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Site specific differential activation of ras/raf/ERK signaling in rabbit isoproterenol-induced left ventricular hypertrophy

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Abstract

To understand better the mediating role of *ras/raf*/ERK signaling pathway in development of cardiac hypertrophy and cerebrovascular events *in vivo*, the molecular mechanism of the pathway in heart and cerebral arteries after isoproterenol (ISO) induced β-adrenergic receptor (βAR) stimulation was examined in rabbit as animal model. Compared with the heart, our findings indicate that ISO-stimulation results in increase in mRNA levels of *ras*, *raf*, and immediate-early genes in the cerebral arteries. Conversely, the *ras* and *raf* protein expression levels (determined by Western blot) and the *ras*-GTP level (determined by pull-down assay) in the heart, but not the cerebral arteries, are markedly elevated after treatment. In addition, despite constant ERK1/2 abundance, phosphorylated ERK (*p*ERK) activity was elevated at both sites with prominent effect on heart following stimulation. Opposing to the PKA and PKC, as upstream contributors in the pathway, which seem to be similarly affected at both sites following ISO-stimulation, the results imply that the downstream candidates *ras* and *raf*, as well as immediate-early genes, have different responses at both sites post-stimulation. The results provide an evidence of site-dependent differential response of *ras*/*raf*/ERK pathway after cardiac hypertrophy-induced by ISO-stimulation. This varied response may account for underlying mechanisms of development of cardiac hypertrophy and cerebrovascular events in heart and cerebral arteries, respectively.

Keywords: Cardiac hypertrophy; β-adrenergic receptor; Cerebrovascular event; *ras*/*raf*/MAPK pathway

1. Introduction

Although the contribution of cardiac hypertrophy as prognostic clues in aggravation of cerebral damage still equivocal, several experimental and clinical data [1–6] suggest that left ventricular hypertrophy (LVH) is associated with cerebral damage even in the absence of clinical symptoms. Cardiac remodeling associated with cardiac hypertrophy involves alterations in specific signaling molecules and their respective downstream pathways in individual myocytes [7–9].

These hypertrophic stimuli affect the necessary alterations in gene expression by receptor-mediated activation of downstream kinase cascades [9,10]. Norepinephrine, as one of neurohormones, has been implicated as mediators of cardiac hypertrophy and failure. In cardiac hypertrophy and failure, the G-dependent pathways activated by this neurotransmitter stimulate mitogen-activated protein kinases (MAPK); the elusive signaling pathway responsible for many cerebral and brain injury [11–13]. Despite of that the common link between cardiac hypertrophy endothelial dysfunction, MAPK activation and cerebrovascular events still speculative [14]. Moreover, there is possible cross-talk between the mitogen-activated protein kinase (MAPK) and protein kinase C (PKCα) during the ISO-induced hypertrophic response [15,16]. In addition, the target of both protein kinase A (PKA) and PKCα is thought to be the *ras*/*raf*/MEK/ERK cascade in a variety of cell types [17–20]. Although this diverse array of βAR stimulated interacting cascades was shown to induce cardiac remodeling, it is not determined yet whether chronic βAR stimulation alters genes or proteins expression in remote vascular bed, such as the cerebral arteries, with possible contribution in development of cerebrovascular events [5,6].

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Repeated or continuous injection of the βAR agonist isoproterenol (ISO) in animals induces marked cardiac hypertrophy within days without systolic hypertension [21–25], making it a useful tool in experimental model. ISO interacts with βARs of cardiomyocyte sarcolemmal membrane, leading to the activation of different intracellular signaling pathways [26,27]. β-Adrenergic stimulation leads to activation of many signals that modulate the expression of proto-oncogenes, such as c-*fos*, c-*jun*, and c-*myc*, with subsequent induction of cardiac hypertrophy [28]. Previously, we provided evidence that prolonged ISO infusion in a rabbit model leads to functional impairment of the coronary arteries, which implies a novel mechanism for reduced coronary reserve during cardiac hypertrophy [29]. Since full understanding of this signaling pathway is the first step for better intervention of βAR-induced cardiac hypertrophy and reliable interpretation of possible involvement in cerebrovascular events. We therefore postulate possible central role of *ras*/*raf*/MEK/ERK cascade in the development of both cardiac hypertrophy and cerebrovascular events after β-adrenergic receptor (βAR) stimulation using βAR agonist isoproterenol (ISO) in rabbit model.

This study addresses questions about the *in situ* local effect of ISO-stimulation, with cardiac hypertrophy end result, on the *ras*/*raf*/MAPK pathway in heart and its remote impact on the cerebral arteries. The contribution of PKA and PKCα in this cascade and the downstream activation of c-*fos*, c-*jun*, and c-*myc,* as early response genes, at both sites were also tested.

