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Functional aspects of creatine kinase isoenzymes in endothelial cells

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Decking, Ulrich K. M., Christiane Alves, Theo Wallimann, Markus Wyss, and Jürgen Schrader. Functional aspects of creatine kinase isoenzymes in endothelial cells. *Am J Physiol Cell Physiol* 281: C320–C328, 2001.—To characterize the isoenzyme distribution of creatine kinase (CK) in endothelial cells (ECs) and its functional role during substrate depletion, ECs from aorta (AECs) and microvasculature (MVECs) of pig and rat were studied. In addition, high-energy phosphates were continuously monitored by ^{31}P NMR spectroscopy in pig AECs attached to microcarrier beads. CK activity per milligram of protein in rat AECs and MVECs (0.08 ± 0.01 and 0.15 ± 0.08 U/mg, respectively) was <3% of that of cardiomyocytes (6.46 ± 1.02 U/mg). Rat and pig AECs and MVECs displayed cytosolic BB-CK, but no MM-CK. Gel electrophoresis of mitochondrial fractions of rat and pig ECs indicated the presence of mitochondrial Mi-CK, mostly in dimeric form. The presence of Mi_a-CK was demonstrated by indirect immunofluorescence staining using Mi_a-CK antibodies. When perifused with creatine-supplemented medium, phosphocreatine (PCr) continuously increased with time (1.2 ± 0.6 nmol·h^{-1}·mg protein^{-1}), indicating creatine uptake and CK activity. Glucose withdrawal from the medium induced a rapid decrease in PCr, which was fully reversible on glucose addition, demonstrating temporal buffering of an energy deficit. Because both cytosolic and mitochondrial CK isoforms are present in ECs, the CK system may also contribute to energy transduction ("shuttle hypothesis").

endothelium; transport; energy metabolism; phosphorus-31 nuclear magnetic resonance spectroscopy

CELLS AND TISSUES WITH INTERMITTENTLY high and fluctuating energy requirements, such as skeletal and cardiac muscle, brain, retina, and spermatozoa, depend on the immediate availability of large amounts of energy. In these cells and tissues, the enzyme creatine kinase (CK; ATP:creatine *N*-phosphoryl transferase, EC 2.7.3.2.) has been proposed to play a key role in energy metabolism (38). This role may involve *1*) the replenishment of ATP through transfer of the phosphoryl group from phosphocreatine (PCr) to ADP ("buffer function") but also *2*) a facilitated intracellular energy transduction ("creatine kinase shuttle hypothesis"), linking mitochondrial ATP generation to cytosolic sites of ATP consumption via mitochondrial and cytosolic CK isoenzymes (2, 25, 38).

Two cytosolic subunit isoforms, "ubiquitous" brain-type B-CK and "sarcomeric" muscle type M-CK as well as two mitochondrial subunit isoforms, ubiquitous Mi_a-CK and sarcomeric Mi_b-CK, are synthesized in a tissue-specific manner. The cytosolic subunits combine to form enzymatically functional homodimers and heterodimers, MM-CK, MB-CK, and BB-CK isoenzymes, whereas the mitochondrial isoforms form either homodimers or homooctamers (26).

The vascular endothelium is generally regarded as a nonexcitable tissue with a rather constant energy demand imposed by processes as diverse as cytoskeletal contraction (myosin ATPase), protein synthesis, and ion transport (Na^+-K^+-ATPase) (5). The endothelium is characterized by a high glycolytic capacity and can survive extended periods of hypoxia (18). These properties suggest that cytosolic isoforms of CK are not essential in endothelial cells (ECs) and may not be present at all, as is the case, e.g., in hepatocytes (12). It was therefore rather surprising that almost equal amounts of BB-CK and MM-CK were suggested to be present in human umbilical vein ECs (15). BB-CK activity, but no MM-CK, was also observed in rat liver ECs (35), while no evidence of mitochondrial CK was obtained. In fact, the release of BB-CK from liver has even been used as an index of endothelial cell injury (24, 35).

While there appears to be agreement that ECs contain CK, several important questions remain. *1*) What is the functional role of CK in ECs? *2*) Do vascular ECs contain both MM-CK and BB-CK or do they display BB-CK activity only? In normal adult hearts, in the absence of hypertrophy, cardiac myocytes predominantly express MM-CK and MB-CK. Thus, in the case where ECs would exclusively express BB-CK, one might be able to use a release of this latter CK isoform to discriminate between cardiac myocyte and endothelial cell injury. *3*) Are there differences between mac-

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rovascular and microvascular ECs, e.g., between ECs from aorta and coronary capillaries? *4*) Are both cytosolic and mitochondrial isoforms of CK expressed? In this case, a prerequisite for the CK shuttle hypothesis would be fulfilled. If only the cytosolic isoform were present, the CK system might simply operate as a temporary high-energy phosphate buffer.

To address these questions, microvascular ECs from the coronary system of pig and rat as well as macrovascular aortic ECs from these two species were analyzed and compared with rat cardiomyocytes. Isoenzyme distribution and total CK activity were assessed. Moreover, the role of CK in the intact cell was investigated by ^{31}P-NMR spectroscopy of perifused porcine aortic ECs grown on microcarrier beads; in this system the total creatine pool was either increased by creatine supplementation or the cells were subjected to a reduced energy status by withdrawing glucose supply.

