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Biochimica et Biophysica Acta 1502 (2000) 471–480

BIOCHIMICA ET BIOPHYSICA ACTA

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Acute changes of myocardial creatine kinase gene expression under β-adrenergic stimulation

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Received 11 January 2000; received in revised form 27 July 2000; accepted 9 August 2000

Abstract

Creatine kinase (CK) plays a crucial role in myocardial energy metabolism. Alterations in CK gene expression are found in hypertrophied and failing heart, but the mechanisms behind these changes are unclear. This study tests the hypothesis that increased adrenergic stimulation, which is observed in heart failure, induces changes of myocardial CK-activity, -isoenzyme distribution and -gene expression that are characteristic of the failing and hypertrophied heart. Isolated rat hearts were perfused (constant pressure of 80 mmHg) with red cell suspensions. Following a 20-min warm-up period, perfusion for 3 h with 10^{-8} M (iso 3 h) or without (control 3 h) isoproterenol was started or experiments were immediately terminated (control 0 h). Left ventricular tissue was analyzed for total CK-activity, CK-isoenzyme distribution and, by use of quantitative RT–PCR, for B-CK, M-CK, mito-CK and GAPDH- (as internal standard) mRNA. After β-adrenergic stimulation (iso 3 h) but not after control perfusion (control 3 h) a roughly threefold increase in B-CK mRNA levels and a decrease in M-CK mRNA levels by 18% was found. There were no significant differences among the three groups in total CK-activity and in distribution of CK-MM, CK-BB, CK-MB and mito-CK. Thus, β-adrenergic stimulation induces a switch in CK gene expression from M-CK to B-CK, which is characteristic for the hypertrophied and failing heart. This may be interpreted as an adaptive mechanism making energy transduction via CK more efficient at times of increased metabolic demand.

Keywords: Creatine kinase; Gene expression; β-Adrenergic stimulation; Isoproterenol; Isolated heart

1. Introduction

Creatine kinase (CK, ATP; creatine *N*-phosphotransferase, EC 2.7.3.2), which catalyzes the phosphoryl exchange between ADP and creatine, is highly expressed in the myocardium [1]. It plays a crucial role in myocardial energy metabolism and in energy transduction from the sites of ATP production to the sites of ATP utilization [2]. The CK gene family codes for four subunit CK isoforms, that are expressed in a tissue-specific manner [2]. There are two cytosolic forms, M-CK and B-CK, and two mitochondrial forms [3–6]. M-CK and B-CK subunits combine to form the dimeric cytosolic isoenzymes, MM-, MB- and BB-CK [2]. A developmental transition from BB- via MB- to the MM-CK is found during muscle cell differentiation [3]. The mitochon-

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0925-4439/00/$ – see front matter
PII: S0925-4439(00)00070-3

drial isoforms occur mostly as octamers. Two distinct mitochondrial CK isoenzymes exist, ubiquitous and a sarcomeric mito-CK [7,8], but in heart all mito-CK activity arises from the sarcomeric isoform.

Changes in myocardial CK isoenzyme distribution and/or CK gene expression have been reported for left ventricular hypertrophy and/or failure induced by pressure overload [9–12], volume overload [13], in heart failure due to rapid pacing [14], LAD occlusion [15,16] or mitral regurgitation [17]. The mechanisms by which hemodynamic alterations in these animal models evoke changes in CK gene expression and isoenzyme distribution are not completely understood.

Left ventricular hypertrophy and failure are closely related to adrenergic stimulation. General activation of the sympathetic nervous system occurs in heart failure [18], resulting in increased plasma concentrations of norepinephrine [19]. A close correlation between norepinephrine levels and mortality has been reported. Increased release of norepinephrine [20] and reduced myocardial norepinephrine stores [21] are both found in patients with heart failure. Furthermore, β- as well as α-adrenergic stimulation cause hypertrophy and induction of proto-oncogenes in myocyte cell culture [22,23], in isolated hearts [24] and in the in vivo heart of several animal species [25,26].

The present study examines the hypothesis that acute β-adrenergic stimulation, which is known to induce proto-oncogene induction and subsequent hypertrophy, also mediates changes in CK isoenzyme distribution. Using an isolated erythrocyte suspension perfused Langendorff rat heart model, the effects of acute β-adrenergic stimulation on CK isoenzyme distribution and gene expression can be studied without influence of any other neurohumoral mechanisms that may be activated in a whole animal model.