2. Materials and methods

2.1. Induction of cardiac hypertrophy in an animal model

White male New Zealand rabbits (0.8–1.0 kg) were used in the study. Cardiac hypertrophy was induced in the tested group by daily isoproterenol administration (10 mg/kg body weight, i.v.) for 7 days [29]. The control group was treated similarly with 0.9% saline (1 ml/kg body weight). At the end of experimental period cardiac hypertrophy was confirmed as previously described [29] in the tested group. Only those proved to exhibit cardiac hypertrophy were considered in tested group. At the end of the experimental period, samples of heart and cerebral arteries were collected. To improve the protein yield of cerebral arteries total cerebral arteries, e.g., anterior, middle and posterior cerebral arteries were included in the study.

2.2. PKA and PKCα assay activities

The Pep Tag assay (Promega, Madison, WI) was used to measure PKA and total PKC activity, according to the manufacturer's instructions. Briefly, PKA and PKC were isolated by tissue homogenization in extraction buffer A (10 mM HEPES [pH 7.9], 10 mM KCL, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.4% IGEPAL) at 4 °C using cold Polytron homogenizer. The homogenate was then centrifuged (14,000×*g*) for 5 min at 4 °C. The recovered supernatant was passed through 1 ml DEAE cellulose column that was pre-equilibrated with extraction buffer. The column was then washed and eluted with 200 mM NaCl. In the reaction buffer, the purified PKA and PKC samples were incubated for 30 min at 37 °C with the specific substrate in the presence of activating solution. The reaction was stopped in a heating block at 95 °C for 10 min. The Pep Tag assay utilizes brightly colored, fluorescent peptides as alternative substrates that are highly specific for the kinases in question: L-R-R-A-S-L-G peptide for PKA and P-L-S-R-T-L-S-V-A-A-K peptide for PKC. Therefore, the activity was measured as a function of the phosphorylation of alternative substrates used by the assay. The PKA or PKC-induced phosphorylation specifically changes the net charge of the fluorescent peptide substrate from +1 to −1. Consequently peptides were separated according to their net charges via electrophoresis in 0.8% agarose horizontal gel at 100 V for 15 min. The phosphorylated species migrated toward the anode whilst non-phosphorylated to the cathode. The ratio between phosphorylated to non-phosphorylated peptides was quantified using a densitometer (Gel Doc 1000 Darkroom; Bio-Rad, Hercules, CA).

To quantify the concentration of heart PKCα soluble (cytoplasm) and particulate (membrane) fractions, the previously frozen tissues were homogenized in double volume ice-cold extraction buffer A, using a liquid nitrogen pre-cooled mortar and pestle. A handheld micro-tissue grinder was ultimately used to homogenize the cerebral arteries. The homogenates were then centrifuged (15,000×*g*) for 5 min at 4 °C. The recovered supernatant was used to determine the soluble PKCα (cytosol) concentration. The resulting pellet was re-suspended by stirring for 1 h at 4 °C in extraction buffer B (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF), and re-centrifuged at 30,000×*g* at 4 °C. The resulting supernatant was used to determine the particulate PKCα (membrane) concentration. Expressed protein in each fraction was determined by Western blot using specific anti-PKCα Ab.

2.3. Real-time quantitative PCR

Total RNA was extracted from the heart and cerebral arteries using RNA-Bee reagent (Tel-Test; Iso-Tex Diagnostics, Houston, TX). Changes in the mRNA levels of H-*ras*, *raf*-1, c-*fos*, c-*jun*, and c-*myc* were examined using quantitative real-time PCR. After treatment with deoxyribonuclease I (Invitrogen, Carlsbad, CA), total RNA was reverse transcribed to cDNA using SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed using the iCycler iQ system (Bio-Rad, Hercules, CA). The primers and probes for each gene were designed using Beacon Designer 2.06 software (Premier Biosoft International, CA). All primers and probes were designed using gene-specific sequences deposited in GenBank. The gene expression data were normalized to mammalian GAPDH [30]. The primer sequences used for each gene are shown in Table 1.

2.4. Western blot analysis

Twenty micrograms of protein from each tissue were immunoblotted using antibodies against H-*ras* (Santa Cruz Biotechnology, Santa Cruz, CA), *raf*-1 (Santa Cruz Biotechnology), PKCα (Upstate Biotechnology, Lake Placid, NY), ERK1/2 (Abcam, Cambridge, UK), phospho-ERK1/2 (Upstate Biotechnology),

