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Differential Activation of Ras/Raf/MAPK Pathway between Heart and Cerebral Artery in Isoproterenol-induced Cardiac Hypertrophy

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Cardiac hypertrophy contributes an increased risk to major cerebrovascular events. However, the molecular mechanisms underlying cerebrovascular dysfunction during cardiac hypertrophy have not yet been characterized. In the present study, we examined the molecular mechanism of isoproterenol (ISO)-evoked activation of Ras/Raf/MAPK pathways as well as PKA activity in cerebral artery of rabbits, and we also studied whether the activations of these signaling pathways were altered in cerebral artery, during ISO-induced cardiac hypertrophy compared to heart itself. The results show that the mRNA level of c-fos (not c-jun and c-myc) in heart and these genes in cerebral artery were considerably increased during cardiac hypertrophy. These results that the PKA activity and activations of Ras/Raf/ERK cascade as well as c-fos expression in rabbit heart during cardiac hypertrophy were consistent with previous reports. Interestingly, however, we also showed a novel finding that the decreased PKA activity might have differential effects on Ras and Raf expression in cerebral artery during cardiac hypertrophy. In conclusion, there are differences in molecular mechanisms between heart and cerebral artery during cardiac hypertrophy when stimulated with β_2 adrenoreceptor (AR), suggesting a possible mechanism underlying cerebrovascular dysfunction during cardiac hypertrophy.

Key Words: Cardiac hypertrophy, Cerebrovascular events, Ras/Raf/MAPK pathway

INTRODUCTION

Cardiac hypertrophy is a fundamental adaptation process to an increased workload, whether pressure- or volume-overloaded (Wankerl et al, 1995). These hypertrophic responses are mediated by hemodynamic as well as humoral factors that may play synergistic role in modifying the cardiac contraction. Pressure- and volume-overload-induced cardiac hypertrophy is a well-recognized risk factor for cerebrovascular diseases (Verdecchia et al, 2001; Selvetella et al, 2003). Studies with the general population and cohorts of hypertensive patients have shown a several-fold increase in the risk of cardiovascular events in subjects with cardiac hypertrophy. Therefore, in oeder to prevent or treat these cardiovascular events as well as cardiac hypertrophy, it is important and necessary to fully understand molecular mechanisms of these diseases.

It is known that chronic infusions of an β_2-adrenoreceptor (β_2AR) agonist, isoproterenol (ISO) induce typical hypertrophic responses (Zierhut et al, 1989; Boluyt et al, 1995). Binding of ISO to β_2ARs stimulates adenylyl cyclase (AC) through interaction with G protein Gαs, which increases intracellular cAMP levels and finally activates protein kinase A (PKA). β_2ARs can also be phosphorylated by PKA, which can lead to feedback desensitization of β_2ARs signaling. The PKA-mediated β_2AR desensitization pathway may also affect the Ras/Raf/MEK/ERK pathway by coupling to Gi/Go via Src/Sos pathway. The target of PKA in the MAPK pathway was shown to be Raf-1. Activated Raf-1 phosphorylates and activates the dual-specificity theronine/tyrosine kinase MEK. MEK in turn phosphorylates ERK1/2, resulting in increased expression of early response genes such as c-fos, c-jun and c-myc. cAMP/PKA has been reported to have an inhibitory effect on activation of Raf-1 and mitogen-activated protein kinase (MAPK) by interfering with Ras-Raf-1 association in a variety of cell types such as Rat-1 cells, smooth muscle cells, Chinese hamster ovary cells, COS-7, and adipocytes (Burgering et al, 1993; Cook et al, 1993; Wu et al, 1993; VanRenterghem et al, 1994; D'Angelo et al, 1997; Hecquet et al, 2002). On the contrary, however, in some cell types such as PC12 cells, Swiss-3T3 cells, and S49 mouse lymphoma cells, cAMP activates ERKs and potentates the effects of growth factors on differentiation and gene expression (Nagano et al, 1992; Faure et al, 1994; Froedin et al, 1994; Faure et al, 1995; Yao et al, 1995; Yamazaki et al, 1997; Wan et al, 1998): On activation, EKR 1/2 are translocated to nucleus and activate transcription of immediate early genes

