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Ischemic preconditioning exaggerates cardiac damage in PKC-δ null mice

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Mayr, Manuel, Bernhard Metzler, Yuen-Li Chung, Emma McGregor, Ursula Mayr, Helen Troy, Yanhua Hu, Michael Leitges, Otmar Pachinger, John R. Griffiths, Michael J. Dunn, and Qingbo Xu. Ischemic preconditioning exaggerates cardiac damage in PKC-δ null mice. *Am J Physiol Heart Circ Physiol* 287: H946–H956, 2004; 10.1152/ajpheart.00878.2003.—Ischemic preconditioning confers cardiac protection during subsequent ischemia-reperfusion, in which protein kinase C (PKC) is believed to play an essential role, but controversial data exist concerning the PKC-δ isoform. In an accompanying study (26), we described metabolic changes in PKC-δ knockout mice. We now wanted to explore their effect on early preconditioning. Both PKC-$\delta^{-/-}$ and PKC-$\delta^{+/+}$ mice underwent three cycles of 5-min left descending artery occlusion/5-min reperfusion, followed by 30-min occlusion and 2-h reperfusion. Unexpectedly, preconditioning exaggerated ischemia-reperfusion injury in PKC-$\delta^{-/-}$ mice. Whereas ischemic preconditioning increased superoxide anion production in PKC-$\delta^{+/+}$ hearts, no increase in reactive oxygen species was observed in PKC-$\delta^{-/-}$ hearts. Proteomic analysis of preconditioned PKC-$\delta^{+/+}$ hearts revealed profound changes in enzymes related to energy metabolism, e.g., NADH dehydrogenase and ATP synthase, with partial fragmentation of these mitochondrial enzymes and of the E_2 component of the pyruvate dehydrogenase complex. Interestingly, fragmentation of mitochondrial enzymes was not observed in PKC-$\delta^{-/-}$ hearts. High-resolution NMR analysis of cardiac metabolites demonstrated a similar rise of phosphocreatine in PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts, but the preconditioning-induced increase in phosphocholine, alanine, carnitine, and glycine was restricted to PKC-$\delta^{+/+}$ hearts, whereas lactate concentrations were higher in PKC-$\delta^{-/-}$ hearts. Taken together, our results suggest that reactive oxygen species generated during ischemic preconditioning might alter mitochondrial metabolism by oxidizing key mitochondrial enzymes and that metabolic adaptation to preconditioning is impaired in PKC-$\delta^{-/-}$ hearts.

protein kinase C; proteomics; metabolomics; ischemia-reperfusion; mouse model

BRIEF EPISODES OF ISCHEMIA-REPERFUSION protect myocardial tissue from a subsequent prolonged ischemia by delaying cardiomyocyte death. This phenomenon, called ischemic preconditioning, was first described by Murry et al. (32). Since then, many publications have reported possible mechanisms and various signaling pathways involved in preconditioning (1, 6, 18, 21, 33, 39, 50). One of the most important signal transducers is protein kinase C (PKC), which is believed to play a pivotal role in mediating both the early and late phases of ischemic preconditioning (4, 5, 35, 36, 39, 40, 47).

So far, several PKC isoforms have been suggested as mediators of preconditioning. Whereas the protective role of PKC-ε in preconditioning is well established, controversial results exist concerning the cardioprotective effects of PKC-δ (19, 49). For instance, Wang et al. (44) demonstrated that mitochondrial translocation of PKC-δ is related to cardioprotection induced by ischemic preconditioning, whereas Fryer et al. (13) reported that PKC-δ plays an important role in pharmacological-induced preconditioning but not in ischemic preconditioning (12).

To get a better understanding of the role of PKC in preconditioning, we created knockout mice lacking PKC-δ (23), established a mouse model of heart ischemia-reperfusion (29), and analyzed the protein and metabolite profiles of preconditioned and nonpreconditioned hearts. We provide firm evidence that hearts lacking PKC-δ lose cardioprotection by ischemic preconditioning and demonstrate preconditioning-associated changes at a proteomic and metabolomic level.

EXPERIMENTAL PROCEDURES

Mice and surgical procedures. All procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. The PKC-δ-deficient (PKC-$\delta^{-/-}$) mice were generated by targeted disruption of the endogenous PKC-δ gene. Genotypic characterization of adult mice with a background of 129/SVxO1a was performed by Southern blot analysis of *Eco*RI-digested genomic DNA (23).

Preconditioning protocols. The procedure for myocardial ischemia-reperfusion in mice used in the present experiment was similar to that described previously (29). Briefly, mice were anesthetized with phenobarbital (100 mg/kg ip), and respiration was maintained by a Harvard Apparatus rodent ventilator (model 687). After the chest was opened by a lateral cut along the left side of the sternum and incision of the pericardial sac, the visible left descending artery was ligated with a placement of a polyethylene (PE)-10 tube on top of the artery. After occlusion for the prescribed periods, reperfusion was followed by opening the knot of the PE-10 tube. For preconditioning, mice underwent three cycles of 5-min artery occlusion/5-min reperfusion, respectively. Ten minutes later, the animals underwent 30 min of coronary occlusion of the artery, followed by 2 h of reperfusion. This protocol was found to be effective in inducing preconditioning in mice (30). Control groups ($n = 9$) were subjected to 30 min of coronary occlusion followed by 2 h of reperfusion. Blood pressure measurements were obtained by cannulating the common carotid artery with a PE-10 catheter (46). The arterial catheter was connected to a pressure transducer (COBE; Lakewood, CO) and a blood pressure analyzer (Micro-MED; Louisville, KY). Measurements were recorded every 30 s for 40 min.