MATERIALS AND METHODS

Preparation of macrovascular ECs from porcine aortas. Macrovascular ECs were isolated from porcine thoracic aortas obtained from a local slaughterhouse as described in detail before (30). In brief, isolated cells were suspended in culture medium [M199 with Earle's salts (GIBCO, Karlsruhe, Germany) containing 20% newborn calf serum (NCS), 250 IU/ml penicillin, 250 μg/ml streptomycin, 12.5 μg/ml amphotericin B, and 50 μg/ml gentamicin] and plated onto 100-mm plastic culture dishes (Falcon, type 3003). Cultures were incubated at 37°C with 5% CO_2 in room air. Four hours after plating, the culture medium was renewed. After 2 days of incubation the medium was renewed again, reducing the concentration of antibiotics to 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B. Gentamicin was omitted at this time. Five to seven days after plating, cells reached confluency and were harvested at this point. Cultures were washed twice with PBS, pH 7.4, scraped off the dishes with a rubber policeman, immediately frozen in MSH buffer (in mM: 220 mannitol, 70 sucrose, 10 HEPES, 0.2 EDTA, 1 2-mercaptoethanol, and 1 sodium azide, pH 7.4) and stored at −70°C. This procedure was repeated for all cell types.

For ^{31}P NMR spectroscopy perifusion experiments, macrovascular ECs from porcine aorta (AECs) were grown to confluence on dishes for 7 days as described above and transferred to microcarrier beads (5×10^6 cells/g carrier; NUNC, Wiesbaden, Germany) in stirring flasks (350 ml; Techne, Cambridge, UK). With the use of the same medium as before, AECs formed a confluent monolayer on the microcarrier surface within 7 days. In one set of NMR experiments the culture medium was supplemented with 0.5 mM creatine for the final 48 h of culture before starting the perifusion.

Preparation of microvascular ECs from porcine hearts. Microvascular ECs (MVECs) were isolated from porcine hearts obtained from a local slaughterhouse. The procedure used was similar to the isolation of MVECs from rat hearts described by Piper et al. (22). In brief, an artery of the right ventricle was cannulated and rinsed with ice-cold Krebs-Henseleit solution [containing (in mM) 116 NaCl, 4.7 KCl, 1.1 $MgSO_4$, 1.17 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, 8.3 glucose, and 2 pyruvate, pH 7.4, when equilibrated with carbogen (95% O_2-5% CO_2)] to remove the remaining blood and to define the area of tissue supplied by this vessel. This piece of the right ventricle was cut out and mounted onto a perfusion device. The flow was adjusted to ~10 ml/min. After an initial perfusion period with Krebs-Henseleit buffer at 37°C, the perfusate was switched to a buffer, containing (in mM) 110 NaCl, 2.6 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, and 11 glucose and continuously gassed with carbogen through the tip of a Pasteur pipette to maintain a pH of 7.4. The effluent was collected in a beaker placed underneath the tissue and was discarded. After 20 min the perfusion was switched to a recirculating mode and continued for another 45 min with 80 ml of fresh buffer containing 20 mg of collagenase and 15 μl of a 100 mM $CaCl_2$ stock solution. The ventricular tissue was taken off the cannula, treated with a tissue chopper, and transferred into a glass beaker containing the recirculated medium and 800 mg of BSA, fraction V. During an incubation period of 30–40 min at 37°C the mixture was gassed with carbogen and frequently resuspended with a 10-ml disposable pipette. The dissolved tissue was filtered through a nylon mesh, and the filtrate was centrifuged at 25 *g* for 5 min. The supernatant was transferred to a Teflon beaker, and 300 mg of BSA, 15 mg of trypsin (1:250), and 45 μl of $CaCl_2$ stock solution (100 mM) were added. During an incubation period of 30 min at 37°C, the mixture was gassed again with carbogen and stirred in a shaking water bath. The cell suspension was centrifuged at 250 *g* for 10 min and the pellet resuspended in a culture medium, similar to that of porcine aortic ECs but containing 10% NCS, 10% FCS, and no amphotericin B. After another centrifugation step and resuspension, the cells were plated onto 100-mm plastic culture dishes and handled as indicated above.

Preparation of ECs from rat aortas. Thoracic aortas were isolated from 200- to 300-g male Wistar rats following removal of the hearts (see above). The vessels were immersed in ice-cold PBS, and adjacent connective and adipose tissue were removed. The aortas were opened in a longitudinal direction, and segments of ~2–3 mm length were cut. They were placed with the intima facing downward into a 100-mm plastic culture dish and were allowed to adhere to the bottom of the dish by incubation at 37°C (with 5% CO_2) for about 1 h. Culture dishes were cautiously filled with culture medium (see *Preparation of macrovascular ECs from porcine aortas*) and incubated again at 37°C (with 5% CO_2). Every other day, culture medium containing reduced concentrations of antibiotics was renewed. When ECs started to grow from underneath the aortic segments, the tissue was removed and cells were cultured until they reached confluency.

Preparation of microvascular ECs and cardiomyocytes from rat hearts. Microvascular ECs and adult cardiac myocytes were isolated from 200- to 300-g male Wistar rats as described by Piper et al. (21–23) and kindly provided by T. Stumpe from our laboratory. In brief, following perfusion with a Ca^{2+}-free Krebs-Henseleit buffer, hearts were treated with collagenase (0.1%) in a recirculating mode, and then were minced and filtered. Centrifugation enabled the separation of ECs (supernatant) and cardiomyocytes (pellet). Cardiomyocytes were washed with increasing Ca^{2+} concentrations and purified by centrifugation (4% BSA). The rat heart ECs were incubated with trypsin for 30 min, centrifuged, and transferred to culture plates. Culture medium and incubation conditions were similar to those for pig heart MVECs. Aliquots of cell suspensions obtained from different experiments were immediately frozen and stored at −70°C.

Purity of cell culture. Purity of EC cultures was determined by phase-contrast microscopy and immunofluorescence. The uptake of low-density lipoproteins (LDLs) in live ECs was performed with indocarbocyanin-coupled acetylated LDLs (10 μg/ml, for 4 h at 37°C; Paesel+Lorei, Frankfurt, Germany) (36). Contamination with fibroblasts or smooth muscle cells was studied by immunofluorescent staining with a

monoclonal mouse antibody to α-smooth muscle actin (1:50, 30 min; Sigma, Deisenhofen, Germany) (1). Endothelial cultures used in this study were >95% pure.