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ABBREVIATIONS: ISO, isoproterenol; AR, adrenoreceptor; AC, adenylyl cyclase; PKA, protein kinase A, MAPK, mitogen-activated protein kinase; PLC, phospholipase C.

such as c-fos, c-jun, and c-myc. However, the molecular mechanisms of β_2ARs induced Ras/Raf/MAPK activation remain largely unknown. In the present study, we examined the molecular mechanism of ISO-evoked activation of Ras/Raf/MAPK pathways as well as PKA activity in cerebral artery of rabbits. We also studied whether the activation of these signaling pathways was altered in cerebral artery, during ISO-induced cardiac hypertrophy compared to heart itself.

METHODS

Cardiac hypertrophy induction

Isoproterenol (300 μg/kg/day) was injected to New Zealand White rabbits (700±50 g body weight) to produce LVH, and rabbits were then studied 7 days after the injection, when documented LVH had developed. Rabbits were anesthetized with an intravenous injection of 50 mg/kg phentobarbital sodium, and hearts and cerebral arteries were then rapidly dissected out. Body weight was measured prior to the removal of heart, ventricle was trimmed free of any other material, then blotted dry and weighted. The ventricular weights (g) were divided by the body weight (kg) for each animal, and an increase of this ratio was taken to represent a cardiac hypertrophy.

Isolation of protein from tissue samples

Snap-frozen hearts were homogenized in a 1:2 volume of ice-cold protein isolation solution (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.4% IGEPAL) using motor and pestle pre-cooled with liquid nitrogen. Cerebral arteries were homogenized in a hand-held micro-tissue grinder in a 1 : 2 volume of ice-cold isolation solution (see above). The homogenate was then centrifuged at 15,000 g for 5 min at 4°C, and the supernatant was removed and stored at −80°C until use for Western blot analysis.

Western blot analysis

Twenty micrograms of protein from each tissue were resolved on 10% SDS-polyacrylamide electrophoresis gels. The proteins on the gels were transferred to Immobilon-P membranes (Millipore, Mass., USA), which were blocked overnight in Tris-buffered saline (20 mM Tris and 150 mM NaCl, pH 8.0) containing 5% non-fat dry milk, and then probed with β-Tubulin (Sigma Chemicals, MO, USA), H-ras (Santa Cruz, California, USA), Raf-1 (Santa Cruz, California, USA), ERK1/2 (Abcam, Cambridge, England), and phospho-ERK1/2 (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1 μg/ml at room temperature for 1 h. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1 : 2000 for 1 h at room temperature. Immunoreactivity was visualized with an ECL Western blotting detection kit (Amersham Biosciences, Buckinghamshire, UK).

Ras activation assay

Ras activity was examined by using a ras activation assay kit (Upstate Biotechnology, Lake Placid, NY). In brief, each tissue extract was incubated with 15 μl of 50% slurry of Raf-1 RBD-agarose for 45 min at 4°C. The samples were then boiled in sample buffer, resolved on 12% SDS gel, transferred to a PDVF membrane, and probed with a monoclonal anti-Ras antibody (1 μg/ml). The blotted membrane was then reacted with horseradish peroxidase-comjugated goat antimouse secondary antibody and detected with the ECL Western blotting detection kit (Amersham Biosciences, Buckinghamshire, UK).