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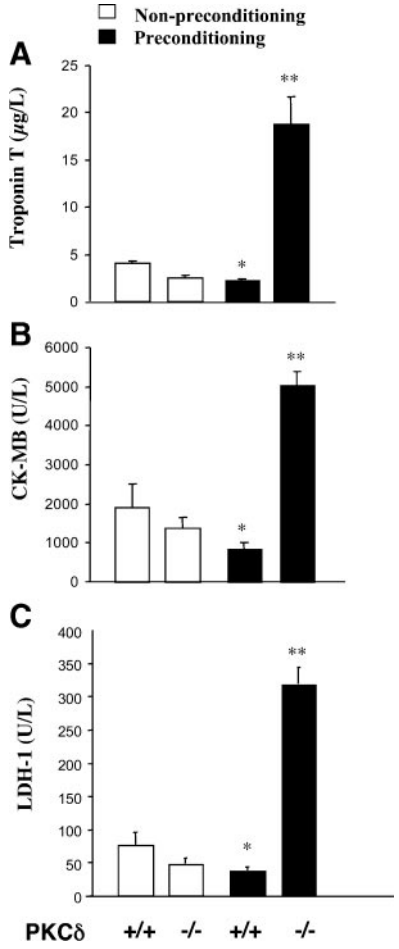

Fig. 1. Elevated plasma troponin T, creatine kinase-MB (CK-MB), and lactate dehydrogenase isoenzyme 1 (LDH-1) concentrations. Troponin T, CK-MB, and LDH-1 enzymes were measured using commercial kits according to the manufacturer's instruction. *Significant difference from wild-type mice without preconditioning, $P < 0.05$; **significant difference from all other groups, $P < 0.01$.

Determination of troponin T, creatine kinase-MB, and lactate dehydrogenase isoenzyme 1. Heparinized blood was collected, and troponin T (TnT) was measured as an index of cardiac cellular damage using the quantitative rapid assay kit (Roche). Creatine kinase isoenzyme MB (CK-MB) and lactate dehydrogenase isoenzyme 1 (LDH-1) were electrophoretically analysed using commercially available kits (Paragon; Fullerton, CA).

Measurement of myocardial infarct size. After reperfusion, the heart was harvested, fixed in 4% paraformaldehyde, and embedded in paraffin (51). Sections were stained with hematoxylin and eosin for histological evaluation of tissue damage. The infarct area was measured as described previously (29). Additionally, the heart was cut into three slices proceeding transversely from the base to apex. The slices were incubated with triphenyltetrazolium chloride (10 mg/ml) in 0.2 mol/l phosphate buffer solution for 30 min. Noninfarcted myocardium, which contains LDH, stained brick red by reacting with triphenyltetrazolium chloride, whereas the necrotic (infarcted) tissue remained unstained. The infarcted area (uncolored), area at risk (uncolored and brick red), and nonoccluded areas (blue) were measured. The ratio of the infarct size to area at risk was calculated.

Measurement of O_2^-. O_2^- was quantified spectrophotometrically by monitoring of the superoxide dismutase-inhibitable cytochrome *c* reduction according to Zhou et al. (50). Briefly, after being preconditioned, hearts were harvested and protein extracts were prepared as described previously (24). Fifty microliters of 40 μmol/l cytochrome *c* and 500 μl of sample were mixed immediately and incubated for 10 min at room temperature, and the absorbance measured at 550 nm. Reduced cytochrome *c* was calculated, and the results were expressed as nanomoles per milligram of protein.

Proteomic analysis of heart tissue. Heart tissues for proteomic and metabolomic analysis were rinsed thoroughly with cold PBS to remove any blood components within the heart chambers, and whole hearts were frozen immediately in liquid nitrogen to avoid protein or metabolite degradation. The procedure used for two-dimensional (2-D) electrophoresis has been described previously (10, 26, 27). Protein extracts were loaded on nonlinear immobilized pH gradient

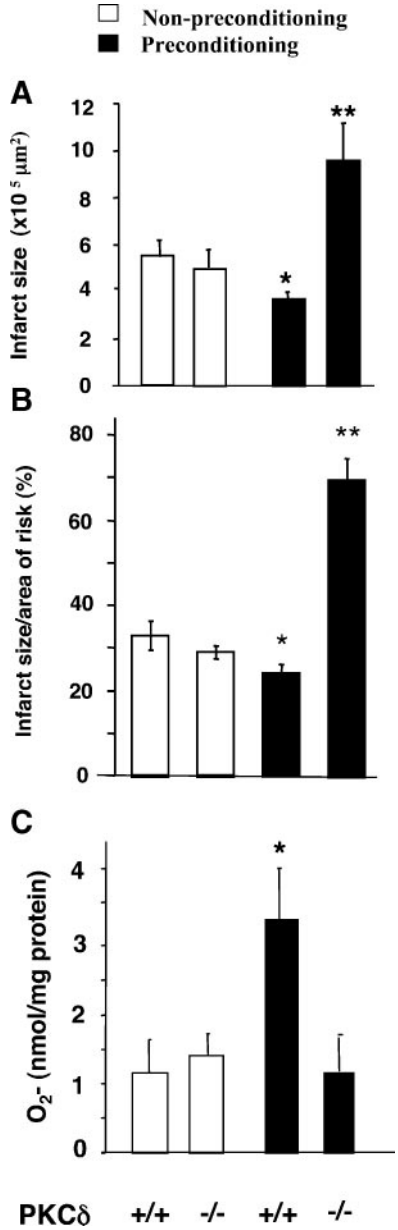

Fig. 2. Myocardial damage. Hearts were harvested after the left descending artery was occluded for 30 min and reperfused for 2 h with or without preconditioning. *A*: infarct size; *B*: infarct size normalized to the region at risk. *Significant difference from wild-type mice without preconditioning, $P < 0.05$; **significant difference from all other groups, $P < 0.05$. *C*: superoxide dismutase-inhibitable cytochrome *c* reduction ($n = 4$ mice/group). *Significant difference from all other groups, $P < 0.05$.

(IPG) 18-cm strips (3–10, Amersham Pharmacia Biotech) using an in-gel rehydration method. Strips were focused at 0.05 mA/IPG strip for 60 kV·h at 20°C. SDS-PAGE was carried out overnight at 20 mA/gel at 8°C. 2-D electrophoresis protein profiles were visualized by silver staining. Spot patterns were analyzed using Progenesis (Nonlinear Dynamics), Proteomweaver (Definiens), and PDQuest software (Bio-Rad). Quantitative estimates for individual spots were obtained after each gel was normalized according to the total number of spots using a scaling factor of parts per million. Spots showing a statistical difference in intensity were excised for identification.

