Enhanced gene expression of calcium regulatory proteins in stunned porcine myocardium

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Objective: Increasing evidence points to a molecular disturbance of Ca^{2+} homeostasis in stunned myocardium. The aim of this study was therefore to investigate the expression of mRNAs for Ca^{2+} binding proteins related to the sarcoplasmic reticulum in a porcine model of myocardial stunning. **Methods:** In 22 anaesthetised pigs, stunning was achieved by one or two cycles of 10 min left anterior descending coronary artery occlusion and reperfusion. Hearts were excised at various timepoints of the protocol. Total RNA was extracted from stunned (experimental) as well as normally perfused (control) myocardium. **Results:** Northern blot analysis using radioactive cDNA probes revealed that the Ca^{2+}-ATPase mRNA levels increased 1.6-fold compared to the control value at 90 min of the second reperfusion. The steady state level of phospholamban mRNA rose 2.5-fold at 180 min of reperfusion. A 2.3-fold increase in calsequestrin mRNAs was observed after 90 min of the second reperfusion. The calmodulin and α,β myosin heavy chain mRNA levels were unchanged. A glyceraldehyde-3-phosphate dehydrogenase cDNA probe served as a reference system. Nuclear run-on assays showed increased transcription for Ca^{2+}-ATPase and calsequestrin at 90 min of reperfusion, supporting the view that increased mRNA levels seen with northern hybridisation were due to increased transcription of the respective gene. **Conclusions:** The results suggest specific repair mechanisms of stunned myocardium and point to the involvement of calcium regulatory proteins related to the sarcoplasmic reticulum in the pathogenesis of myocardial stunning.
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Myocardial stunning has been defined as a long lasting (hours to days) contractile dysfunction in the absence of cell necrosis, following brief coronary occlusions.[1 2] The relatively long time necessary for restoration of contractile function (several hours to several days) suggests the existence of molecular damage and subsequent repair processes on the transcriptional and translational level. The molecular mechanisms involved in the pathogenesis of stunning are incompletely understood but alterations in calcium homeostasis have been implicated, since it was found by Ito *et al* that intracoronary calcium infusions restore contractility.[3] Two contrasting hypotheses were put forward: the first suggests that decreased Ca^{2+} sensitivity of the contractile apparatus causes stunning,[4] and the second implies partial failure of normal calcium cycling at the level of the sarcoplasmic reticulum.[5]

Three predominant proteins of the cardiac sarcoplasmic reticulum have been included in this study. The sarcoplasmic reticular Ca^{2+}-ATPase plays a substantial role in the diastolic Ca^{2+} sequestration, lowering the high intracellular levels of Ca^{2+} present during systole to submicromolar levels.[6] Phospholamban, an integral protein of the sarcoplasmic reticular membrane, regulates Ca^{2+}-ATPase activity.[7] Calsequestrin, anchored inside the junctional sarcoplasmic reticulum, binds Ca^{2+} with high capacity.[8] In hearts hypertrophied by pressure overload a reduction of the number of functionally active Ca^{2+}-ATPase molecules was accompanied by a decrease in Ca^{2+}-ATPase and phospholamban mRNA under *chronically* altered conditions.[9 10] Rohrer and Dillmann showed that the adaptation of the heart to differing thyroid hormone levels results in *acute* changes in Ca^{2+}-ATPase mRNA.[11]

Myocardial stunning has not yet been investigated by employing a molecular biological approach to the study of the adaptive repair process in reperfusion. A study of the expression of Ca^{2+} binding protein genes may also help to provide a better understanding of the aetiology of stunning. An increase in a specific mRNA may indicate repair of the respective protein damaged during the brief ischaemia. Beside sarcoplasmic Ca^{2+}-ATPase, phospholamban, and calsequestrin, calmodulin was included as the major intracellular Ca^{2+} receptor.[12] We also studied the expression of the myosin heavy chain gene in order to evaluate the alterations in contractile protein expression.

Methods

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 (DHEW Publication No (NIH) 80-23, 1980) and under regulations of the animal care committee of the Erasmus University Rotterdam.

Tissue Preparation

Animals were prepared as previously described.[13 14] Briefly, 22 cross breed Landrace x Yorkshire pigs weighing 22-40 kg were sedated and anaesthetised with 20 $mg\cdot kg^{-1}$ pentobarbitone intravenously. Animals were mechanically ventilated. The heart was exposed through a midline thoracotomy. The left anterior descending coronary artery was gently dissected free for later placement of an arterial clamp distal to its first diagonal branch. Myocardial function in the experimental area (E), as well as in the control area (C) perfused by the left circumflex coronary

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artery, was recorded by measuring myocardial wall thickness with sonomicrometry. The experimental design is depicted in fig 1. After a stabilisation period of 30 min the pigs were subjected to one or two 10 min occlusions each followed by 30 min reperfusion periods. The second reperfusion was also extended by an additional 90, 180, or 360 min. As shown in fig 1, the pigs were killed at various timepoints of the protocol, hearts were removed, and approximately 3-4 g of tissue from the ischaemic left anterior descending coronary artery region (experimental) and from the circumflex coronary artery region (control) were excised transmurally. The tissue was frozen separately in liquid nitrogen and stored at –70°C until further processing. Six sham operated pigs were handled as described above but without coronary occlusion; their hearts were excised after 80 or 440 minutes of normal perfusion (fig 1).

