COMPARATIVE PROTEOMICS ANALYSIS OF DIFFERENTIALLY EXPRESSED PHOSPHOPROTEINS IN ADULT RAT VENTRICULAR MYOCYTES SUBJECTED TO DIAZOXIDE PRECONDITIONING

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SUMMARY

Mitochondrial ATP sensitive potassium channels (mitoK_{ATP} channels) are involved in the cardioprotection afforded by ischemic preconditioning (IPC) and diazoxide, a selective mitoK_{ATP} channel opener. The activation of some kinases, including phoshoprotein kinase (PKC)-ε and mitogen-activating protein kinases (MAPK), is involved in signal conduction of preconditioning downstream from mitoK_{ATP} channel opening. Diazoxide can open mitoK_{ATP} channels and activate PKC-ε, which will phosphorylate some substrate proteins. These proteins that exhibit altered post-translational modification via phosphorylation due to diazoxide pretreatment may be the target molecules and play an important role in cellular protection after mitoK_{ATP} channel opening. To analyze and identify the phospho-proteins associated with diazoxide preconditioning, phosphoprotein

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enrichment and comparative two-dimensional gel electrophoresis (2D-GE) were used. Cultured adult rat ventricular myocytes were pretreated in the presence and absence of 100 μmol/l diazoxide for 10 min and enriched phosphoproteins from control myocytes and those pretreated with 100 μmol/l diazoxide were separated by 2D-GE and stained with a silver staining kit. Phosphoproteins of interest were further identified by matrix-assisted laser desorption ionization tandem mass spectrometry (MALDI-TOF MS). Eight protein spots with different abundance were found, of which six differentially expressed proteins were identified by MALDI-TOF MS. They included 94 kDa glucose-regulated protein, calpactin I heavy chain, chaperonin containing TCP-1 zeta subunit, hypothetical protein XP_346548, ferritin light chain and ferritin light chain 2. These findings provide new clues to understanding the mechanism of ischemic preconditioning in cardiomyocytes downstream from mitoK_{ATP} channel opening.

KEY WORDS

phosphoproteome, cardiomyocytes, diazoxide, preconditioning

INTRODUCTION

It is generally accepted that the mitochondiral ATP sensitive potassium channel (mitoK_{ATP} channel) is involved in the cardioprotection afforded by ischemic preconditioning (IPC) and diazoxide, a selective mitoK_{ATP} channel opener. Several reports support an involvement of the mitoK_{ATP} channel as both a trigger and end effector of IPC and mitoK_{ATP} openers, such as diazoxide /1,2/, while it is still unclear which target molecules participate in IPC and play an important role in cellular protection after mitoK_{ATP} channel opening.

A recent study by Liu *et al.* /3/ demonstrated that phoshoprotein kinase (PKC)-ε is downstream from mitoK_{ATP} activation in apoptosis-limiting effects of IPC. In addition, both IPC and diazoxide treatment activated PKC-ε in the particulate fraction. These results were also supported by data provided by Wang and Ashraf /4/ who demonstrated that pretreatment with diazoxide did indeed mediate translocation of specific PKC isoforms.

Another study places mitogen-activating protein kinase (MAPK) activation downstream from mitoK_{ATP} channel opening. Using THP-1 cells, Samavati *et al.* /5/ demonstrated that diazoxide induces mitochondrial reactive oxygen species (ROS) production. Moreover, the increase in ROS resulted in an increase in the phosphorylation of ERK, a member of the MAPK family.

These data suggest that kinase activity and the phosphorylation status of target substrates may act as critical effectors or important regulators of IPC after mitoK_{ATP} channel opening.

To date, proteomic analysis of preconditioned cardiomyocytes has been very limited. Arrell *et al.* /6/ used comparative two-dimensional gel electrophoresis (2D-GE) to examine post-translational modification of rabbit ventricular myocytes after adenosine preconditioning, and found that exposure of isolated rabbit ventricular myocytes to adenosine at levels which confer cardioprotection produced a small but significant increase in the levels of ventricular MLC1 phosphorylation. Mayr *et al.* /7/ and Zhang *et al.* /8/ found by functional proteomic analysis that PKC-δ and PKC-ε were important signaling molecules for cardiac protection. Changes in phosphorylation in cardiomyocytes after mitoK_{ATP} channel opening are still unclear.