Matrix-assisted laser desorption/ionization mass spectrometry. Gel pieces containing selected protein spots were treated overnight with modified trypsin (Promega) according to a published protocol (37) and as described previously (26). Matrix-assisted laser desorption/ionization mass spectrometry was performed using an Axima CFR spectrometer (Kratos; Manchester, UK). The resulting peptide masses

Fig. 3. Two-dimensional (2-D) electrophoresis map of heart proteins before and after preconditioning. Protein extracts were separated on a pH 3–10 nonlinear IPG strip, followed by a 12% SDS-polyacrylamide gel. Spots were detected by silver staining. A direct overlay of PKC$\delta^{+/+}$ hearts with and without preconditioning is shown. Each average gel was created from 4 single gels (total $n = 8$). Differentially expressed spots are highlighted in color (blue and orange for PKC-$\delta^{+/+}$ hearts with and without preconditioning, respectively). Proteins identified by MALDI-MS are marked with numbers and listed in Table 1.

were searched against databases using the MASCOT program (34). One missed cleavage per peptide was allowed, and carbamidomethylation of cysteine as well as partial oxidation of methionine were assumed.

Proton NMR spectroscopy. Snap-frozen hearts were extracted in 6% perchloric acid (2). Neutralized extracts were freeze dried and reconstituted in D_2O. Extracts (0.5 ml) were placed in 5-mm NMR tubes. ^1H NMR spectra were obtained using a Bruker 500-MHz spectrometer. The water resonance was suppressed by using gated irradiation centered on the water frequency. Sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate was added to the samples for chemical shift calibration and quantification. Immediately before the NMR analysis, the pH was readjusted to 7 with PCA or KOH.

Statistical analysis. Statistical analysis was performed using ANOVA and Student's *t*-test, respectively. Results are given as means ± SD. A *P* value of <0.05 was considered significant.

RESULTS

Myocardial damage. Ischemia-reperfusion injury was performed by 30 min of occlusion of the left descending coronary artery, followed by 2 h of reperfusion. This procedure caused a myocardial infarct in wild-type and PKC-$\delta^{-/-}$ mice. Infarct size as well as serum markers were measured for the accurate assessment of cardiac damage (28, 29). As expected, preconditioning with three cycles of 5-min artery occlusion/5-min reperfusion before the prolonged ischemia significantly reduced serum levels of all three cardiac damage markers in wild-type mice ($P < 0.05$) but unexpectedly caused a fivefold enhanced release in PKC-$\delta^{-/-}$ mice (TnT, 2.5 ± 0.3 vs.18.8 ± 2.9; CK-MB, 1,376 ± 286 vs. 5,021 ± 355; LDH-1 48.5 ± 8.2 vs. 319 ± 25, $P < 0.001$; Fig. 1). No differences in blood pressure were observed between wild-type and PKC-$\delta^{-/-}$ mice during the preconditioning procedure (mean arterial pressure: 95 ± 13 and 92 ± 9 mmHg for PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ mice, respectively, $n = 3$).

Histological examinations of cardiac tissues confirmed that the infarction area was significantly larger in PKC-$\delta^{-/-}$ mice treated with preconditioning compared with all other groups

Table 1. *Differences in protein profiles between PKC-$\delta^{+/+}$ hearts with and without preconditioning*

Spot Number	Protein Identity	Δ	*P* Value	NCBI Entry Number	Function	Theoretical pI/Molecular Mass, Da ($\times 10^3$)	Observed pI/Molecular Mass, Da ($\times 10^3$)	Sequence Coverage/ Mascot Score
				Respiratory chain/TCA cycle				
1	ATP synthase, H^+-transporting mitochondrial F_1 complex, β-subunit	−4.3	0.006	12832739	ATP generation, respiratory chain	5.4/56.9	5.0/57.4	37%/200
2	ATP synthase, H^+-transporting mitochondrial F_1 complex, α-subunit (fragment)	+2.9	0.018	6680748	ATP generation, respiratory chain	9.2/59.7	9.1/27.0	26%/172
3	NADH dehydrogenase (ubiquinone) Fe-S protein 1	−2.1	0.013	21704020	Respiratory chain	5.5/79.7	5.3/75.2	31%/205
4	NADH dehydrogenase (ubiquinone) Fe-S protein 1 (fragment)	Greater than +10	0.013	21704020	Respiratory chain			12%/68
5a	Malate dehydrogenase, soluble	−3.9	0.008	6678918	TCA cycle	6.2/36.4	5.9/34.8	14%/63
5b	Malate dehydrogenase, soluble	−2.9	0.033	6678918	TCA cycle	6.2/36.4	5.8/34.8	17%/65
				Glucose metabolism				
6	Glyceraldehyde-3-phosphate dehydrogenase	+1.9	0.001	20832385	Glycolysis	8.3/24.6	8.3/26.5	22%/64
7a	Dihydrolipoamide *S*-acetyltransferase (*fragment a*)	+2.0	0.039	31542559	E_2 component of pyruvate dehydrogenase	8.8/67.9	5.3/43.3	15%/97
7b	Dihydrolipoamide *S*-acetyltransferase (*fragment b*)	+2.3	0.008	31542559	E_2 component of pyruvate dehydrogenase	8.8/67.9	6.6/22.9	13%/72
				Lipid metabolism				
8	3-Hydroxyacyl-CoA dehydrogenase type II	−8.6	0.001	7949047	Short chain/hydroxy-steroid dehydrogenase	8.8/27.4	9.5/24.6	36%/96
				Cytoskeleton				
9	t-Complex protein 1	−2.7	0.004	228954	Actin/tubulin folding	5.8/60.5	5.9/60.9	11%/65
10	mDj11	+2.6	0.038	6567178	DNAJ (Hsp40) homolog	6.1/56.4	6.4/54.4	22%/76
11	F-actin capping protein, β_1-subunit	−2.8	0.036	1345668	Cytoskeletal regulation	5.5/31.3	5.4/33.2	32%/111
12	α-Actin, cardiac (mouse)			627834	Cytoskeleton	5.2/41.8	5.2/44.7	27%/96
13	Similar to surfeit 3 protein	−4.4	0.019	20825556	Cytokeletal regulation	10.0/31.6	9.5/25.4	17%/65
14	Riken cDNA D930010J01	+3.1	0.038	19527384	Surfeit protein	8.3/27.1	8.4/24.2	23%/72
				Others				
15	Atrial natriuretic factor precursor	−6.5	0.002	113865	Vasodilatative peptide	6.7/16.6	5.3/15.9	28%/80
16	Selenium-binding protein 1	−3.4	0.021	134259	Intracellular selenium transport	6.0/52.3	5.9/52.8	16%/77
17	Unknown (protein for MGC:62540)	−1.5	0.095	33243954	Splice isoform of carbonic anhydrase 2	6.5/29.0	6.7/26.3	33%/120
18	Riken cDNA 2310005O14	−2.2	0.004	20885971	Unknown function	5.6/35.1	5.0/32.4	30%/120

Δ, fold increased/decreased expression in preconditioned PKC-$\delta^{+/+}$ hearts compared with nonpreconditioned PKC-$\delta^{+/+}$ hearts. pI, isoelectric point; TCA, tricarboxylic acid.

