Identification of ϵ PKC Targets During Cardiac Ischemic Injury

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Background: Epsilon-protein kinase C (ϵ PKC) protects the heart from ischemic injury. However, the mechanism(s) of ϵ PKC cardioprotection is still unclear. Identification of the ϵ PKC targets may aid in elucidating the ϵ PKC-mediated cardioprotective mechanisms. Previous studies, using ϵ PKC transgenic mice and difference in gel electrophoresis, identified proteins involved in glucose metabolism, the expression of which was modified by ϵ PKC. Those studies were accompanied by metabolomic analysis, suggesting that increased glucose oxidation may be responsible for the cardioprotective effect of ϵ PKC. Whether these ϵ PKC-mediated alterations were because of differences in protein expression or phosphorylation was not determined.

Methods and Results: In the present study, we used an ϵ PKC -specific activator peptide, $\psi\epsilon$ RACK, combined with phosphoproteomics, to find ϵ PKC targets, and identified that the proteins whose phosphorylation was altered by selective activation of ϵ PKC were mostly mitochondrial proteins. Analysis of the mitochondrial phosphoproteome led to the identification of 55 spots, corresponding to 37 individual proteins, exclusively phosphorylated, in the presence of $\psi\epsilon$ RACK. The majority of the proteins identified were involved in glucose and lipid metabolism, components of the respiratory chain as well as mitochondrial heat shock proteins.

Conclusions: The protective effect of ϵ PKC during ischemia involves phosphorylation of several mitochondrial proteins involved in glucose and lipid metabolism and oxidative phosphorylation. Regulation of these metabolic pathways by ϵ PKC phosphorylation may lead to ϵ PKC-mediated cardioprotection induced by $\psi\epsilon$ RACK. (*Circ J* 2012; 76: 1476–1485)

Key Words: Epsilon-protein kinase C (ϵ PKC); Ischemia; Mitochondria; Phosphorylation

We previously developed and used an epsilon-protein kinase C (ϵ PKC) isoenzyme-selective activator peptide and found that ϵ PKC activation reduces cardiac cell death induced by ischemia.^{1,2} To provide insight into ϵ PKC-mediated cytoprotective mechanisms, we used a proteomic approach combining antibodies that specifically recognize proteins phosphorylated at the PKC consensus phosphorylation site and an ϵ PKC activator peptide.^{3,4} This approach led to the identification of mitochondrial aldehyde dehydrogenase 2 (ALDH2) as an ϵ PKC substrate, the phosphorylation and activation of which is necessary and sufficient to induce cardioprotection during an ischemic injury.³ We also demonstrated that the cytoprotective mechanism of ϵ PKC is mediated, at least in part, by ALDH2-mediated detoxification of reactive aldehydes, such as 4-hydroxy-2-nonenal (4-HNE), that accumulate in the heart during ischemia.^{3,5,6} Studies by others, using transgenic and dominant negative ϵ PKC mice, identified

other ϵ PKC signaling complexes, composed of proteins involved in glucose and lipid metabolism, and proteins related to transcription/translation, suggesting that ϵ PKC-mediated cytoprotection involves regulation of other cellular processes.^{7–9} A study using difference in gel electrophoresis (DIGE) compared the hearts of mice overexpressing catalytically active and dominant negative ϵ PKC and identified alterations in the levels of proteins involved in glucose metabolism. Metabolomic studies confirmed that during ischemia/reperfusion glucose is metabolized faster in animals expressing constitutively active ϵ PKC.¹⁰ However, these studies did not clarify whether the differences in the identified proteins were because of differential expression or phosphorylation levels. Overexpression of ϵ PKC lead to its mislocalization¹¹ and a compensatory effect observed by δ PKC overexpression.¹⁰ Therefore, some of the targets identified in this study could possibly have been phosphorylated by overexpressed δ PKC or mislocalized ϵ PKC. Nevertheless,

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those studies suggested that the cardioprotective mechanism of εPKC is also related to the regulation of glucose and lipid metabolism.

In the present study we used an adult rat heart Langendorff coronary perfusion system, and treated isolated hearts with an εPKC specific activator peptide (ψεRACK) prior to ischemia, to determine phosphorylation events following selective activation of εPKC. We chose to use ψεRACK to detect εPKC targets, which would help elucidate the protective mechanisms of εPKC activation, because preconditioning with ψεRACK is cardioprotective.

Methods

Ex Vivo Rat Heart Model of Cardiac Ischemia

Animal protocols were approved by the Stanford University Institutional Animal Care and Use Committee. The hearts from adult Wistar rats (250–300 g), each group consisting of 3 rats, were perfused via the aorta at a constant flow rate of 10 ml/min with oxygenated Krebs-Henseleit buffer (in mmol/L: 120 NaCl, 5.8 KCl, 25 NaHCO₃, 1.2 NaHCO₃, 1.2 MgSO₄, 1.2 CaCl₂, and 10 dextrose, pH 7.4) at 37°C. After a 20-min equilibration period, hearts were subjected to 35 min of global, no-flow ischemia. The εPKC-selective agonist ψεRACK peptide [HDAPIGYD]¹² fused to the cell permeable Tat protein transduction domain peptide; amino acids 47–57¹³ (1 mmol/L) was perfused for 10 min immediately prior to ischemia onset.

