ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 10, pp. 1140-1146. © Pleiades Publishing, Ltd., 2011.
Published in Russian in Biokhimiya, 2011, Vol. 76, No. 10, pp. 1398-1406.

Identification of Proteins Responding to Adrenergic Receptor Subtype-Specific Hypertrophy in Cardiomyocytes by Proteomic Approaches

Ning Liu[1]*#, Kai-zheng Gong[2,3#], Yuan-bin Cai[2], and Zijian Li[2]*

[1]*Central Laboratory, Jilin University Second Hospital, Changchun 130041, P.R. China; fax: +86 (431) 8879-6510; E-mail: liu_ning@jlu.edu.cn*
[2]*Institute of Vascular Medicine, Peking University Third Hospital and Key Laboratory of Cardiovascular Molecular Biology and Regulatory Peptides, Ministry of Health and Key Laboratory of Molecular Cardiology, Ministry of Education, Beijing 100091, P.R. China; fax: +86 (10) 6201-7700; E-mail: lizijian@bjmu.edu.cn; caiyuanbin@bjmu.edu.cn*
[3]*Department of Cardiology, The First People's Hospital of Yangzhou, Yangzhou 225001, P.R. China; E-mail: yungkzh@163.com*

Received December 17, 2010
Revision received April 25, 2011

Abstract—The individual signaling pathways underlying cardiac hypertrophy, which is induced by either α or β adrenergic receptor (AR), are different. Activation of different AR subtypes couples to different G proteins and induction of specific signaling pathways, which ultimately results in subtype-specific regulation of cardiac function. We present the first proteomics study identifying proteins that are related to AR subtype-specific hypertrophy in cardiomyocytes by comparing the two-dimensional electrophoresis patterns between neonatal rat cardiomyocytes treated by phenylepinephrin (PE) and by isoproterenol (ISO). An improved 2-DE strategy was used in these comparative experiments. Twenty-five differentially expressed proteins in cardiomyocytes treated by PE or treated by ISO were successfully analyzed and identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry, especially those that might be responsible to intracellular oxidative stress such as dismutase, peroxiredoxin, and thioredoxin-like protein p46. In addition, induced reactive oxygen species were also found to be AR subtype-specifically relevant to endoplasmic reticulum proteinase ERK1/2 phosphorylation during the development of hypertrophy induced by different AR subtypes. The results will help to better understand the underlying mechanisms of different adrenergic receptor subtype-induced hypertrophy.

DOI: 10.1134/S0006297911100075

Key words: adrenergic receptors, cardiac hypertrophy, proteomics, reactive oxygen species

Adrenergic receptors (ARs) are members of the G-protein-coupled receptor superfamily. There are two major groups of adrenergic receptors, namely α-AR and β-AR subtypes, which regulate a wide variety of physiological and pathological responses through their specific signaling pathways. Both of the two main groups of AR subtypes play important roles in promoting cardiac hypertrophy [1].

Activation of different AR subtypes couples to different G proteins and induction of specific signaling pathways, which ultimately results in subtype-specific regulation of cardiac function. α-ARs couple to a broad spectrum of signaling pathways – including protein kinase C, mitogen-activated protein kinase (MAPK), and Ca^{2+} channels—by activating G proteins in the Gq family. The acute effects of α-AR stimulation on the contractile properties of the heart are complex and dependent on the species being studied. Chronic activation of α-AR stimulates cardiac myocyte hypertrophy in animals.

β-AR mainly couples to the stimulatory Gs protein, which leads to activation of adenylyl cyclase and production of cAMP. The cAMP-dependent protein kinase A phosphorylates various substrates, including the L-type Ca^{2+} channel and phospholamban and troponin I and C proteins. *In vivo* and *in vitro* assays show that β-AR plays the predominant role in modulating the heart rate and the force of contraction in the mouse. Increased protein syn-

Abbreviations: AR, adrenergic receptor; DPI, diphenyliodonium; eIF-5, eukaryotic translation initiation factor 5; ERK, endoplasmic reticulum kinase; ISO, isoproterenol; MAPK, mitogen-activated protein kinase; NAC, N-acetyl cysteine; PE, phenylepinephrin; ROS, reactive oxygen species.