($9.6 \times 10^5 \pm 1.6$ μm^2 vs. $3.7 \times 10^5 \pm 0.3$ μm^2, $P < 0.05$; Fig. 2*A*). While the ratio of the infarct size (in μm^2) to area of risk was similar in nonpreconditioned PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ mice (33.2 ± 3.4% vs. 29.3 ± 1.6%), preconditioning significantly reduced this ratio in wild-type hearts but increased it in PKC-$\delta^{-/-}$ mice (24.2 ± 2.1% vs. 69.7 ± 5.1%; Fig. 2*B*). These data demonstrate that ischemic preconditioning was not cardioprotective but had the opposite effect in PKC-$\delta^{-/-}$ hearts.

Reactive oxygen species are known to be crucial triggers of cardioprotection after ischemic preconditioning (31, 41, 42, 50). To investigate whether PKC-δ deficiency alters reactive oxygen species production after preconditioning, we determined O_2^- concentrations using protein extracts from cardiac tissues. The data presented in Fig. 2*C* demonstrate that preconditioning treatment increases O_2^- production in PKC-$\delta^{+/+}$ but not PKC-$\delta^{-/-}$ hearts, indicating the importance of PKC-δ for the preconditioning-induced increase in oxidative stress.

Proteomic changes in preconditioned PKC-$\delta^{+/+}$ hearts. Whole hearts were directly harvested after preconditioning for proteomic analysis by 2-D electrophoresis. Average gels of preconditioned and nonpreconditioned PKC-$\delta^{+/+}$ hearts were created from 4 gels/group. A direct overlay is presented in Fig. 3. With the use of a broad-range pH gradient (pH 3–10 nonlinear), 2-D electrophoresis gels compromised ~1,400 protein features. Differentially expressed spots were highlighted in color (orange and blue indicate an increase in nonpreconditioned and preconditioned PKC-$\delta^{+/+}$ hearts, respectively). Numbered spots were excised and subject to in-gel tryptic digestion. Protein identifications as obtained by MALDI-MS are listed in Table 1.

Protein changes were observed for enzymes involved in aerobic metabolism and cytoskeletal proteins (Table 1). For instance, isoforms of NADH dehydrogenase and ATP synthase decreased in intensity in preconditioned hearts, whereas proteolytic fragments of these mitochondria-located enzymes became detectable by MALDI-MS. These findings are consistent with a recent study by Da Silva et al. (8) demonstrating an impaired respiratory chain reaction in isolated mitochondria from preconditioned hearts with preferential inhibition of mitochondrial complex I. Notably, two complimentary fragments of dihydrolipoamide *S*-acetyltransferase were observed after

Match to: **gil31542559**
dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex) [Mus musculus]
Nominal mass (Mr): **68473**; Calculated pI value: **8.81**

Sequence coverage for Spot 7a:

```
  1 MWRVCARRAR SAVPRDGFRA RWAALKEGPG APCGSPRIGP AAVRCGSGIP
 51 RYGVRSLCGW SSGSGTVPRN RLLRQLLGSP SRRSYSLPPH QKVPLPSLSP
101 TMQAGTIARW EKKEGEKISE GDLIAEVETD KATVGFESLE ECYMAKILVP
151 EGTRDVPVGS IICITVEKPQ DIEAFKNYTL DLAAAAAPQA APAAAPATAA
201 APAAPSASAP GSSYPTHMQI VLPALSPTMT MGTVQRWEKK VGEKLSEGDL
251 LAEIETDKAT IGFEVQEEGY LAKILVPEGT RDVPLGAPLC IIVEKQEDIA
301 AFADYRPTEV TSLKPQAAPP APPPVAAVPP TPQPVAPTPS AAPAGPKGRV
351 FVSPLAKKLA AEKGIDLTQV KGTGPEGRII KKDIDSFVPS KAAPAAAAAM
401 APPGPRVAPA PAGVFTDIPI SNIRRVIAQR LMQSKQTIPH YYLSVDVNMG
451 EVLLVRKELN KMLEGKGKIS VNDFIIKASA LACLKVPEAN SSWMDTVIRQ
501 NHVVDVSVAV STPAGLITPI VFNAHIKGLE TIASDVVSLA SKAREGKLQP
551 HEFQGGTFTI SNLGMFGIKN FSAIINPPQA CILAIGASED KLIPADNEKG
601 FDVASVMSVT LSCDHRVVDG AVGAQWLAEF KKYLEKPITM LL
```

Fig. 4. Fragmentation of mitochondrial enzymes after ischemic preconditioning.