Preparation of probes
The hybridisation probes used in this study were 100% homologous to porcine sequences, with the exception of the rat-specific Ca^{2+}-ATPase cDNA probe.[15] The myosin heavy chain cDNA clone containing an insert of 1.6 kb was isolated from an oligo(dT)-primed porcine heart cDNA library.[16] The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe[17] used to rehybridise membranes for reference purposes was purchased from American Type Culture Collection, Bethesda, USA. The hybridisation probes for calsequestrin, phospholamban and calmodulin were prepared using the reverse transcription polymerase chain reaction (RT-PCR).

Reverse transcription polymerase chain reaction and sequencing
The reverse transcription of 2 μg of total RNA template from normal porcine heart (left ventricle) was carried out as described by Kawasaki.[18] For RT-PCR amplification of calmodulin, calsequestrin, and phospholamban the following pairs of sense and antisense oligonucleotide primers were synthesised on a Cyclone DNA synthesiser (Biosearch Inc, USA).
– Calmodulin (human cDNA sequence[19]): sense primer: nucleotides +198 – +127: 5'-AGGAGTTGGGGACAGTGATG-3'; antisense primer: nucleotides +479 – +498: 5'-ATGTCAGCCTCCCTGATCAT-3' (product size: 300 base pairs).
– Calsequestrin (canine cDNA sequence[20]): sense primer: nucleotides +443 – +462: 5'-AAGCTTGCCAAGAAGCTGGG-3'; antisense primer: nucleotides +944 – +963: 5'-GCAAAGGCCACAATGTGGAT-3' (product size: 520 base pairs).
– Phospholamban (porcine cDNA sequence[21]): sense primer: nucleotides +121 – +140: 5'-TCAGCTTTCTCTTGACGGCT-3'; antisense primer: nucleotides +330 – +349: 5'-GGAGATTGT-GGCAGAACTTC-3' (product size: 228 base pairs).

An aliquot of the cDNA products equivalent to 0.5 μg total RNA was amplified as described by Kawasaki.[18] The annealing temperature was 5°C lower than the calculated melting temperature of the primers, ie, 51°C for calmodulin, 55°C for calsequestrin, and 52°C for phospholamban. Asymmetric RT-PCR was employed using a sense and antisense primer ratio of 1:50 for 37 cycles. Asymmetrically amplified single stranded DNA (ssDNA) was purified and sequenced by the dideoxy-chain-termination method using a T_7-sequencing kit (Pharmacia).

Isolation of total RNA and northern hybridisation analysis
Total RNA was isolated by the acid guanidinium isothiocyanate method[22] and 15 μg of total RNA were electrophoresed in formaldehyde on 1% agarose gels.[23] Pairs of total RNA from the C and E region of the same heart were always handled in parallel. The RNA was vacuum blotted to a nylon membrane (Hybond N, Amersham, Braunschweig, Germany) and fixed to it by ultraviolet light. Hybridisation was performed for 16 h under standard conditions[23] with the particular cDNA probe labelled with Klenow fragment after priming with random hexamers by use of [^{32}P]dCTP (Multiprime labelling kit, Amersham-Buchler, Braunschweig, Germany). The hybridised membranes were washed under appropriate conditions of stringency. Membranes were then exposed to X-OMAT AR film (Kodak) at –80°C for 4 to 36 h. The membranes were dehybridised after each hybridisation in boiling 0.01 × standard saline citrate and 0.01% sodium dodecyl sulphate for 20 min, and rehybridised with the radiolabelled GAPDH cDNA probe.

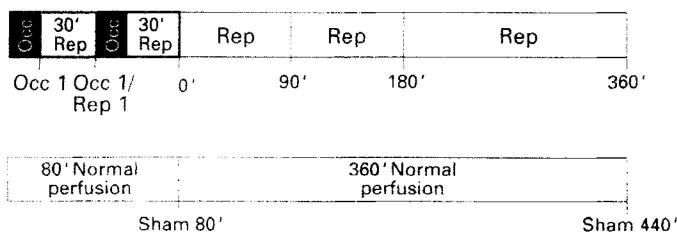

Figure 1 *Protocol for myocardial stunning in pigs. The experimental design is depicted as a temporal axis. Two cycles of left anterior descending coronary artery occlusion (10 min Occ) and reperfusion (30 min Rep) are followed by 90, 180 or 360 min of additional reperfusion. The sham operated pigs were killed after 80 or 440 min of normal perfusion. Sham 80', n = 4; Occ 1, n = 3; Occ 1/Rep 1, n = 4; 0'Rep, n = 2; 90'Rep, n = 3; 180'Rep, n = 3; 360'Rep, n = 1; Sham 440', n = 2.*

