JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1993, 44, 4, 333—344

# Original articles

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## REPERFUSION AFTER BRIEF REPETITIVE ISCHEMIA IN PORCINE MYOCARDIUM DOES NOT ALTER EXPRESSION OF CREATINE KINASE MM OR MITOCHONDRIAL ATPase mRNAs[1]

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We examined whether the mRNA expression of creatine kinase MM isozyme (CKMM) and mitochondrial F1-ATPase, the key ezymes of intracellular energy transduction, are altered in porcine myocardium subjected to repeated brief periods of ischemia followed by reperfusion. The left anterior descending coronary artery was occluded for two cycles of 10 min with 30 min reperfusion in between, followed by the reperfusion up to 210 min. Systolic wall thickening was significantly decreased at 30 min reperfusion after both occlusions and remained depressed during reperfusion. In Northern blot analysis 1.5 kb CKMM and 1.9 F1-ATPase mRNA species were detected in sham, nonischemic and ischemic myocardial tissues. Densitometric analysis of signals showed a 30% decrease of the CKMM mRNA expression ($p < 0.05$ as compared to nonischemic area of the same heart and sham operated animals) only during the first period of ischemia. Reperfusion as well as the subsequent period of ischemia did not alter expression of CKMM mRNA. The expression of F1-ATPase mRNA remained unchanged during ischemia and reperfusion. We conclude that reperfusion after brief myocardial ischemia in swine is not associated with changes in CKMM and F1-ATPase mRNA expression. Our findings would support the hypothesis that myocardial stunning is not caused by altered expression of energy transducing enzymes.

Key words: *myocardial ischemia and reperfusion; gene expression; creatine kinase; mitochondrial F1-ATPase; myocardial stunning; ischemic preconditioning*

[1] This work was presented in part at Satellite Symposium of the XIV World Congress of the International Society for Heart Research "Preconditioning, Stunning and Concepts of Cardioprotection" in Sapporo, Japan (J Mol Cardiol 24 Suppl II: P-2, 1992)

## INTRODUCTION

We have previously shown that two periods of 10 min coronary artery occlusion separated by 30 min of reperfusion not only results in protection against irreversible damage during a subsequent 60 min occlusion (preconditioning) but also leads to prolonged dysfunction of the postischemic myocardium in the absence of necrosis (stunning), (1–3). The mechanism of myocardial stunning is still incompletely understood but several authors have suggested that an altered calcium homeostasis is involved (for recent reviews see 4–7), while oxidative phosphorylation has been found to be intact (8). In support of a normal energy state of stunned myocardium we observed a normal energy charge and elevated levels of creatine phosphate after two cycles of 10 min coronary artery occlusions and 30 min reperfusion (2). In addition, we noted that stimulation of stunned myocardium with dobutamine increased regional myocardial contractile function with an increase in lactate consumption (9). These findings suggest that performance of the key enzymes for energy production and transfer to the myofibrills, mitochondrial ATPase and cytosolic creatine kinase, are maintained in stunned myocardium. If this were true, the levels of mRNA encoding these enzymes would be expected to be unchanged. To test this hypothesis we investigated the expression of mRNA encoding cytosolic creatine kinase MM isozyme and mitochondrial F1-ATPase during brief repetitive coronary artery occlusions followed by reperfusion and showed unaltered tissue levels of mRNA encoding both enzymes during myocardial stunning.

## MATERIALS AND METHODS

### *Experimental model*

All experiments were performed in accordance with the Guiding Principles in the Care and Use of Animals as approved by the council of the American Physiological Society and under regulations of the Animal Care Committee of the Erasmus University Rotterdam, The Netherlands. Cross-bred Landrace x Yorkshire pigs (H. V. C. Hedel, The Netherlands) of eiher sex (n = 41, 22–40 kg) were fasted overnight and sedated with an intramuscular injection of 2–3 mg/kg azaperone ("Stresnil" Janssen Pharmaceutica, Beerse, Belgium), anesthetized with an intravenous injection of 20 mg/kg sodium pentobarbital ("Norvasc", B. V., Arnhem, The Netherlands), and intubated for artificial ventilation. Fluid filled catheters (7F) were positioned in the superior caval vein for infusion of 15 mg/kg/h sodium pentobarbital and administration of 4 mg of the muscle relaxant pancuronium bromide ("Pavulon", Organon Teknika B. V., Boxtel, The Netherlands) prior to thoracotomy. A 7F Sensodyn micromanometer-tipped catheter (B. Braun Medical B. V., Uden, The Netherlands) was inserted into the left ventricle through the left carotid artery, to measure left ventricular blood pressue and its first derivative (LVdP/dt). An 8F catheter was inserted into the aorta, to record the central aortic blood pressure (50 AD presure transducer, Spectramed, Bilthoven, The Netherlands). Following a midsternal thoracotomy the