* To whom correspondence should be addressed.
# These authors contributed equally to this work.

thesis in cardiac myocytes induced by both α- and β-AR agonists has also been observed [2-5]. So far, the mechanisms of both α- and β-AR contribution to cardiac myocyte hypertrophy are controversial and less well understood. Therefore, characterization of subtype-specific adrenergic signaling in neonatal cardiac myocytes will be needed to generate a more comprehensive picture of adrenergic signaling pathways. Although some proteomic work in heart disease have been recently published [6-8], our knowledge about protein changes involved in cardiac hypertrophy, especially when induced by different subtype receptors agonists, still remains scarce.

Our previous work showed that cardiac hypertrophy can be induced clearly by different subtype receptors agonists [9]. To further explore the protein expression profile that is involved in AR-mediated subtype-specific cardiac hypertrophy, we adopted 2-DE/MS based proteomic approaches to identify a panel of proteins that were differentially expressed due to AR subtype-specific stimulations—phenylephrine (PE), a specific α_1-adrenoceptor agonist, and isoproterenol (ISO), a specific β-adrenoceptor agonist. The results implied that the intracellular oxygen environment during the subtype-specific adrenoceptor-mediated hypertrophy seemed to be different. As previously reported [10], reactive oxygen species (ROS) can trigger both endoplasmic reticulum stress response and oxidative phosphorylation response during the development of hypertrophy mediated by norepinephrine, a kind of agonist of both α_1- and β-adrenoceptors. Therefore, in the subtype-specific point of view, we further investigated the subtype-specific effects of the intracellular ROS from different source on regulation of endoplasmic reticulum kinase (ERK), mainly by checking whether ERK1/2 phosphorylation can be blocked by inhibitors of ROS production from different sources, such as mitochondrial respiration or NADPH oxidase. Our data show AR subtype-specific differences in cellular proteome during the course of development of AR-mediated hypertrophy, of which the mechanisms were that ERK1/2 pathway was activated by the AR subtype-specific intracellular sources of induced ROS.

MATERIALS AND METHODS

Cultures of rat neonatal cardiomyocytes were established as described previously [11]. Briefly, ventricles of 1-to-2-day-old neonatal Sprague–Dawley rats were excised and cardiomyocytes were isolated using 0.1% trypsin (HyClone) and 80 units/ml collagenase (Worthington Biochemical Corp.) in a Hanks' balanced salt solution (calcium-free; HyClone). To purify the cardiac myocytes from non-myocytes, isolated cells were pre-plated for 90 min. The enriched cardiomyocyte fractions were cultured in DMEM (Sigma, USA) supplemented with 10% FBS for 24 h. Bromodeoxyuridine was added at 100 μM during the first 48 h to prevent the proliferation of non-cardiomyocytes. Culture media were replaced with serum-free media at 24 h. After 24 h, hypertrophy was induced by the addition of either PE (10 μM) or ISO (1 μM) to the cell culture medium 48 h before lysing the cells that were to be used for proteomic analysis.

To examine subtype-specific effects of ROS induced by either PE or ISO on ERK1/2 phosphorylation during AR-mediated cardiomyocyte hypertrophy, cardiomyocytes were preincubated with either of the two ROS specific inhibitors—rotenone (inhibitor of mitochondrial respiration) or diphenyliodonium (DPI) (inhibitor of NADPH oxidase)—for 30 min, and then treated with either PE or ISO for 48 h.

To examine the subtype-specific effects of a ROS scavenger (N-acetyl cysteine, NAC) on ERK1/2 phosphorylation induced by either PE or ISO during AR-mediated cardiomyocytes hypertrophy, cardiomyocytes were preincubated with NAC for 30 min, and then treated with either PE or ISO for 48 h. The lysates from cell samples were analyzed by Western blot with anti-p44/42 MAP kinase (Cell Signaling, No. 9102), anti-phospho-p44/42 MAPK (Cell Signaling, No. 9101), and anti-eIF-5 (Santa Cruz Biotechnology, sc-28309) using an enhanced-chemiluminescence system (ECL kit, Pierce).

To assess the purity of the isolated cardiomyocytes, aliquots of cardiomyocytes were cultured on chamber slides ($3 \cdot 10^5$ cells/well) for 24 h. Further, the cardiomyocytes were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min. Anti-sarcomeric α-actinin monoclonal antibody and IgG biotinylated secondary antibody were used for α-actinin staining.