Quantitation
The hybridisation signals were quantitated by densitometry (Elscript 400, Hirschmann, Unterhaching, Germany). For each pair of northern blot bands from one heart (E; C) the induction of each gene was expressed by dividing the normalised total absorption from E by the normalised total absorption from C. The values were expressed as percentage of this ratio with SEM. For normalisation, the total absorption of each band was divided by the total absorption of the matching GAPDH signal. Single bands were quantified. Where more than one band was detected the uppermost band was used for quantification after exclusion of differential expression of the multiple bands. Calibration curves for the exposure times of the autoradiography and the amount of total RNA used for northern hybridisation have been performed (data not shown).

Nuclear run-on assays
To evaluate whether the changed mRNA levels detected by northern blotting were due to accelerated transcription, we performed nuclear run-on assays. Nuclei were isolated from the experimental as well as from the control areas of the myocardium originating from the same pig. The isolation of the myocyte nuclei was performed according to the non-enzymatic procedure of Liew *et al*[25] and Boheler *et al*.[26] The nuclear transcription reaction was performed according to the protocol of Cox *et al*[27] and Boheler *et al*.[26] Nascent labelled RNA was isolated.[22] Equal amounts of radioactive RNA from experimental and control tissue were hybridised to the Ca^{2+}-ATPase cDNA and the calsequestrin RT-PCR product blotted onto separate nylon membranes (Hybond N). Hybridisation was carried out for 2-3 d under standard conditions.[23] The filters were washed under appropriate conditions of stringency and exposed to X-OMAT AR films (Kodak) at –70°C for one week. The films were quantitated by densitometry.

Statistical analysis
Myocardial contractile function was assessed by analysis of variance for repeated measures, followed by a paired two tailed *t* test with the Bonferroni correction for multiple comparisons. Expression of calcium regulatory protein genes was assessed by an unpaired two tailed *t* test. Values are expressed as mean(SEM).

Results

Regional contractile function
Within seconds after the first occlusion there was a complete loss of regional systolic wall thickening of the myocardium perfused by the left anterior descending coronary artery [from 34(SEM 7)% to –3(10)%, fig 2] which partially recovered during the first 30 min of reperfusion to 13(9)%. Following the second course of occlusion and reperfusion, recovery of function was retarded by an additional 10%. During the subsequent 180 min and 360 min of reperfusion there was a gradual increase to 19(9)% and 24%. Systolic wall thickening of the control area did not change significantly during the course of the experiments. (For systemic haemodynamics see Brand *et al*.[14])

Reverse transcription polymerase chain reaction
RT-PCR amplification of porcine ventricular RNA with primers specific for phospholamban, calsequestrin, and calmodulin yielded products corresponding to the respective expected size. Asymmetric PCR amplified products of the same size and additional bands of ssDNA. Partial sequencing of the phospholamban ssDNA showed 100% homology with the known porcine sequence of phospholamban.[21] In addition, partial sequencing of calsequestrin and calmodulin ssDNAs and comparison of the sequence to the published

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respective cDNA sequences[19 20] confirmed their specific amplification (data not shown).

Northern blot analyses

Sarcoplasmic Ca^{2+}-ATPase – The 1.2 kb restriction fragment encoding rat cardiac sarcoplasmic Ca^{2+}-ATPase bound to one message just below 28S RNA (4.4 kb), and the hybridisation pattern seen was in good agreement with previously reported data about the expression of the sarcoplasmic Ca^{2+}-ATPase gene 2 in porcine myocardium[28 29] (fig 3). During the first occlusion and after the first reperfusion period only minor differences in the mRNA levels were detectable. The second cycle of occlusion/reperfusion reduced the Ca^{2+}-ATPase mRNA levels by a factor of 0.6-fold of control; however at 90 min of reperfusion the mRNA levels had increased by 1.6-fold (SEM 0.2). After 180 min and 360 min of reperfusion the mRNA levels had decreased again to 1.2-fold (SEM 0.2) and 0.8-fold respectively (table).

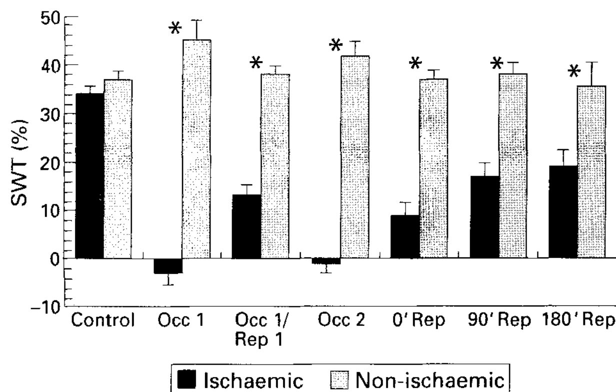

Figure 2 *Systolic wall thickening (SWT) of ischaemic and non-ischaemic myocardium during the cycle described in fig 1. Occ = occlusion; Rep = reperfusion. Columns are means, bars = SEM. Values are percent change from baseline of the same segment.*
$^*p < 0.05$ v ischaemic myocardium.