In this study, we isolated and identified the phosphoproteome associated with diazoxide preconditioning. Phosphoprotein enrichment and comparative 2D-GE were used to profile phosphoprotein expression. Proteins detected by silver staining were identified by matrix-assisted laser desorption ionization tandem mass spectrometry (MALDI-TOF MS). The set of proteins that exhibited altered post-translational modification after diazoxide pretreatment included several proteins related to protein catabolism, protein folding, and other functions.

MATERIALS AND METHODS

Materials

Acrylamide, methylene *bis*-acrylamide, glycine, Tris, SDS, urea, glycerol, bromophenol blue, Triton X-100, IPG buffers, IPG strips and 2-D Quant kit were purchased from Amersham Biosciences. Diazoxide, collagenase type II, hyaluronidase, Pharmalyte, TEMED, CHAPS, thiourea, iodoacetamide, ammonium persulfate, silver stain

kit and In-Gel Digest kit were from Sigma. DTT was from Promega. Dulbecco's modified Eagle's medium and fetal bovine serum were from Invitrogen. Phosphoprotein Enrichment Kit 635624 was from BD Biosciences.

Isolation and preconditioning of adult rat ventricular myocytes

All protocols were reviewed and approved by the Animal Use and Care Committee of the Third Military Medical University. Adult male Sprague-Dawley (SD) rats (weighing 200 to 250 g) from the Center for Experimental Animals at the University were anesthetized with pentobarbital and heparinized (200 U i.v.), and the heart was rapidly removed and perfused using the Langendorff technique with collagenase (type II, Sigma) and hyaluronidase (Sigma) solution, as described previously /9,10/. Myocytes were then mechanically dispersed in a shaking water bath followed by washing in Tyrode solution. Typically, there was 70-90% yield of quiescent, rod-shaped cells. Cultured ventricular myocytes were randomly assigned to two groups, the control group and the diazoxide preconditioning group, which were pretreated in the absence and presence respectively of 100 μmol/l diazoxide for 10 min. After washout of diazoxide, the ventricular myocytes were cultured for another 20 min, and then cardiomyocytes from both groups were collected on ice.

Phosphoprotein enrichment

To obtain cell lysates enriched for phosphoproteins, Phosphoprotein Enrichment Kit 635624 was purchased from BD Biosciences and used in accordance with the manufacturer's instructions. Briefly, cells were washed, lysed using the supplied lysis buffer, and centrifuged to remove insoluble material. Protein lysate was loaded onto the supplied phosphoprotein binding columns, washed, and eluted. The eluate containing putative phosphoproteins was concentrated and desalted using ultrafiltration columns (10 kDa cut-off) purchased from Millipore. Samples were frozen and stored at -80°C until analyzed by 2D-GE.

Two-dimensional gel electrophoresis

2D-GE was performed as previously described /11/. IEF was carried out using a Protean IEF cell (Amersham Biosciences) according to the manufacturer's protocol. Immobilized pH gradient (IPG) Ready Strips (130 mm, pH 3 to 10 linear gradient, Amersham Pharmacia Biotech) were actively rehydrated at 30 V for 12 hours to enhance protein uptake, then subjected to the following conditions using a rapid-voltage ramping method: 200 V for 1 h, 500 V for 1 h, 8,000 V ramp for 0.5 h, and 8,000 V for 6 h. A temperature control platform maintained gels at 20°C throughout IEF. Focused gels were stored at -20°C before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For SDS-PAGE, IPG strips were incubated for 10 min in equilibration buffer (50 mmol/l Tris-HCl [pH 8.8], 6 mol/l urea, 30% vol/vol glycerol, 2% wt/vol SDS) supplemented with 10 mg/ml DTT, followed by 10 min incubation in equilibration buffer supplemented with 25 mg/ml iodoacetamide, then rinsed once with SDS-PAGE buffer (25 mmol/l Tris, 192 mmol/l glycine [pH 8.3], 0.1% wt/vol SDS). IEF strips were then embedded in a 5% acrylamide stacking gel, and the proteins were separated by 12.5% SDS-PAGE using a Protean II XL system (Amersham Biosciences). Electrophoresis was carried out at 10 mA for 15 min, followed by 25 mA for 3-4 hours. 2D-GE gels were scanned with an ImageScanner (Amersham Biosciences) and analyzed using Image Master 2D Elite 3.10 software (Amersham Biosciences). Differences in the abundance of different protein spots were analyzed using Student's t-test; a p value <0.05 was considered significant.