Preparation of mitochondrial and cytosolic extracts. Mitochondrial and cytosolic extracts from rat or pig heart muscle and brain were prepared as described previously (41). In brief, following tissue homogenization and one centrifugation step (750 g), the supernatant was centrifuged at 6,000 *g*, separating mitochondria (pellet) and cytosolic extract (supernatant). The pellet was washed and centrifuged (6,000 *g*) two times before the mitochondria were resuspended in 5–30 ml of buffer and subsequently extracted. Fractions were stored in MSH buffer at −70°C until used as reference samples for cellulose polyacetate electrophoresis.

NMR spectroscopy of ECs. ECs grown on microcarrier beads were studied as previously described (6). They were transferred to a 10-mm NMR tube that contained a bottom filter (pore diameter 35 μm); a central capillary passing through the filter was used as outflow line. Within the NMR-sensitive volume, a standard capillary containing a defined amount of methylenediphosphonic acid (MDP) was fixed close to the center of the tube. Cells on microcarriers sedimented rapidly and formed a homogeneous column that was perfused from top to bottom at 1 ml/min with a HEPES buffer, containing (in mM) 150 NaCl, 2.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$, 5.6 glucose, 0.5 creatine, and 10 HEPES, as well as 10% NCS, adjusted to pH 7.4 and equilibrated with room air. The NMR tube was placed inside the magnet, and temperature was maintained at 37°C. Protein content per gram of carrier was determined in every experiment (16). For this purpose an aliquot of the cells was washed in serum-free HEPES buffer.

All NMR spectra were obtained on an AMX 400 WB NMR spectrometer (Bruker, Karlsruhe, Germany) that was connected to an Oxford/Spectrospin 9.4 Tesla wide-bore magnet. A 10-mm broadband probe head (Bruker) was employed. The homogeneity of the magnetic field was adjusted by optimizing the free induction decay (FID) of the water proton signal. A near-Lorentzian line shape with a line width at half height of 12–20 Hz was achieved in every experiment. Each FID consisted of 2,048 data points. Data were subsequently zero-filled to 4,096, followed by exponential multiplication (line broadening 25 Hz), Fourier transformation and manual phasing.

The NMR-sensitive volume (1.75 ml) contained ~5.4 mg protein of ECs on 1.04-g microcarriers. Fully relaxed spectra were the result of 2,048 90° scans (pulse width 27 μs) with a pulse repetition time of 15 s. Partially saturated spectra were obtained using 1,800 70° scans and a pulse interval of 3 s. Peak areas were determined after manual zero and first-order baseline correction for each individual peak and referenced to the external standard (MDP) that contained a known amount of phosphates, using the integration software of the NMR spectrometer (UXNMR; Bruker). Because the longitudinal relaxation time T_1 of MDP was measured to be 5.68 s, an appropriate saturation factor (0.933) was taken into account in spectra obtained at a pulse interval of 15 s.

HPLC. To compare the NMR measurements with conventional biochemical techniques, aliquots of ECs were extracted with 1 M perchloric acid. The extracts were neutralized and centrifuged, and the supernatant was injected and separated on a μBondapak C18 4-μm column (Waters, Eschborn, Germany) while ultraviolet absorption at 254 nm was detected. A linear elution gradient was employed changing from 36.8 mM KH_2PO_4/2.95 mM tetrabutylammoniumsulfate (TBAS), pH 3.0, to 14.7 mM KH_2PO_4/2.95 mM TBAS, pH 5.4, and finally to 70% methanol. Chromatogram peaks were identified by comparing the retention times of the samples with those of external standards containing AMP, ADP, and ATP, and peaks were quantified by comparing the integrated peak areas with those of the external standards.

CK activity. CK activity was determined according to the method of Szasz et al. (32). A commercially available test kit (Boehringer Mannheim, Germany) was used for these assays. Thawed samples were sonicated and, following centrifugation, the supernatants were used for measurements of enzyme activity. Protein content of all cell suspensions was determined according to the Lowry method.

Cellulose polyacetate electrophoresis. Cellulose polyacetate electrophoresis was performed on Cellogel strips (Bio-tec-Fischer, Reiskirchen, Germany) in veronal buffer (pH 8.6) at room temperature as described earlier (40). CK isoenzymes were separated at a constant voltage of 150 V for 45 min (see Figs. 4 and 5) or at 100 V for 90 min (see Figs. 6 and 7). Separated isoenzymes were visualized by an overlay gel technique (39) in the presence and absence of 15 μM P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A), which inhibits adenylate kinase activity (14).

Immunofluorescence labeling. ECs were grown in monolayers on glass coverslips placed in plastic culture dishes (35 mm, Falcon). Cells were fixed overnight with 3% paraformaldehyde and 0.2% glutaraldehyde in ice-cold PBS, pH 7.4. The fixative was exchanged with a mixture of 0.3% paraformaldehyde and 0.02% glutaraldehyde in ice-cold PBS, and cells were stored at 4°C until use. All immunolabeling steps were carried out at room temperature. Fixed cells were washed three times with Tris-buffered saline (TBS) containing 50 mM Tris·HCl and 150 mM NaCl, pH 7.4, and the fixative was quenched by incubation for 10 min with TBS supplemented with 0.1 M glycine. After permeabilization with 0.2% Triton X-100 in TBS for 20 min, cells were incubated for 20 min with TBS containing 0.5% BSA, 0.2% gelatin, and 2% horse serum (referred to as TBG) to saturate nonspecific protein-binding sites. After a 90-min incubation period in a moist chamber with either rabbit anti-chicken B-CK or anti-chicken ubiquitous mitochondrial CK (Mi_a-CK) antibodies and, in parallel, also with the corresponding preimmune IgG [previously prepared and characterized in the lab of T. Wallimann (10)], each diluted 1:500 with TBG, cells were washed three times for 10 min each with 0.2% Triton X-100 in TBS and three times with TBS to remove unbound antibodies. The cells were then incubated for 75 min with rhodamine-conjugated goat anti-rabbit IgG (Pierce) diluted 1:500 with TBG. After additional washing steps (as the ones described above), the cells were mounted in buffered polyvinyl alcohol Lennette medium (13) containing *p*-phenylenediamine (1 mg/ml; Sigma) as anti-fading agent. Photographs were taken on a Zeiss universal fluorescence microscope using Kodak T-Max 400 DIN black/white film.