Cell extract preparation. After being washed three times with solution containing 10 mM Tris, 1 mM EDTA, and 250 mM sucrose, which was adjusted to pH 7.0-7.5, the cells were dislodged from the bottom of the dishes with a cell scraper and pelleted by centrifugation. The cells were lysed in ice-cold buffer containing 6 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT, 25 mM spermine, 0.2% (w/v) Bio-Lyte, pH 3-10. The resulting lysate was then centrifuged at 40,000*g* for 60 min. The supernatant was collected, and an aliquot of 25 μl was used for determination of protein concentration. The remaining supernatant was then divided into aliquots and stored at −80°C until used for 2-DE.

Two-dimensional electrophoresis. Proteins were separated by two-dimensional electrophoresis. Isoelectric focusing was carried out using a Protean IEF Cell (Bio-Rad, USA). Samples containing 200 μg protein for analytical gels or up to 1 mg for micro-preparative gels were diluted to 300 μl with rehydration solution (6 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, 0.5% v/v pH 3-10 Bio-Lyte, trace of bromophenol blue) and applied to IPG strips (pH 3-10, 17 cm; Bio-Rad) for 14 h in passive mode. The isoelectric focusing was performed at 20°C over 24 h for a total of 70,000 V-h. After being equilibrat-

ed in equilibration solution containing 0.1% SDS, gel strips were applied on second-dimensional PAGE with 12% acrylamide.

For the second dimensional SDS-PAGE, a novel 2-DE strategy, namely "mirror images in one gel" was developed. Because the majority of the protein spots visualized by 2-DE are located in the range between pH 4 and 8, the strips were cut at the zone of pH 4 and 8. Since the strips we used are a kind of linear type, in which the pH of the immobilized ampholytes was distributed in a linear gradient pattern along the strip, it is easy to locate the zone of pH 4 and 8 in the strip for cutting. The resulting shorter strips were about 8.5 cm each, corresponding to the pH range between pH 4 and 8. Strips with the same pH range coverage (pH 4-8) were then loaded reversely side-by-side on top of a second SDS-PAGE gel with 12% polyacrylamide. As the two cut IPG strips representing differential protein expression were run on one SDS-PAGE, variations in gel shape, size and different developing time during silver stain could be greatly eliminated. This can improve matching efficiency of 2-DE comparing to the traditional one gel on one SDS-PAGE protocol. Separation was then carried out on a Protean II xi electrophoresis system (Bio-Rad) at a current setting of 7.5 mA/gel for the initial 2 h and 15 mA/gel until the bromophenol blue reached the bottom of the gel. After two-dimensional gel electrophoresis, the proteins were stained with silver or Coomassie blue G-250 for subsequent mass spectrometry. As a result, the stained gels represented bilateral symmetry like mirror images.

The stained gels were scanned with a high-resolution scanner (Umax 1120), and the gel images were analyzed using PDQuest software (v.7.1.1; Bio-Rad) according to the instructions provided by the manufacturer. To accurately compare spot quantity between gels, a normalization based on the total density on each gel was applied for each gel. Student's *t*-test was performed. The significantly differentially expressed protein spots ($p < 0.05$) with 2-fold increased or decreased intensity were selected and subject to further identification by MALDI-MS.

Sample preparation and MALDI-TOF-MS. Protein spots of interest visualized with colloidal Coomassie blue G-250 staining were excised and transferred to 1.5 ml siliconized Eppendorf tubes. After being washed and then destained by 50% ACN, the gel pieces were dried in a vacuum centrifuge. The dried gel-pieces were incubated in digestion solution containing 50 mM NH_4HCO_3 and 0.1 mg/ml TPCK-trypsin for 12 h at 37°C. The resulting peptides were extracted three times by 50 µl aliquots of 5% trifluoroacetic acid in 60% acetonitrile. Combined extracts were concentrated in a SpeedVac to 3-5 µl.

The concentrated tryptic peptide mixture was mixed with a saturated CHCA (α-cyano-4-hydroxycinnamic acid) matrix solution and vortexed gently. A volume (1 µl) of the mixture containing CHCA matrix was loaded on a 96 × 2 well hydrophobic plastic surface sample plate (Applied Biosystems, USA) and air-dried. The samples were analyzed with a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems) fitted with a 337-nm nitrogen laser. Spectra were acquired using the instrument in reflectron mode and calibrated using a standard peptide mixture. Database searching with the monoisotopic peptide masses was performed against the NCBInr database by using the peptide search engines ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound) and Mascot (http://www.matrixsciece.com).

