injury are poorly understood. Here, we demonstrate by the novel approach of $^{18}$O/$^{31}$P NMR analysis the importance of intracellular phosphotransfer pathways in producing a stress-tolerant cellular energetic phenotype. Preconditioning induced redistribution of high energy phosphoryl transfer through individual phosphotransfer reactions catalyzed by creatine kinase, adenylate kinase, and glycolytic enzymes, leading to an improved intracellular metabolic communication and preservation of cellular ATP synthesis and ATP consumption processes. Thus, the present study establishes that adaptive remodeling of the cellular energetic system is an integral mechanism in ischemic preconditioning-induced cell resistance to stress.

The energetic homeostasis of the cell and, consequently, energy-driven cellular functions require tight coordination between ATP utilization and ATP generation (38–40). Such coordination is believed to be mediated by phosphotransfer relays composed of creatine kinase, adenylate kinase, and glycolytic enzymes that facilitate high energy phosphoryl delivery and removal of ATPase end-products (12, 36, 37, 41). Here, phosphotransfer dynamics were dissected by $^{18}$O/$^{31}$P NMR spectroscopy, which allowed simultaneous capture of net phosphotransfer flux through individual enzymes, as well as total ATP production and consumption rates in intact heart muscle. In nonconditioned hearts, a prolonged ischemia-reperfusion challenge produced a marked reduction in the rates of creatine kinase, adenylate kinase, and glycolysis-catalyzed phosphotransfer, contributing to energetic and contractile dysfunction of the post-ischemic myocardium. Phosphotransfer deficit further disrupted intracellular handling of P$_i$, resulting in the accumulation of nascent P$_i$ at ATPase sites and impaired de-
livery to mitochondrial ATP synthase. A restricted mobility of intracellular P\textsubscript{i} could be the result of functional entrapment and/or physical diffusional restriction due to the viscosity and high structural organization of the muscle cytosol (15, 18). This would negatively impact ATPase activity responsible for efficient contraction-relaxation cycles and contribute to reduced ATP synthesis by oxidative phosphorylation (41). With short-term hypoxia, the drop in creatine kinase flux is usually compensated for by increased adenylate kinase and/or glycolytic flux (22, 37, 42). However, with prolonged metabolic stress, uncompensated deficits in phosphotransfer enzymes do develop, as observed here and as previously reported in severe cardiac conditions such as heart failure and various forms of myocardial insufficiency (11, 16–19). The deletion of genes encoding creatine kinase or adenylate kinase compromises the ability of a muscle to sustain cellular energetic economy under metabolic stress (14, 20–23, 43). Thus, defective phosphotransfer networking, associated with a reduced rate of ATP turnover, precipitates poor myocardial recovery in the nonconditioned myocardium following ischemia-reperfusion.

Preconditioning improves myocardial post-ischemic contractile recovery and provides protection from metabolic injury (4, 8, 9, 24). In the present study, by \textsuperscript{18}O/\textsuperscript{31}P NMR analysis of myocardial phosphotransfer dynamics we found that preconditioning up-regulates creatine kinase phosphotransfer flux associated with higher levels of CrP and a CrP/P\textsubscript{i} ratio. This finding supports the notion that preconditioning-induced creatine phosphate overshoot is required for generation of the protective phenotype (24–27, 44). In addition to the creatine kinase system, preconditioning also markedly increased glycolytic phosphotransfer associated with higher lactate and α-glycerophosphate levels. Previous studies have

**FIG. 5. Preconditioning protects ATP production in post-ischemic hearts.** A, \textsuperscript{18}O/\textsuperscript{31}P NMR spectra of \textsuperscript{18}O-labeled γ-ATP in control (CR, left panel) and preconditioned (PR, right panel) hearts following ischemia-reperfusion. \textsuperscript{18}O incorporation induces an isotope shift of 0.0228 ppm in \textsuperscript{31}P NMR spectra. \textsuperscript{18}O\textsubscript{1}, \textsuperscript{18}O\textsubscript{2}, \textsuperscript{18}O\textsubscript{3}, and \textsuperscript{18}O\textsubscript{4} designate γ-ATP phosphoryls with 0, 1, 2, and 3 atoms of \textsuperscript{18}O. B, \textsuperscript{18}O labeling of γ-ATP, a measure of cellular ATP synthesis, in control and preconditioned hearts prior to (C and P, respectively) and following (CR and PR, respectively) ischemia-reperfusion. C, correlation between ATP synthesis expressed as \textsuperscript{18}O labeling of γ-ATP and cardiac performance expressed as the rate pressure product (RPP). Both linear and sigmoid functions provided tight fits of experimental data. D, cellular ATP levels in C, P, CR, and PR hearts. ‡, indicates significant difference between C and CR or PR hearts; *, significant difference between CR and PR hearts.