AA 91-430: Nominal mass (Mr): **35.3**; observed: **43.0**
Calculated pI Da(x10^3): **5.17;** observed: **5.3**
pfam:BIOTIN_LIPOYL pos. 91-165 and 218-292

Sequence coverage for Spot 7b:

```
  1 MWRVCARRAR SAVPRDGFRA RWAALKEGPG APCGSPRIGP AAVRCGSGIP
 51 RYGVRSLCGW SSGSGTVPRN RLLRQLLGSP SRRSYSLPPH QKVPLPSLSP
101 TMQAGTIARW EKKEGEKISE GDLIAEVETD KATVGFESLE ECYMAKILVP
151 EGTRDVPVGS IICITVEKPQ DIEAFKNYTL DLAAAAAPQA APAAAPATAA
201 APAAPSASAP GSSYPTHMQI VLPALSPTMT MGTVQRWEKK VGEKLSEGDL
251 LAEIETDKAT IGFEVQEEGY LAKILVPEGT RDVPLGAPLC IIVEKQEDIA
301 AFADYRPTEV TSLKPQAAPP APPPVAAVPP TPQPVAPTPS AAPAGPKGRV
351 FVSPLAKKLA AEKGIDLTQV KGTGPEGRII KKDIDSFVPS KAAPAAAAAM
401 APPGPRVAPA PAGVFTDIPI SNIRRVIAQR LMQSKQTIPH YYLSVDVNMG
451 EVLLVRKELN KMLEGKGKIS VNDFIIKASA LACLKVPEAN SSWMDTVIRQ
501 NHVVDVSVAV STPAGLITPI VFNAHIKGLE TIASDVVSLA SKAREGKLQP
551 HEFQGGTFTI SNLGMFGIKN FSAIINPPQA CILAIGASED KLIPADNEKG
601 FDVASVMSVT LSCDHRVVDG AVGAQWLAEF KKYLEKPITM LL
```

AA431-642: Nominal mass (Mr): **22.9**; observed: **22.9**
Calculated pI Da(x10^3): **6.58**; observed: **6.6**
pfam:2_OXOACID_DH pos. 412-642

preconditioning (Tab. 1). The E_2 component of the pyruvate dehydrogenase complex is particularly susceptible to reactive oxygen species due to its lipoic acid moieties (17). On the basis of the sequence coverage obtained by tryptic digest peptides, fragmentation of dihydrolipoamide *S*-acetyltransferase seemed to occur between amino acid residues 430–436 (Fig. 4), a sequence containing cleavage sites for trypsin and chymotrypsin. Abnormal proteins generated by oxidative damage are rapidly eliminated by the proteasome, an essential proteolytic complex in eukaryotic cells with trypsin and chymotrypsin-like activity (14). In fact, it has been demonstrated that mild tryptic digestion of the pyruvate dehydrogenase complex gives rise to two fragments of 45 and 22 kDa (22), respectively, which correspond to the observed molecular mass of the preconditioning-induced fragments on 2-D electrophoresis gels. Thus oxidative stress might compromise the integrity of mitochondrial enzymes in preconditioned tissues.

Other differences induced by ischemic preconditioning included changes in cytoskeletal proteins or proteins associated with cytoskeletal remodeling, i.e., a threefold decrease of F-actin cap-

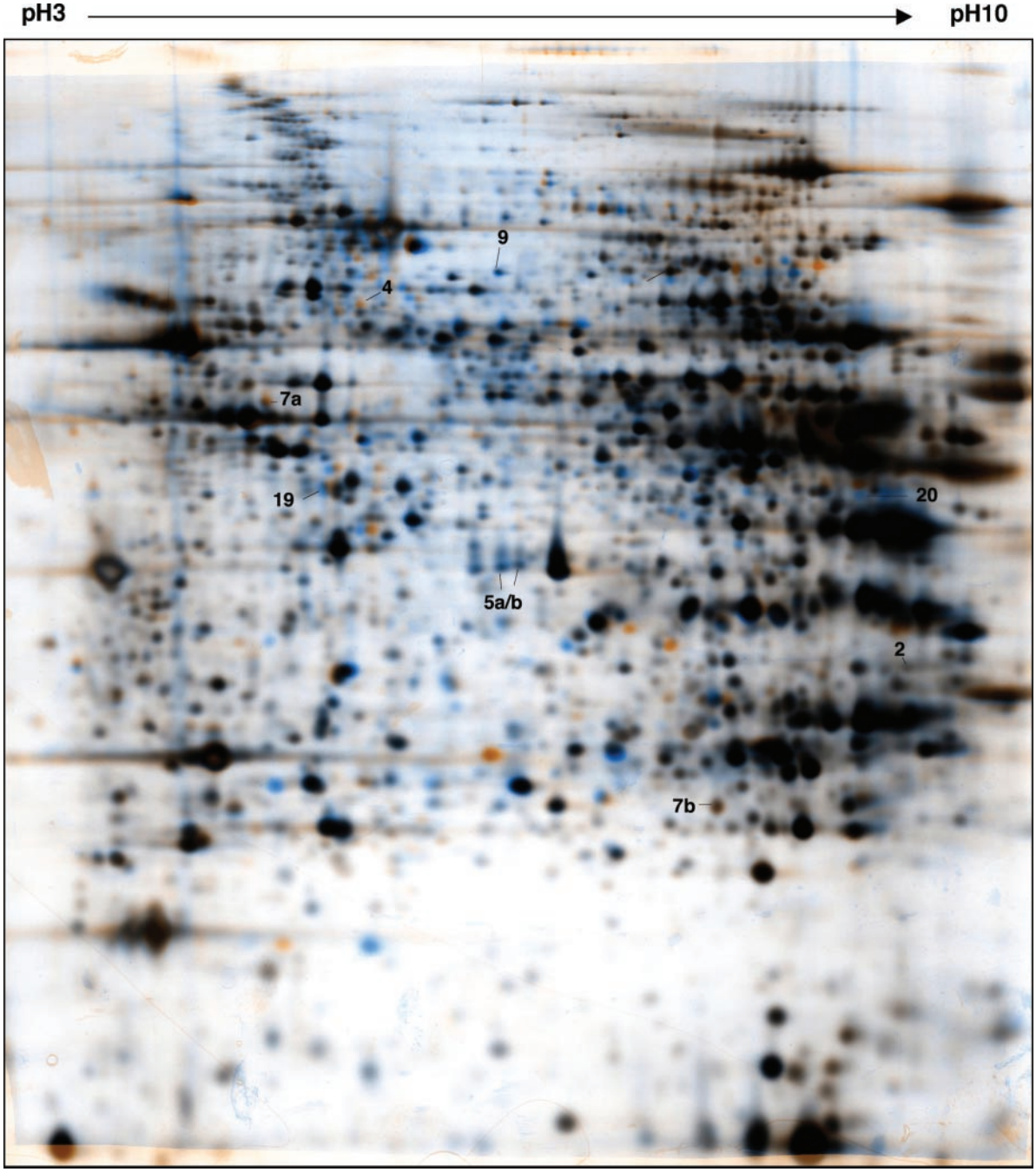

Fig. 5. 2-D electrophoresis map of heart proteins from preconditioned PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts. A direct comparison of preconditioned PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts is presented in Fig. 5. Each average gel was created from 4 single gels (total n = 8). Differentially expressed spots are highlighted in color (orange and blue for preconditioned PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts, respectively). Proteins identified by MALDI-MS are marked with numbers and listed in Table 2.