Preparation of Heart Lysates and Subcellular Fractionation

At the end of the period of ischemia, the hearts were removed from the cannula and immediately homogenized on ice to obtain total and mitochondrial fractions. To obtain the total lysate fraction, heart ventricles were homogenized in Buffer A [7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 5 mmol/L magnesium acetate, 17 μg/ml PMSF and phosphatase inhibitor cocktail diluted 1:300 (Sigma # P8340 and Sigma # P5726)]. To obtain the mitochondrial fraction, heart ventricles were homogenized in ice-cold mannitol-sucrose (MS) buffer [210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L MOPS and 1 mmol/L EDTA containing protease) and phosphatase inhibitors as above]. The homogenate was centrifuged at 700 g for 10 min (to pellet the cytoskeletal fraction), the resultant supernatant was filtered through gauze, and re-centrifuged at 10,000 g for 10 min (to pellet the mitochondrial fraction). The mitochondrial pellet was washed 3 times in MS buffer before the pellet was resuspended in DIGE buffer.

2D Gel Electrophoresis

Protein samples (300 μg for analytic gels and 500 μg for preparative gels of total heart lysate and 250 μg for analytic/preparative gels of mitochondrial fraction) were applied onto 3–10 linear immobilized pH gradient strips (13 cm; GE Healthcare Life Science). Strips were rehydrated for 16 h at room temperature. Isoelectric focusing was performed on an IPG phor III apparatus (GE Healthcare Life Science) at 17 KVh. For the second dimension, strips were incubated at room temperature for 20 min in equilibration buffer [6 mol/L urea, 2% (w/v) SDS, 50 mmol/L Tris-HCl, pH 6.8, 30% (v/v) glycerol, 0.001% (w/v) bromophenol blue] with 2% (w/v) DTT, followed by incubation with 4% (w/v) iodoacetamide in equilibration buffer for 20 min. The second dimension was separated using vertical SDS-PAGE. Experiments were performed in triplicate. Phosphoproteins were detected by staining with Pro-Q Diamond (Invitrogen) per the manufacturer's instructions. Gels were scanned using a Typhoon TRI scanner (GE

Healthcare Life Science), stained with Coomassie Brilliant Blue G250 (CBB)¹⁴ and scanned using a UTA-1100 scanner and Labscan v 5.0 software (GE Healthcare Life Science).

Image analysis was performed using Image Master Software v.5.01 (GE Healthcare Life Science). For each pair of samples analyzed, individual spot volumes of replicate gels were determined in Pro-Q Diamond stained gels (phosphoproteins), followed by normalization (individual spot volume/volume of all spots ×100). Spots (of treated samples) that appeared or showed a change in spot volume of least 1.5-fold as compared to samples of hearts submitted to ischemia alone were excised from CBB-stained preparative gels and identified by mass spectrometry. Differences between experimental groups were evaluated by the Mann-Whitney t-test for proteomic analysis. A P value <0.05 was considered statistically significant.

"In Gel" Protein Digestion and MALDI-TOF/TOF MS

Digestion of selected spots was performed as previously described.¹⁵ Matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS) as analysis was executed as previously described.¹⁶ MASCOT MS/MS Ion Search (www.matrixscience.com) software was used to blast sequences against the SwissProt and NCBI nr databanks. Combined MS-MS/MS searches were conducted with parent ion mass tolerance at 50 ppm, MS/MS mass tolerance of 0.2 Da, carbamidomethylation of cysteine (fixed modification) and methionine oxidation (variable modification). According to MASCOT probability analysis, only hits with significant P<0.05 were accepted. Spots from total lysates were identified at the Mass Spectrometry Facility, Stanford University (mass-spec.stanford.edu).

Results

Identification of Phosphoproteins

Hearts were exposed to global, no-flow ischemia (35 min) in the presence or absence of ψεRACK (1 μmol/L) applied for normoxia 10 min prior to an ischemic onset, with no wash-out, as previously described.¹⁷ Groups submitted to ischemia had a 20-min equilibration period, after which hearts were subjected to 30 min of global, no-flow ischemia. For one of the groups of hearts the εPKC-selective agonist peptide, ψεRACK, was perfused for 10 min (1 μmol/L), immediately prior to ischemia onset and maintained throughout ischemia. Total lysate of 3 hearts, from 3 independent experiments, were prepared, and run individually on 2D gels. Considering phosphorylated spots that had at least a 1.5-fold increase, we compared phosphorylated spots from hearts of animals subjected to ischemia only and ψεRACK+ischemia. The phosphorylation of 20 spots increased only in ischemic hearts treated with ψεRACK. Of these, 18 spots were identified by mass spectrometry (Figures 1,2; Table 1).

Because the majority of the proteins (~70%) identified were mitochondrial proteins and because a number of previous studies demonstrated that εPKC can interact with and phosphorylate mitochondrial proteins,^{5,7,18} we set out to analyze the εPKC phosphoproteome in isolated mitochondria.