Calsequestrin – A transcript between the 18S and 28S band was detected, which corresponds to the 2.9 kb message reported earlier in canine myocardium[20] (fig 4). After the first 10 min of occlusion the mRNA content was 1.3-fold *v* control at which level it remained during the first reperfusion period (table); the second occlusion-reperfusion decreased the mRNA levels equivalent to the time course of the preceding mRNAs. At 90 min after the second reperfusion, a peak of 2.3-fold (SEM 0.2) was recorded. mRNA levels returned to below sham levels at 360 min of reperfusion.

Phospholamban – The sequence verified PCR product from the coding region of the porcine cardiac phospholamban bound to two transcripts of 0.9 and 2.8 kb (fig 5) which is in agreement with the reported porcine hybridisation pattern for phospholamban in myocardium.[21] However a variable degree of background was detected by this hybridisation. As depicted in the table, the mRNA levels for phospholamban did not change during the first occlusion period, whereas during the first reperfusion it increased by 1.9-fold (SEM 0.7) in experimental *v* control tissue. The phospholamban mRNA levels decreased after the second course of occlusion/reperfusion. Over 180 min of reperfusion, the mRNA content of stunned myocardium rose gradually [2.1(0.5)-fold and 2.5(1.1)-fold] but had nearly returned to sham levels after 360 min of reperfusion. The mRNA accumulations described could not be verified by statistical analysis.

Calmodulin – Three transcripts for calmodulin were detected (0.8, 1.4, 2.3 kb), the size of which appear to be nearly identical with those reported for human calmodulin isolated from a teratoma cell line (T_1, T_2)[19] (fig 6). Calmodium was expressed with a 1.2-1.3-fold difference of the stunned *v* normally perfused areas throughout the time course (table). Again the quantitation represents the densitometry of the uppermost band and no differential expression of the three transcripts was detected.

Myosin – One major band of approximately 6.5 kb was detected by our myosin cDNA probe (data not shown). No changes in myosin expression throughout the time course were noted.

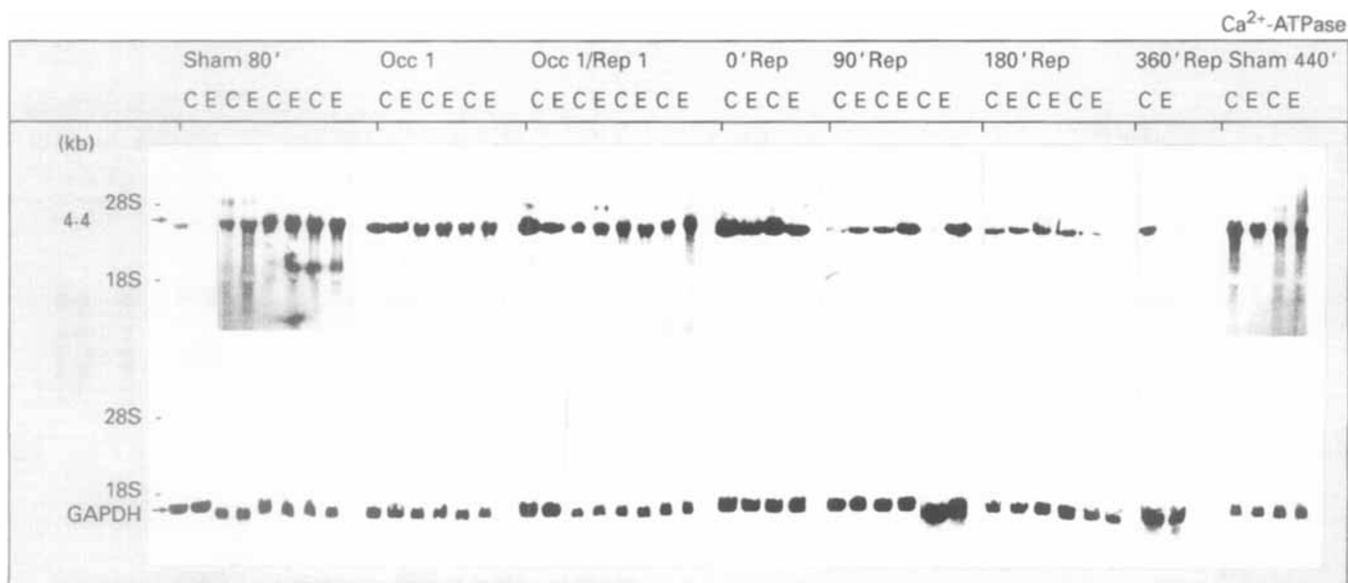

Figure 3 *Northern blot analysis of Ca^{2+}-ATPase. Total RNA from stunned myocardium (E) as well as from normally perfused myocardium (C) was electrophoresed on 1% agarose gels as pairs from one heart, blotted to nylon membranes, and hybridised with radiolabelled hybridisation probes encoding the respective Ca^{2+} regulating protein and GAPDH. Approximate size of the respective band expressed in kilobases (kb). The positions of 18S, 28S, and GAPDH bands are indicated. Occ = occlusion; Rep = reperfusion (see fig 1).*