Statistics. Data are given as means ± SD. Differences in CK activities between different types of ECs were evaluated using Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

^{31}P NMR of porcine aortic ECs. Energy metabolism of perifused porcine macrovascular ECs from the aorta (AECs) was investigated by ^{31}P NMR spectroscopy. In fully relaxed spectra, peak areas represent the concentration of phosphorus nuclei of the respective compounds. A representative example is shown in Fig. 1. Resonances of inorganic phosphate, PCr, NAD, and nucleotide triphosphates (α-, β- and γ-phosphate reso-

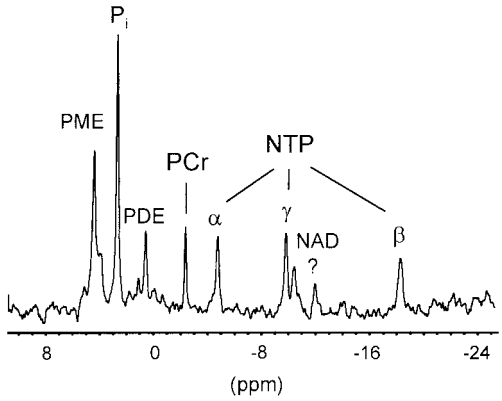

Fig. 1. Representative fully relaxed ^{31}P NMR spectrum of perifused endothelial cells from porcine aorta; 2,048 90° scans with a pulse interval of 15 s were acquired. Line broadening was 20 Hz. PCr, phosphocreatine; PME, phosphomonoester region; PDE, phosphodiester region; NTP, nucleotide triphospates; ppm, parts per million.

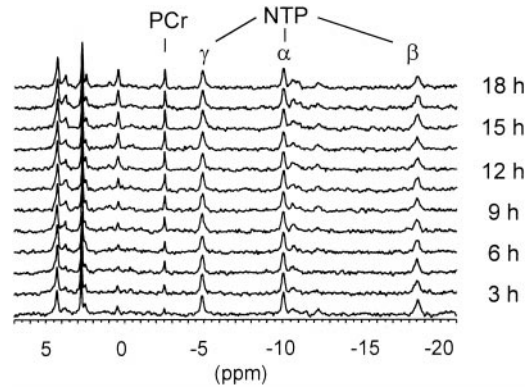

Fig. 2. Stack plot of partially saturated ^{31}P NMR spectra of perifused porcine aortic endothelial cells. Cells had been previously cultured with standard medium including 20% newborn calf serum. Each spectrum was obtained within 90 min and was the result of 1,800 70° pulses with a pulse interval of 3 s. Line broadening was 10 Hz.

nances) were identified, as well as peaks in the phosphomonoester and phosphodiester region. The β-phosphate of the nucleotide triphosphates was quantified to be 22.8 ± 3.8 nmol/mg protein (n = 3), similar to ATP measurements obtained in acid extracts of the same cell batches by HPLC. Thus ATP is the dominant nucleotide in AECs. While ADP was below the NMR detection threshold in perifused AECs, the ADP/ATP ratio in extracts was ~1:7. This suggested that a considerable portion of ADP is protein bound and NMR invisible. A further peak at −12.3 parts per million (ppm) could not be identified and may represent uridine diphosphoglucose. The inorganic phosphate peak represents almost exclusively the inorganic phosphate of the perifusion medium (1.2 mM KH_2PO_4), since the intracellular volume of the ECs (~30 μl) was small compared with the surrounding medium. The amount of phosphomonoesters, i.e., metabolites like glucose 6-phosphate and fructose 6-phosphate, was about twice the size of the endothelial ATP pool, consistent with the high glycolytic capacity of EC.

As can be seen in Fig. 1, porcine AECs contain a considerable amount of PCr, suggesting the presence of CK. The PCr content was determined by ^{31}P NMR to be 19.5 ± 0.5 nmol/mg protein. This value represents the mean content determined during the entire acquisition period (~8.5 h) of fully relaxed spectra. However, when cells previously cultured in the absence of creatine were perifused with medium containing 0.5 mM creatine, the PCr concentration was not constant. When studying energy metabolism in creatine-supplemented perifused AECs with a time resolution of 1.5 h, a continuous, steady increase in PCr content was observed over a period of 18 h while ATP remained constant (Fig. 2). The increase in PCr was linear for the entire acquisition period and calculated to be 1.2 ± 0.6 nmol·h^{-1}·mg protein^{-1} (n = 5). It was confirmed by HPLC measurements made in parallel experiments outside the magnet (data not shown), thus confirming that these cells possess a creatine transport system (9, 27). When the medium was devoid of creatine, the rate of PCr accumulation was reduced by >90%.

When cells were cultured in the presence of creatine (0.5 mM) during the final 48 h before perifusion, a considerable PCr peak was observed already in the initial ^{31}P-NMR spectrum acquired. This peak remained constant during the following 4 h of perifusion (Fig. 3). When, under these conditions, the energy status and turnover were compromised by withdrawing glucose supply for 2 h, a near-complete disappearance of PCr was observed while ATP remained unaltered (n = 3). On reperfusion with glucose-containing medium, PCr fully recovered within 2 h, indicating substantial CK activity and possibly creatine transporter activity as well (Fig. 3).