left anterior descending coronary artery was dissected free just distal from its first diagonal branch for the placement of the arterial clamp. Regional myocardial function was estimated from recordings of myocardial wall thickness obtained with sonomicrometry (Triton Model 20, Triton Technology, San Diego, Ca, USA). Two pairs of ultrasonic crystals were implanted, one pair in the area perfused by the left anterior descending coronary artery (LAD) and another pair in the area perfused by the left circumflex coronary artery (LCX). Of each pair, one crystal (4 mm diameter) attached to a dacron patch was sutured onto the epicardial surface, while other crystal (2 mm diameter) was inserted tangentially through the myocardium and positioned in the subendocardial layer underneath the epicardial crystal. The correct positionof the crystals was confirmed at the end of each experiment. From the stripchart recording, the end-diastolic (EDT) and end-systolic (EST) wall thickness were measured (10) and systolic wall thickening (SWT) was calculated as: SWT (%) = $100 \times (EST-EDT)/EDT$.

## *Experimental protocol*

After a stabilization period of at least 30 min, following completion of the instrumentation, baseline measurements of systemic hemodynamics and regional myocardial wall thickness were obtained. Subsequently, the LAD was completely occluded just distal to its first diagonal branch for 10 min followed by 30 min of reperfusion. Then the LAD was again occluded for 10 min and reperfused for either 30 min, 120 min, or 210 min. Systemic hemodynamics and myocardial wall thickness were recorded throughout the experimental protocol. At each of these timepoints experiments were terminated in a number of pigs and tissue from the LAD and LCX regions from the same heart were excised, washed free of blood in 0.9% NaCl, quickly cut into small pieces (100 mg each), snap-frozen in liquid nitrogen and stored at $-80°C$ until analysis. In eleven sham-operated animals, effects of the surgical procedure on the induction of gene expression in the areas perfused by the LAD and LCX were assessed at baseline ($n = 3$) and at timepoints corresponding to 30 min ($n = 5$) and 210 min ($n = 3$) of the final reperfusion period. Since ventricular fibrillation occured in five pigs during reperfusion following the first period of LAD occlusion, we also assessed the effect of a single direct current countershock on gene expression in three additional sham operated animals. In these animals ventricular fibrillation was induced by placing two electrodes attached to a 6 V battery onto the myocardium (at timepoint corresponding to early reperfusion after first period of ischemia), which was followed by defibrillation within 20 seconds. In these animals myocardial specimen were taken at timepoint corresponding to 30 min of the final reperfusion period.

## *RNA isolation and Northern blot analysis*

Total RNA was isolated according to the method of Chomczynski and Sacchi (11). Samples of total RNA (15 µg) were electrophoresed in 1% agarose gels, vacuum-transfered to Hybond-N membranes and fixed by UV-light using a Stratalinker (model 1800, Stratagene, Heidelberg, FRG). Membranes were prehybridized in hybridization buffer (50% deoinized formamide, 1 M NaCl, 0.2% polyvinyl-pyrollidone, 0.2% ficoll, 50 mM Tris-Cl, pH 7.5, 0.1% $Na_4P_2O_7$, 1% SDS, 10% dextransulfate, 250 µg/ml denatured salmon sperm DNA) at 42°C for 4 hour. Thereafter the hybridization bufer was changed and the labelled cDNA probe was added ($1 \times 10^6$cpm/ml). The cDNA probes were labelled in vitro with Klenow fragment after priming with random hexamers using $^{32}$P-dCTP (3000 Ci/mmol, Amersham-Buchler, Braunschweig). The CKMM cDNA probe was a 1160 bp HindIII/EcoRI insert encoding human CKMM, and the F-1ATPase cDNA probe was a 1.8 kb EcoR1 insert encoding human F1-ATPase, beta subunit (both purchased from American Type Tissue Collection, Rockville, USA). As a standard we used