2. Materials and methods

2.1. Isolated red blood cell perfused rat heart

2.1.1. Animals and isolated heart preparation

Male Wistar rats (ca. 300 g) were obtained from Charles River Laboratories (Kißlegg, Germany). Rats were anesthetized by intraperitoneal injection of 10 mg/100 g body weight pentobarbital sodium. One hundred IU heparin were injected intravenously. The thorax was rapidly opened. The heart was excised and placed into ice-cold buffer. The aorta was dissected and the heart was subjected to retrograde perfusion with erythrocyte suspension via the aortic cannula of the perfusion apparatus. The perfusion pressure was set to 80 mmHg. Less than 30 s elapsed between opening of the thorax and initiation of perfusion. A small vent was inserted into the left ventricular apex to drain the flow from Thebesian veins. A second vent was placed into the right ventricle via the pulmonary artery to drain coronary circulation. A water-filled latex balloon was inserted into the left ventricle through an incision in the left atrial appendage, via the mitral valve and secured by a ligature. The balloon was connected to a pressure transducer (Statham P23Db Gould Instruments, Glen Burnie, MD) via a small bore polyethylene tubing for continuous recording of left ventricular pressure and heart rate. Coronary flow, heart rate and left ventricular pressure were continuously recorded. The heart was placed in a water-jacketed constant temperature chamber and maintained at 37°C. The experiments were started after a 20-min stabilization period.

2.1.2. Continuous pressure perfusion system

The perfusion system consisted of a water-jacketed 'venous reservoir', a variable flow pump, an oxygenator, a water-jacketed 'arterial reservoir' and a 20-μm pore size filter. The oxygenator consisted of semipermeable tubes which allow gas equilibration. The perfusate was equilibrated with a gas mixture of N_2, O_2 and CO_2 (77%, 20% and 3%, respectively). The gases were adjusted to achieve perfusate pO_2 of 120–140 mmHg and perfusate pH between 7.37 and 7.43. Blood gas analysis was performed every 15 min (ABL 30, Radiometer, Copenhagen, Denmark). Perfusion pressure was adjusted by varying the height of the 'arterial reservoir' and monitored via a side arm of the aortic cannula with a pressure transducer (Statham P23Db; Gould Instruments).

2.1.3. Preparation and storage of bovine erythrocytes

Fresh whole cow blood was obtained from a local slaughterhouse. Blood was collected from the jugular veins. Immediately after collection 0.25 vol. of a sta-

bilization solution containing sodium citrate 50 mM, citric acid 23 mM and glucose 74 mM as well as heparin 7500 IU/l and tobramycin 20 mg/l were added to prevent coagulation, hemolysis and bacterial growth. This mixture was transported on ice and was centrifuged at 4°C and 2500×*g* for 20 min. The supernatant consisting of plasma and buffy coat was discarded. The packed cells were washed twice with 1 vol. of 10 mM phosphate-buffered isotonic sodium chloride solution and twice with 1 vol. of Krebs–Henseleit buffer. Finally, erythrocytes were resuspended in 1 vol. storage solution containing sodium chloride 151 mM, glucose 45 mM, mannitol 29 mM and adenine 0.25 mM. Ten mg/l tobramycin was added. This suspension was stored at 4°C for no longer than 6 days.

2.1.4. Perfusate preparation

The perfusate was prepared daily. The suspension of erythrocytes and storage solution was centrifuged for 10 min, 2500×*g* at 4°C. The packed cells were washed with Krebs–Henseleit buffer and then resuspended with a modified Krebs–Henseleit buffer containing additional 40 g/l fatty acid-free bovine serum albumin, glucose 5.5 mM, pyruvate 2.2 mM, and tobramycin 20 mg/l, which was passed through a 0.45-μm Millipore filter. The hematocrit was adjusted to 40% and the concentration of free calcium to 1.1 mM.

2.2. Total creatine kinase activity and creatine kinase isoenzyme distribution

Intact left ventricular tissue (5–10 mg) was homogenized in 0.1 M phosphate buffer (pH 7.4) containing 1 mM EGTA and 1 mM β-mercaptoethanol. Aliquots for measurement of protein content [27] were taken. 0.1% Triton X was added. Samples were kept on ice. Total CK activity was measured using an Ultraspec III spectrophotometer (Pharmacia Biosystems, Freiburg, Germany) as previously described [16]. CK isoenzymes were separated using the Rapid Electrophoresis System (REP, Helena Diagnostika) as a separation unit. The REP CK Isoforms kit was used for agarose gel and incubation solution. The Electrophoresis Data Center (EDC, Helena Diagnostika) automatically quantified the isoenzyme bands. This way the relative activities of the isoenzymes were determined simultaneously. Since the specific activities of the isoenzymes are similar, relative activities correspond to relative enzyme concentrations.