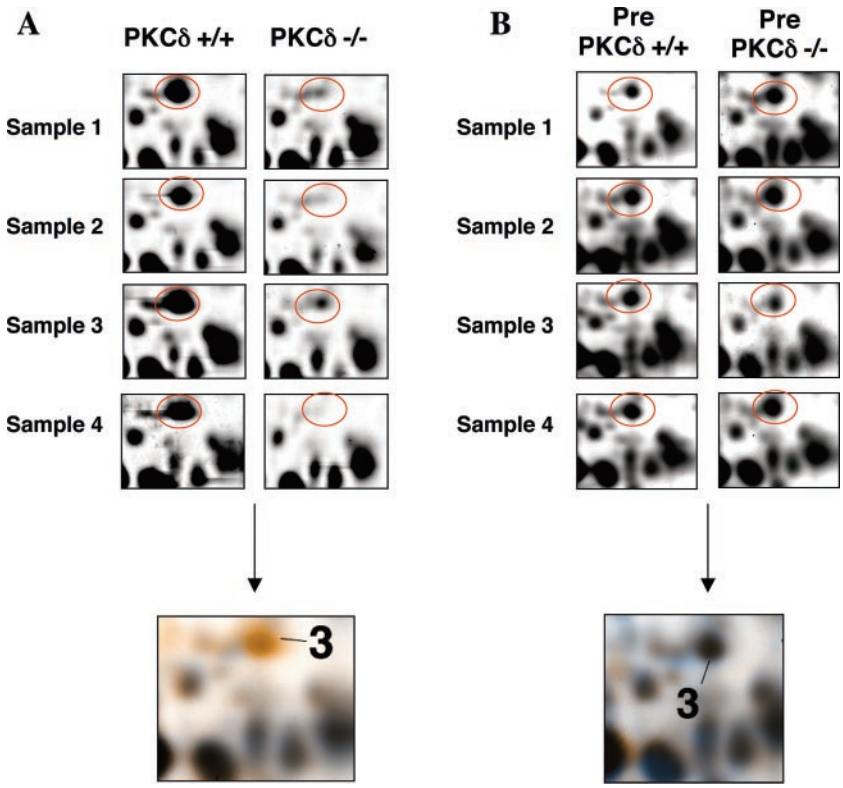

Fig. 6. Proteomic changes of LDH-1. *A*: enzymatic isoform expression of LDH-1 in PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts. *B*: enzymatic isoform expression of LDH-1 in preconditioned PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts. Note that differences for LDH-1 between PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts under normoxic conditions disappear in response to ischemic preconditioning, indicating that proteomic changes on 2-D gels can reflect posttranslational modifications related to enzymatic activity. *Bottom*: corresponding overlay of average gels based on the 4 biological replicates shown above (see *part I, spot 3*).

ping protein (β_1-isoform), and a decrease in the atrial natriuretic factor (ANF) precursor. This finding is consistent with a previous report (7) showing that vasodilatator peptides, such as ANF, are rapidly released in response to preconditioning.

Proteomic changes in preconditioned PKC-$\delta^{-/-}$ hearts. Average gels of preconditioned PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts are presented as a direct overlay in Fig. 5. Most of the differences described for normoxic hearts (26) persisted after

Table 2. *Differences in protein profiles between hearts of PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ mice after preconditioning*

Spot Number	Protein Identity	Δ	*P* Value	NCBI Entry Number	Function	Theoretical pI/Molecular Mass, Da ($\times 10^3$)	Observed pI/Molecular Mass, Da ($\times 10^3$)	Sequence Coverage/ Mascot Score
					Respiratory chain			
2	ATP synthase, H^+-transporting mitochondrial F_1 complex, α-subunit (fragment)	−2.2	0.043	6680748	Respiratory chain	9.2/59.7	9.1/27.0	26%/172
4	NADH dehydrogenase (ubiquinone) Fe-S protein 1 (fragment)	−9.9	0.013	21704020	Respiratory chain	5.5/79.7	5.6/55.9	12%/68
5a	Malate dehydrogenase, soluble	+3.1	0.040	6678918	TCA cycle	6.2/36.4	5.9/34.8	14%/63
5b	Malate dehydrogenase, soluble	+2.4	0.049	6678918	TCA cycle	6.2/36.4	5.9/34.8	15%/65
					Glucose metabolism			
7a	Dihydrolipoamide *S*-acetyltransferase (*fragment a*)	−2.8	0.002	31542559	E_2 component of pyruvate dehydrogenase	8.8/67.9	5.3/43.3	15%/97
7b	Dihydrolipoamide *S*-acetyltransferase (*fragment b*)	−1.7	0.040	31542559	E_2 component of pyruvate dehydrogenase	8.8/67.9	6.6/22.9	13%/72
					Cytoskeleton			
9	t-Complex protein 1	+2.9	0.000	228954	Actin/tubulin folding	5.8/60.5	5.9/60.9	12%/68
19	Tubulin α_4 (fragment)	+10	0.004	6678467	cytoskeleton	5.0/49.9	5.5/38.3	11%/65
					Others			
20	MCG37309	+10	0.010	23274114	Similar to heterogeneous nuclear ribonucleoprotein A_3	8.5/37.1	8.4/38.0	26%/78

Δ, Fold increased/decreased expression in preconditioned PKC-$\delta^{-/-}$ hearts compared with preconditioned PKC-$\delta^{+/+}$ hearts. pI, isoelectric point; TCA, tricarboxylic acid.