a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe of 780 bp cDNA encoding the human gene (12). A 1.9 kb BamH1 insert encoding human β-actin was used as an additional standard (13). After hybridization for 16 h membranes were washed under stringent conditions and exposed to X-OMAT AR film (Kodak) at −80°C for 6 to 24 hours. After autoradiography, the hybridization signals were quantitated by densitometry (Elsprit 400, Hirschmann Unterhaching, FRG, absorbance vallues at 546 nm). The filters were probed with GAPDH before the first and after the last hybridization. Induction of genes was expressed as fold induction of mRNA signal (±SEM) in the ischemic, experimental (E) area, and compared to the fold induction in the nonischemic, control (C), area. For normalisation, the data of each hybridization signal were divided by the absorption of the matching GAPDH and β-actin mRNA expression signals.

### *Satistical analysis*

Data are presented as mean±SEM. Statistical significance was assessed by analysis of variance for repeated measures followed by paired t-test with Bonferroni correction for multiple comparisons. Significance was declared at $p<0.05$.

## RESULTS

### *Systemic hemodynamics*

During the first LAD occlusion there were decreases in mean arterial blood pressure from 81(2) mmHg to 74(2) mmHg ($p<0.05$) and left ventricular $dP/dt_{max}$ from 2280 (130) $mmHgs^{-1}$ to 1520 (110) $mmHgs^{-1}$ ($p<0.05$), an increase in left ventricular end-diastolic pressure from 13 (1) mmHg to 18(1) mmHg ($p<0.05$), while heart rate did not change from its baseline value of 92(3) $beatsmin^{-1}$. Left ventricular $dP/dt_{max}$ remained depressed but the other variables returned to values that were not different from baseline (*Table 1*). During the second occlusion the hemodynamic changes were very similar to those observed during the first occlusion. During the second reperfusion, $LVdP/dt_{max}$ remained depressed while the other variables again recovered. In the sham-operated animals systemic hemodynamics remained stable during the experimental protocol (not shown).

### *Systolic wall thickening*

Occlusion of the LAD caused a complete loss of systolic wall thickening of the myocardium perfused by the LAD (*Table 1*). During subsequent reperfusion, there was a recovery to 50% of its baseline value. A similar loss and recovery of wall function was observed during the second cycle of occlusion and reperfusion. In the control area perfused by the LCX systolic wall thickening did not change significantly over the course of the experiments, although it tended to increase during the LAD occlusions. In the sham-operated animals systolic wall thickening in both the LAD and LCX areas did not change during the experimental protocol (not shown).

*Table 1.* Systemic hemodynamics and regional myocardial wall thickening in anesthetized open-chest pigs during two periods of 10 min LAD occlusions separated by 30 min of reperfusion

| | Baseline n = 35 | 10′ OCC1 n = 27 | 30′ REP1 n = 23 | 10′ OCC2 n = 18 | 30′ REP2 n = 12 | 120′ REP2 n = 8 | 210′ REP2 n = 4 |
|---|---|---|---|---|---|---|---|
| Heart rate (beats/min) | 92 (3) | 97 (3) | 91 (5) | 96 (5) | 92 (4) | 97 (7) | 91 (4) |
| Mean aortic pressure (mmHg) | 81 (2) | 74 (2) * | 78 (2) | 70 (3) * | 77 (2) | 73 (3) | 86 (3) |
| LVEDP (mmHg) | 13 (1) | 18 (1) | 14 (1) | 16 (1) | 14( 1) | 12 (4) | 12 (2) |
| LVdP/dtmax (mmHg/s) | 2280 (125) | 1520 (110) * | 1800 (94) * | 1430 (119) * | 1540 (101) * | 1560 (2107) * | 1650 (134) * |
| SWT LAD (%) | 33 (1) * | - 2(1) * | 15 (2) * | -1(2) * | 12 (2) * | 17 (3) * | 97 (3) * |
| SWT LCX (%) | 29(2) | 34(2) | 30(2) | 33(3) | 34(2) | 39(1) | 37(4) |