2.3. Quantification of creatine kinase mRNA (reverse transcription–polymerase chain reaction)

Quantification of mRNA was performed by use of the methods which were previously described [16].

2.3.1. RNA preparation

RNA from frozen left ventricular tissue was prepared using the TRIZOL Reagent (GibcoBRL Life Technologies, Eggenstein, Germany) according to the single-step RNA isolation method [28]. In brief, frozen left ventricular tissue was placed in 1 ml of ice-cold TRIZOL reagent and homogenized with a polytron device for 30 s. After 5 min incubation at room temperature 0.2 ml chloroform was added. The samples were centrifuged (15 min, 4°C, 12 000×*g*) after 3 min incubation at room temperature. The aqueous phase was transferred into a fresh tube. RNA precipitation was performed with 1.5 ml isopropyl alcohol. After centrifugation (10 min, 4°C, 12 000×*g*), the RNA was washed with 2 ml 75% ethanol. The RNA pellet (after centrifugation 5 min, room temperature, 7500×*g*) was then dissolved in 20 μl water. The RNA concentration was determined spectrophotometrically, and the purity and quality of RNA was checked by measuring the ratio of the absorption at 260 and 280 nm, which was 1.8 to 2.0 in all cases, by determining the absorption spectra, and by RNA electrophoresis using a denaturating 3-(*N*-morpholino)propanesulfonic acid (MOPS) gel. The 260/280 nm ratio was 1.8–2.0 and typical RNA spectra were found in all cases. The MOPS gel demonstrated the characteristic three RNA bands.

2.3.2. Reverse transcription (RT)

One μg of total RNA was used for reverse transcription into cDNA. Twenty pmol random hexamers (Boehringer, Mannheim, Germany) were added, the mixture was heated to 70°C for 10 min and chilled one ice. The final reaction volume of 20 μl contained 0.5 mM dNTP, 0.01 M DTT, 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 U RNAsin

(Promega, Mannheim, Germany) and 100 U Superscript II reverse transcriptase (GibcoBRL Life Technologies), pH 8.3. Reverse transcription was performed at 42°C for 60 min. Reactions containing no reverse transcriptase were done as negative controls.

2.3.3. Quantitative PCR reactions

Forward and reverse oligonucleotide primer pairs were synthesized to match the sequence of the rat M-, B-, sarcomeric CK [8,29] and glyceraldehyde phosphate dehydrogenase (GAPDH) as a standard [30]. The primer sequences and positions as well as the expected product sizes and the annealing temperatures are summarized in Table 1.

The transcript obtained from 50 ng RNA (i.e., 1/20 of the reverse transcribed cDNA) were used for polymerase chain reaction (PCR) reactions. The 50 μl reaction volume contained 0.8 μM of the respective oligonucleotide primers, 2 U *Taq* DNA polymerase (Boehringer), 300 μM dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$. 0.3 μCi α-[^{32}P]dCTP (Amersham Pharmacia Biotech, Buckinghamshire, UK) were present for quantification of PCR products. A GeneAmp 2400 thermal cycler (Perkin–Elmer, Weiterstadt, Germany) was used. Denaturation was done at 94°C for 15 s (5 min first cycle). The following 15-s annealing step was carried out at the temperature given in Table 1. The temperature of the extension step (30–60 s, 10 min last cycle) was 72°C. All samples were assayed for each gene using a single master reaction mixture. The reaction products were separated by vertical 5% polyacrylamide gel electrophoresis. The ^{32}P content of the appropriate PCR products was determined with a Molecular Dynamics phosphoimager.

RNA of all samples was reverse transcribed three times. PCR reactions for each gene were done with the transcript of each of the three transcriptions. This way, each tissue sample was assayed for the expression of each gene three times.