Table 1
The forward and reverse primers used for quantitative real-time PCR

Gene	GenBank Accession no.	Primer sequence	
		Forward	Reverse
H-*ras*	X57125	5′-TGAAAGACTCGGACGACGTG-3′	5′-TCGATATAGGGGACGCCGTA-3′
raf-1	NM012639	5′-ATGTCCACATGGTCAGCACC-3′	5′-GGCTGAAGGTGAGGCTGATT-3′
c-*fos*	AB020214	5′-TCCGAGGAGCCTTTCAGTCT-3′	5′-GCTCCCATCTCGGCATAGAA-3′
c-*jun*	AB020219	5′-TTCTTGGGGCACAGGAACT-3′	5′-ACAGAGCATGACCCTGAACC-3′
c-*myc*	AB019241	5′-TCAGAGAAGCTGGCCTCCTA-3′	5′-TCGTTGGAGGAGAGCAGAGA-3′

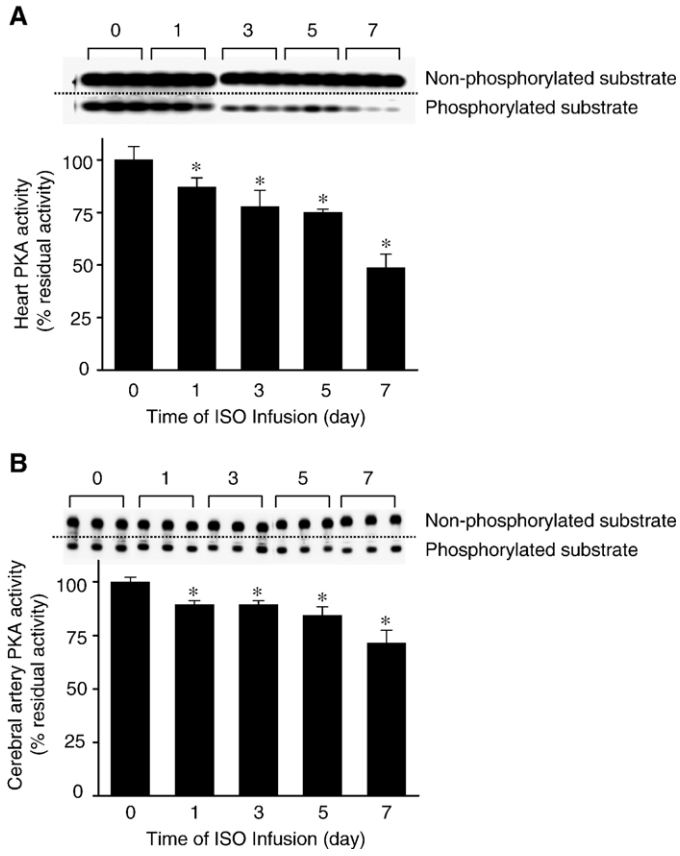

Fig. 1. Effect of prolonged ISO-stimulation on PKA activity in the heart and cerebral arteries. PKA activities of heart (A) and cerebral artery (B) showing the time course of PKA activity following ISO infusion. Upper panels: horizontal electrophoresis of PKA-specific non-phosphorylated and phosphorylated substrate (Pep Tag assay) in 0.8% agarose gel. Densitometric analysis of band intensities at each time point was determined using Multi Gauge V2.2. Lower panels: The percentage ratio of phosphorsubstrate/non-phosphosubstrate at each time point vs. that of the control value at day zero (100% residual activity) was used as function of PKA activity. (Data represent the mean±SE, $n=3$ per group at each time point; *$P<0.05$). Over time, there is a significant decrease in PKA activity in both the heart and cerebral arteries when compared with the control (day 0).

and the housekeeping reference protein β-tubulin (Sigma Chemicals, St. Louis, MO). Immunoreactivity was visualized with an ECL detection kit (Amersham Biosciences, Buckinghamshire, UK).

2.5. ras activity assay

The *ras* activity was analyzed with a *ras* activation (*ras* pull-down) assay kit (Upstate Biotechnology), according to the manufacturer's instructions. In brief, each tissue extract was incubated with 15 μl of a 50% slurry of *raf*-1 binding domain (RBD) conjugated with agarose beads. After extensive wash, the active Ras protein (*ras*-GTP) bound by the RBD was eluted with sample buffer, and subjected to Western blot analysis for *ras*. The *ras*-GTP levels were related to the total *ras* protein levels as determined by anti-H-*ras* immunoblotting in the corresponding total-cell lysates.

2.6. Determination of ERK1/2 activity

To measure the ERK1/2 activity, the concentration of phosphorylated ERK (*p*ERK) from tissue lysates was used as a function of ERK activity. The anti-phospho-p44/42 ERK monoclonal antibody was used in Western blot.