PKA assay

Tissues were homogenized in cold extraction buffer (25 mM Tris-HCl, pH7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and centrifuged at 13,000 g for 15 min. The supernatant was used as a tissue extract for determination of kinase activity. PepTag Assay (Promega, Madison, Wis) was used to measure activity of PKA. The PepTag assay utilizes a brightly colored, florescent peptide substrate that is highly specific to PKA. Phosphorylation by PKA changes the net charge of the substrate from +1 to −1, thereby allowing the phosphorylated and nonphosphorylated forms of the substrate to be separated on an agarose (0.8%) gel. The phosphorylated species migrates toward positive electrode, and the phosphorylated peptide in the band can then be visualized under UV light. Thus, total lysates (20 μg in 10 μl) were incubated with PKA reaction mixture (25 μl) according to the manufacturer's protocol at room temperature for 30 min, and the reactions were stopped by placing the tubes in a boiling water bath. After adding 80% glycerol (1 μl), the samples were loaded onto an agarose gel (0.8% agarose in 50 mM Tris-HCl, pH 8.0), resolved on the agarose gel in the same buffer at 100 V for 15 min, and the bands were visualized under UV light.

Real-time quantitative PCR

Changes in the mRNA expression of c-fos, c-jun, and c-myc were examined by real-time quantitative PCR methods, using an iCycler iQ system (BioRad, Hercules, CA, USA). In brief, total RNA was extracted from heart and cerebral artery of control and cardiac hypertrophy, using the RNA-Bee reagent (TEL-TEST). The rate of accumulation of amplified DNA was measured by continuously monitoring SYBR Green I fluorescence. Melting curves of the reaction products were generated, and fluorescence data were collected at a temperature above the melting temperature of nonspecific products. Specifically, quantitative real-time

Table 1. Sequences of primers for GAPDH, c-fos, c-jun, and c-myc

Gene	Sequence	GenBank Acc. No.
GAPDH	Forward: AAGGCCATCACCATCTTCCA Reverse: GTTCACGCCCATCACAAACA	L23961
c-fos	Forward: TCCGAGGAGCCTTTCAGTCT Reverse: GCTCCCATCTCGGCATAGAA	AB020214
c-jun	Forward: TTCTTGGGGCACAGGAACT Reverse: ACAGAGCATGACCCTGAACCeC	AB020219
c-myc	Forward: TCAGAGAAGCTGGCCTCCTA Reverse: TCGTTGGAGGAGAGCAGAGA	AB019241

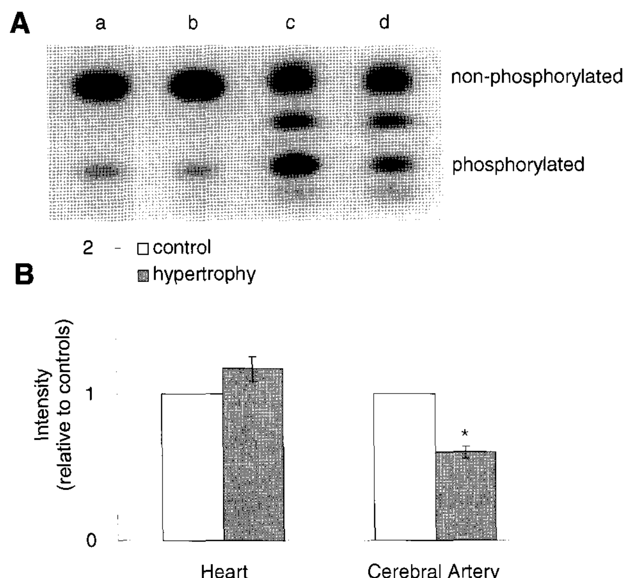

Fig. 1. PKA activity in heart and cerebral artery during isoproterenol-induced cardiac hypertrophy. Tissue extracts were prepared as described under "METHODS," and were subjected to the PepTag nonradioactive PKA assay. (A) Phosphorylated peptide migrated toward the anode (+), while nonphosphorylated peptide migrated toward the cathode (−). a; heart from control, b; heart from cardiac hypertrophy, c; cerebral artery from control, d; cerebral artery from cardiac hypertrophy. (B) The intensity of the phospho-PKA bands was determined by Multi Gauge V2.2, and the column represents mean±S.E., n=4 rabbits. $^*P<0.05$ compared with the control.