RESULTS AND DISCUSSION

In this study, neonatal rat cardiomyocytes were isolated and cultured as an *in vitro* model system for studying the adrenergic receptor subtype-specific changes in protein-expression profiles. To eliminate the interference of other types of the cells in the heart, such as cardiac fibroblasts, the purity of the cardiomyocytes was confirmed by immunohistochemical analysis. The result demonstrated that the purity of the cardiomyocytes was more than 95% as shown by the staining of the sarcomeric α-actinin, a protein specifically expressed in cardiomyocytes (Fig. S1 in Supplement; see on-line site of *Biochemistry (Moscow)* http://protein.bio.msu.ru/biokhimiya).

Although the cell signaling of adrenergic receptors in hypertrophy has been widely studied, the differences of protein alterations at proteome scale mediated by the two main adrenergic receptor subtype groups (α and β) have not been investigated comprehensively. We used an improved proteomic approach to profile the AR subtype-specific differences in protein expression in cardiomyocytes induced by specific agonist for either α or β adrenergic receptor. In our 2-DE based analysis, right after IEF, IPG strips that were loaded with either of the two different samples were cut into slightly shorter strips (with approximate range of pH 4-8) and placed reversely side-by-side on one SDS-PAGE with mirror symmetry and run on one SDS-PAGE. Well-resolved and reproducible 2-DE patterns were obtained as displayed in Fig. 1.

For each cell model, a triplet of gel images was assigned for a group using PDQuest software. Thus, two groups, namely one containing the gels of cardiomyocytes treated by PE and one containing the gels of cardiomyocytes treated by ISO, were summarized to a statistical analysis set. The analysis revealed that 25 reproducible protein spots had abundance greater than 2-fold different between cardiomyocytes treated by PE and cardiomyocytes treated by ISO, including 20 upregulated proteins and 5 downregulated proteins in cardiomyocytes treated by PE compared with those treated by ISO. Moreover, to obtain information regarding individual effect of the two agonists on cardiomyocytes, separate comparisons of cells treated by either PE or ISO with the untreated control cells were made, as illustrated in Figs. S2 and S3, respec-