2.7. Statistical analysis

All results are expressed as the mean±SE. Differences between the ISO infusion test group and the control were analyzed using an unpaired *t*-test. The time course data were compared using a repeated measures one-way analysis of variance (ANOVA). Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. PKA and PKCα assays

The time course of PKA activity changes in the heart and cerebral arteries after prolonged ISO infusion are shown in Fig. 1A and B, respectively. The results clearly show that the PKA activity in both the heart and cerebral arteries decreased gradually with time. In contrast, the PKC activity did not change in either the heart or cerebral arteries following ISO-stimulation (Fig. 2A). To evaluate whether any relationship

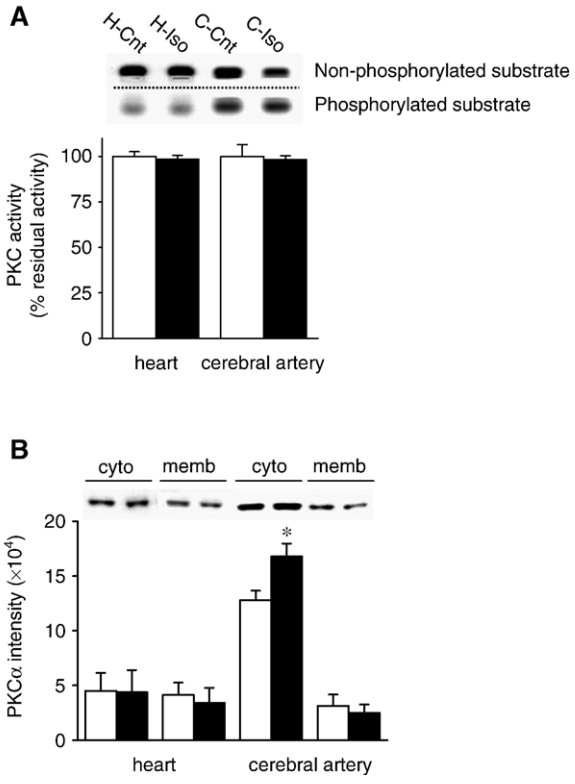

Fig. 2. Effect of prolonged ISO-stimulation on PKC activity (A) and PKCα concentration (B), in heart and cerebral arteries. (A) Horizontal electrophoresis of PKC-specific non-phosphorylated and phosphorylated substrate (Pep Tag assay) in 0.8% agarose gel (upper panel). Densitometric analysis at each time point was determined using Multi Gauge V2.2. Lower panel: The percentage ratio of phosphorsubstrate/non-phosphosubstrate after ISO-stimulation vs. control (100% residual activity) was used as direct function of PKC activity. There was no significant change in PKC activity in either heart or cerebral arteries following ISO-stimulation. (B) Upper panel: PKCα abundance as determined by Western blotting for either the cytosol or membrane fractions in heart and cerebral arteries. Lower panel: presented data as the corresponding band intensity. It is clear that the soluble fraction of the cerebral arteries has a marked elevation in PKCα abundance above the control value, which was already significantly higher compared to all the other fractions. The data represent the mean±SE of five independent experiments; *$P<0.05$.

Table 2
Quantitative analysis of the relative changes in H-*ras* mRNA expression levels using real-time quantitative PCR during isoproterenol-induced cardiac hypertrophy

H-*ras*	Gene	C_T [a]	ΔC_T [b]	$\Delta\Delta C_T$ [c]	Expression relative to control [d]
Heart					
Cardiac Hypertrophy	GAPDH	12.78±0.62			
	H-*ras*	22.65±0.49	10.16±0.12	−0.31±0.19	1.24
Control	GAPDH	12.01±0.31			
	H-*ras*	22.09±0.25	10.01±0.16		
Cerebral arteries					
Cardiac Hypertrophy	GAPDH	16.87±0.78			
	H-*ras*	24.29±0.71	7.43±0.26	−0.95±0.26	1.93
Control	GAPDH	14.82±0.27			
	H-*ras*	23.18±0.25	8.37±0.07		

[a] The average of the C_T data for each sample.
[b] The ΔC_T value is calculated by the subtraction of the GAPDH C_T from each sample C_T.
[c] The $\Delta\Delta C_T$ value is calculated by subtraction of the control ΔC_T from each ISO-treated sample ΔC_T.
[d] The expression relative to control is calculated using the equation $2^{-\Delta\Delta CT}$.

exists between PKC activity and PKCα abundance in the cytosol and membrane fractions, we determined the level of PKCα expression. Interestingly, despite the stability of PKCα expression in the heart cytosol and membrane fractions after ISO-stimulation (Fig. 2B), the cerebral arteries soluble fraction showed a significant increase after stimulation above its basal control value. This basal value, however, was relatively higher than other measured basal values of other fractions in heart and cerebral arteries.