CK activity in ECs of rats and pigs. CK activities were measured in cell preparations from rat and pig (Table 1). In the rat, ECs isolated from hearts (MVECs) or thoracic aortas (AECs) showed a significantly lower CK activity compared with cardiomyocytes. Rat MVEC CK activity was ~1/40 and AEC CK activity only ~1/80 of that of cardiomyocytes. Enzyme activities of macro-

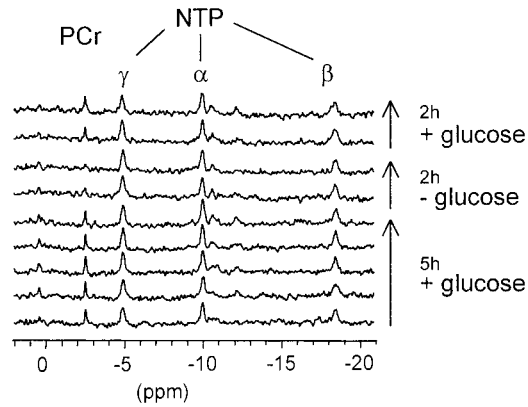

Fig. 3. Partially saturated ^{31}P NMR spectra of perifused porcine aortic endothelial cells. The culture medium had been supplemented with 0.5 mM creatine for the final 48 h of culture. Perifusion was performed with standard medium (5.6 mM glucose) for 5 h, followed by 2 h of glucose withdrawal and 2 h of reperfusion. Each spectrum was obtained within 60 min and was the result of 1,200 70° pulses with a pulse interval of 3 s. Line broadening was 10 Hz.

Table 1. *Creatine kinase activities of different cell preparations of rat and pig*

Cell Type	Concentration, U/mg protein	*n*
Rat		
Cardiomyocytes	6.46 ± 1.02	5
MVEC	0.15 ± 0.03*	6
AEC	0.08 ± 0.01	6
Pig		
MVEC	0.36 ± 0.03*	3
AEC	0.06 ± 0.01	6

Values are means ± SD; *n* = no. of individual cell preparations. MVEC, microvascular heart endothelial cells; AEC, macrovascular endothelial cells from the aorta. *$P < 0.005$ for MVEC vs. AEC.

vascular ECs from pig and rat were in a similar range (0.06 and 0.08 U/mg protein). Both in pig and rat, microvascular ECs displayed a higher CK activity than the corresponding AECs.

Cytosolic CK isoenzymes in ECs. Extracts of macro- and microvascular ECs from rat and pig were analyzed by cellulose polyacetate electrophoresis to separate cytosolic and mitochondrial CK isoenzymes. Tissue extracts of heart and brain from both species were included as standards. As can be seen in Fig. 4*A*, macro- and microvascular ECs from rat contain mainly cytosolic BB-CK, thereby corresponding in enzyme pattern to rat brain (*lane 4*). MM-CK, typical for heart (*lane 3*) and skeletal muscle, as well as the heterodimer MB-CK could not be detected. Similar results were obtained with AECs and MVECs from pig (Fig. 4*B*). The fainter bands seen especially in MVECs from rat (Fig. 4*A, lane 2*) and pig (Fig. 4*B, lane 2*) represent adenylate kinase (see below; Fig. 5).

To inhibit adenylate kinase, which migrates similarly to MM-CK, gels were stained in the presence of 15 μM Ap_5A. Figure 5*B* demonstrates the effect of Ap_5A on the actual staining pattern of ECs, cardiomyocytes, and rat heart extracts compared with an identical experiment where Ap_5A had been omitted from the staining solution (Fig. 5*A*). In extracts of AECs from pig (*lane 1*) and MVECs from rat (*lane 2*), bands (Fig. 5*A*) corresponding to the intensely stained bands of MM-CK in cardiomyocytes (*lane 3*) and heart muscle (*lane 4*) proved to be due to adenylate kinase activity, since the bands disappeared in the presence of Ap_5A (Fig. 5*B*). Additionally, when the coupled CK assay was performed in the absence of PCr (Fig. 5*C*), the same bands appeared at the equivalent places, also indicating the presence of adenylate kinase. Loike et al. (15) reported in 1992 almost equal amounts of BB-CK and MM-CK for human umbilical vein ECs. This discrepancy might be due to species differences. Alternatively, it is well conceivable that the band taken to be MM-CK represented in fact the dimeric form of Mi_a-CK (see below; Fig. 7).

Mitochondrial CK isoenzymes in ECs. Analysis of enriched mitochondrial fractions from rat and pig micro- and macrovascular ECs revealed some enzymatic activity at cathodic positions indicative for mitochondrial Mi-CK isoenzymes, which have a higher isoelectric point than the cytosolic isoforms (26) (Fig. 6). Although these Mi-CK activity bands are rather weak, no obvious differences between micro- and macrovascular ECs of the same species were noticeable (*lanes 1* and *4* and *2* and *3*). However, the bands of mitochondrial CK isoenzymes obtained from rat displayed a more cathodic position than those of pig.

Cardiac muscle and brain from chicken (26, 40) and rat (41) contain two different Mi-CK isoenzymes that both form dimeric and octameric isoforms that are interconvertible under certain conditions (40). As depicted in Fig. 7 (*lanes 1* and *4*), similar results have been obtained for pig tissue. The isoenzyme Mi_a-CK is found in brain, whereas Mi_b-CK is present in the heart. When the electrophoretic pattern of AEC (*lane 2*) and MVEC extracts (*lane 3*) is compared with those obtained for cardiac muscle and brain, no definite assignment can be made, since the stained bands do not correspond exactly. However, it seems most likely that both EC fractions contain the ubiquitous brain-type Mi_a-CK isoenzyme, since the bands labeled by a star correspond best to Mi_a-CK dimers (Mi_a-D). The stronger more anodal bands again correspond to adenylate kinase. To obtain conclusive evidence for this deduction, immunolabeling experiments with antibodies were performed.