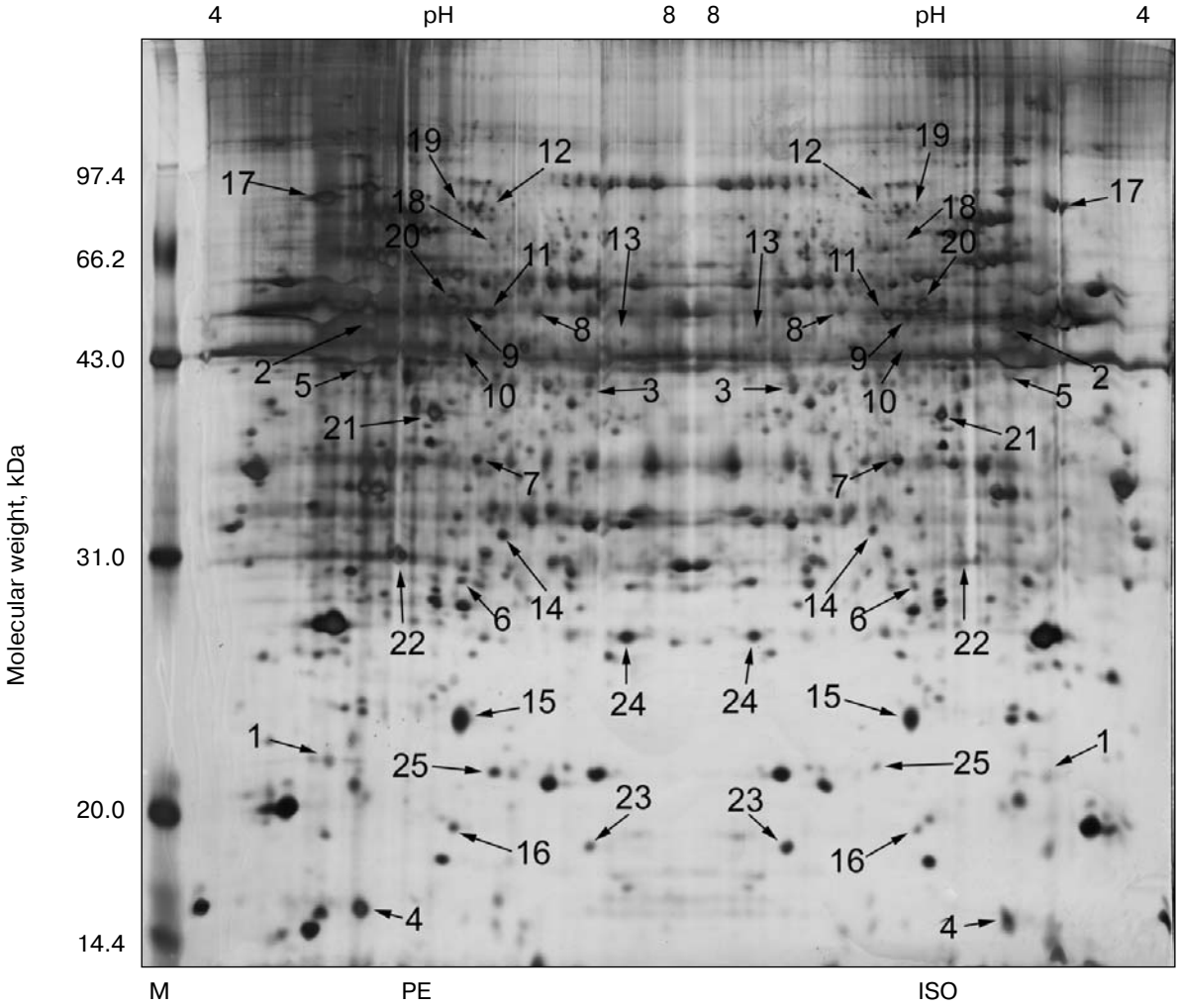

Fig. 1. A representative gel image of cardiomyocytes treated with PE and cardiomyocytes treated with ISO. The isoelectric focusing was carried on a 17 cm IPG strip with a pH range from 3 to 10. The two cut IPG strips representing pH range from 4 to 8 were reversely applied on a second dimensional SDS-PAGE with acrylamide of 12%. Numbers associated with the spots on the gel images refer to the identified proteins listed in the table.

tively, in the supplementary materials (see Supplement). All these protein spots were successfully identified with high confidence using in-gel trypsin digestion followed by mass spectrometry. The identifications of all the regulated proteins, as well as regulations of the individual proteins upon stimulation by either PE or ISO, are provided in detail in the table. For all the proteins identified, both database search engines (Profound and Mascot) gave the same protein hits with high confidence scores. Some of these proteins were previously described as diagnostic and prognostic markers of cardiovascular diseases or associated with cardiac hypertrophy. For example, myosin, which comes in a greater variety than actin, is found to be associated with dilated cardiomyopathy in rats [13] and humans [14] by 2-DE studies. Another protein known to be involved in hypertrophy in rat cardiomyocytes is dismutase [15], which serves as an important antioxidant in almost all cells exposed to oxygen. Additionally, peroxiredoxin as well as thioredoxin-like protein p46 is also shown to be more upregulated in α-AR mediated hypertrophy. We suggested that the oxidation environment in the two hypertrophied cells might be different due to the different expression levels of dismutase, peroxiredoxin, and thioredoxin-like protein p46 between the hypertrophied cells mediated by different adrenoceptor subtypes.

We previously demonstrated that reactive oxygen species (ROS) were involved in the ERK pathway during the development of hypertrophy in cultured cardiomyocytes when both α_1- and β-adrenoceptors were simultaneously stimulated [10]. Therefore, considering that the oxidation environment in the two hypertrophied cells might be different as implied from our proteomics results, we further investigated the subtype-specific effects of the intracellular ROS from different sources on regulation of

Identified proteins in relation to AR subtype-specific hypertrophy in cardiomyocytes