3.2. H-ras and raf-1 assay

Chronically enhanced catecholaminergic stimulation of the myocardium has been linked to MAPK activation. To investigate the upstream MAPK cascade regulatory mechanism, the H-*ras* and *raf*-1 mRNA expression level and their corresponding protein concentration were analyzed using real-time PCR and Western blot, respectively. We performed real-time PCR using GAPDH as a reference housekeeping gene. Using real-time PCR, the H-*ras* and *raf*-1 mRNA expression levels in the cerebral arteries were significantly elevated after stimulation (Tables 2 and 3). Contrastingly, the final outcome of this mRNA elevation was significant decrease in H-*ras* and *raf*-1 protein levels as shown by Western blot (Fig. 3A and B) that confirmed by decreased *ras* activity, demonstrated by the lowered H-*ras*-GTP level (Fig. 3C). This was not the case in the heart after ISO-stimulation, where the expressed H-*ras* and *raf*-1 protein concentrations were markedly elevated (Fig. 3A and B) with increased *ras* activity (Fig. 3C) without an initial mRNA induction (Tables 2 and 3).

3.3. ERK1/2 and phospho-ERK1/2

The classical ERKs, ERK2 or p42 and ERK1 or p44, are positioned downstream from *raf*-1 and MEK1. Together, these comprise an orderly signaling cascade in response to a variety of extracellular stimuli. To examine the possible ISO-evoked H-*ras* and *raf*-1 activation, the concentration and activity of ERK1/2 has to be determined in both heart and cerebral arteries. Therefore, Western blot was performed using ERKs p42 and

Table 3
Quantitative analysis of the relative changes in *raf*-1 mRNA expression levels using real-time quantitative PCR during isoproterenol-induced cardiac hypertrophy

raf-1	Gene	C_T [a]	ΔC_T [b]	$\Delta\Delta C_T$ [c]	Expression relative to control [d]
Heart					
Cardiac Hypertrophy	GAPDH	12.86±0.73			
	raf-1	20.63±0.88	7.77±0.33	−0.45±0.33	1.37
Control	GAPDH	11.54±0.36			
	raf-1	19.75±0.46	8.22±0.16		
Cerebral arteries					
Cardiac Hypertrophy	GAPDH	15.59±0.65			
	raf-1	21.36±0.72	5.76±0.25	−0.92±0.25	1.89
Control	GAPDH	14.27±0.38			
	raf-1	21.00±0.42	6.68±0.07		

[a] The average of the C_T data for each sample.
[b] The ΔC_T value is calculated by the subtraction of the GAPDH C_T from each sample C_T.
[c] The $\Delta\Delta C_T$ value is calculated by subtraction of the control ΔC_T from each ISO-treated sample ΔC_T.
[d] The expression relative to control is calculated using the equation $2^{-\Delta\Delta CT}$.

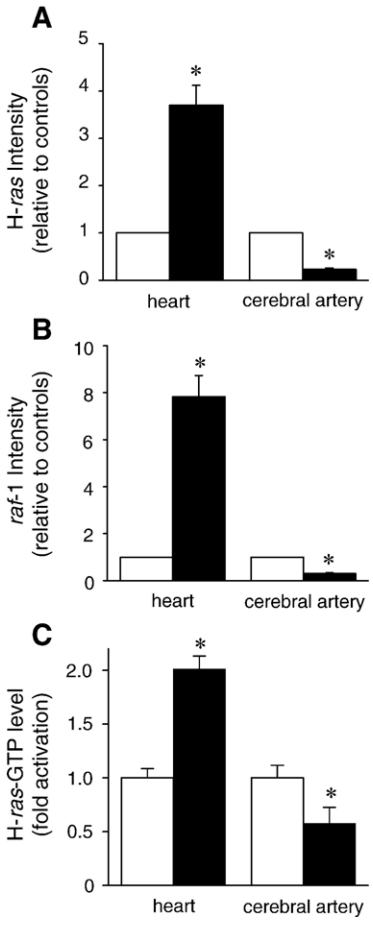

Fig. 3. The protein expression levels of H-*ras* and *raf*-1 (A, B), and H-*ras*-GTP level in ISO-stimulated heart and cerebral arteries (C). A and B represent the Western blot data of H-*ras* and *raf*-1 expressed proteins, respectively. (C) Western blot of affinity-precipitated active Ras. Active Ras chemiluminescent immunoblot data in densitometry units. The intensity was determined using Multi Gauge V2.2. Columns represent the mean±SE, $n=5$ independent experiments. □, control group; ■, ISO group. *$P<0.05$ compared to control.

p44 antibodies for heart and cerebral arteries. To determine the degree of ERK1/2 phosphorylation as a direct function of ERK1/2 activity, immunoblotting was conducted using antibodies against phospho-ERKs p42 and p44. However, ISO-stimulation had little impact on the level of ERK1/2 expression (Fig. 4A and B), the activity of ERK1/2 markedly increased after ISO-stimulation (Fig. 4A and B). Note that there was a major shift in ERK1/2 activity in the heart that increased as a consequence of ISO-stimulation compared to the basal control value before stimulation.