In all experiments, a template-free negative control, RNA template not subjected to reverse transcription and known amounts of plasmids containing the respective sequences were amplified to check the accuracy and efficiency of the PCR. The number of cycles required to obtain a reproducible amplification in the exponential range was determined for each PCR product individually, as described earlier [31]. The cycle number, indicated in Table 1, was selected to remain well within the exponential amplification range. For each PCR product, the amplifications occurred with similar efficiency and the products were well within the linear range of curves with standard amounts of tissue RNA as detailed by Engelhardt et al. [32]. Each PCR was simultaneously performed in all samples of all experimental groups ($n = 21$). The activity of the PCR product of each sample was counted using a phosphoimager. The activity of the sample with the highest activity was set to 1. The activity of the remaining samples was given as fraction of 1 (relative activity). The relative activities of three PCRs per gene (B-, M-, mito-CK and GAPDH) were averaged. This way the averaged relative activity ranges between 0 and 1, independent from the total mRNA content. The averaged relative activities of the B-, M- and mito-CK-mRNA were

Table 1
Sequences of oligonucleotide primers and conditions used in quantitative PCR reactions

mRNA	Orientation	Primer sequence	Position in coding sequence	Distance (bp)	Cycle number	Annealing temperature (°C)
B-CK	Forward	5′-GCCGCCATGCCCTTCTC-3′	89	807	28	58
	Reverse	5′-CTTAGACTTGAAGAGAGTTTC-3′	896			
M-CK	Forward	5′-CCACAACAAGTTCAAGCTGAA-3′	113	773	22	60
	Reverse	5′-AAGATCTCCTCAATCTTCTGC-3′	886			
Mito-CK (sarcomeric)	Forward	5′-CTGTGGGCATGGTGGCTG-3′	514	699	22	60
	Reverse	5′-TGGCTGAGCTTTGGGATCC-3′	1213			
GAPDH	Forward	5′-GCTGCCTTCTCTTGTGACAAA-3′	55	531	21	60
	Reverse	5′-CACGCCACAGCTTTCCAGA-3′	586			

related to the averaged relative activity of GAPDH-mRNA. This way the mRNA levels of all CK-isoenzymes related to GAPDH mRNA represent a value which ranges between 0 and 1 and do not reflect absolute amounts of mRNA. It allows comparison of mRNA content of each CK isoenzyme between the experimental groups. It does not allow comparison of total mRNA content between the different isoenzymes.

2.4. Experimental protocol

After a 20-min warm-up and stabilization period, isoproterenol (final perfusate concentration 10^{-8} M, $n=8$) or vehicle solution (control, $n=7$) were infused over a period of 3 h. Isoproterenol was diluted in Krebs–Henseleit buffer in a concentration of 1.01×10^{-6} M. This solution was infused into the perfusion line immediately proximal of the aortic cannula. The infusion rate of the isoproterenol solution was adjusted every 15 s. automatically according to the coronary flow and set to 1/100 of the measured coronary flow. This way the perfusate isoproterenol concentration was 10^{-8} M. Krebs–Henseleit buffer without isoproterenol was used for perfusion in the control group. Left ventricular tissue was washed in ice-cold saline solution and rapidly frozen in liquid nitrogen for analysis after the 3 h recording time (isoproterenol 3 h group and control 3 h group) or for measurement of baseline values immediately after the warm-up period at $t=0$ (control 0 h group, $n=6$).

2.5. Statistical analysis

The data are given as mean ± S.E.M. Comparison between experimental groups was performed by Student's t-test with Bonferroni correction.

3. Results

3.1. Mechanical function

Fig. 1 presents the time course of left ventricular pressures, heart rate and coronary flow. No significant differences were observed among the three groups for these parameters at $t=0$. Left ventricular