Spot number on 2-D gels	Accession number	Protein information	Z score estimated by Profound	Mowse score estimated by Mascot	Sequence coverage, %	p*I*/Mr, kDa	Regulation of protein expression by ISO treatment*	Regulation of protein expression by PE treatment	Differential ratio (PE/ISO), folds
1	2	3	4	5	6	7	8	9	10
1	NP_036738.1	myosin, light polypeptide 3	2.41	98	38	5.03/22.03	=	↑	4.36
2	XP_225257.2	thioredoxin-like protein p46	2.43	73	47	5.58/46.35	=	↑	3.24
3	XP_194144.3	RIKEN cDNA 4930473A06	2.43	85	41	5.20/42.42	↓	↓	0.13
4	AAH34302.1	cytochrome *c* oxidase, subunit Va	2.43	112	56	6.08/16.10	↓	↓	3.15
5	CAA56429.1	α-actin cardiac	2.43	63	38	5.23/42.02	=	↑	2.52
6	AAK28338.1	LASP-1	2.43	138	49	6.61/29.97	↓	↑	3.64
7	AAH57149.1	Cd1d2 protein	2.43	82	27	6.38/33.80	=	↓	0.41
8	NP_035577.1	SRY-box containing gene 8	2.43	88	27	6.64/49.88	↓	↑	3.45
9	AAO85477.1	hemopoietic lineage switch protein 5	2.43	106	37	6.73/57.35	↓	↓	0.42
10	NP_032303.1	4-hydroxyphenylpyruvic acid dioxygenase	2.38	126	39	6.58/45.05	=	↓	0.48
11	S10246	phosphopyruvate hydratase α	2.34	86	45	6.37/47.12	↓	↓	2.56
12	P48679	lamin A	2.30	63	42	6.54/74.32	=	↑	4.26
13	XP_215069.2	similar to RIKEN cDNA 2300002G02	2.38	94	41	7.23/49.52	=	↓	0.42
14	AAH59122.1	peroxiredoxin 4	2.34	102	33	6.18/31.01	↓	↑	3.43
15	P54370	ornithine decarboxylase antizyme (ODC-Az)	2.43	76	32	6.71/25.23	=	↑	2.76
16	AAB37470.1	myosin light chain 2 isoform	2.38	116	37	4.86/18.86	↑	↑	4.17
17	AAH62017.1	heat shock 70-kDa protein 5	2.13	106	41	5.07/72.35	↓	=	3.57
18	1703420A	non-muscle caldesmon	2.43	92	28	6.36/61.66	=	↓	0.45
19	A34337	propionyl-CoA carboxylase α-chain precursor	1.94	146	39	6.33/77.61	=	↑	3.67

Table (Contd.)

1	2	3	4	5	6	7	8	9	10
20	AAH42187.2	heterogeneous nuclear ribonucleo-protein H1	2.26	76	16	5.89/49.20	=	↑	2.25
21	AAH61551.1	ornithine amino-transferase	2.43	96	27	6.53/48.33	↓	↑	4.74
22	NP_114039.1	prohibitin	1.65	136	24	5.57/29.82	↓	↑	1.92
23	CAA29121.1	dismutase	2.43	98	40	5.89/15.58	↓	↓	0.22
24	AAH61727.1	adenylate kinase 2	2.43	122	29	7.01/25.53	↓	↑	3.14
25	AAP31995.1	αB-crystallin	2.43	93	68	6.76/20.09	=	↑	8.54

* Designations =, ↑, ↓ mean that protein expression level did not change, increased, and decreased, respectively.

ERK, mainly by studying whether ERK1/2 phosphorylation can be blocked by source-specific inhibitors of ROS production from different sources, such as rotenone (inhibitor of mitochondrial respiration) and DPI (inhibitor of NADPH oxidase). As shown in Fig. 2, activity of ERK1/2 was increased when either of the two α_1- and β-adrenoceptor subtypes was stimulated by PE and ISO, respectively. The increased activity of ERK1/2 induced by PE can be blocked by either rotenone or DPI (Fig. 2a), suggesting that both mitochondrial and NADPH-oxidase ROS are involved in ERK/MAPK activation induced by α_1-adrenoceptor stimulation. The increased activity of ERK1/2 induced by ISO can only be blocked by DPI but not rotenone (Fig. 2b), indicating that the principal ROS involved in ERK/MAPK activation induced by β-AR stimulation is mainly of NADPH-oxidase origin rather than from mitochondrial respiration. To further investigate the subtype-specific relevance of ROS induced by either