3.4. c-fos, c-jun, and c-myc mRNA

The above actions stimulate, at least in part, the translocation of activated MAPK into the nucleus, where it phosphorylates and thereby activates nuclear transcription factors and consequently mediates transcription of the immediate-early response oncogenes c-*fos* and c-*jun*. The mRNA levels of c-*fos*, c-*jun*, and c-*myc* in heart and cerebral arteries are shown in Tables 4, 5, and 6, respectively. However there is no change in mRNA level of c-*jun* or c-*myc*, the level of c-*fos* mRNA was increased after prolonged ISO-stimulation in heart. In contrast, marked increase in the mRNA levels of c-*fos* and c-*myc* mRNA genes was observed in the cerebral arteries after prolonged ISO-stimulation.

4. Discussion

Although the ISO-stimulated activation of the *ras*/*raf*/ERKs pathway has been previously investigated [27,31], its detailed role in development of cardiac hypertrophy with possible *in vivo* contribution to cerebrovascular events remains unclear. This work was done to understand better the effect of long term ISO-stimulation with cardiac hypertrophy end result on *ras*/*raf*/ERKs signaling pathway in both heart and cerebral arteries.

As with many G_s protein-coupled receptors, long term ISO-stimulation of βAR receptor results in PKA-dependent receptor phosphorylation with consequent desensitization after previous activation [32]. Therefore there is observed decrease, rather than increase, in PKA activity in both heart and cerebral arteries after several days of receptor activation. Previous peaking, however, was possibly too short to be recognized one day of ISO-stimulation. It is not clear whether this decrease in PKA activity; as innate inhibitor of *Ras* /*raf* pathway [33], indirectly contributes to their activation. Moreover, recent finding provides evidence that PKA activation pathway is unlikely contribute to *ras/raf* activation pathway [34]. *Ras* and *raf* are two proto-oncogenes that activated during hypertrophy [27,31,35] and involve in growth modulation, cell proliferation, and transformation. The increase in their protein expression and activity (*ras*-GTP) in heart rather than cerebral arteries confirms their site specific differential response to ISO stimulation. Their important roles in organization of cytoskeletal proteins [36] and modulation of actin cytoskeleton in many cell types [37] via Rho-GTPase activation afford an explanation of their elevated levels in heart; the place where cardiac hypertrophy occurs.

Another major finding of this study is that the mismatch on different molecular levels, e.g. the mRNA and protein level between ras and raf at both sites after ISO-stimulation. Since RT-PCR technique was used to estimate the mRNA copy numbers on transcriptional base regardless to translational, post-translational or “translation on demand” [38] regulations level, this discrepancy between constitutive mRNA expression and its corresponding final protein outcome is not unlikely to be found [39,40]. On the other hand, prenylation and subsequent methylation are essential for posttranslational modification events affecting many signaling regulatory proteins, therefore *ras*, like many guanine nucleotide-binding proteins, is prenylated and undergoes methylation [41,42]. Prenylated methylated protein methyl esterase (PMPMEase) readily hydrolyzes its protein substrate esters as a reversible and possibly regulatory step. Since PMPMEase is recently isolated from the rat brain [43], its presence can account for accelerated hydrolysis of preformed, prenylated active *ras* in cerebral arteries with consequent cellular autodegradation. Moreover, the role of GTPase activating proteins that terminates *ras* signaling by stimulating intrinsic GTPase activity [44] cannot