Silver stain and In-Gel digest

Silver stain kit and In-Gel digest kit were purchased from Sigma and used in accordance with the manufacturer's instructions. Prior to spotting for MALDI analysis, samples were cleaned using ZipTips (Millipore, Billerica, MA) following the manufacturer's recommended procedure.

Mass spectrometry and protein identification

After the peptides were eluted in turn with TFA of different concentrations, the peptide mixture was measured on a MALDI-TOF

mass spectrometer (Manchester, UK) according to the method of Jungblut and Thiede /12/. The peptide mass fingerprint data from MALDI-TOF MS were analyzed by searching against an NCBInr database using MASCOT search software.

RESULTS

Differential expression of phosphoproteins

The higher stability and reproducibility of 2D-GE are the important basis for proteomic analysis. The match rate of protein spots among different 2D-GE maps is an effective index of reproducibility. In the present study, both the phosphoprotein enrichment and 2D-GE protocol were performed using a standardized procedure. Based on Image-Master 2D Elite software analysis, 214 ± 15 and 208 ± 12 spots were detected in the control and diazoxide preconditioning group gels, respectively, by silver staining. The match rate of gels in the control group was 82.74 ± 10.29%, and that in the diazoxide preconditioning group was 78.66 ± 8.57%. The expression patterns of phosphoproteins are shown in Figure 1. Most of the protein spots were distributed in the region of pH 4-8 and had M_r between 20 and 90 kDa. The differences in protein profiles between the control and diazoxide preconditioning groups were detected by image analysis. Diazoxide preconditioning resulted in phosphorylation of protein spots 4 and 5, and less phosphorylation of spots 1, 2, 3, 6, 7 and 8, as shown in Figure 1.

Identification of differentially expressed phosphoproteins

The differentially expressed proteins were analyzed by MALDI-TOF MS. Peptide mass data are shown in Table 1. One of the mass spectrum maps obtained from spot 5 is displayed in Figure 2. After database searching, six protein spots were successfully identified. The proteins are listed in Table 2. They included 94 kDa glucose-regulated protein, calpactin I heavy chain, chaperonin containing TCP-1 zeta subunit, hypothetical protein XP_346548, ferritin light chain and ferritin light chain 2. The other two spots, nos. 3 and 6, are not yet identified.

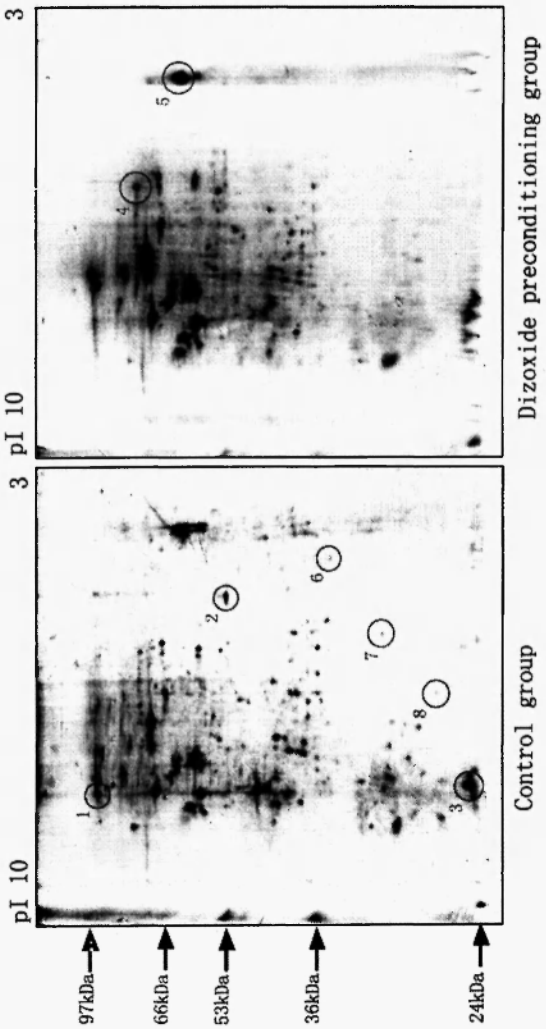

Fig. 1: 2D-GE maps of phosphoproteins in adult rat ventricular myocytes. Phosphoproteins (100 μg) were loaded onto pH 3-10 linear IPG strips (13 cm), with 12.5% vertical SDS-PAGE as the second dimension. The gels were visualized by silver staining.