Identification of Phosphoproteins in Mitochondrial Fractions

Mitochondria from ischemia only and ψεRACK+ischemia treated hearts were isolated as described above. In a previous study we verified the purity of our mitochondrial preparation by electron microscopy and western blot analysis of specific mitochondrial proteins.⁵ Mitochondrial proteins were separated

Figure 1. Detection of direct and indirect ϵ PKC substrates in total rat heart lysates. Representative 2D gels ($n=3$ hearts of individual animals) of lysates from control hearts (**A,D**), hearts subjected to ischemia alone (**B,E**) and ischemia+ $\psi\epsilon$ RACK (**C,F**). Coomassie blue G250 stained gels (**A–C**) and gels stained with phosphospecific dye Pro-Q Diamond (**D–F**). Spots used to align gels are labeled (**A,D**). ϵ PKC, epsilon-protein kinase C.

Figure 2. Coomassie blue G250 stained gel of total heart lysate treated with $\psi\epsilon$ RACK+ischemia indicating the spots identified by mass spectrometry, the phosphorylation of which significantly increased in hearts from rats treated with $\psi\epsilon$ RACK+ischemia as compared to hearts subjected to ischemia alone. For the annotation of the proteins identified see [Table 1](#).

Table 1. Proteins Identified by Mass Spectrometry Whose Phosphorylation Increased in Total Heart Lysates of Hearts Subjected to ψεRACK+Ischemia Relative to Ischemia Alone

Spot no.	Protein	Accession no.	Peptide count	MASCOT prot. score	Theoretical		Experimental		% Vol	Location	P value
					MW	pl	MW	pl			
1	ATP synthase subunit β, mitochondrial precursor	P10719	16	589	56kDa	5.19	42kDa	5.23	2.3	Mitochondria	0.02*
2	Myosin light polypeptide 3	P16409	12	495	22kDa	5.03	23kDa	4.94	2.6	Cytosol	0.02*
3	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor	Q920L2	29	693	71kDa	6.75	65kDa	7.32	2.0	Mitochondria	0.03*
4	Creatinekinase, sarcomeric mitochondrial precursor	P09605	15	309	47kDa	8.76	45kDa	7.99	2.2	Mitochondria	0.04*
5	Short-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	P15651	11	280	44kDa	8.47	31kDa	9.06	2.5	Mitochondria	0.08
6	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	P45953	23	300	70kDa	9.01	59kDa	8.00	1.8	Mitochondria	0.01*
7	Mitochondrial inner-membrane protein	Q8CAQ8	17	225	83kDa	6.18	75kDa	6.35	5.1	Mitochondria	0.02*
8	Propionyl-CoA carboxylase α chain, mitochondrial precursor	P14882	12	78	77kDa	6.33	69kDa	6.41	5.1	Mitochondria	0.02*
9	Dihydrolipoyl dehydrogenase, mitochondrial precursor	Q6P6R2	13	207	54kDa	7.96	48kDa	7.33	4.1	Mitochondria	0.01*
10	ATP synthase subunit α, mitochondrial precursor	P15999	20	694	59kDa	9.22	45kDa	9.14	3.0	Mitochondria	0.04*
11	Creatine kinase M-type	P00564	14	568	43kDa	6.58	39kDa	6.87	4.0	Mitochondria	0.01*
12	Pyruvate dehydrogenase E1 component subunit α, mitochondrial precursor	P26284	13	266	43kDa	8.49	44kDa	8.06	3.3	Mitochondria	0.03*
13	Actin, α-cardiac muscle 1	P68035	18	1,030	41kDa	5.23	40kDa	5.14	6.5	Cytosol	0.04*
14	Ezrin	P31977	12	93	69kDa	5.83	55kDa	5.80	3.5	Cytosol	0.03*
15	Acetyl-coenzyme A synthetase 2-like, mitochondrial precursor	Q99NB1	10	91	74kDa	6.51	66kDa	6.40	5.8	Mitochondria	0.09
16	Pyruvate kinase isozymes M1/M2	P11980	26	790	57kDa	6.63	46kDa	7.01	1.7	Mitochondria	0.01*
17	Phosphatidyl ethanolamine-binding protein 1	P31044	5	326	20kDa	5.48	19kDa	4.65	6.0	Cytosol	0.01*
18	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	P08733	15	409	18kDa	4.86	19kDa	4.35	2.7	Cytosol	0.01*

Identified proteins indicated in Figure 2, together with uniprot accession number, number of peptides identified, MASCOT score, theoretical and experimental molecular weights (MW) and isoelectric (pl) point, %volume of ischemia where ischemia=normoxia (average of 3 experiments) and P-values as determined by Whitney t-test where *P<0.05 are indicated.