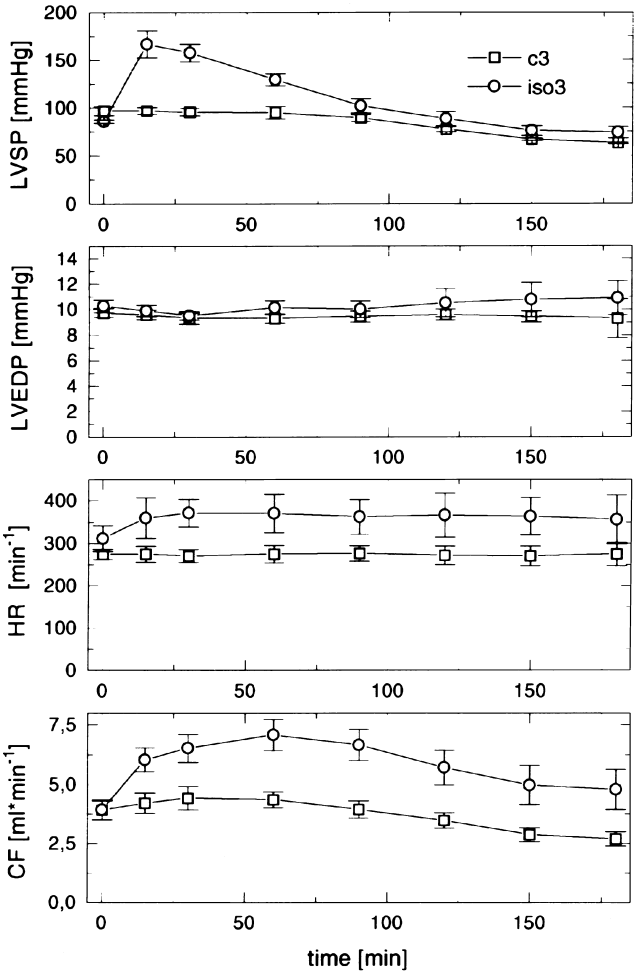

Fig. 1. Mechanical function. Time course of left ventricular systolic and end-diastolic pressure (LVSP, LVEDP), of coronary flow (CF) and of heart rate (HR) of the control $t=3$ h group (control 3 h) and of the isoproterenol group (iso 3 h). The corresponding mean values of the control at $t=0$ averaged 95.8 ± 6.5 mmHg, 10.1 ± 0.45 mmHg, 3.4 ± 0.4 ml min^{-1} and 297.5 ± 23.6 min^{-1}.

end-diastolic pressure (about 10 mmHg) was constant in the control group during the whole 3 h recording time, indicating that no impairment of diastolic relaxation occurred. Also, heart rate remained constant at about 275 min^{-1} throughout the experiments. Left ventricular systolic pressure, left ventricular developed pressure and coronary flow of the control 3 h group remained constant over the first 90 min. Thereafter, a continuous decrease was observed. Left ventricular developed pressure and coronary flow pressure declined by 38% and 31% from 87 ± 4.9 mmHg and 3.9 ± 0.40 ml min^{-1} ($t=0$) to

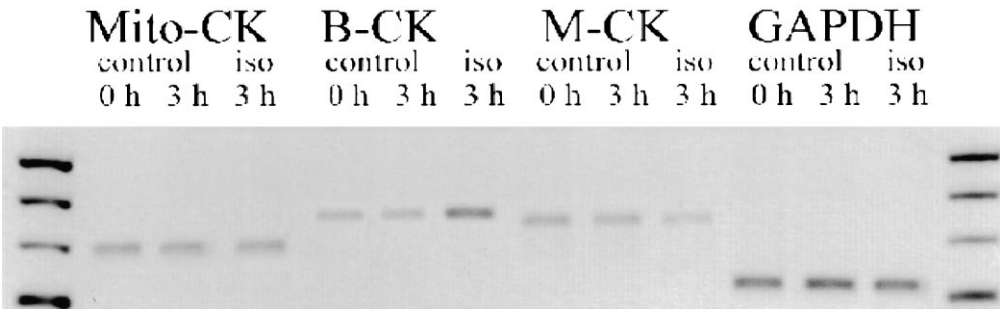

Fig. 2. PCR products of B-CK, M-CK, mito-CK, and GAPDH-mRNA obtained from a control $t=0$ h (control 0 h), control $t=3$ h (control 3 h) and isoproterenol (iso 3 h) heart. Reverse transcription and PCR were performed as described. An ethidium bromide-stained agarose gel of the reaction products is shown; the left and right traces indicate DNA standards (from the top (bp): 1114, 900, 692, 501).

54 ± 1.3 mmHg and 2.7 ± 0.29 ml min^{-1} after 3 h. There was no myocardial lactate production and no significant release of CK or alanine aminotransferase into the perfusate, indicating that there was no anaerobic metabolism or severe tissue damage. The start of isoproterenol administration caused a rapid increase in heart rate to about 360 min^{-1} and in left ventricular systolic pressure to about 170 mmHg. Heart rate remained elevated during the whole 3 h recording time. Left ventricular systolic and developed pressures declined towards baseline values. As in the control group, the left ventricular end-diastolic pressure remained unchanged during the whole experiment under isoproterenol stimulation. Also, there was no lactate production and no release of CK or alanine aminotransferase into the perfusate in the isoproterenol group.

3.2. Wet-to-dry-weight ratio

Wet-to-dry-weight ratios averaged 4.47 ± 0.22, 4.64 ± 0.14 and 4.42 ± 0.36 in the control 0 h, control 3 h and in the isoproterenol 3 h groups. Thus, relevant edema formation did not occur in this isolated heart model over a time period of 3 h. These data correspond to results of other studies using isolated red cell perfused rat heart [33].