Data are presented as mean (±SEM); *$p<0,05$ versus Baseline; n = number of experimental observations; OCC = occlusion; Rep = reperfusion; LVEDP = left ventricular end-diastolic pressure; SWT LAD and SWT LCX = systolic wall thickenning in left anterior descending coronary artery and left circumflex coronary artery perfusion areas, respectively, calculated as described in Materials and methods.

*mRNA expression of creatine kinase MM isozyme and mitochondrial ATPase*

Northern blot analysis revealed dominant bands of 1.5 kb creatine kinase MM isozyme (CKMM), 1.4 kb glyceraldehyde- 3-phosphate dehydrogenase (GAPDH), 1.9 kb mitochondrial F1-ATPase, and 1.8 kb β-actin mRNA in the control and the experimental area at all timepoints, which are illustrated by the representative examples in *Fig. 1.* Densitometric analysis showed that at

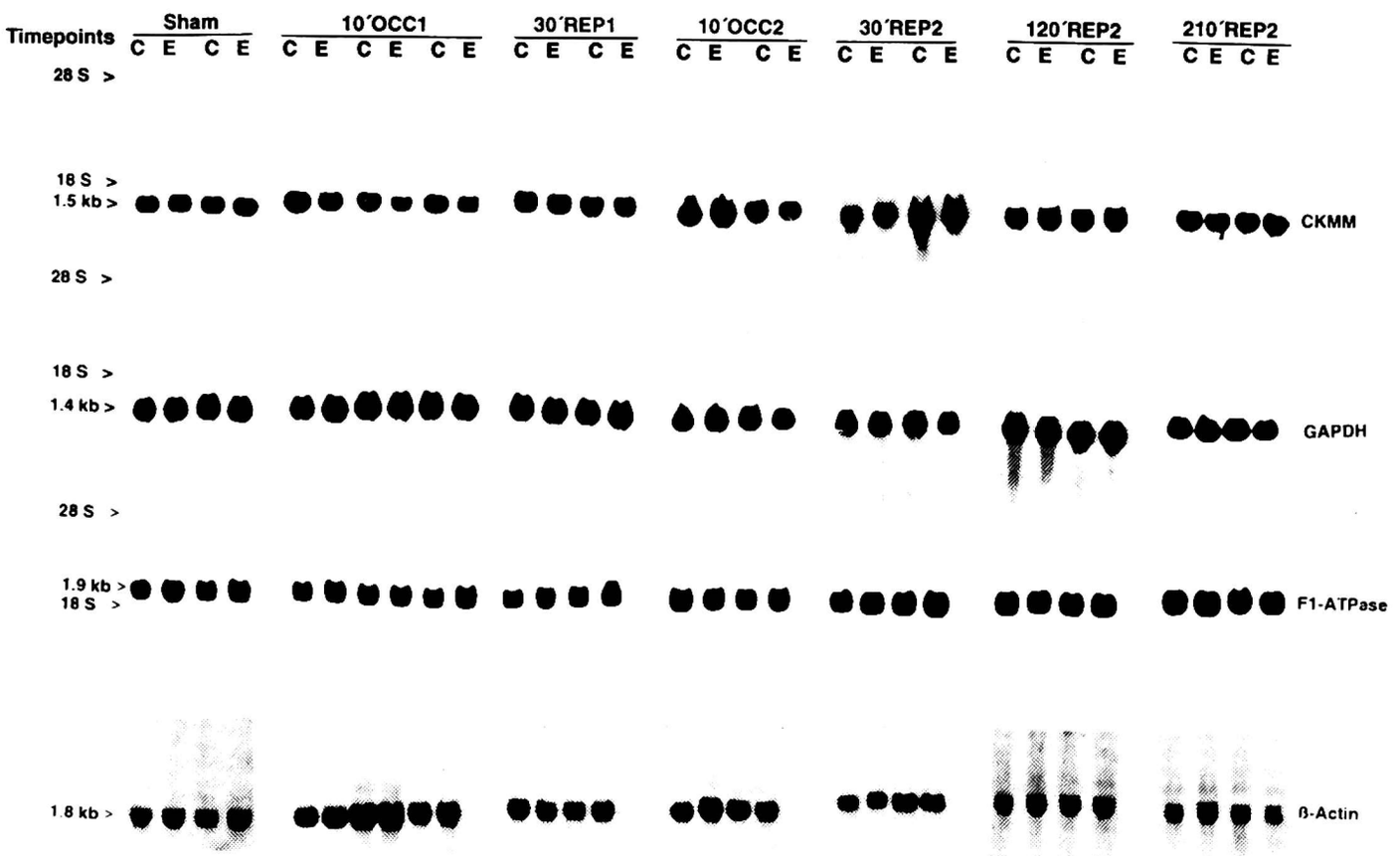


*Fig. 1.* Northern blot analysis showing representative examples of CKMM and F1-ATPase mRNA expression in normally perfused, control (C) and ischemic, experimental (E) myocardial tissues taken from the same heart at different experimental timepoints. Additionally, two sham-operated animals are presented. For control purposes, the blots were also hybridized to the glyceraldehyde-3-dehydrogenase (GAPDH) and β-actin cDNA probes. The position of 18S and 28S RNAs are indicated as size markers for each probe. OCC = occlusion; REP = reperfusion.

baseline and in sham operated animals the levels of mRNA encoding CKMM in the experimental area were not different from the control area (*Fig. 2,* panel A), but after 10 min of LAD occlusion levels in the experimental area were 30% lower than in the control area ($p<0.01$). This difference was not longer present at the end of the first 30 min reperfusion period and was also not observed at the end of the second occlusion and subsequent reperfusion period. Levels of CKMM mRNA expression in experimental as well as in control areas were not altered by the fibrillation-defibrillation procedure (not shown).