Immunofluorescence staining of porcine aortic ECs. Indirect immunofluorescence staining of porcine aortic ECs with anti-B-CK antibodies revealed a relatively homogeneous, rather strong staining of all ECs sparing the nuclei (Fig. 8*A*). In contrast, the same staining procedure for ubiquitous Mi_a-CK (Fig. 8*B*) revealed a rather weak, mostly perinuclear and spotted staining, typical for mitochondria (7), while no staining was seen in the control (Fig. 8*C*). This indicated (in line with the biochemical data) that B-CK is the prominent cytosolic CK isoform in ECs, with only minor amounts of Mi_a-CK present as the ubiquitous mitochondrial CK isoform in ECs. The presence of both B-CK and Mi_a-CK

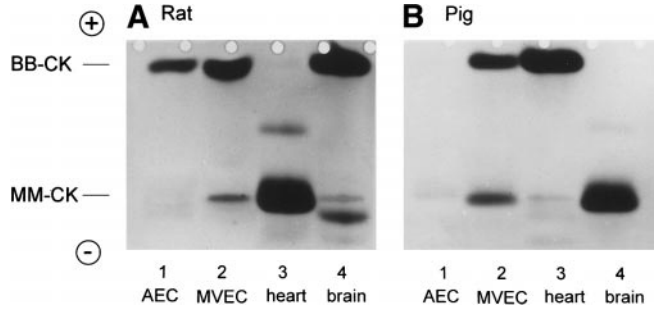

Fig. 4. Creatine kinase (CK) isoenzymes in endothelial cells (ECs) from rat and pig. Cellulose polyacetate electrophoresis was performed for 45 min at 150 V. CK isoenzymes were stained in the presence of 15 μM P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A) to suppress adenylate kinase activity. *A*: rat tissue. *Lane 1*, ECs from aorta (AECs); *lane 2*, ECs from microvasculature (MVECs); *lane 3*, cytosolic fraction from heart; *lane 4*, cytosolic fraction from brain. *B*: pig tissue. *Lane 1*, AECs; *lane 2*, MVECs; *lane 3*, cytosolic fraction from heart; lane 4, cytosolic fraction from brain. O, origin; +, anode; −, cathode.

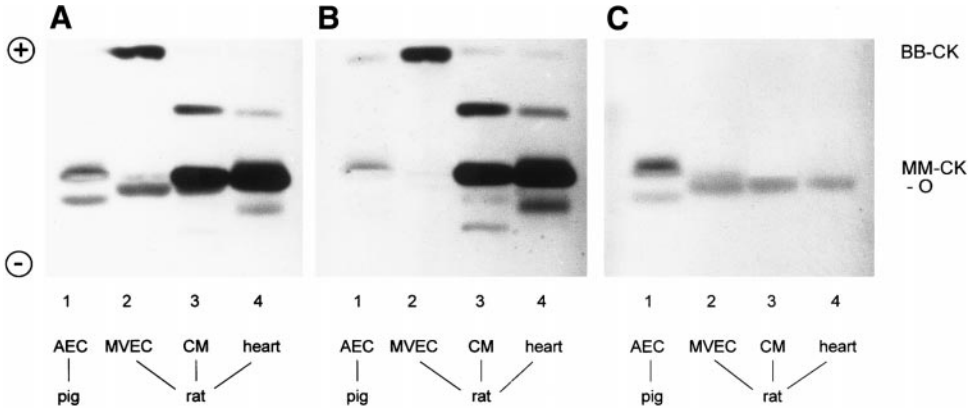

Fig. 5. Effect of Ap_5A on electrophoretic appearance of CK isoenzymes. Cellulose polyacetate electrophoresis was performed for 45 min at 150 V. CK isoenzymes were stained in the absence (*A*) and presence (*B*) of 15 μM Ap_5A. *C*: a negative control since staining was performed in the absence of phosphocreatine. *Lane 1*, AECs from pig; *lane 2*, MVECs from rat; *lane 3*, cardiomyocytes (CM) from rat; *lane 4*, rat heart; O, origin. Note that the staining in the region of MM-CK in *lane 1* (AECs from pig) is due to deposited material remaining at the origin during electrophoresis; +, anode; −, cathode.

would indicate a prerequisite fulfilled for a PCr circuit working in these ECs.

DISCUSSION

By monitoring the cellular energy status noninvasively by ^{31}P-NMR spectroscopy, the present study demonstrates reversible alterations in PCr concentration and thus functional CK activity in ECs. The only cytosolic isoform detected in cultured macro- and microvascular ECs of pig and rat was BB-CK. In addition, the presence of a mitochondrial isoform of CK, most likely Mi_a-CK, was observed in ECs of both rat and pig. The finding of both mitochondrial (albeit at low levels) and cytosolic BB-CK (at significantly higher levels) isoforms in conjunction with functional data strongly suggests that the PCr circuit is operative also in ECs.

CK isoforms in ECs. In the past, conflicting evidence had been provided regarding the cytosolic CK isoforms expressed in ECs. While some groups observed exclusively BB-CK in the endothelial compartment (35), others reported the presence of both BB-CK and CK-MM in human ECs derived from the umbilical vein (15). This was surprising, considering the fact that MM-CK is predominantly expressed in skeletal and cardiac muscle. However, since ECs are also characterized by a rather large contribution of myosin ATPase to total energy turnover (5), a functional similarity of ECs to muscular cells may be assumed. In the light of the present data, it seems likely that the previous identification of MM-CK in ECs was confounded by a similar migration pattern of CK and adenylate kinase: in our hands, the putative MM-CK band disappeared when gels were treated with Ap_5A to inhibit adenylate kinase activity. Moreover, the band developed also in the absence of creatine phosphate. Thus the only cytosolic isoform present in pig and rat ECs was BB-CK.