by 2D gel electrophoresis and phosphoproteins stained with Pro-Q Diamond. Of the 183 spots that appeared or were increased in gels of mitochondria from hearts of animals treated with ψεRACK+ischemia, 62 spots were visible by CBB and 56 spots corresponding to 39 different proteins were identified by in-gel excision followed by mass spectrometry (Figures 3,4; Tables 2,3). Of the 27 mitochondrial proteins, 9 were mitochondrial inner-membrane proteins and 1 was an outer-membrane protein. Proteins involved in fatty acid oxidation, electron transport chain (complexes I–IV), and heat shock proteins (HSP), as well as structural proteins, were also identified. Interestingly, protein disulfide-isomerase A3 precursor, oxoglutarate (α-ketoglutarate) dehydrogenase (lipoamide), tubulin α 1A, mitochondrial aconitase, creatine kinase, mitochondrial 2, acyl-Coenzyme A dehydrogenase very long-chain, 3-oxoacid CoA transferase 1, carnitine palmitoyltransferase II, electron transfer flavoprotein-ubiquinone oxidoreductase, succinate dehydrogenase complex, subunit A, flavoprotein (Fp), glyceraldehyde 3-phosphate-dehydrogenase, desmin, ubiquinol-cytochrome c reductase core protein I and Coq9 protein had a change in more than 1 phospho-spot, indicative of multiple phosphorylation sites.

Recently, we showed that translocation of εPKC to the mitochondria is mediated by HSP90, so the identified substrates could be direct targets of εPKC.⁵ Using scansite ([http://scansite.](http://scansite.mit.edu/)

[mit.edu/](http://scansite.mit.edu/)) we predicted the PKC phosphorylation sites of the mitochondrial proteins that had increased phosphorylation increased after treatment with ψεRACK. All identified mitochondrial proteins had putative PKC phosphorylation, some which matched phosphorylation sites deposited in <http://www.phosphosite.org/> (Table S1).

Discussion

Several lines of evidence suggest that selective εPKC activation reduces cardiac damage caused by ischemic injury. Activation of εPKC reduces infarct size and improves functional recovery of the heart.^{1–3} whereas εPKC inhibition or knockout negates the infarct-sparing effect of ischemic preconditioning.^{3,10,19–21} A number of mechanisms have been proposed for εPKC-mediated cardioprotection, including regulation of sarcolemmal and/or mitoK_{ATP} channels,^{18,22,23} regulation of gap-junction permeability phosphorylation of connexin 43,²⁴ modulation of proteasomal activity¹⁷ or regulation of mitochondrial permeability transition pore (MPTP) opening through direct phosphorylation of MPTP components.⁷ We recently identified mitochondrial ALDH2 as a direct εPKC substrate, the phosphorylation and activation of which is essential for εPKC-mediated cardioprotection.³ The cytoprotective mechanism of ALDH2 activation by εPKC is related to the increased metabo-

Figure 3. Detection of direct and indirect ϵ PK substrates in isolated rat heart mitochondria. Representative 2D gels (n=3 hearts of individual animals) of lysates from control hearts (A,D), hearts subjected to ischemia alone (B,E) and ischemia+ $\psi\epsilon$ RACK (C,F). Coomassie blue G250 stained gels (A–C) and gels stained with phosphospecific dye Pro-Q Diamond (D–F). Spots used to align gels are labeled (A,D). ϵ PK, epsilon-protein kinase C.

Figure 4. Detection of direct and indirect ϵ PK substrates in isolated rat heart mitochondria. Representative 2D gels (n=3 of mitochondria isolated from individual animals) of lysates from hearts subjected to ischemia and $\psi\epsilon$ RACK+ischemia as indicated in Figure 1. Coomassie blue G250 stained gels (Upper) and gels stained with phosphospecific dye Pro-Q Diamond (Lower).

Table 2. Proteins Identified by Mass Spectrometry Whose Phosphorylation Increased in Mitochondria Isolated From Hearts Subjected to ψ RACK+Ischemia Relative to Ischemia Alone