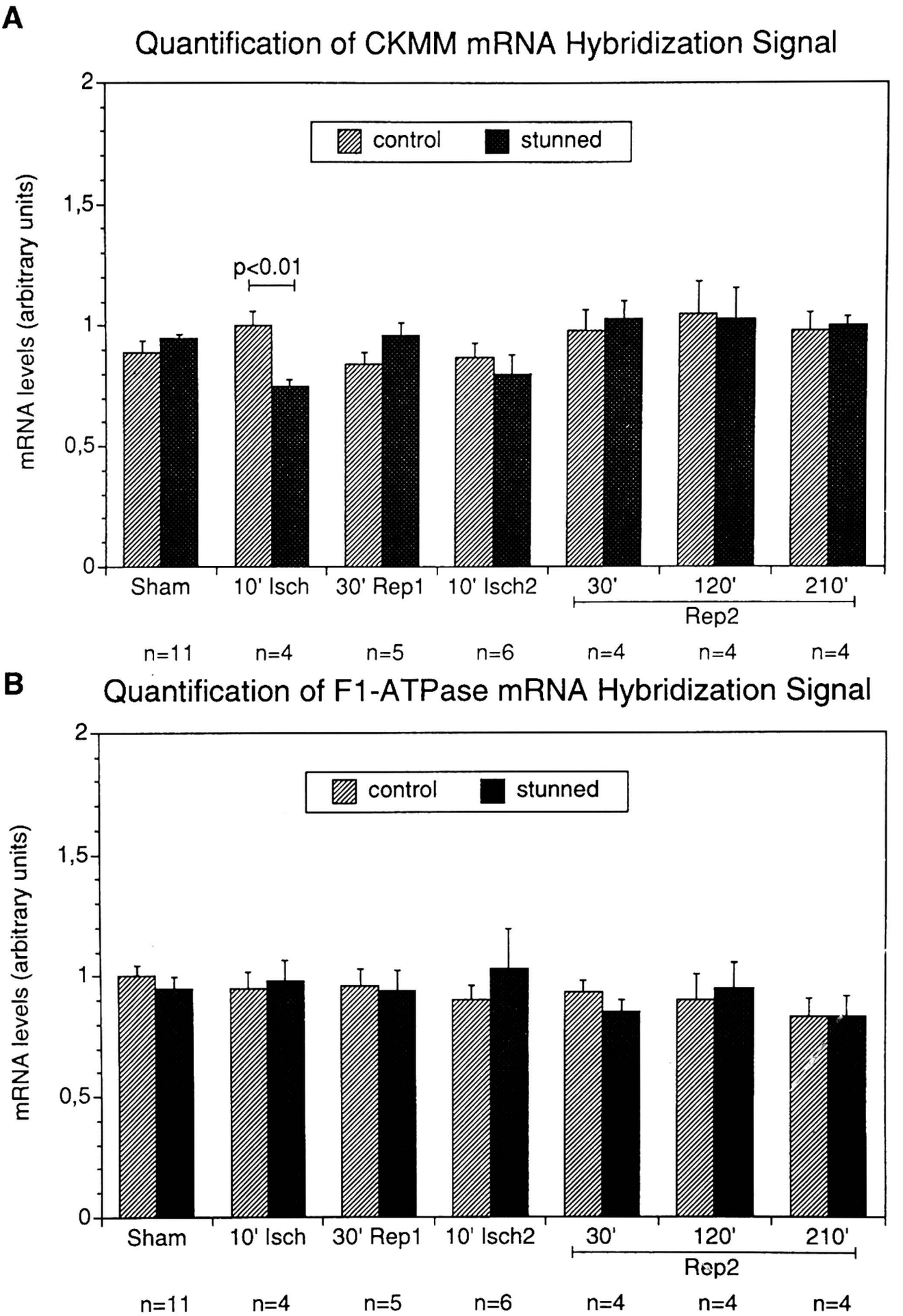


*Fig. 2.* Bar graphs showing densitometric analysis of CKMM (Panel A) and F1-ATPase (Panel B) mRNAs expression in control and stunned myocardial tissues. Densitometric values were corrected with GAPDH expression and presented in columns (mean ± SEM) as a fold induction of mRNA expression in control and stunned areas of the same heart. Data collected from sham operated animals at the three timepoints (baseline, 30' Rep2, 210' Rep2) did not differ, and were combined as sham. Isch = ischemia; Rep = reperfusion; n = number of experimental animals.

At baseline, expression of F1-ATPase mRNA in the experimental area was not different from that in the control area. Levels of mRNA in both areas remained unchanged in sham-operated animals and were not affected by the occlusion-reperfusion protocol (*Fig. 2*, panel B), and were also not altered by the fibrillation-defibrillation procedure (not shown).

## DISCUSSION

The major finding of our study is that the mRNA expression of two important enzymes of intracellular energy transduction, creatine kinase MM isozyme and mitochondrial ATPase, remain unchanged during reperfusion after repetitive brief ischemia in porcine myocardium.

### *Effect of short repetitive ischemia and reperfusion on the expression of creatine kinase MM mRNA*

Among the various isoenzymes of creatine kinase, the cytoplasmic creatine kinase MM isoezyme (CKMM) is most abundant in mature heart tissue (14). It is also bound to the myofibrills and uses phosphocreatine (PCr) to regenerate ATP from ADP at sites of energy utilization (15).

Ischemic intervals of short duration cause a reversible sarcolemmal leakage and macromolecular egress through the cell membrane (16, 17). An example of this is the release of creatine kinase from stunned myocardium (18, 19). In fact, Greenfield and Swain (20) have argued that creatine kinase dissociates from myofibrills and is therefore a cause for stunning. This dissociation and release of creatine kinase from cells could be a cause for increased production of new transcript encoding the lost protein. However, in our experimental model, the mRNA expression of CKMM in the postischemic myocardium was unchanged, a finding, which could indicate the absence of cellular injury. During the first period of ischemia CKMM mRNA expression was depressed, which is consistent with earlier findings in dogs (21) and could reflect attenuated transcription of mRNA in response to ischemic stress. During the second occlusion, we did not observe changes in CKMM mRNA expression, what suggests normal function of the transcription machinery during subsequent period of ischemia. Consistent with this findings could be the observation that mammalian cells *in vitro* previously exposed to a brief heat shock, recover protein synthesis after a subsequent heat shock more quickly than cells after only one hyperthermic insult (22).