The expression and activity of CK varies between different vascular beds. Both in pig and rat, the BB-CK expression in microvascular ECs by far exceeded the expression in macrovascular ECs derived from the aorta. In fact, the presence of BB-CK in pig aortic ECs was hardly discernible (see Figs. 4 and 5). The different expression correlated with CK activity, which in the pig was sixfold greater in microvascular than in macrovascular ECs (Table 1). The major quantitative differences in CK activity imply differences in energy turnover and cellular function. It is well conceivable that ECs from the coronary circulation, playing a substantial role in the regulation of coronary flow by the formation of various mediators, are characterized by a higher energy turnover or a more fluctuating energy demand than cells from the aorta.

Previous studies did not report the presence of the mitochondrial isoform of CK in ECs of hepatic and myocardial origin (3, 28, 35). Lack of mitochondrial CK

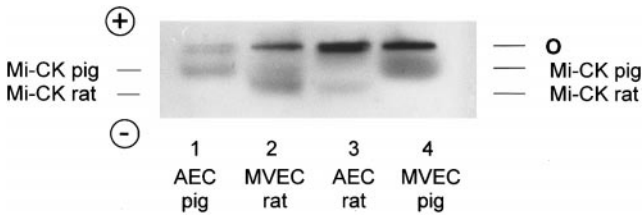

Fig. 6. Mitochondrial CK isoenzymes in enriched mitochondrial preparations of micro- and macrovascular endothelial cells from rat and pig. Cellulose polyacetate electrophoresis was performed for 90 min at 100 V. CK isoenzymes were stained in the presence of 15 μM Ap_5A. *Lane 1*, AECs from pig; *lane 2*, MVECs from rat; *lane 3*, AECs from rat; *lane 4*, MVECs from pig. Note differentially migrating faint bands toward the cathode (−) of pig and rat Mi-CK indicated by lines, whereas adenylate kinase stained next to the origin (O).

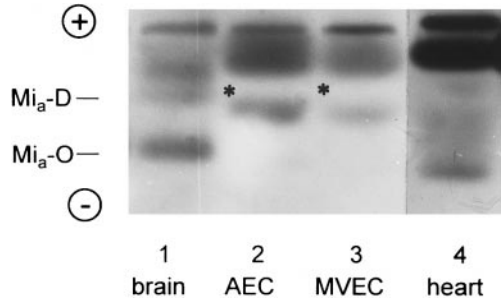

Fig. 7. Comparison between mitochondrial brain-type and heart muscle-type CK isoenzymes from pig. Cellulose polyacetate electrophoresis was performed for 90 min at 100 V. *Lane 1*, mitochondrial extract of pig brain; *lane 2*, AECs; *lane 3*, MVECs; *lane 4*, mitochondrial extract of pig heart muscle. Note that the weak cathodic bands of AECs and MVECs in *lanes 2* and *3* (asterisks) most closely correspond to the ubiquitous Mi_a-CK dimer (Mi_a-D). Mi_a-O, Mi_a-CK octamer.

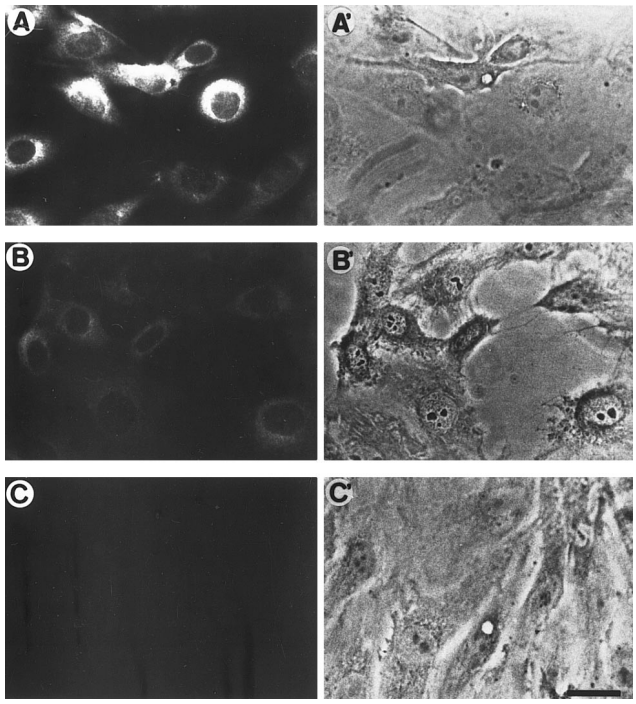

Fig. 8. Immunofluorescent staining of CK isoenzymes in porcine aortic ECs. Cultured ECs from pig aorta were stained with polyclonal rabbit anti-chicken B-CK antibody (*A, A′*), anti-ubiquitous mitochondrial CK antibody (Mi_a-CK; *B, B′*), and control serum (*C, C′*), followed by rhodamine-conjugated secondary antibody. Note the significant labeling by anti-B-CK antibodies throughout the entire cell body but sparing the nuclei (*A*), whereas a faint spotted staining, mostly perinuclear and typical for mitochondria, can be seen with the anti-Mi_a-CK antibody (*B*). No staining is seen in the control. *A, B, C* are immunofluorescence images, and *A′, B′, C′* are the corresponding phase-contrast images. Bar, 20 μm.

would imply that the cytosolic CK could only operate as a temporal energy buffer. In the present work, however, weak bands were detected in both rat and pig ECs, clearly representing mitochondrial CK. Although no definite assignment of the mitochondrial isoform was feasible on the basis of gel electrophoresis, indirect immunofluorescence staining for ubiquitous Mi_a-CK revealed the presence of this mitochondrial isoform in porcine ECs (Fig. 7).