TABLE 1

Peptide masses for eight differentially expressed phosphoproteins from cardiomyocytes

Protein spots	Peptide masses for selected protein spots
1	1767.89 1786.00 1864.05 1878.95 1917.02 2030.11 2052.1 2068.08 2111.11 2250.11 2262.10 2272.12 2288.08 2356.16 2372.16 2713.85 2728.44 2737.49 2753.49 2766.39 3104.61 3232.69
2	833.05 842.50 855.02 870.49 893.43 1035.52 1050.53 1060.06 1094.50 1111.54 1128.55 1133.54 1149.49 1222.56 1244.65 1282.60 1405.70 1427.71 1443.67 1460.70 1476.70 1483.79 1498.69 1524.79 1542.88 1564.89 1580.78 1588.82 1604.77 1668.95 1684.94 1706.88 1722.87 1764.92 1772.02 1777.90 1799.87 1815.85 1825.93 1863.89 1908.92 1921.01 1946.94 1954.03 2065.00 2103.96 2211.10 2225.07 2249.14 2284.17 2838.36 2876.32 2938.37 2976.30
3	775.43 842.50 855.02 870.55 945.57 976.47 1028.64 1045.61 1060.04 1082.08 1091.64 1107.61 1127.73 1164.70 1179.65 1185.04 1198.75 1210.79 1277.78 1287.77 1307.79 1315.76 1345.67 1353.81 1359.69 1383.71 1434.87 1444.03 1471.82 1475.85 1486.81 1611.01 1707.88 1716.94 1718.96 1731.99 1837.96 1866.98 1874.94 1913.00 1926.97 1939.00 1940.99 1960.98 1975.01 1976.94 1986.05 1990.95 2025.90 2083.03 2121.09 2185.09 2211.10 2225.15 2239.13 2247.19 2249.13 2283.22 2299.17 2313.21 2341.17 2383.96 2417.24 2490.19 2497.26 2510.14 2521.34 2564.18 2573.38 2625.35 2705.17 2748.24 2781.41 2807.22 2921.24 3021.68 3035.42 3191.47 3312.23 3348.32
4	800.46 842.50 870.55 904.50 937.53 1075.57 1191.52 1246.64 1255.75 1447.82 1481.76 1498.77 1665.79 1761.92 1767.98 1866.90 1882.92 2045.01 2112.02 2127.99 2143.99 2201.09 2211.10 2225.15 2234.25 2279.29 2283.20 2300.18 2315.25 2331.23 2403.37 2439.36 2452.27 2544.36 2583.42 2807.36 2890.41 2921.66 3348.79
5	1278.69 1281.56 1297.57 1306.61 1318.62 1337.61 1487.70 1637.83 1649.89 1659.78 1671.78 1675.79 1702.85 1722.32 1723.20 1736.90 1748.92 1948.18 1984.09 1996.15 2000.11 2041.16 2219.05 2235.10 2332.02 2469.10 2503.29 2579.49 3377.72
6	842.50 856.52 868.54 870.55 973.56 1004.60 1028.66 1045.62 1060.06 1111.63 1157.66 1165.70 1179.68 1234.74 1277.79 1294.76 1306.78 1323.72 1337.81 1357.77 1365.76 1383.78 1427.94 1434.85 1440.86 1475.85 1493.85 1513.81 1521.88 1637.87 1707.87 1716.96 1728.99 1740.99 1744.98 1768.95 1791.86 1838.04 1941.00 1948.17 1952.12 1994.04 2000.13 2083.04 2126.09 2211.10 2225.15 2233.15 2239.16 2249.13 2283.25 2299.20 2367.30 2383.99 2408.10 2417.27 2510.16 2564.16 2705.09 2807.23 3311.92 3348.26
7	714.45 833.06 842.50 855.05 864.54 870.54 973.55 1045.58 1055.58 1060.07 1071.51 1179.63 1183.68 1234.74 1277.76 1307.71 1357.74 1389.72 1405.71 1427.75 1434.79 1461.74 1475.79 1480.77 1493.79 1686.85 1705.98 1716.91 1727.95 1743.93 1791.81 1837.97 1875.91 1897.98 1913.91 1960.01 1973.92 1982.04 1994.01 2082.99 2120.97 2211.10 2225.11 2233.12 2249.10 2299.14 2330.10 2383.98 2400.28 2416.22 2705.16 2807.23 3119.31
8	833.08 839.09 842.50 855.07 864.55 871.05 888.65 904.73 931.23 1044.09 1055.55 1060.07 1071.53 1116.28 1249.07 1270.97 1321.59 1461.76 1475.94 1480.76 1686.86 1705.97 1743.92 1875.88 1913.88 1973.91 2211.03 2225.08 2249.07 2330.08 2383.93