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Nuclear run on assays
We confirmed our results obtained by northern hybridisation concerning calsequestrin and Ca^{2+}-ATPase employing reproducible nuclear run-on assays, two of which have been shown in fig 7. At 90 min of reperfusion the transcriptional activity for calsequestrin RNA from the experimental region myocardium differed by a factor of 1.6 *v* control tissue. In the case of Ca^{2+}-ATPase, transcription was increased 4.2-fold in the experimental *v* control myocardium at the same time point.

Expression of calcium regulatory protein genes in stunned versus control areas of pig heart. Values are means (SEM).

	n	*Ca-ATPase*	*Phospholamban*	*Calsequestrin*	*Calmodulin*
Sham 80'	4	100(40)	112(39)	84(22)	136(37)
OCC 1	3	93(26)	109(34)	129(12)*	96(12)
OCC 1/REP 1	4	110(19)	186(69)	120(20)	123(14)
OCC 2/REP 2					
0' REP	2	64(1)	137(32)	71(2)	120(30)
90' REP	3	158(20)†	213(47)	235(15)**††	130(6)
180' REP	3	123(16)	247(111)	74(19)	126(11)
360' REP	1	80	150	36	117
Sham 440'	2	86(27)	114(109)	88(23)	96(36)

n = number of experimental observations; OCC = occlusion; REP = reperfusion; sham = sham operated control.
Calcium regulatory protein gene expression was determined by densitometry, and data of the experimental signals were divided by data of their corresponding control signals normalised by the matching glyceraldehyde-3-phosphate dehydrogenase signal (see Methods).
†$p < 0.1$ *v* value for Sham 440'; *$p < 0.1$ *v* Sham 80'; **$p < 0.002$ *v* value for Sham 80'; ††$p < 0.001$ *v* Sham 440'.

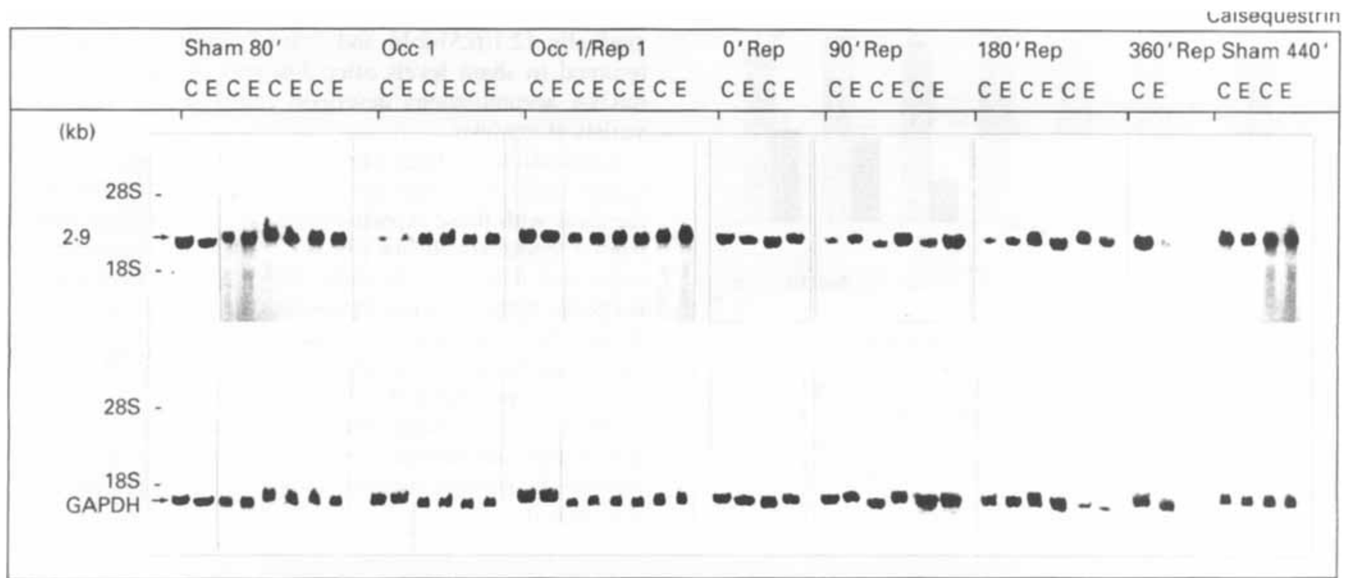

Figure 4 *Northern blot analysis of calsequestrin. For description, see legend to fig 3.*