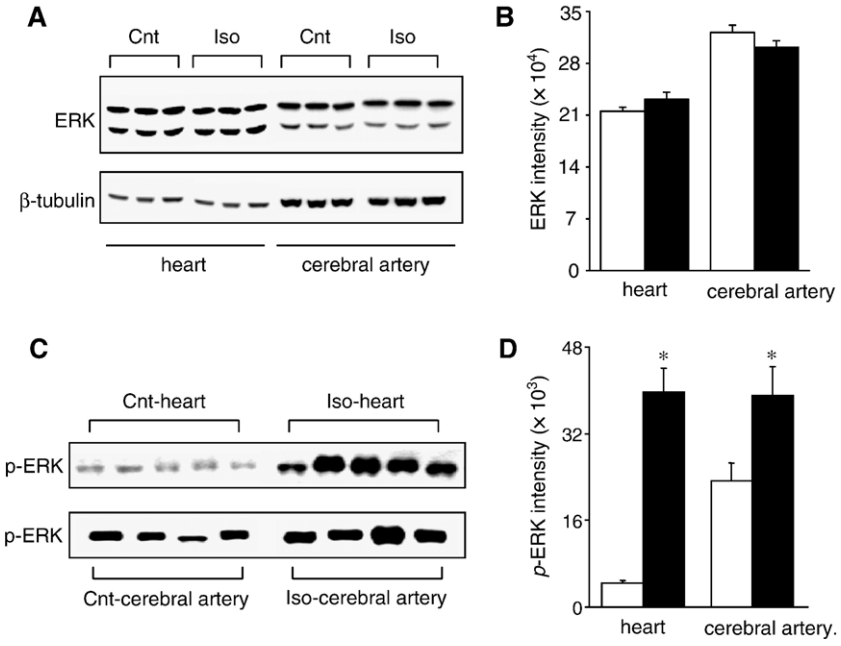

Fig. 4. Abundance and activity of ERK1/2 in the heart and cerebral arteries during isoproterenol-induced cardiac hypertrophy. (A) Whole ERK1/2 abundance in whole extracts was measured using Western blot analysis with anti-ERK1/2 antibody using β-tubulin as house keeping reference protein. (B) The intensity of the ERK1/2 bands was determined using Multi Gauge V2.2. The columns represent the mean±SE, $n=5$ independent experiments. □, control groups; ■, hypertrophic groups. *$P<0.05$ compared to control. (C) Phospho-ERK1/2 (as a function of activity) in whole extracts was measured using Western blot analysis with phospho-specific ERK1/2 antibody. (D) The intensity of the phospho-ERK1/2 bands was determined using Multi Gauge V2.2. The columns represent the mean±SE, $n=5$ independent experiments. □, control groups; ■, hypertrophic groups. *$P<0.05$ compared to control.

be ruled out in cerebral arteries. If this is the case it can afford a reasonable explanation of the decreased activity and concentration of *ras*, despite of previous high mRNA expression in cerebral arteries. These assumptions, however, remain to be resolved.

It was proved that the effect of β-adrenoceptor activation on ERKs activity is generally stimulatory [45,46]. In agreement with that ERKs activation is the final stimulatory outcome observed in both heart and cerebral arteries. *Ras*-GTP loading translocates *raf* family mitogen-activated protein kinase (MAPK) kinase kinases to the membrane, and initiates the activation of *raf*, thereby activating the extracellular signal-regulated kinase 1/2 (ERK1/2) cascade. The increased concentration of *ras* and *raf* as well as *ras* activity observed in heart assist their consequent downstream activation of signal transducing ERKs protein in heart rather than cerebral arteries. Therefore, the increase in the phosphorylation rate of ERK, was much higher in the heart, where the regulatory role of the cytoskeleton is prominent, than in the cerebral arteries, which may be more vulnerable to mitogenic effects.

Nevertheless, the higher basal level of *p*EKR in cerebral arteries evidently exposes the constitutive role of *p*EKR in

Table 4
Quantitative analysis of the relative changes in c-*fos* mRNA expression levels using real-time quantitative PCR during isoproterenol-induced cardiac hypertrophy

c-*fos*	Gene	C_T [a]	ΔC_T [b]	$\Delta\Delta C_T$ [c]	Expression relative to control [d]
Heart					
Cardiac hypertrophy	GAPDH	14.18±1.34			
	c-*fos*	23.10±1.48	8.92±0.33	−1.35±0.33	2.55
Control	GAPDH	13.87±1.17			
	c-*fos*	24.16±1.02	10.27±0.35		
Cerebral arteries					
Cardiac hypertrophy	GAPDH	20.76±1.85			
	c-*fos*	27.65±1.95	11.40±0.28	−4.51±0.32	22.76
Control	GAPDH	13.83±0.39			
	c-*fos*	25.23±0.54	6.89±0.32		

[a] The average of the C_T data for each sample.
[b] The ΔC_T value is calculated by the subtraction of the GAPDH C_T from each sample C_T.
[c] The $\Delta\Delta C_T$ value is calculated by subtraction of the control ΔC_T from each ISO-treated sample ΔC_T.
[d] The expression relative to control is calculated using the equation $2^{-\Delta\Delta CT}$.

Table 5
Quantitative analysis of the relative changes in c-*jun* mRNA expression levels using real-time quantitative PCR during isoproterenol-induced cardiac hypertrophy

c-*jun*	Gene	C_T [a]	ΔC_T [b]	$\Delta\Delta C_T$ [c]	Expression relative to control [d]
Heart					
Cardiac hypertrophy	GAPDH	12.15±0.15			
	c-*jun*	22.96±0.32	10.78±0.37	0.03±0.23	0.98
Control	GAPDH	13.66±1.09			
	c-*jun*	24.44±1.23	10.81±0.23		
Cerebral arteries					
Cardiac hypertrophy	GAPDH	15.38±0.68			
	c-*jun*	22.88±0.62	11.44±0.34	−0.63±0.23	1.55
Control	GAPDH	14.35±0.30			
	c-*jun*	25.79±0.58	10.81±0.23		