Spot no.	Protein	Accession no.	Peptide count	Ion score	Theoretical		Experimental		Coverage (%)	Vol (% ischemia)
					MW	pl	MW	pl		
1	Acetyl-CoA dehydrogenase, medium chain	gi:8392833	9	214	46kDa	8.63	39kDa	7.53	13	Appeared
2	Sorting and assembly machinery component 50 homolog	gi:51948454	4	57	52kDa	6.34	59kDa	6.51	9	Appeared
3	Dihydrolipoamide dehydrogenase	gi:40786469	5	102	54kDa	7.96	61kDa	6.43	9	Appeared
4	Hydroxysteroid dehydrogenase like 2 [<i>Rattus norvegicus</i>]	gi:71043858	3	49	58kDa	5.85	85kDa	6.2	6	Appeared
5	Protein disulfide-isomerase A3 precursor	gi:1352384	8	116	57kDa	5.88	66kDa	5.92	11	Appeared
6	Protein disulfide-isomerase A3 precursor	gi:1352384	10	329	57kDa	5.88	66kDa	5.95	23	Appeared
7	Aconitase 2	gi:18079339	8	163	85kDa	8.05	105kDa	6.4	8	Appeared
8	Oxoglutarate (α -ketoglutarate) dehydrogenase (lipoamide)	gi:62945278	6	171	12kDa	6.3	174kDa	5.83	8	Appeared
9	Oxoglutarate (α -ketoglutarate) dehydrogenase (lipoamide)	gi:62945278	6	44	12kDa	6.3	174kDa	5.93	8	Appeared
10	Vimentin	gi:14389299	5	147	54kDa	5.06	67kDa	4.85	5	Appeared
11	Tubulin α 1A	gi:38328248	4	32	50kDa	4.94	64kDa	5.24	10	Appeared
12	Tubulin α 1A	gi:38328248	4	36	50kDa	4.94	64kDa	5.31	11	Appeared
13	Pyruvate dehydrogenase (lipoamide) β	gi:56090293	5	247	39kDa	6.2	40kDa	5.47	20	Appeared
14	Branched-chain ketoacid dehydrogenase E1, β polypeptide	gi:158749538	4	267	43kDa	6.41	42kDa	5.48	13	Appeared
15	Striated-muscle α tropomyosin	gi:207349	9	95	37kDa	4.71	38kDa	4.07	13	Appeared
19	Mitochondrial aconitase	gi:10637996	9	196	85kDa	7.87	105kDa	7.16	12	Appeared
20	Mitochondrial aconitase	gi:10637996	9	190	85kDa	7.87	105kDa	7.29	12	Appeared
21	Mitochondrial aconitase	gi:10637996	8	229	85kDa	7.87	104kDa	5.23	12	Appeared
22	Mitochondrial aconitase	gi:10637996	8	325	85kDa	7.87	104kDa	7.71	13	Appeared
23	Annexin A2	gi:9845234	8	442	39kDa	7.55	48kDa	7.1	30	Appeared
24	Aldolase A	gi:202837	4	125	40kDa	8.3	39kDa	8.04	22	Appeared
25	Creatine kinase, mitochondrial 2	gi:38259206	6	326	47kDa	8.64	46kDa	7.57	21	Appeared
26	Creatine kinase, mitochondrial 2	gi:38259206	9	442	47kDa	8.64	45kDa	8.65	26	Appeared
27	Acyl-Coenzyme A dehydrogenase, very long-chain	gi:6978435	7	125	71kDa	9.01	71kDa	7.62	18	Appeared
28	Acyl-Coenzyme A dehydrogenase, very long-chain	gi:6978435	5	181	71kDa	9.01	71kDa	7.42	13	Appeared
29	3-oxoacid CoA transferase 1	gi:189181716	8	463	57kDa	8.7	61kDa	7.52	23	Appeared
30	3-oxoacid CoA transferase 1	gi:189181716	4	238	57kDa	8.7	61kDa	7.35	13	Appeared
31	ATP synthase α subunit precursor	gi:203055	8	327	59kDa	9.22	59kDa	8.25	20	Appeared
32	Pyruvate dehydrogenase E1 α form 1 subunit	gi:57657	5	211	43kDa	8.32	66kDa	6.48	7	Appeared
33	Carnitine palmitoyltransferase II	gi:1850592	8	257	74kDa	7.02	74kDa	7.1	7	Appeared
34	Carnitine palmitoyltransferase II	gi:1850592	7	125	74kDa	7.02	74kDa	7.12	15	Appeared
35	Carnitine palmitoyltransferase II	gi:1850592	7	174	74kDa	7.02	74kDa	7.27	13	Appeared
36	Electron transfer flavoprotein-ubiquinone oxidoreductase	gi:52000614	6	158	61kDa	7.33	70kDa	7.11	15	Appeared
37	Electron transfer flavoprotein-ubiquinone oxidoreductase	gi:52000614	6	321	61kDa	7.33	70kDa	7.18	14	Appeared
38	Vinculin (predicted), isoform CRA_a	gi:149031250	5	41	123kDa	5.54	146kDa	5.84	5	Appeared
41	Heat shock protein 1, β (HSP90)	gi:40556608	7	268	83kDa	4.97	105kDa	4.65	9	Appeared
42	Heat shock protein 5 (HSP70 ptn5) glucose regulated protein	gi:25742763	9	296	72kDa	5.07	84kDa	4.7	14	Appeared
43	70-kDa heat shock cognate protein	gi:178847300	9	309	60kDa	5.91	72kDa	5.13	20	Appeared
44	DNA K-type molecular chaperone HSP72-ps1	gi:347019	8	369	71kDa	5.43	73kDa	5.21	16	Appeared
45	grp75	gi:1000439	8	414	74kDa	5.87	79kDa	5.24	16	Appeared
46	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa	gi:53850628	7	283	80kDa	5.65	81kDa	5.14	12	Appeared
47	Isocitrate dehydrogenase 3 (NAD $^{+}$) α	gi:16758446	7	221	40KDa	6.47	41kDa	6.3	26	Appeared
48	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	gi:18426858	10	249	72kDa	6.75	72kDa	6.45	20	Appeared

(Table 2 continued the next page.)