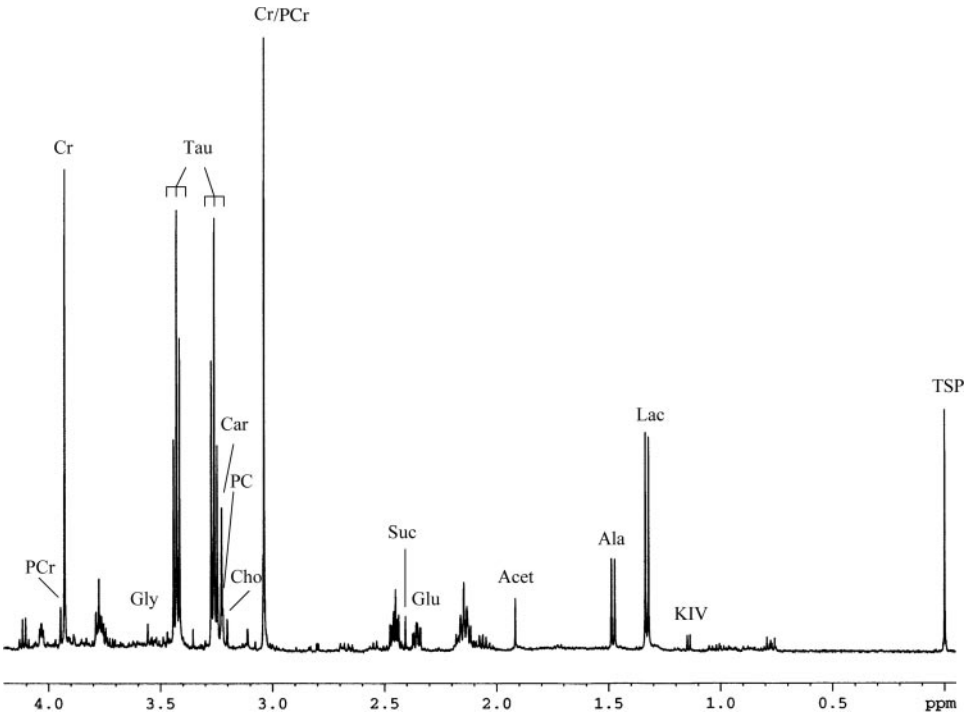

Fig. 7. NMR spectrum from a preconditioned PKC-$\delta^{+/+}$ heart. Within the aliphatic region (−0.05 to 4.2 ppm) of the NMR spectra, resonances have been assigned to α-ketoisovalerate (KIV), lactate (Lac), alanine (Ala), acetate (Acet), glutamate (Glu), succinate (Suc), creatine (Cr), choline (Cho), phosphocholine (PC), carnitine (Car), taurine (Tau), glycine (Gly), and phosphocreatine (PCr) (11). Sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP) was added to the samples for calibration.

preconditioning, but, strikingly, differences in glycolytic enzymes, e.g., LDH-1, disappeared (see *Part I, spot 3*, and Fig. 6). Similar findings were obtained for pyruvate kinase (*Part I, spot 2*), glycerol-3-phosphate dehydrogenase (*Part I, spot 1*), and putative glucose dehydrogenase (*Part I, spot 4*). Notably, changes that were found to be associated with preconditioning in PKC-$\delta^{+/+}$ hearts were absent in PKC-$\delta^{-/-}$ hearts, including increased fragmentation of mitochondrial enzymes (Table 2).

Metabolic changes after preconditioning. Cardiac metabolites were measured with high-resolution NMR spectroscopy (Fig. 7) (11). Ischemic preconditioning induced the characteristic rise in phosphocreatine in PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts, demonstrating the effectiveness of the preconditioning procedure in our animal model. Furthermore, preconditioning caused a significant increase in alanine, which was less pronounced in PKC-$\delta^{-/-}$ hearts (1.857 ± 0.246 vs. 1.449 ± 0.187, $P = 0.18$), and a drop in succinate, the oxidation of which is directly associated with respiratory chain reactions. Other significant changes, such as a rise in carnitine, phosphocholine, and glycine concentrations, were only observed in wild-type hearts (Table 3). Strikingly, among all the metabolites shown in Table 3, only lactate levels were considerably higher in preconditioned PKC-$\delta^{-/-}$ than PKC-$\delta^{+/+}$ hearts, indicating that the loss of PKC-δ broadly impairs the metabolic response to ischemic preconditioning.

DISCUSSION

Activation of PKC is thought to be a crucial step in ischemic preconditioning. PKC-δ and PKC-ϵ are among the predominant isoforms of PKC in cardiac ventricles. Whereas PKC-ϵ has been implicated as a mediator of ischemic preconditioning in different models (9, 25, 35, 38), controversial results exist concerning the PKC-δ isoform (12, 45, 49). In the present

Table 3. *Differences in metabolites between PKC-$\delta^{+/+}$ and PKC-$\delta^{-/-}$ hearts*

	PKC-$\delta^{+/+}$	PKC-$\delta^{-/-}$	Preconditioned PKC-$\delta^{+/+}$	Preconditioned PKC-$\delta^{-/-}$	*P* Value
Alanine	1.220 (±0.080)	0.997 (±0.221)	1.857 (±0.246)*	1.449 (±0.187)	0.043*
Creatine	5.691 (±0.391)	5.158 (±0.559)	6.855 (±0.665)	6.538 (±0.237)	0.376
Choline	0.152 (±0.037)	0.210 (±0.124)	0.169 (±0.019)	0.112 (±0.042)	0.733
Carnitine	0.563 (±0.100)	0.409 (±0.085)	0.891 (±0.113)*	0.538 (±0.101)	0.039*
Taurine	14.892 (±1.513)	14.012 (±2.229)	19.803 (±2.160)	14.829 (±1.052)	0.149
Glutamate	1.510 (±0.286)	1.310 (±0.378)	1.393 (±0.089)	1.091 (±0.059)	0.682
Lactate	7.152 (±1.132)	5.800 (±0.993)	2.393 (±0.966)*	3.459 (±0.400)	0.017*
Acetate	0.549 (±0.097)	1.001 (±0.326)	0.752 (±0.159)	0.645 (±0.101)	0.368
Succinate	0.607 (±0.213)	0.409 (±0.101)	0.180 (±0.028)*	0.195 (±0.024)	0.084*
α-ketoisovalerate	0.243 (±0.076)	0.683 (±0.351)	0.508 (±0.210)	0.409 (±0.141)	0.492
Glycine	0.216 (±0.019)	0.227 (±0.034)	0.502 (±0.085)*	0.309 (±0.048)	0.011*
Phosphocholine	0.114 (±0.006)	0.117 (±0.004)	0.224 (±0.018)*	0.128 (±0.030)	0.001*
Phosphocreatine	ND	ND	0.803 (±0.336)*	0.962 (±0.452)*	0.051*

Data are means ± SE (in μmol/g wet wt); $n = 5$ PKC-$\delta^{+/+}$ hearts and 3 PKC-$\delta^{-/-}$ hearts. ND, not detectable. *P* values were derived from ANOVA tables. *Significant difference from nonpreconditioned hearts using the Fisher protected least-significant difference test.

study, we investigated the role of PKC-δ in preconditioning using PKC-δ knockout mice. We characterized proteomic as well as metabolomic changes after preconditioning and provided the first evidence that mutational ablation of the PKC-δ gene is associated with a loss of preconditioning-induced cardioprotection.