In the liver, the release of BB-CK in reperfusion following ischemia has been taken as a marker of endothelial cell injury (24, 35). Similarly, in the heart, following cardioplegia during valve replacement surgery, substantial differences in the time course of serum BB-CK and MM-CK activity were reported (33), suggesting these isoforms to be released from different compartments. Consistent with the smaller size of the endothelial compartment and the lower CK activity, the arterio-venous difference for BB-CK was much smaller than for MM-CK. A distinct release of BB-CK has also been observed following myocardial infarction (17, 34). However, in contrast to the liver, BB-CK release from the heart is unlikely to be an exclusive marker of endothelial injury. BB-CK is also present in smooth muscle cells (11) and cardiomyocytes, as indicated, e.g., by the faint BB-CK band in Fig. 5*B*. Moreover, it is well known that cardiac hypertrophy is often associated with a fetal shift in CK isoenzyme distribution, i.e., an increased myocardial expression of BB-CK (29). Finally, when comparing BB-CK and MM-CK release following cardioplegia (33), a ratio of 1:10 was observed. Because the endothelial compartment comprises only 3% of the total cardiac volume and endothelial CK activity per mg protein is rather low, it is unlikely that the endothelium contributed up to 10% to total cardiac CK release. This further underlines the notion that BB-CK release into the coronary sinus is not a selective marker of endothelial injury.

CK activity and functional role in ECs. Compared with cardiomyocytes, ECs are characterized by a rather low activity of CK (2.5% of cardiomyocytes). Considering the different cellular functions of ECs and cardiomyocytes, the lower CK activity in ECs may be considered to be paralleled by a lower energy turnover. However, several lines of evidence suggest that the energy turnover of ECs is in the same range as that of cardiomyocytes. In the presence of physiological substrate concentrations, the endothelial oxygen consumption was reported to be 8 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ (18), which is about one-third of that of stimulated cardiomyocytes (31). Moreover, when total energy turnover of perifused ECs on microcarrier beads was determined by microcalorimetry, a total heat flux of 230 μW/mg protein was found (5). This is comparable to the total energy turnover of cardiomyocytes, as revealed by the following calculation: cardiac oxygen consumption being ~5 $\mu mol \cdot min^{-1} \cdot g^{-1}$ (110 $\mu l \cdot min^{-1} \cdot g^{-1}$) and the average caloric equivalent being 20 kJ/l O_2 gives an approximate energy turnover of 2.2 $J \cdot min^{-1} \cdot g^{-1}$ (37 $mJ \cdot s^{-1} \cdot g^{-1}$). Assuming a protein content of 160 mg/g wet weight, an energy turnover of 231 $mJ \cdot s^{-1} \cdot mg\ protein^{-1}$ can be estimated, virtually identical to the total heat flux of ECs. Therefore, it is not only the total CK activity but also the ratio of CK activity to energy turnover that is rather low in ECs. This, together with the low CK turnover, made it impossible to use ^{31}P NMR magnetization transfer to measure the CK flux in perifused endothelial cell (unpublished results).

These data indicate distinct differences in the role of CK in ECs and cardiomyocytes. In the heart (representing mainly cardiomyocytes), total CK turnover exceeds the rate of oxidative phosphorylation (ADP → ATP) by approximately 5- to 10-fold (19) and may thus play a significant role in the energy transduction process linking mitochondria to sites of ATP consumption. In the endothelium with a low CK activity, CK turnover may be estimated to be 1/40 of that of cardiomyocytes, assuming no differences in substrate concentrations. Because there are no major differences in energy turnover (see above), CK turnover appears to be well below the rate of ATP formation in ECs, suggesting only a minor role in energy transfer. However, under conditions of a sudden decrease in energy supply, the dephosphorylation of PCr catalyzed by CK may represent an important energy reserve. Evidence for this latter function is demonstrated in Fig. 3. During 2 h of

substrate deprivation, PCr was almost completely broken down, while ATP content remained unchanged. However, the constant ATP concentration can only be partially explained by rephosphorylation of ADP via CK. Due to the high energy turnover of ECs (5), there is a high turnover of the ATP pool as well, suggesting that an intracellular energy reserve in the form of a phosphorylated compound alone cannot maintain a constant ATP level during 2 h of reduced energy supply. In fact, in a recent study it was demonstrated that complete substrate deprivation results in a controlled downregulation of diverse cellular energy-consuming processes (4).

As demonstrated, ECs are indeed able to take up creatine from the perifusion medium, thus possessing a creatine transporter, as described previously for muscle cells (8, 9). Because, in vivo, creatine is taken up from the blood into muscle cells, ECs will contribute to this process. The creatine transporter is a Na^+-Cl^- cotransporter, most likely coupled to the Na^+-K^+-ATPase (20). Thus, besides being involved in the energetics of ion transport and homeostasis (37), an additional function for the CK system could be proposed in conjunction with the energetics of creatine uptake, whereby CK would form a tight functional compartment with the Na^+-K^+-ATPase (9, 20).

When endothelial energy metabolism was studied in the past, adenine nucleotide content was classically determined in acid extracts of ECs, resulting in ATP/ADP ratios ranging from 7:1 (this study) to 5:1 (18). Studying the energy status of perifused ECs on microcarrier beads by ^{31}P-NMR spectroscopy offers new insights in this respect. Because the γ-ATP signal is at least 10-fold greater than the noise (Fig. 1), two ADP signals (α- and β-ADP) may also be expected. However, in contrast to spectra of acid extracts (data not shown), there is no ADP visible in intact ECs, clearly suggesting that a major portion of ADP is protein bound, possibly to the myosin ATPases. The presence of CK will enable the calculation of thermodynamic indexes like the concentration of free cytosolic ADP, the ATP/ADP ratio, and the free energy of ATP hydrolysis (ΔG_{ATP}) in future studies.

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