Spot no.	Protein	Accession no.	Peptide count	Ion score	Theoretical		Experimental		Coverage (%)	Vol (% ischemia)
					MW	pI	MW	pI		
49	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	gi:18426858	10	364	72kDa	6.75	71kDa	6.56	19	Appeared
50	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	gi:18426858	10	355	72kDa	6.75	71kDa	6.82	21	Appeared
51	$\delta(3,5)$ - $\delta(2,4)$ -dienoyl-CoA isomerase: Precursor	gi:6015047	8	219	36kDa	8.13	33kDa	6.47	35	Appeared
52	Glyceraldehyde 3-phosphate-dehydrogenase	gi:56188	4	265	36kDa	8.43	47kDa	7.43	3	Appeared
53	Glyceraldehyde 3-phosphate-dehydrogenase	gi:56188	3	74	36kDa	8.43	47kDa	7.65	17	Appeared
54	ATP synthase β subunit	gi:1374715	6	238	51kDa	4.92	65kDa	4.87	20	Appeared
55	Tubulin, β , 2	gi:5174735	6	110	50kDa	4.79	66kDa	4.75	11	Appeared
56	Desmin	gi:11968118	27	65	53kDa	5.21	64kDa	4.87	44	Appeared
57	Desmin	gi:11968118	28	72	53kDa	5.21	64kDa	5.12	53	Appeared
58	Ubiquinol-cytochrome c reductase core protein I	gi:51948476	22	38	53kDa	5.57	51kDa	5.43	32	Appeared
59	Ubiquinol-cytochrome c reductase core protein I	gi:51948476	7	385	53kDa	5.57	52kDa	5.59	21	Appeared
60	Coq9 protein	gi:51259441	2	62	35kDa	5.5	30kDa	4.87	10	Appeared
61	Coq9 protein	gi:51259441	8	ND	35kDa	5.5	30kDa	5.09	25	19.5*
62	Polymerase I and transcript release factor	gi:6679567	3	46	44kDa	5.43	64kDa	3.31	7	19.5*

Identified proteins indicated in [Figure 4](#) are shown, together with Uniprot accession number, number of peptides identified, MASCOT score, theoretical and experimental molecular weights (MW) and isoelectric point (pI), %volume of control (average of 3 experiments). *P<0.05 as determined by Whitney t-test.

lism of reactive aldehydes, such as 4-HNE, which are produced as a by-product of reactive oxygen species (ROS)-induced lipid peroxidation,²⁵ and accumulate in the ischemic/reperfused heart.²¹ In the present study, we used the Pro-Q Diamond phosphospecific staining method to label proteins that had phosphorylation increased by ψ eRACK during ischemia. The majority (~70%) of the ϵ PKC phosphoproteins identified in total heart homogenates treated with ψ eRACK during ischemia were mitochondrial proteins. The observation that ϵ PKC activation and cytoprotection results in phosphorylation of mitochondrial proteins is consistent with other studies reporting that ϵ PKC-mediated cardioprotection is mediated by phosphorylation of mitochondrial proteins.^{3,10,19–21}

To provide a more extensive analysis of the ϵ PKC mitochondrial phosphoproteome, we repeated the Pro-Q Diamond analysis on the cardiac mitochondrial-enriched subfraction. In the presence of ψ eRACK we saw the appearance of 182 phosphorylated spots, suggesting that ϵ PKC activation results in phosphorylation of a number of mitochondrial proteins. We identified novel mitochondrial ϵ PKC phosphoproteins involved in lipid oxidation, glycolysis, electron transport chain (including proteins from complexes I–IV), ketone body metabolism, and HSPs.

We found an increase in the phosphorylation of inner-mitochondrial protein components of the respiratory chain, (complexes I, II and III), NADH dehydrogenase (ubiquinone) Fe-S protein, electron transfer flavoprotein-ubiquinone oxidoreductase, succinate dehydrogenase complex, subunit A, flavoprotein (Fp) and ubiquinol-cytochrome c reductase core protein I. Our results are in agreement with a number of biochemical and functional analyses that found ϵ PKC interacts with, and phosphorylates, inner-mitochondrial proteins involved in mitochondrial respiration.^{7,9,10,26} Further, the presence of ϵ PKC in a highly purified mitochondrial inner-membrane preparation has already been previously demonstrated.²² An increase in the activity of the electron transport chain and activation of

cytochrome c oxidase subunit IV (COX) by direct ϵ PKC phosphorylation has also been previously demonstrated.²⁷ COX activation was suggested to be a cardioprotective mechanism of ϵ PKC, possibly because of increased electron flux through the electron transport chain, resulting in enhanced ATP generation and reduced ROS generation.^{20,27,28} An ϵ PKC-mediated increase in cytochrome c oxidase activity was also shown to protect the lens from ischemic damage.²⁹ Selective activation of ϵ PKC with ψ eRACK increased the phosphorylation and activity of complexes I, III and IV in synaptic mitochondria, indicating that other components of the electron transport chain are also regulated by ϵ PKC phosphorylation,³⁰ and ϵ PKC activation led to a decrease in the mitochondrial ROS generation of neuronal mitochondria.³⁰ In agreement with a role for ϵ PKC in mitochondrial respiration, hearts of constitutively active ϵ PKC transgenic mice demonstrate preserved coupling of oxidative phosphorylation, maintained mitochondrial membrane potential and decreased cytochrome c release induced by ischemia and reperfusion.³¹ Those ϵ PKC transgenic mice have a mutation of Ala¹⁵⁹ to Glu in ϵ PKC, resulting in constitutively active ϵ PKC and increased resistance to cardiac ischemia/reperfusion.⁷ Interestingly, in constitutively active ϵ PKC transgenic mice, mitochondrial PKC expression is preferentially increased over cytosolic expression, suggesting that the active form of PKC results in its mitochondrial translocation.⁷ Taken together, these data suggest that phosphorylation of intra-mitochondrial targets is crucial for ϵ PKC-mediated cytoprotection. In the present study we identify other components of the respiratory chain and inner-mitochondrial phosphorylated proteins. However, whether there is a direct physical association between ϵ PKC and each of the inner-mitochondrial ϵ PKC phosphoproteins identified here, and whether these are direct or indirect ϵ PKC substrates, remains to be determined. Nevertheless future studies can be directed by the results obtained here.