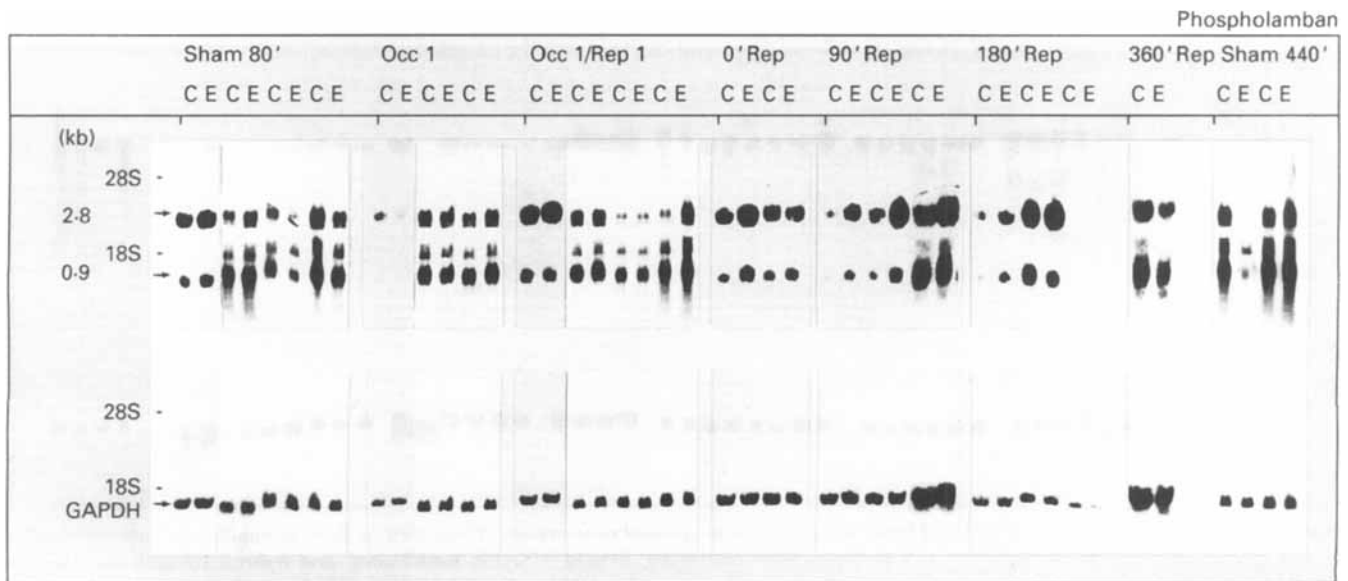

Figure 5 *Northern blot analysis of phospholamban. For description, see legend to fig 3.*

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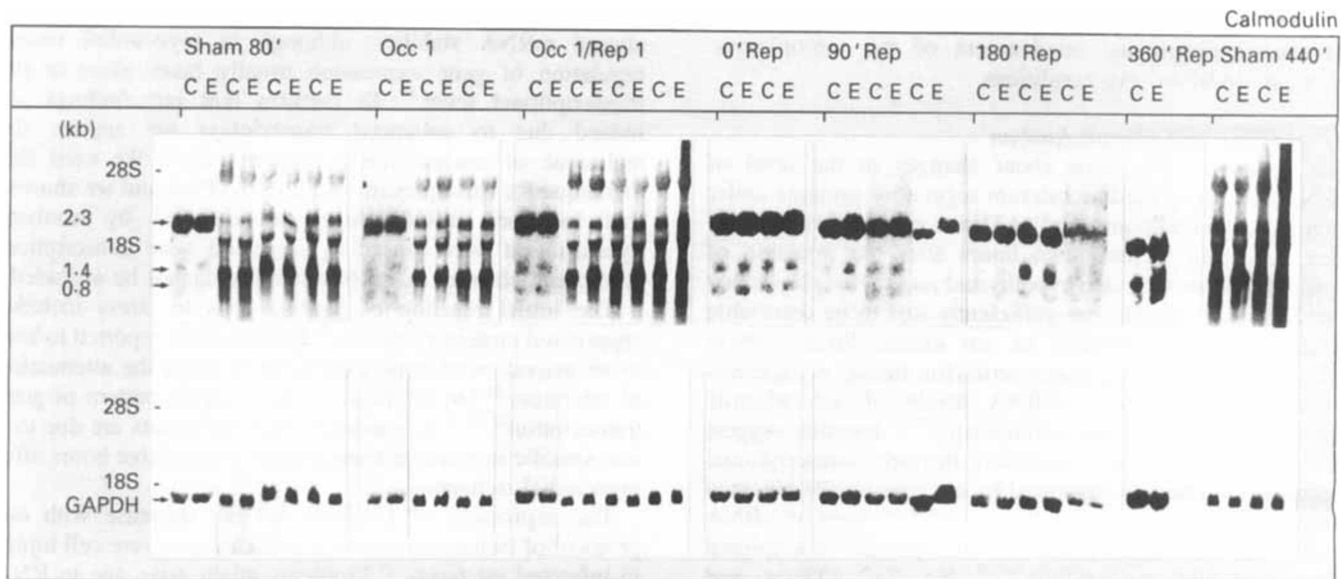