3.3. Total CK activity and isoenzyme distribution

Table 2 summarizes total tissue CK activity and relative and absolute activities of CK isoenzymes. Control values at $t=0$ and after 3 h did not differ significantly. Total CK activity (about 7 IU/mg protein) as well as isoenzyme distribution with $\sim$1% BB-CK, 13–15% MB-CK, 47–48% MM-CK and 37–38% mito-CK are similar to previously reported data [34,35]. Total CK activity as well as CK isoenzyme distribution remained unchanged in the control 3 h group, indicating stability of the preparation for these parameters. Isoproterenol stimulation over 3 h did not affect total CK activity or isoenzyme distribution.

Table 2
Total CK activity, CK isoenzyme distribution and CK isoenzyme activity

		Control 0 h	Control 3 h	Iso 3 h
Total CK	IU/mg protein	6.5 ± 0.4	7.5 ± 0.4	7.3 ± 0.7
BB-CK	%	1.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.2
MB-CK	%	13.7 ± 0.9	14.6 ± 0.6	12.9 ± 0.3
MM-CK	%	47.8 ± 1.4	47.2 ± 1.4	47.1 ± 0.7
mito-CK	%	37.1 ± 1.6	36.8 ± 1.4	38.5 ± 0.9
B-CK	IU/mg protein	0.53 ± 0.03	0.66 ± 0.04	0.58 ± 0.06
M-CK	IU/mg protein	3.6 ± 0.3	4.1 ± 0.2	3.9 ± 0.3
Mito-CK	IU/mg protein	2.4 ± 0.1	2.8 ± 0.1	2.8 ± 0.3

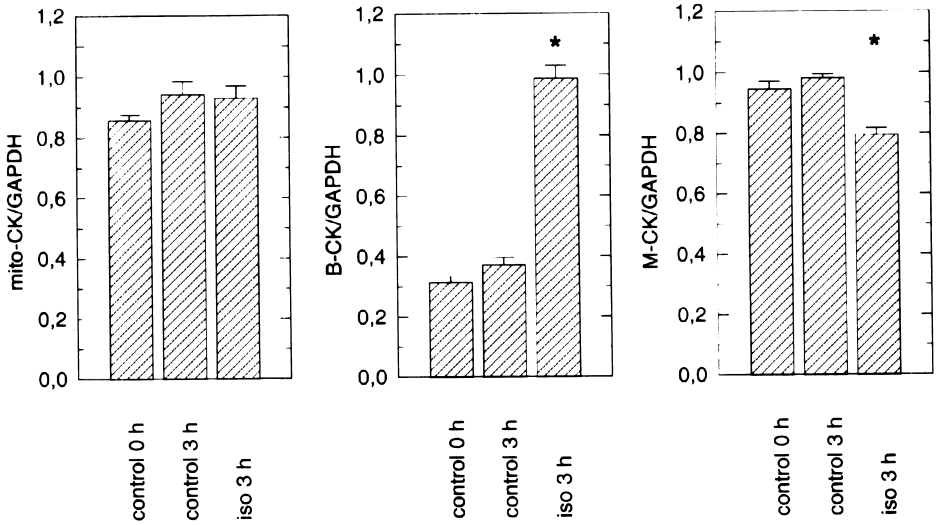

Fig. 3. CK gene expression of control groups at $t=0$ (control 0 h) or 3 h (control 3 h) and after 3 h isoproterenol infusion (iso 3 h). The CK-mRNA levels were determined by quantitative PCR and normalized to the respective levels of the GAPDH-mRNA. *$P<0.05$ versus control $t=0$ and versus control $t=3$ h ($P<0.025$ for each of the two t-tests, according to the Bonferroni correction).

3.4. CK mRNA levels

Messenger RNAs of the CK system were measured by use of quantitative PCR. The PCR products, which were obtained using the conditions given in Table 1, of B-CK, M-CK and sarcomeric mito-CK mRNA are demonstrated in Fig. 2. The PCR products of a representative heart of each group are shown. The PCR products are found in appropriate size according to their length given in Table 1. There was no difference in the intensity of the GAPDH signal among groups. The B-CK signal of the isoproterenol-stimulated heart is markedly more intense than those of the control groups, whereas the M-CK signal of the isoproterenol-stimulated heart appears less intense. Incorporation of α-[^{32}P]dCTP was used to allow for quantification of the PCR products by phospho-imaging. To account for variations in sample treatment and RNA preparation, normalization to GAPDH mRNA PCR products measured in the same sample as an internal standard was performed [31,32]. Absolute values for GAPDH averaged 87.1 ± 6.4 in the control 0 h group, 82.6 ± 5.6 in the control 3 h group and 82.1 ± 2.8 phospho-image units in the isoproterenol 3 h group, and were not significantly different among groups, supporting the validity of this internal standard.