RT-PCR on the iCycler iQ was performed with 2 μl of template cDNA per 20 μl reaction. Reactions consisted of iQ supermix (BioRad, Hercules, CA, USA) at a final concentration of 1, 10 nM fluorescein calibration dye, 1 μl of a 1:1,500 dilution of 10,000 SYBR Green I stock, 500 nM each of Gene-specific primers (Table 1), and 2 μl of cDNA. To control pipetting losses, 19 μl each of 20 μl reaction was amplified in a 96-well thin-wall PCR plate (BioRad, Hercules, CA, USA) using the following PCR parameters : 95°C for 5 min followed by 45 cycles of 95°C for 20 sec, 57°C (GAPDH), and 56°C (c-fos, c-myc & c-jun) for 20 sec, and 72°C for 30 sec. Melting-curve analysis was performed immediately following amplification, by increasing the temperature in 0.5°C increments starting at 55°C, for 80 cycles of 7 sec each.

RESULTS

PKA activity

Changes of the PKA activity in control and cardiac hypertrophied groups in heart and cerebral artery are shown in Fig. 1. As seen in the figures, the activity in heart did not changed among control and cardiac hypertrophied groups, however, it decreased in cerebral artery of cardiac hypertrophy, compared to the control.

Western blot analysis of H-ras and Raf-1

Changes of H-ras and Raf-1 protein levels in heart and cerebral artery between control and cardiac hypertrophied groups are shown in Fig. 2. In hypertrophied heart, the

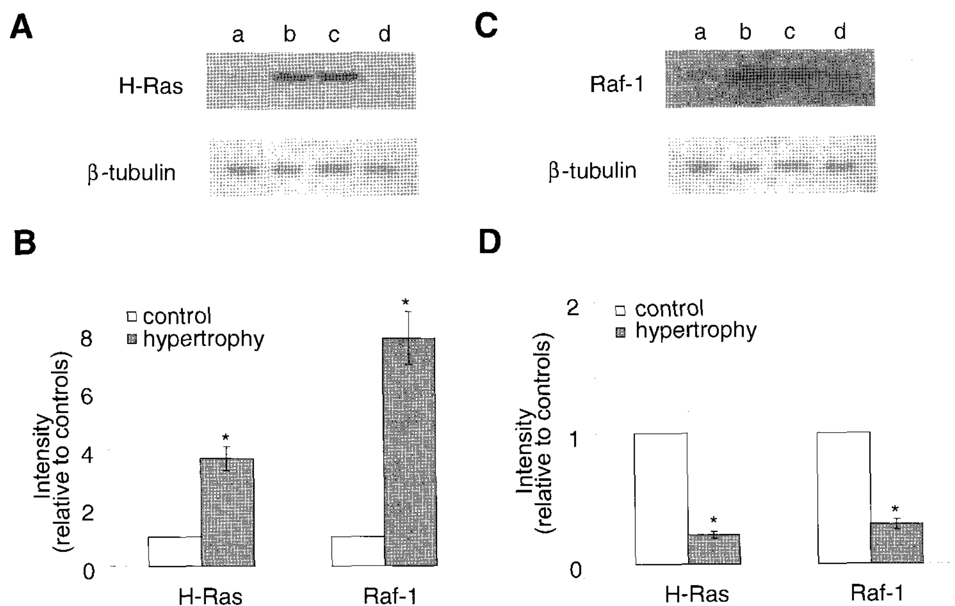

Fig. 2. Comparisons of H-ras and Raf-1 protein expressions in heart and cerebral artery during isoproterenol-induced cardiac hypertrophy. H-ras and Raf-1 activities in whole extracts were measured through Western blot analysis with H-ras (A) and Raf-1 (C) antibodies. The intensity of the H-ras and Raf-1 bands were determined by Multi Gauge V2.2, and the column represents mean±S.E., n=4 rabbits (B, D). a; heart from control, b; heart from cardiac hypertrophy, c; cerebral artery from control, d; cerebral artery from cardiac hypertrophy. $^*P<0.05$ compared with the control.

expression levels of H-ras and Raf-1 protein were significantly increased compared to the control. Interestingly, the expression levels of H-ras and Raf-1 proteins in cerebral artery were significantly decreased during the cardiac hypertrophy compared to the control.