Figure 6 *Northern blot analysis of calmodulin. For description, see legend to fig 3.*

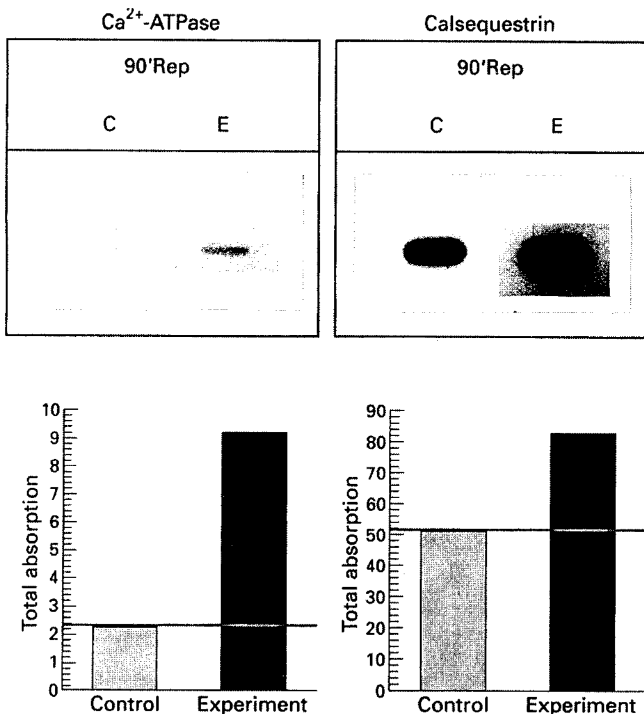

Figure 7 *Nuclear run-on assays of Ca^{2+}-ATPase and calsequestrin. Nuclear run-on assays from experimental and control myocardial tissue were performed using cDNA probes for Ca^{2+}-ATPase and calsequestrin bound to a nylon membrane which were hybridised with radioactivity labelled total RNA obtained from isolated nuclei.*

Discussion

Our main findings are the increased transcription of the calcium regulating protein genes Ca^{2+}-ATPase and calsequestrin during the reperfusion phase following brief coronary occlusions, whereas the transcription of the calmodulin and myosin heavy chain (α and β) genes remained unchanged. Phospholamban mRNA accumulation cannot be verified as statistically significant. This is of interest because several recent studies reported an impaired calcium homeostasis following brief coronary occlusions/reperfusions.[3–5 30–32]

We showed earlier in identical experiments that the heat shock protein genes hsp-70 and ubiquitine and the proto-oncogenes c-*fos*, *Egr-1*, *junB*, and c-*jun* are also upregulated, but the expression of creatine kinase and of c-*myc* remained unchanged,[14 33 34] ie, a specific pattern of gene expression appears following brief coronary occlusions.

Our interpretation of these findings is that molecular damage has occurred which is either repaired (renatured by hsp-70), or completely destroyed (after ubiquitination) by proteolysis, and that the calcium regulating proteins may belong to those damaged by ischaemia/reperfusion. This would agree with functional measurements.[5 30]

We do not know at present whether "damage", "repair", or other ischaemia/reperfusion related factors are triggers for the increased transcriptional activity. We find it noteworthy that the transcriptional control regions of the three genes in question do not exhibit sequence motifs corresponding to CRE (= Ca^{2+} and cAMP response element), ARE (= antioxidant response element), SRE (= serum response element), and AP-1 sites. Furthermore a computer scan revealed no convincing structural homology between the three calcium regulating genes suggestive of a novel response element, although sequence homologies (up to 70%) exist with any two pairs of the three molecules. This would make it rather unlikely that an "ischaemia response element" could be identified unless several different ones exist that produce the described ischaemia/reperfusion induced pattern of gene transcription.

Decreased calcium sensitivity or impaired calcium homeostasis in stunned myocardium?

The contractile failure following brief coronary occlusions could have been caused either by a decrease of the myofibrillar calcium sensitivity[4 35] or by damage to the sarcoplasmic reticulum, decreasing the activation of the contractile proteins.[3 5 30 36] Both hypotheses have been proposed and the controversy is difficult to solve because the models used differ too. Generally, in vitro models of stunning show more damage (tension development is reduced) than in vivo models, where tension development is normal and only shortening is impaired. Phenomena observed in vitro may thus represent a more advanced degree of damage, and both hypotheses may be valid, that is, decreased activation may cause the milder and decreased sensitivity the more severe

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form of stunning. Our present results would favour the hypothesis of primary involvement of the sarcoplasmic reticulum under in vivo conditions.