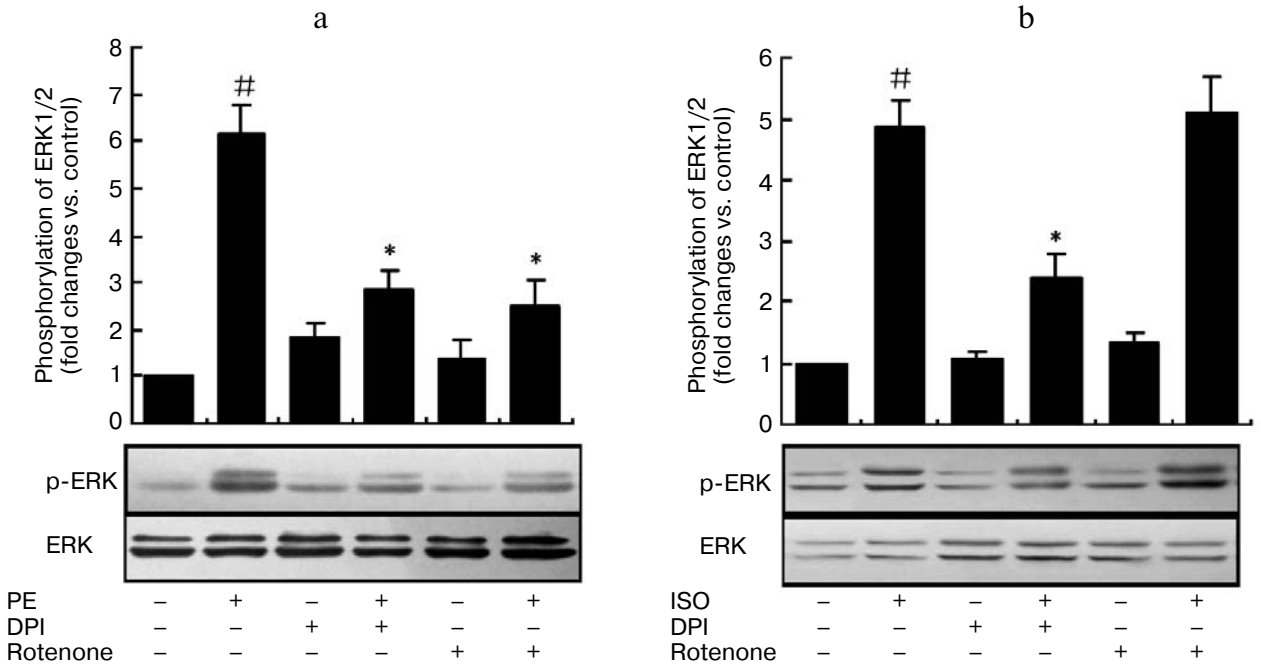

Fig. 2. Subtype-specific effects of AR-induced ROS on ERK/MAPK signaling pathway. Cardiomyocytes were treated with drugs and inhibitors to investigate the effects of DPI and rotenone on ERK/MAP kinase activation by either PE (a) or ISO (b). Activated ERK1/2 was detected by Western blot analysis using antibodies against phospho-ERK1/2 and total ERK1/2. eIF-5 was used as a loading control. Fold activations of phospho-ERK compared to control are expressed as mean ± SD of three independent experiments.

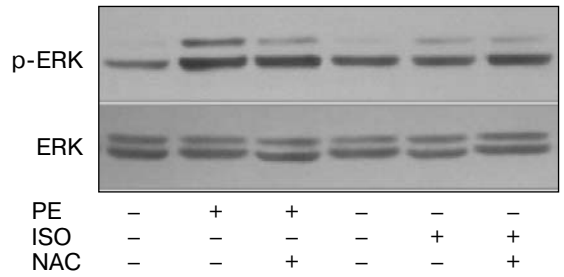

Fig. 3. Subtype-specific effects of a ROS scavenger N-acetyl cysteine (NAC) on ERK/MAPK signaling pathway. Cardiomyocytes were preincubated with NAC and then treated with either of the two agonists to investigate the effects of NAC on ERK/MAP kinase activation by either PE or ISO. Activated ERK1/2 was detected by Western blot analysis using antibodies against phospho-ERK1/2 and total ERK1/2.

PE or ISO to ERK signaling, NAC was used as a nonspecific ROS scavenger to assess the effects of ROS on PE/ISO-induced ERK phosphorylation. The results showed that ERK phosphorylation induced by either PE or ISO can be partially inhibited in the presence of NAC. In particular, the inhibitory effect of NAC on ERK phosphorylation induced by PE is more significant than that of NAC on ERK phosphorylation induced by ISO, as indicated in Fig. 3. Our data present direct evidence of activation of ROS production after addition of agonists, which shows subtype-specific relevance to ERK signaling.