*Effect of ischemia and reperfusion on the expression of mitochondrial F1-ATPase mRNA*

According to the chemiosmotic hypothesis, the electron transfer system in mitochondria is functionally linked to an ATP-synthesizing system, in which mitochondrial ATPase (F1-ATPase complex) reversibly phosphorylate ADP to ATP (23, 24). The complicated structure of this enzyme has been recently clarified and many cDNAs have been cloned and sequenced, thus allowing for molecular biological approaches to be used for study of its expression and regulation (25). The cDNA insert used here is derived from the catalytic β-subunit of the F1 portion of the enzyme (26).

It is not known whether mitochondrial ATPase mRNA expression changes during stunning, a phenomenon known to cause long term depression of steady state levels of tissue ATP, which recover very slowly (27, 28). In our experimental model the expression of ATPase mRNA remained unchanged during ischemia as well as in the postischemic, stunned and preconditioned myocardium.

*Critique of methodology and explanations*

The method we used to study gene expression that is based on the measurement of steady state levels of mRNA in tissue has some drawbacks. First, we do not know whether measured values express the increased rate of transcription or reflect the stability of mRNA. However, regulation of gene expression in the heart usually takes place at the transcriptional level (29), and because the isolated porcine mRNA appears to be stable and not degraded we like to conclude that we are measuring changes in gene transcription. Secondly, we assume that the standards for quantification of mRNA expression (here GAPDH and β-actin mRNA expression) do not change under our experimental conditions. The GAPDH mRNA expression has been used as a standard by other groups studying gene expression in myocardial stunning (30) and end-stage heart failure (31), and remained unchanged in these studies. In addition, expression of mRNAs encoding contractile proteins such as myosin (Frass et al. unpublished) and β-actin (shown here) remained unchanged in our experimental model. A third problem is the amount of mRNA, which could be washed out during stripping of radioactivity from the filter. In our hands the reprobed GAPDH mRNA signal on the filter, which was previously hybridized with CKMM and F1-ATPase cDNAs, was slightly (5% as compared to the initial GAPDH hybridization signal) and uniformly reduced, and thus has no influence on the calculated values of mRNA expression.

## CONCLUSIONS

Our group has recently shown that gene expression is markedly changed in stunned myocardium (1, 7, 32, 33) but the implications of these changes are still unclear. We hypothesize that myocardial stunning results in the reversile injury of intracellular proteins (misfolding, phosphorylation or changes in conformational state), which must either be replaced by new protein synthesis or repaired in order for normal function to recover. In the present study we did not observe any changes in the expression of creatine kinase MM or mitochondrial F1-ATPase during reperfusion after repetitive brief ischemia in porcine myocardium. The lack of changes in expression of creatine kinase could indicate that the hypothetical damage occuring during stunning does not occur at the cellular level but rather at the subcellular or molecular level. The targets of this injury are at thus far unknown, but possible candidates include the intracellular calcium binding proteins (33, 34).

Unchanged expression of mitochondrial ATPase mRNA would support the hypothesis of an intact energy state of the postischemic, stunned myocardium (35). It also indicates that well known changes in tissue levels of high energy phosphates during ischemia and reperfusion, are not associated with the changes of F1-ATPase mRNA expression. We conclude from the present study that ischemia/reperfusion injury resulting in the contractile dysfunction observed in stunned myocardium is not associated with changes in the expression of creatine kinase MM and mitochondrial F1-ATPase mRNAs. This suggests unaltered expression of other "housekeeping" enzymes in stunned porcine myocardium and might support the hypothesis that stunned myocardium is not energy deficient.

*Aknowlegement:* We thank Dr Cynthia Jeck for helpful discussions and critical reading of the manuscript, Elfriede Neubauer and Claudia Ulmann for technical assistance, Annemarie Möbs and Gerd Stämmler for help with the graphic presentation and statistical analysis. Dr. J. Andres was supported by Alexander von Humboldt Foundation throughout this study.

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Received: June 3, 1993
Accepted: August 20, 1993

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