Quantitative PCR results are presented in Fig. 3. No differences were found between both control groups, indicating stability of the preparation concerning CK gene expression. B-CK mRNA values of the isoproterenol group were increased ~3-fold in comparison to both control groups. The M-CK mRNA levels of this group, on the other hand, were significantly reduced by about 18%.

4. Discussion

This study demonstrates acute changes in CK mRNA expression due to β-adrenergic stimulation, which in part reproduced previously described effects on CK caused by chronic hemodynamic and metabolic alterations with or without left ventricular hypertrophy and failure. Both, an increase in B- and a decrease in M-CK-mRNA was found in intact residual post-myocardial infarction ventricular tissue two months following myocardial infarction [16]. A reduction of M-CK mRNA levels is described due to acute ischemia in dog myocardium [36] and following 1 month of diabetic metabolic conditions [37] but this stands in contrast to experiments using pressure overload by aortic banding, in which increased M-, B- and mito-CK-mRNA levels were found after 4 days [12]. Upregulation of B-CK mRNA is reported in chronic volume overload due to mitral regurgitation in dogs after 4 months [17], after the onset of pacing-induced heart failure in dogs [14]

and in response to pressure overload [12]. More previous studies investigated CK isoenzyme activity changes in response to cardiac injury. An increase in B-subunit-containing isoform activity is found due to left ventricular hypertrophy induced by renal hypertension in the dog [13] and by aortic banding in the rat [10,11,13], due to LAD occlusion [15] and due to volume overload induced by aortocaval fistula in the dog [14].

All these studies investigated the influence of hemodynamic alterations on CK isoenzyme gene expression or CK activity and were performed in whole animal models. Here, the occurrence of left ventricular hypertrophy and/or heart failure is accompanied by the activation of local and systemic neurohumoral mechanisms, such as the renin-angiotensin, sympathetic or endothelin systems. Furthermore, changes in receptor distribution as well as in post-receptor signaling may occur during development of left ventricular hypertrophy and heart failure in chronic models. For these reasons these previous studies are limited in their ability to identify single humoral stimuli which may induce the changes in CK isoenzyme gene expression.

The present study focused on the effects of β-adrenergic stimulation. An isolated heart model was selected to examine the effects of the β_1/β_2-adrenergic agonist isoproterenol independent from other circulating mediators. β-Adrenergic stimulation has been studied previously concerning its effect on proto-oncogene expression and subsequent involvement in cardiac hypertrophy. Several studies in whole animals and in cell culture report an increase in cardiac protein synthesis 60 min and an increase in proto-oncogene expression as short as 15–30 min after the onset of β-adrenergic stimulation [38–41]. Proto-oncogene induction is likely to contribute to the regulation of transcription of cardiac-specific genes such as the CK-isoenzyme genes and may precede the induction or suppression of these genes [42,43]. Therefore, isoproterenol-induced changes in CK genes expression are expected to occur later than proto-oncogene induction.

Continuous administration of isoproterenol over 3 h as performed in our study, therefore, was regarded as feasible to induce changes of CK gene expression in the isolated heart preparation. Perfusion with red cell suspension was performed to provide physiological coronary perfusion flow rate. This way endothelial stimulation and/or damage which may occur due to high perfusion rates in crystalloid perfused preparations are avoided. Furthermore, perfusion with red cell suspensions was expected to reduce extracellular edema formation. In the present study, a measurable increase in extracellular fluid during the 3 h protocol did not occur as indicated by the unchanged wet-to-dry-weight ratio. There was no release of intracellular enzymes (CK or alanine aminotransferase) into the perfusate and no myocardial lactate production ruling out significant cell damage and anaerobic metabolism due to disturbances in local perfusion. Disturbances of left ventricular diastolic function did not occur as left ventricular diastolic pressure remained constant during the 3 h recording time. Also, heart rate was stable over the 3 h protocol. Left ventricular systolic and developed pressure as well as coronary flow, however, declined by 38% and 30% during the protocol after being stable for the first 90 min. Limited stability in systolic performance is a problem that is commonly observed in isolated heart protocols lasting more than 90 min. Eberli et al. [33] used isolated red cell perfused rat hearts in an 100 min protocol. They found a 9% decrease in the left ventricular developed pressure after 100 min in control experiments. This corresponds to the present study in which a 9% decrease was found at $t = 90$ min. In isolated red cell perfused rabbit hearts, Eberli et al. [44] report a significant 14% and 32% decrease in left ventricular developed pressure and coronary flow after 180 min but not lactate release, only minimal release of CK and alanine aminotransferase and constant tissue ATP, phosphocreatine and glycogen content. Despite the changes in left ventricular developed pressure and coronary flow, they regarded the system as metabolically stable [44]. Considering these data and the present results, the late decrease in left ventricular developed pressure and coronary flow represents a minor and unavoidable limitation of the study. As there were no differences in CK and GAPDH gene expression between the 0 and 3 h control groups, results of the study concerning isoproterenol-induced changes in CK gene expression are unaffected by the decreased left ventricular developed pressure and coronary flow.