Detection of Ras activity

The amount of Ras-GTP was measured using Raf-1-Ras binding domain, which was conjugated to agarose beads, to pull down active Ras (Fig. 3). Ras activity in heart was also significantly increased during the cardiac hypertrophy, compared with control. In contrast, the ras activity in cerebral artery was significantly decreased during cardiac hypertrophy, compared with control.

Western blot analysis of ERK 1/2 phosphorylation

To determine whether ERK 1/2 in heart and cerebral artery were activated in response to ISO-evoked H-ras and raf-1 activation, we performed Western blot analysis with anti-phosphospecific ERK 1/2 (Fig. 4) There was significant activation of phospho-ERK 1/2 in cerebral artery as well as heart during cardiac hypertrophy, compared to control, in agreement with the results of other investigators (1.9-fold increase in heart, 6.9-fold increase in cerebral artery).

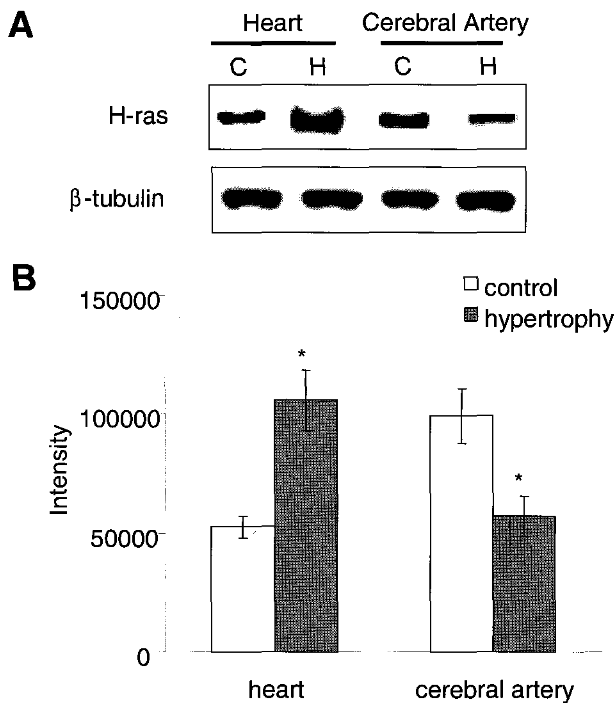

Fig. 3. Ras activation assay in heart and cerebral artery during isoproterenol-induced cardiac hypertrophy. 10 μg of Raf-1 RBD-conjugated agarose was added, and they were then incubated at 4°C for 30 min. The affinity precipitated active Ras was then separated by SDS-PAGE and immunoblotted with an anti-Ras antibody. (A) Representative Western blot of affinity-precipitated active Ras. (B) The intensity of the Ras bands was determined by Multi Gauge V2.2, and the column represents mean±S.E., n=6 rabbits. *$P<0.05$ compared with the control.