Less cardiac damage in PKC-δ$^{-/-}$ hearts without preconditioning. PKC-δ and PKC-ϵ are classified as novel PKC isoforms. Both isoforms might have distinct roles in response to ischemic injury: PKC-ϵ activation but PKC-δ inhibition are thought to be involved in myocardial salvage (19). In a transgenic mouse model, PKC-δ translocation inhibitors exerted even better cardioprotection from ischemic injury than PKC-ϵ activation (16). Similarly, in nonpreconditioned PKC-δ$^{-/-}$ hearts, serum concentrations of TnT, CK-MB, and LDH-1 suggested a minor reduction in infarct size after ischemia-reperfusion injury (TnT, 4.1 ± 0.2 vs. 2.5 ± 0.3 μs/l; CK-MB, 1,989 ± 622 vs. 1,376 ± 286 U/l; LDH-1, 75.8 ± 20 vs. 48.5 ± 8.2 U/l; Fig. 1), but this trend failed to reach statistical significance. In contrast to its possible cardioprotective effects in nonpreconditioned hearts, loss of PKC-δ was associated with exaggerated postischemic injury in preconditioned hearts.

Protective effects of preconditioning in PKC-δ$^{+/+}$ hearts. Compared with other studies (15), the observed reduction in infarct size after preconditioning was rather modest in wild-type mice. It has to be noted that other investigators applied six cycles of short ischemia-reperfusion, followed by 30 min of prolonged ischemia and 4 h of reperfusion (15), whereas we used only three cycles and 2 h of reperfusion. Using the longer protocol, we found that the reduction in infarct size in wild-type mice became more pronounced after preconditioning (data not shown), which is consistent with a previous report (3). For proteomic and metabolomic analysis, however, it was obviously preferable to limit the time of open-chest surgery, and the shorter protocol was found to be sufficient to induce maximum damage in PKC-δ$^{-/-}$ hearts.

Preconditioning induces reactive oxygen species. Paradoxically, the transient increase in reactive oxygen species during preconditioning results in cardioprotection (31, 42, 50). While antioxidants block the beneficial effects of preconditioning, exposure to exogenous oxidants can mimic preconditioning in intact hearts (6, 41). Reactive oxygen species have been shown to activate putative mediators of preconditioning, including PKC (41). Furthermore, it has been recently suggested that PKC-δ activation is required for the increase in O_2-derived free radical generation from mitochondria (42, 45). Support for these findings comes from our observation that increased oxidative stress and fragments of mitochondrial enzymes were only observed in preconditioned PKC-δ$^{+/+}$ hearts. Dependent on the respiratory activity, oxidative modifications of mitochondrial enzymes occur even under normoxic conditions (48), but increased oxidation during preconditioning might contribute to metabolic adaptation in preconditioned hearts.

Metabolic effects of ischemic preconditioning. Under ischemic conditions, exact adjustment of the Embden-Meyerhof pathway (EMP) is essential because anaerobic glycolysis is the major pathway for production of high-energy phosphates. Ischemic preconditioning changes the regulatory properties of EMP enzymes, and some of these changes are not exclusively on the basis of substrate kinetics (43). Strikingly, differences in glycolytic enzymes, as observed under normoxic conditions, disappeared in preconditioned PKC-δ$^{-/-}$ hearts. Concomitantly, alanine, a surrogate marker for glycolytic activity in the metabolomic analysis pathway, increased in PKC-δ$^{-/-}$ hearts. Hence, the anaerobic mode of glycolysis might not be impaired by PKC-δ deficiency. This would explain why PKC-δ$^{-/-}$ hearts had a similar rise in phosphocreatine levels after preconditioning. The preischemic "phosphocreatine overshoot" is normally attributed as a metabolic sign of successful preconditioning (20, 21). However, despite this rise in phosphocreatine, ischemic preconditioning failed to protect PKC-δ$^{-/-}$ hearts.

Enhanced cardiac damage in PKC-δ$^{-/-}$ hearts after preconditioning. Mutational ablation of PKC-δ had profound effects on cardiac metabolism (26): PKC-δ$^{-/-}$ mice compensated their impairment in aerobic glycolysis by increased fatty acid oxidation. However, this compensation mechanism was overwhelmed by the preconditioning procedure. Under normoxia, the ratio of (alanine + lactate)/(acetate) was significantly decreased in PKC-δ$^{-/-}$ hearts (26), indicating their dependence on fatty acids. Under hypoxic conditions, this ratio became similar: preconditioned wild-type hearts increased acetate, which was accompanied by an increase of carnitine, required for mitochondrial transport of long-chain fatty acids, of phosphocholine, a precursor for the synthesis of membrane phospholipids, and of α-ketoisovalerate, a regulator of pyruvate dehydrogenase activity. In contrast, preconditioning resulted in a drop of acetate in PKC-δ$^{-/-}$ hearts, and lactate removal was less efficient compared with PKC-δ$^{+/+}$ hearts, which is in agreement with our findings in nonpreconditioned hearts (26). Therefore, ischemic preconditioning might exhaust the metabolic compensation in PKC-δ$^{-/-}$ hearts.

In conclusion, to the best of our knowledge, our study is the first to characterize proteomic as well as metabolomic changes after ischemic preconditioning, and we provided evidence for a loss of preconditioning-induced cardioprotection in PKC-δ null mice. Our findings could be important for a better understanding of cardioprotection and implicate specific targets for new therapeutic strategies in cardiac patients.

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