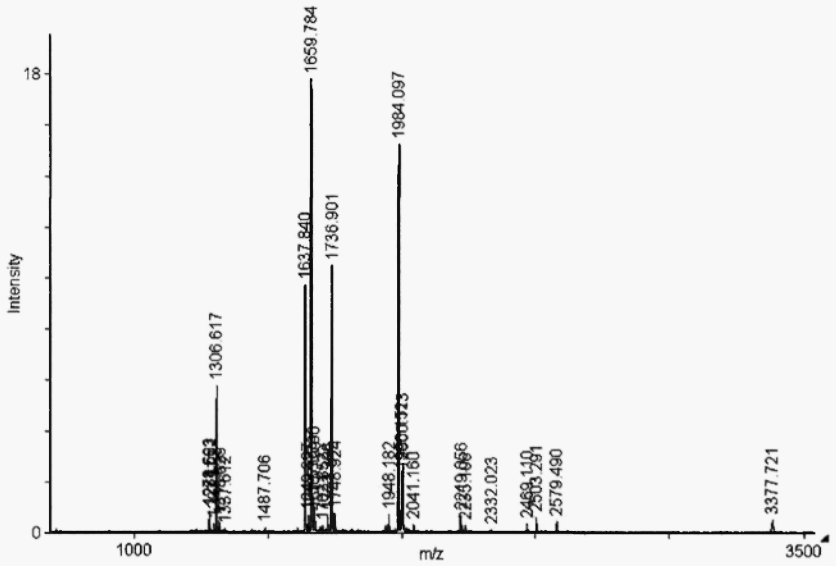

Fig. 2: Peptide mass fingerprint spectrum of protein spot 5.

TABLE 2

Identification of the differentially expressed phosphoproteins from cardiomyocytes

No.	Protein identity	NCBInr index code	Top score	p*I*	M_r (kDa)
1	94 kDa glucose-regulated protein	gi|34862435	205	4.72	92.998
2	calpactin I heavy chain	gi|9845234	164	7.55	38.939
3	not identified			6.62	25.827
4	chaperonin containing TCP-1 zeta subunit	gi|34872057	222	6.63	58.437
5	hypothetical protein XP_346548	gi|34857750	148	8.88	46.646
6	not identified			7.19	43.810
7	ferritin light chain	gi|120527	136	5.99	20.850
8	ferritin light chain 2	gi|6679873	121	6.39	20.887

DISCUSSION

Post-translational modification via phosphorylation underlies various biological processes and particularly the propagation of cellular signals through differential activation or inactivation of specific signaling cascades. This study presents the first phosphoproteome analysis of adult rat cardiomyocytes and the changes in the context of diazoxide preconditioning. Our findings indicate that diazoxide preconditioning is able to change the phosphorylation status of some key substrate proteins involved in cardioprotection in the various subcellular compartments, and these protein substrates, include chaperonin containing TCP-1 and hypothetical protein XP_346548, may undergo post-translational modification via phosphorylation in cardiomyocytes, whereas 94 kDa glucose-regulated protein (GRP94), calpactin I and ferritin may undergo post-translational modification via dephosphorylation. This approach to the analysis of the cardiac phosphoproteome and its dynamic regulation by diazoxide preconditioning provides unique insights into the molecular mechanisms underlying the modulation of preconditioning cardioprotection.