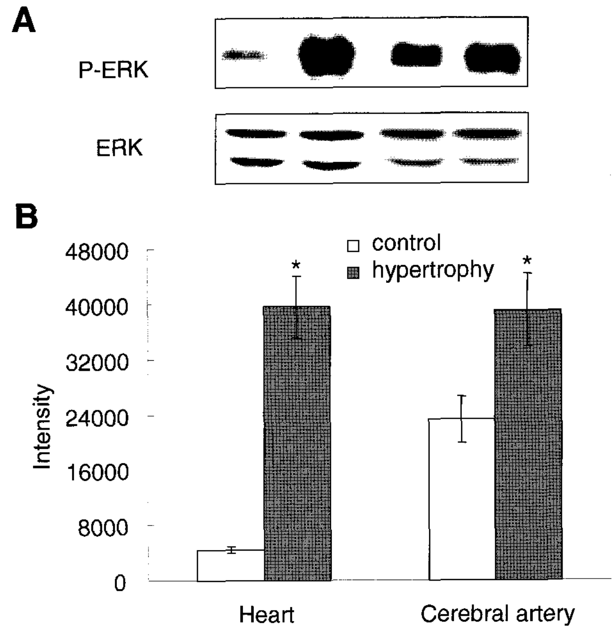

Fig. 4. Activation of ERK 1/2 in heart and cerebral artery during isoproterenol-induced cardiac hypertrophy. (A) ERK 1/2 activity in whole extracts was measured through Western blot analysis with a phosphospecific ERK1/2 antibody. (B) The intensity of the phospho-ERK 1/2 bands were determined by Multi Gauge V2.2, and the column represents mean±S.E., n=4 rabbits. *$P<0.05$ compared with the control.

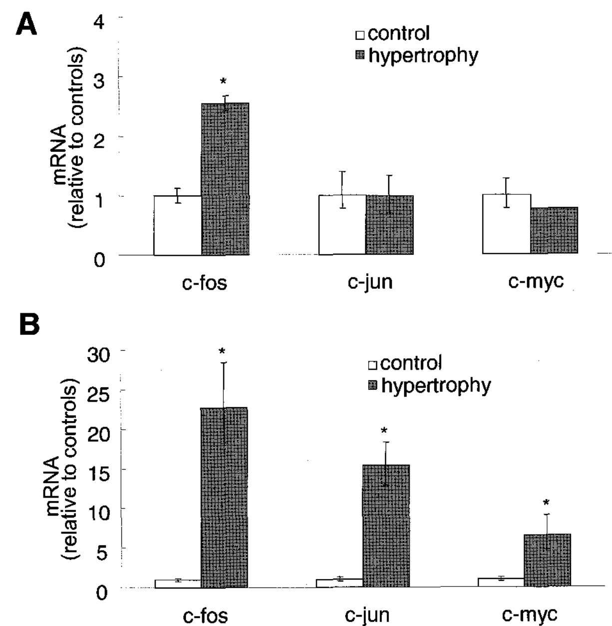

Fig. 5. Quantitative analysis of relative changes in c-fos, c-jun, and c-myc mRNA expression levels by real-time quantitative PCR during isoproterenol-induced cardiac hypertrophy. The samples were analyzed using real-time PCR and the C_T data were imported into Microsoft Excel. The mean fold change in the expression of the target gene at each time point was calculated using $2^{-\Delta\Delta CT}$. Upper panel; summaries of data in the heart, lower panel; summaries of data in the cerebral artery. Column represents mean± S.E., n=6 rabbits. *$P<0.05$ compared with the control.

Real-time PCR analysis for c-fos, c-jun, and c-myc mRNA

The mRNA levels of c-fos, c-jun, and c-myc in heart and cerebral artery are shown in Fig. 5. In heart, the mRNA levels of c-jun and c-myc did not change during cardiac hypertrophy with an exception of c-fos gene: mRNA for c-fos in heart of cardiac hypertrophy increased, compared to control (3.31 ± 2.50, $P<0.05$, Fig. 5A). In cerebral artery, highly marked increases of mRNA for these genes were observed during cardiac hypertrophy, compared to control (27.61 ± 17.94, $P<0.01$, Fig. 5B).