[a] The average of the C_T data for each sample.
[b] The ΔC_T value is calculated by the subtraction of the GAPDH C_T from each sample C_T.
[c] The $\Delta\Delta C_T$ value is calculated by subtraction of the control ΔC_T from each ISO-treated sample ΔC_T.
[d] The expression relative to control is calculated using the equation $2^{-\Delta\Delta CT}$.

cerebral arteries with less sensitivity to ISO-stimulation when compared to that of heart. Moreover, the data suggest that the elevated *p*EKR level in cerebral arteries is more likely a consequence of an ultimate pathway [47] rather than the conventional Ras/Raf-1 activation that proved to be down regulated. Previous data showed that PKCα is the major contributor among PKC isoforms that involved in hypertrophic signaling in cardiomyocytes via the ERK1/2-dependent signaling pathway [48,49]. Regardless to ISO-stimulation, it is of interest to notice that the basal control value of cytoplasmic PKCα, as a possible soluble reserve of active membrane-bounded PKCα, is comparatively higher in cerebral arteries than that of heart. This may be attributed to tissue specificity of cerebral arteries where the regulatory control of smooth muscle contraction takes an advantage. Since translocation of the soluble PKCα into membrane is the first step for its activation, therefore the elevated level of soluble PKCα above the basal level after ISO-stimulation has no impact on final activity of PKCα at both sites. Further study is needed, however, to disclose the cellular benefit behind such adaptation in cerebral arteries.

Immediate-early genes (IEGs; also known as primary (early) response genes) constitute a superfamily of inducible proto-oncogenes, many of which encode nuclear transcription factors that activate the promoters of target genes involved in regulating neuronal function, adaptive processes, or apoptotic cell death [50–53]. As final outcome of increased phosphorylation rate of ERK after βAR stimulation, there is an increase in mRNA levels of *fos* and c-*myc* (as early response genes) in the cerebral arteries and c-*fos* in heart. The increased level of expression of early response genes in cerebral arteries evidently supports their potential phenotypic alteration following ISO-stimulation. The consequent development of cerebrovascular events, however, has not proved in our results.

Although it is known that myocardial hypertrophy is not a prerequisite for changes in IEGs expression *in vivo* [54], it is clear that there is a participation of complex regulatory machinery that controls the overall end result in the form of the activation of IEGs. Considering the stimulatory effect of β-Adrenoceptor on PI3 kinase activity, which is G_i-dependent and generally cell growth stimulatory [55], it is not surprising that

Table 6
Quantitative analysis of the relative changes in c-*myc* mRNA expression levels using real-time quantitative PCR during isoproterenol-induced cardiac hypertrophy

c-*myc*	Gene	C_T [a]	ΔC_T [b]	$\Delta\Delta C_T$ [c]	Expression relative to control [d]
Heart					
Cardiac hypertrophy	GAPDH	12.03±0.15			
	c-*myc*	24.40±0.20	12.37±0.29	0.4±0.29	0.76
Control	GAPDH	12.09±0.34			
	c-*myc*	24.06±0.47	11.97±0.45		
Cerebral artery					
Cardiac hypertrophy	GAPDH	15.06±0.35			
	c-*myc*	25.74±0.53	13.37±0.32	−2.7±0.48	6.50
Control	GAPDH	13.97±0.28			
	c-*myc*	27.35±0.38	10.68±0.48		

[a] The average of the C_T data for each sample.
[b] The ΔC_T value is calculated by the subtraction of the GAPDH C_T from each sample C_T.
[c] The $\Delta\Delta C_T$ value is calculated by subtraction of the control ΔC_T from each ISO-treated sample ΔC_T.
[d] The expression relative to control is calculated using the equation $2^{-\Delta\Delta CT}$.

PKCα in a parallel pathway to PI3Kγ [56] converge to contribute to *fos* and c-*myc* genes activation.

In conclusion the increased concentration and activity of *ras* and *raf* in heart with consequent downstream *p*ERK elevation reflect the importance of *ras*/*raf*/ERK signaling pathway during ISO-stimulation with cardiac hypertrophy end-result. The decrease in their concentration in cerebral arteries, however, clearly disproves their local impact on ERK activation with possible contribution of ultimate regulators. These data give the first insight about site-specific diversity of *ras*/*raf*/ERK signaling pathway as consequence of ISO-stimulation. Increased expression of IEGs in cerebral arteries raises the speculation of ISO-stimulation involvement in cerebrovascular events that warrants further investigation to elucidate the exact mechanism. The mismatch noticed between mRNA transcription level and its final protein outcome suggests post-translation multidisciplinary fine tuning that leads to this discrepancy. From the study attention should be taken to the local final outcome rather than remote intermediate results for better addressing pathophysiological interpretation on molecular basis.

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