We did not detect ALDH2, but this may be because differ-

Table 3. Summary of the Function and Localization of Proteins Whose Phosphorylation Was Unique or Increased 1.5-fold (in 2 of 3 Gels, of Independent Samples) in Mitochondria From Hearts Treated With ψ εRACK+Ischemia Relative to Ischemia Alone (the Biological Process, Mitochondrial Compartment and References to Previous Descriptions of Protein Phosphorylation or Expression Modulated by εPKC Are Shown)

Function/Protein	Localization	Reference
Fatty acidoxidation		
Carnitine palmitoyltransferase II	Mitochondrial inner-membrane	
δ(3,5)-δ(2,4)-dienoyl-CoA isomerase: precursor	Mitochondrial matrix	
Glycolysis/gluconeogenesis		
Aldolase A	Mitochondrial matrix	
Krebs cycle		
Aconitase 2	Mitochondrial matrix	
ATP-specific succinyl-CoA synthase β subunit	Mitochondrial matrix	
Isocitrate dehydrogenase 3 (NAD+) α	Mitochondrial matrix	Previously reported ^{8,10}
Dihydrolipoamide dehydrogenase (E3)	Mitochondrial matrix	
Mitochondrial aconitase	Mitochondrial matrix	
Oxoglutarate (α-ketoglutarate) dehydrogenase (lipoamide)	Mitochondrial matrix	Previously reported ¹⁰
Pyruvate dehydrogenase (lipoamide) β	Mitochondrial matrix	Previously reported ¹⁰
Pyruvate dehydrogenase E1 α form 1 subunit	Mitochondrial matrix	Previously reported ¹⁰
Glyceraldehyde 3-phosphate-dehydrogenase	Mitochondrial matrix	Previously reported ⁸
Electron transport chain		
Electron transfer flavoprotein-ubiquinone oxidoreductase	Mitochondrial inner-membrane	
Complex I		
NADH dehydrogenase (ubiquinone) Fe-S protein	Mitochondrial inner-membrane	
Electron transfer flavoprotein-ubiquinone oxidoreductase	Mitochondrial inner-membrane	
Complex II		
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Mitochondrial inner-membrane	Previously reported ¹⁰
Electron transfer flavoprotein-ubiquinone oxidoreductase	Mitochondrial inner-membrane	
Complex III		
Ubiquinol-cytochrome c reductase core protein I	Mitochondrial inner-membrane	
Electron transfer flavoprotein-ubiquinone oxidoreductase	Mitochondrial inner-membrane	
ATP synthase		
ATP synthase α subunit precursor	Mitochondrial inner-membrane	Previously reported ⁸
ATP synthase β subunit	Mitochondrial inner-membrane	Previously reported ^{8,10}
Ketone body metabolism		
3-oxoacid CoA transferase 1	Mitochondrial matrix	
Branched-chain ketoacid dehydrogenase E1, β polypeptide	Mitochondrial matrix	
Vimentin	Cytosol	Previously reported ^{8,9}
Tubulin α 1A	Cytosol	
Cytoskeletal elements		
Tubulin, β, 2	Cytosol	
Desmin	Cytosol	Previously reported ^{8,9}
Vinculin, isoform CRA_a	Cytosol	Previously reported ^{8,9}
Heat shock protein 1, β (HSP90)	Cytosol	
Heat shock protein		
Heat shock protein 5 (HSP70 ptn5) glucose regulated protein	Mitochondria	
DNA K-type molecular chaperone HSP72-ps1	Mitochondria	Previously reported ^{8,9}
grp75	Mitochondria	
Caveoli		
Polymerase I and transcript release factor (PTRV)	Caveolin	
Annexin A2	Membranes (Caveolin)	Previously reported ^{8,9}
Sorting and assembly machinery component 50 homolog	Mitochondrial outer-membrane	
Hydroxysteroid dehydrogenase like 2 [<i>Rattus norvegicus</i>]	Mitochondrial inner-membrane	
Other		
Protein Coq9 protein	Mitochondrial inner-membrane	
Protein disulfide-isomerase A3 precursor	Endoplasmic reticulum	
Striated-muscle α tropomyosin	Sarcomere	

Reference of previous reports that also found these proteins to be εPKC targets.

ent methods of detecting protein phosphorylation have different sensitivities. Some of the ϵ PKC targets identified can be indirect targets whose phosphorylation may be activated upon ALDH2 activation.