All large-scale analyses of phosphoprotein regulation rely on selective enrichment of phosphoproteins, and recently several strategies to accomplish this have been presented. Immobilized metal affinity chromatography (IMAC) can be employed to retain negatively charged peptides after masking non-phosphate negatively charged residues by methylation /13/. The affinity columns used in the present study offer the advantage of not requiring covalent modification of the sample; this reduces sample processing time, circumvents issues of insufficient blocking or excessive labeling, and avoids creating covalent adducts that can interfere with the interpretation of mass spectra. Similar advantages were noted by Metodiev *et al.* /14/.

Comparative analysis of two-dimensional gels is a common technique for comparing protein expression between two biological conditions. In the present study, silver staining was sufficient to identify a large number of changes in protein abundance. In future studies, difference gel electrophoresis (DIGE) could be used to identify additional smaller alterations /15/.

The glucose-regulated protein GRP94 is an abundant member of the 90-kDa molecular chaperone family in the endoplasmic reticulum

(ER). It is involved in the maintenance of cell survival because it exerts specific protection against stresses due to Ca^{2+} depletion from the ER /16/. Although its precise functional role in cardiac myocytes remains to be determined, it is upregulated after exposure to bacterial lipopolysaccharide and during prenatal development /17/. ER Ca^{2+} depletion and consequent intracellular Ca^{2+} overload have been recognized as stimuli for GRP94 upregulation /16,18/. A recent study by Vitadello *et al.* demonstrated that GRP94 overexpression in both cardiac H9c2 cells and primary cardiomyocytes protects against necrosis induced by simulated ischemia /19/. GPR94 has ATP-binding site(s) and autophosphorylating activity, and it is also a substrate for casein kinase II, so it is able to autophosphorylate itself on serine and threonine residues and be phosphorylated by casein kinase II /20,21/. A recent study demonstrated that phosphorylation of high-mobility GRP94 in Sf21 cells was constitutive, occurring in the absence of hormone stimulation /22/. Though the precise functional role of phosphorylation of GRP94 remains to be determined, constitutive phosphorylation of GRP94 may be present in cardiomyocytes. Our results show that dephosphorylation of GRP94 occurred in cardiomyocytes after diazoxide preconditioning, which may be another mechanism for GRP94 activation to protect cardiomyocytes from death induced by ischemia.

The chaperonin containing TCP-1 (CCT [also known as TRiC]) is the only member of the chaperonin family found in the cytosol of eukaryotes which has been shown to assist the folding and assembly of various newly synthesized polypeptides of some proteins in cytosol, especially the cytoskeletal proteins actin and tubulin /23,24/. CCT plays important roles in the recovery of cells from protein damage by assisting in the folding of proteins that are actively synthesized and/or renatured during this period /25/. Our results show that phosphorylation of CCT in cardiomyocytes was increased by diazoxide preconditioning. However, the functional role of phosphorylated CCT in cardiac myocytes is still unclear.

Ferritin has a large capacity for iron storage. It is a 24 subunit protein composed of two subunit types, termed H and L. The ferritin H subunit has a potent ferroxidase activity that catalyses the oxidation of ferrous iron, whereas ferritin L plays a role in iron nucleation and protein stability. Iron is required for normal cell growth and proliferation. However, excess iron is potentially harmful, as it can

catalyze the formation of toxic reactive oxygen species (ROS) via Fenton reactions. Overexpression of either ferritin H or ferritin L reduced the accumulation of ROS in response to oxidant challenge /26,27/. Calpactin I heavy chain is the protein-tyrosine kinase substrate p36 and contain two Ca^{2+}/phospholipid-binding sites /28/. It is also a substrate for protein kinase C *in vitro* and *in vivo* /29,30/. In the present study, calpactin I heavy chain and ferritin light chain were shown to be dephosphorylated by diazoxide preconditioning in cardiomyocytes. The precise mechanism of dephosphorylation of calpactin I heavy chain and ferritin light chain is unclear, and needs further study.

In conclusion, proteomic analysis of phosphoproteins in cardiomyocytes pretreated with diazoxide at levels that have been established by Kao *et al.* /31/ to confer cardioprotection provided an effective approach in elucidating the molecular mechanisms of mitoK_{ATP} channel opening-induced cardioprotection. Eight protein spots with different abundance were found, of which six differentially expressed proteins were identified by MALDI-TOF MS. The roles of these identified proteins in diazoxide preconditioning remain to be determined. These findings provide new clues to understanding the mechanism of ischemic preconditioning in cardiomyocytes downstream from mitoK_{ATP} channel opening.

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