We presented here the first proteomics study of AR subtype-specific hypertrophy in cardiomyocytes. A list of proteins in relation to AR subtype-specific hypertrophy was identified, especially those that might be involved in regulation of intracellular oxidation environment. Additionally, several proteins that have not been reported so far to be associated with hypertrophy, such as Cd1d2 protein, SRY-box containing gene 8 (*sox8*), prohibitin, and heterogeneous nuclear ribonucleoprotein H1, were also identified. Finally, the specific intracellular sources of induced ROS were found to be AR subtype-specifically relevant to ERK1/2 phosphorylation. Our results provide insights into the differences of the signal transduction pathways between α- and β-AR in cardiac hypertrophy and could help to elucidate the biochemical processes underlying hypertrophy induced by stimulation of adrenergic receptors, discover novel diagnostic markers, and provide the basis for the development of new therapeutic opportunities.

This work was supported by grants from the Major State Basic Research Development Program of China (973 Program) (2011CB503903), the National Science Foundation of China (81070078, 21175055), the Jilin Province Science and Technology Department (20090461, 20110739, 2011713), the Basic Research Foundation of Jilin University (450060445316), and the Beijing Municipal Natural Science Foundation (7102158).

REFERENCES

1. Hunter, J. J., and Chien, K. R. (1999) *N. Engl. J. Med.*, **341**, 1276-1283.
2. Bishopric, N. H., Sato, B., and Webster, K. A. (1992) *J. Biol. Chem.*, **267**, 20932-20936.
3. Pinson, A. K., Schluter, D., Zhou, X. J., Schwartz, P., Kelsser, I. G., and Piper, H. M. (1993) *J. Mol. Cell. Cardiol.*, **25**, 477-490.
4. Yamazaki, T., Komuro, I., Zou, Y. Z., Kudoh, S., Mizuno, T., Hiroi, Y., Shiojima, I., Takano, H., Kinugawa, K., Kohmoto, O., Takahashi, T., and Yazaki, Y. (1997) *J. Mol. Cell. Cardiol.*, **29**, 2491-2501.
5. Barros Rde, A., Okoshi, M. P., and Cicogna, A. C. (1999) *Arq. Bras. Cardiol.*, **72**, 641-656.
6. Zamilpa, R., Lopez, E. F., Chiao, Y. A., Dai, Q., Escobar, G. P., Hakala, K., Weintraub, S. T., and Lindsey, M. L. (2010) *Proteomics*, **10**, 2214-2223.
7. Isserlin, R., Merico, D., Alikhani-Koupaei, R., Gramolini, A., Bader, G. D., and Emili, A. (2010) *Proteomics*, **10**, 1316-1327.
8. Wang, J., Bai, L., Li, J., Sun, C., Zhao, J., Cui, C., Han, K., Liu, Y., Zhuo, X., Wang, T., Liu, P., Fan, F., Guan, Y., and Ma, A. (2009) *Sci. China C Life Sci.*, **52**, 1003-1010.
9. Zhang, Y., Yan, J., Chen, K., Song, Y., Lu, Z., Chen, M., Han, C., and Zhang, Y. (2004) *Clin. Exp. Pharmacol. Physiol.*, **31**, 626-633.
10. Li, Z. J., Liu, N., Zhang, L. S., Gong, K., Cai, Y., Gao, W., Zhang, Y., Liu, Z., Liu, S., Han, Q., and Zhang, Y. (2009) *Proteom.–Clin. Appl.*, **3**, 1407-1421.
11. Ito, H., Hirata, Y., Hiroe, M., Tsujino, M., Adachi, S., Takamoto, T., Nitta, M., Taniguchi, K., and Marumo, F. (1991) *Circ. Res.*, **69**, 209-215.
12. Steinberg, S. F., and Brunton, L. L. (2001) *Annu. Rev. Pharmacol. Toxicol.*, **41**, 751-773.
13. Burniston, J. G. (2009) *Proteomics*, **9**, 106-115.
14. Jager, D., Jungblut, P. R., and Muller-Werdan, U. (2002) *J. Chromatogr. B*, **771**, 131-153.
15. Buscemi, N., Murray, C., Doherty-Kirby, A., Lajoie, G., Sussman, M. A., and van Eyk, J. E. (2005) *Am. J. Physiol. Heart Circ. Physiol.*, **289**, H2325-H2333.