Using DIGE of cardiac mitochondria from transgenic mice expressing constitutively active or dominant negative ϵ PKC, it was found that the majority of spots unique to constitutively active ϵ PKC corresponded to proteins involved in glucose metabolism.¹⁰ Those studies were combined with metabolomic studies, which detected an increase in glucose metabolites in hearts expressing constitutively active ϵ PKC subjected to ischemia/reperfusion.¹⁰ The authors proposed that activating glycolytic pathways during ischemia is a novel mechanism for the cardioprotective role of ϵ PKC. In the present study we used a phosphospecific dye and ψ ERACK to investigate direct protein phosphorylation events mediated by ϵ PKC. Despite the different methods and methodology used to activate ϵ PKC (constitutively active transgenic vs. dynamic activation), we identified many of the same proteins previously described in the DIGE study, including isocitrate dehydrogenase, oxoglutarate (α -ketoglutarate) dehydrogenase, pyruvate dehydrogenase, succinate dehydrogenase (Table S1).^{8–10} We also identified additional ϵ PKC substrates involved in glycolysis and the Krebs cycle, such as aldolase A, ATP-specific succinyl-CoA synthase β subunit, dihydrolipoamide dehydrogenase (E3), mitochondrial aconitase and aconitase 2, confirming that ϵ PKC activation leads to phosphorylation of proteins involved in glycolysis and the Krebs cycle. Our identification of aconitase as an ϵ PKC target suggests that regulation of the Krebs Cycle cycle is mediated by ϵ PKC. Aconitase has been previously identified as a PKC β II substrate in diabetic rats; however, aconitase phosphorylation by PKC β II impaired the Krebs Cycle cycle because there was an increase in reverse activity of aconitase (isocitrate to aconitase).³² Although we identified some proteins that have been identified previously, others were not detected in the present study, such as proteins involved in the malate/aspartate shuttle. This could be explained by the different methodology or sensitivity of the methods (DIGE vs. ProQ Diamond) and that we only identified the more abundant phosphorylated proteins. Alternatively, some of the proteins previously detected could have their expression and not phosphorylation status altered.¹⁰ In a study identifying ϵ PKC complexes, it has been suggested that ϵ PKC may also play a role in regulating transcription and translation processes.⁸ Accordingly, the phosphorylation of Coq9, a key regulator of coenzyme Q synthesis,³³ was also regulated by ϵ PKC in the present study. Further studies should be performed to determine the specific regulation of glycolytic pathways by ϵ PKC phosphorylation and whether different isoenzymes can phosphorylate different sites.

ϵ PKC could also have a direct or indirect role in mitochondrial protein assembly, folding, and import, because we identified 3 mitochondrial HSPs that play a role in the import and folding of proteins inside the mitochondria, and in the sorting and assembly machinery component 50 (SAM50) homolog of a protein involved in the assembly of outer-mitochondrial membrane proteins.³⁴

Cardioprotective signals from G protein coupled receptors (GPCRs), activated for example by bradykinin, propagating from the plasma membrane to the mitochondria through signalosomes, vesicular multimolecular complexes derived from caveoli, have been previously proposed.³⁵ In fact, ϵ PKC was found in signalosomes and inhibition of ϵ PKC by ϵ V1–2 blocks signalosome stimulation of mitoK_{ATP}.³⁵ We found 2 proteins that are found in caveoli, Annexin A2 and PTRF (polymerase

I and transcript release factor), also known as Cavin.³⁶ These proteins could be part of the signalosome, probably co-purified with our mitochondrial fraction. PTRF phosphorylation has been shown to be important in caveoli formation.³⁶

Conclusions

A number of mechanisms have been proposed for ϵ PKC-mediated cardioprotection by preconditioning. In the present study we identified several ϵ PKC phosphoproteins that may be responsible for the cardioprotective effect of ϵ PKC. The ϵ PKC targets identified are in line with many of the previously proposed mechanisms for ϵ PKC-mediated cardioprotection. We identified components of the signalosome, contributing to the idea that ϵ PKC-mediated cardioprotection involves transduction of GPCR signaling to the mitochondria.³⁵ We also found components of lipid and carbohydrate oxidation pathways, consistent with the idea that lipid and carbohydrate metabolism is modulated by ϵ PKC.¹⁰ Activation of the respiratory chain and increased oxygen consumption have also been proposed as protective mechanisms of ϵ PKC during preconditioning, and to this end we identified components of the Krebs cycle, and respiratory chain, the phosphorylation of which was modulated by ϵ PKC.^{27,29,30} The exact mechanisms by which ϵ PKC phosphorylation leads to these different cardioprotective pathways still needs to be elucidated. The data obtained in the present study can therefore direct further studies to characterize the specific role of individual mitochondrial protein phosphorylations in ϵ PKC-mediated cardioprotection. Taken together, our data suggest that ϵ PKC-mediated phosphorylation events in the mitochondria are important for the maintenance of metabolic activity and cardioprotection during ischemic injury.

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Disclosures

Competing interests: D.M.R. is the founder of KAI Pharmaceuticals Inc, a company that aims to bring PKC regulators to the clinic. None of the research performed in her laboratory is in collaboration with or supported by the company. The other authors declare that they have no competing interests.

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Supplemental Files

Supplemental File 1.

Table S1. Predicted PKC Phosphorylation Sites of the Mitochondrial Proteins Phosphorylated Upon Ischemia and ψ RACK

Please find supplemental file(s);
<http://dx.doi.org/10.1253/circj.CJ-11-1360>