As isoproterenol is a non-selective β1/β2-receptor

agonist and as myocardium contains both receptor subtypes (in a approximate ratio of 80:20% [45]), the present study does not answer the question whether isoproterenol-induced changes in CK gene expression are mediated via β1 and/or β2 activation. Both receptor subtypes belong to the G-protein coupled receptors [46]. Although there are differences in the degree of coupling between the β1- and β2-receptors to the adenylate cyclase, the activation of β1- as well as of β2-receptors results via stimulatory G-proteins in adenylate cyclase activation and intracellular cAMP formation (for review see [47]). Cellular cAMP concentration may affect gene expression via the cAMP-response element (CRE), which has been described for many genes [48]. β1- And β2-receptors use the same cAMP-dependent signaling pathway. It may be for this reason that most studies dealing with β-adrenergically induced changes in gene expression have used the a non selective compound such as isoproterenol and have not differentiated between β1- and β2-activation [38,41,49,50]. Some data, however, suggest that the β-adrenergic induced hypertrophic response in rat cardiomyocytes may mediated by β2-receptor stimulation [51].

Previously, changes in CK-mRNA expression in intact residual post myocardial infarction ventricular tissue have been found to correspond to alterations in CK isoenzyme distribution [16]. This was not found in the present isolated heart model of acute β-adrenergic stimulation. Gene expression involves the following processes: (1) transcription of genomic DNA into mRNA, (2) post-transcriptional mRNA processing, (3) translation of the processed, cytoplasmic mRNA into the amino acid sequence, and (4) post-translational regulation. The detected mRNA represents processed, cytoplasmic mRNA. Therefore, the observed discrepancy in alterations of mRNA and specific activity may be explained by a lag of translation or by post-translational regulation, which has been described in osteoblastic cells [52]. Possibly, significant translation would require substantially longer stimulation periods, and this can therefore not be addressed using the present isolated heart model.

As the CK isoenzyme switch from M-CK to B-CK isoenzymes, i.e., the reappearance of a fetal CK pattern, is described for many models of hypertrophy and heart failure [9,53], the observed changes in CK gene expression after 3 h of β-adrenergic stimulation may be regarded as the initiation of the switch to a fetal distribution. This switch has generally been interpreted as an adaptive mechanism, making energy transduction by CK more efficient [54] due to lower K_m of B-CK compared to M-containing isoenzymes. Due to its higher affinity for ADP, the B-containing enzymes may produce ATP more effectively than the MM-isoform [54]. This may be beneficial in situations of increased energy demand (i.e., β-adrenergic stimulation) or under the conditions of reduced energy reserve in the failing heart [54]. Although B-CK activity only comprises a small percentage of total CK activity, changes in the B-CK activity corresponding to the increase in B-CK expression may be relevant, as CK in the cardiomyocyte is operating in a compartmentalized manner [55]. This way, changes in B-CK activity, which only result in small changes of whole cell CK isoenzyme distribution, may be highly relevant if the subsarcolemmal compartment, which is dominated by B-CK, is concerned. Unfortunately, no methodology is currently available to measure compartmentalized reactant concentrations.

The present study demonstrates that β-adrenergic stimulation, which caused increased B-CK and decreased M-CK mRNA expression, evokes changes in the gene expression of functionally relevant myocardial proteins. These isoproterenol-induced changes in CK-gene expression are similar to changes in the distribution of CK-isoenzyme activity which are characteristic of failing and hypertrophied heart.

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