**FIG. 6. Preconditioning improves ATP production in post-ischemic hearts.** A, \textsuperscript{18}O/\textsuperscript{31}P NMR spectra of \textsuperscript{18}O-labeled P\textsubscript{i} in control (CR, left panel) and preconditioned (PR, right panel) hearts following ischemia-reperfusion. \textsuperscript{18}O incorporation induces an isotope shift of 0.0210 ppm in the \textsuperscript{31}P NMR spectra of P\textsubscript{i}. \textsuperscript{18}O\textsubscript{1}, \textsuperscript{18}O\textsubscript{2}, \textsuperscript{18}O\textsubscript{3}, \textsuperscript{18}O\textsubscript{4}, and \textsuperscript{18}O\textsubscript{5} designate P\textsubscript{i} phosphoryls containing 0, 1, 2, 3, and 4 atoms of \textsuperscript{18}O incorporated. B, \textsuperscript{18}O labeling of P\textsubscript{i}, a measure of the cellular ATPase rate, in control and preconditioned hearts prior to (C and P, respectively) and following (CR and PR, respectively) ischemia-reperfusion. C, correlation between the ATPase rate, expressed as \textsuperscript{18}O labeling of P\textsubscript{i}, and cardiac performance, expressed as the rate pressure product (RPP). Both linear and sigmoid functions provided tight fits of experimental data. R\textsuperscript{2}, squared correlation coefficient. D, total ATP turnover expressed as the sum of \textsuperscript{18}O incorporated into cellular high energy phosphoryls, in C, P, CR, and PR hearts. ‡, indicates significant difference between C and P, CR, or PR hearts; *, significant difference between CR and PR hearts.
phosphotransfer reactions are an obligatory route channeling the flow of high energy phosphoryls. Thus, integrated cellular energetics contributes to the preconditioned phenotype by translating metabolic adaptation into increased stress tolerance.

The sequences of events leading to the development of a preconditioning state are still not fully understood. However, a number of signal transduction cascades, including protein kinases and ion channels, have been implicated as triggers and/or effectors of the protective phenotype (4, 8–10). Rapid alterations in cellular energetics induced by ischemic preconditioning could trigger signal transduction events by changing the cellular phosphorylation potential. Indeed, there is a close relationship between creatine kinase phosphotransfer and the activity of protein kinase C, believed to be critical in early stages of preconditioning (49). Moreover, adenylate kinase phosphotransfer, through AMP-driven signaling, modulates the behavior of the AMP-activated protein kinase, a metabolic stress kinase (50). Furthermore, intracellular phosphotransfer reactions regulate the behavior of ATP-sensitive K\(^+\) (K\(_{ATP}\)) channels, an alarm mechanism setting membrane excitability in response to metabolic stress (15, 42, 51–55). In turn, both protein kinase C and AMP-activated protein kinase regulate creatine kinase phosphotransfer rate and other metabolic pathways through phosphorylation of target proteins (49, 56). Thus, the feedback communication between stress-sensitive metabolic and signal transduction events may be central to the generation of the preconditioned state.

In summary, this study has uncovered a homeostatic mechanism by which cells induce a preconditioned energetic state conferring increased tolerance toward injury. This is accomplished by a coordinated redistribution of high energy phosphoryl flux through phosphotransfer enzymes allowing more efficient communication of energetic signals and preservation of ATP generation and consumption processes. In this way, intracellular phosphotransfer reactions emerge as an essential component required for the development of an injury-tolerant state and could thereby serve as a target for regulating the cellular response to stress.

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