DISCUSSION

β_2AR stimulation usually activates an effector enzyme adenylyl cyclase (AC) through Gs, and the activation of AC increases in cAMP levels, in turn activating PKA. However, activated PKA activity leads to β_2AR desensitization through Gi (Yuan et al, 1994; Moffet et al, 1996), and β_2AR-mediated Gi protein activation can directly inhibit PKA activation in subcellular compartments (Meike et al, 1999). In the case of Gq, phospholipase C (PLC) is the principal effector of Gq-mediated signaling. Activated PLC hydrolyzes PIP_2 into DAG and IP_3, and DAG remains membrane bound and promotes translocation of PKC from cytoplasm to membrane and its subsequent activation. Our present study showed that PKA activity decreased in cerebral artery during ISO-induced cardiac hypertrophy, suggesting that chronic stimulation of β_2AR resulted in PKA desensitization, in contrast with heart itself. The desensitization of Gs/cAMP/PKA pathway may increase coupling of β_2AR to Gi and/or Gq. Many studies have shown that activation of PKA inhibits activation of the Raf-1 kinase/ERK cascade in various cell types such as Rat-1 cells, smooth muscle cells, Chinese hamster ovary cells, COS-7 cells, and adipocytes (Burgering et al, 1993; Cook et al, 1993; Wu et al, 1993; VanRenterghem et al, 1994; D'Angelo et al, 1997; Hecquet et al, 2002). However, in other cell types such PC12 cells, S49 mouse lymphoma cells, and Swiss-3T3 cells, PKA activates ERKs and potentiates the effects of growth factors on differentiation and gene expression (Nagano et al, 1992; Faure et al, 1994; Froedin et al, 1994; Faure et al, 1995; Yao et al, 1995; Yamazaki et al, 1997; Wan et al, 1998). Moreover, it has recently been reported that PKA-induced phosphorylation of β_2AR changes the coupling of the receptor from Gs to Gi and activates ERKs through the Src/Ras pathway in HEK293 cells (Daaka Y et al, 1997). In our present study, it increased the mRNA level of c-fos (not c-jun and c-myc) in heart and considerably increased these genes in cerebral artery during cardiac hypertrophy: the PKA activity and activations of Ras/Raf/ERK cascade as well as c-fos expression in rabbit heart during cardiac hypertrophy are consistent with previous reports. Interestingly, we showed in the present study a novel finding that the decreased PKA activity might have differential effects on Ras and Raf expression in cerebral artery during cardiac hypertrophy: in spite of inactivation of these cascades, p-ERK and c-fos expressions were significantly increased in cerebral artery during cardiac hypertrophy, compared to control. Catecholamine-induced cardiac hypertrophy is related to elevated expression of c-fos, c-jun, and c-myc mRNA in neonatal myocyte cell cultures (Starksen NF et al, 1986; Iwaki K et al, 1990), in the hearts of mice, rats and Syrial hamsters (Barka T et al, 1987; Moalic JM et al, 1989), and in Langendorff rat hearts (Moalic JM et al, 1992). These genes induce gene expression, cellular morphology, and new protein synthesis associated with myocardial cell hypertrophy as well as pathophysiological functional alterations. Interestingly, we observed that the expression of all mRNA in LVH cerebral arteries was significantly increased, while the expression of only *c-fos* mRNA was significantly increased in LVH ventricle. The immediate early gene such as *c-fos* plays pivotal roles in a variety of cellular functions, cell proliferation, and cell growth in many cell types. These functions are affected by down-regulation or up-regulation. Because immediate early genes play an important role in normal cellular function, it is important to maintain optimal expression by various stimuli. To confirm the alteration of related gene expression, we examined upstream pathway, including H-*ras* and *raf*-1 protein levels in left ventricle and cerebral arteries using Western blot analysis, and found that, in contrast to left ventricle, H-*ras* and *raf*-1 protein levels were significantly reduced in LVH cerebral arteries, compared to control. Although not clear at present why there are differences in each signaling steps, protein expression, and response ability to stimulation between heart and cerebral artery, our results suggest possible mechanism underlying cerebrovascular dysfunction during cardiac hypertrophy. Further studies are needed to clarify exact mechanisms behind these different results.

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