Ca^{2+}-ATPase and phospholamban

Only a few reports exist about changes in the level of mRNAs encoding cardiac calcium regulatory proteins under pathological conditions. Ca^{2+}-ATPase mRNA levels have been shown to increase two hours after the injection of thyroid hormone (T_3) into hypothyroid rats.[11] This shows that transcriptional changes are sufficiently fast to be detectable within the time constraints of our model. Studies about differentiation of sarcoplasmic reticulum during myogenesis concerning protein and mRNA levels of sarcoplasmic reticular Ca^{2+}-ATPase and calsequestrin[37–39] together suggest that both proteins are regulated through transcriptional mechanisms, which is supported by our own results obtained by nuclear run-on assays. Furthermore the changes of mRNA levels were of linear effect on the specific translational activity of the myocardium.[37,38] No Ca^{2+}-ATPase and calsequestrin isoform switch is reported in ontogenic development and aging.[39]

Ca^{2+}-ATPase and phospholamban gene expression was shown to change in other experimental conditions. Pressure overload induced cardiac hypertrophy in rabbits and rats caused a relative decrease in Ca^{2+}-ATPase and phospholamban mRNA levels while the overall gene expression increased.[9,10,40] In contrast to the *chronic* conditions of pressure overload, the *acute* event of stunning increased Ca^{2+}-ATPase and phospholamban gene expression.

Calsequestrin

Our results indicate that short periods of ischaemia stimulate calsequestrin gene expression by 2.3 times the sham level after 90 minutes of reperfusion. Our nuclear run-on assays confirmed that we detected increased transcriptional activity for calsequestrin by northern hybridisation rather than only an effect of changes in mRNA stability. As far as translation of the mRNA is concerned data obtained during myogenesis[37,38] support our hypothesis of a specific protein repair. It seems that not only the gene expression of the transmembrane proteins, Ca^{2+}-ATPase and phospholamban, but also of the intrasarcoplasmic high capacity Ca^{2+}-binding protein, calsequestrin, is increased in myocardial recovery from stunning.

Myosin

In contrast to the altered gene expression of proteins involved in calcium sequestration we could not find altered mRNA levels for myosin heavy chain in our model of stunned myocardium. Myosin heavy chain was investigated to evaluate the importance of injury to the contractile apparatus. In all species studied the distribution of the cardiac myosin heavy chain isoforms change in response to certain pathological and experimental conditions such as overload,[41] diabetes,[42] and most importantly, changes in thyroid hormone levels.[43] These changes are always regulated at the level of gene transcription.[43,44] These studies justify the conclusion that the unchanged mRNA levels indicate the absence of repair processes for the contractile proteins and that these proteins were most probably not damaged by ischaemia and reperfusion.

Critique of methodology

A general restriction in the interpretation of northern blot analyses lies in the fact that it is not known whether changes in mRNA levels are due to altered gene transcription or altered mRNA stability, although in myocardial tissue, regulation of gene expression usually takes place at the transcriptional level.[45] To confirm that our findings are indeed due to enhanced transcription we applied the technique of nuclear run-on experiments.[46] We used this technique for calsequestrin and Ca^{2+}-ATPase and we showed that increased mRNA levels as detected by northern hybridisation were caused by increased gene transcription. Additional changes in mRNA stability cannot be excluded.

The initial reaction of cardiac cells to stress includes suppressed protein synthesis.[47] This has been reported to lead to an activation of gene expression through the attenuation of inhibitors.[48] On the basis of the complex pattern of gene transcription[14,33,34] it is unlikely that our results are due to a non-specific increase in transcription one to three hours after myocardial ischaemia.

The expression of GAPDH did not decrease with our protocol of ischaemia as reported for more severe cell injury in infarcted rat hearts.[49] Problems might arise due to RNA loss from the filters after dehybridisation. In the present study only northern blot hybridisation signals originating from the same heart (C and E) and from the same blot were compared. Time courses based on 22 pigs and several blots were constructed from relative values calculated as E/C. These steps guarantee that differences between blots do not influence the results.

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Key terms: Ca^{2+}-ATPase; calsequestrin; phospholamban; calmodulin; ischaemia; heart; stunning.

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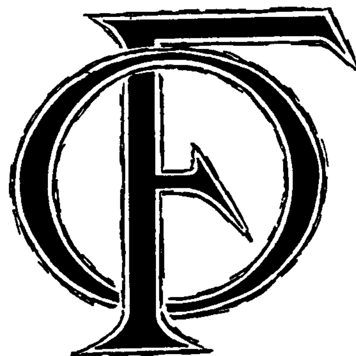

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