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EFFECTS OF NISOLDIPINE ON RECOVERY OF CORONARY BLOOD FLOW, SARCOPLASMIC RETICULUM FUNCTION AND OTHER BIOCHEMICAL PARAMETERS IN POST-ISCHAEMIC PORCINE MYOCARDIUM

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Abstract—The effects of nisoldipine (0.1 μg/kg/min; $n = 9$) or its solvent ($n = 9$) were studied in pigs, in which left anterior descending coronary artery (LADCA) blood flow in both groups was reduced to 20% of baseline for 60 min and reperfused for 2 hr. Infusions were started at 30 min of ischaemia and lasted throughout reperfusion. In both groups, flow reduction abolished regional contractile function and caused similar decreases in the level of creatine phosphate (CP; by 70%) and the energy charge (from 0.91 to 0.69), mean arterial blood pressure (by 25%), $LVdP/dt_{max}$ (by 30%) and cardiac output (by 30%). During ischaemia LADCA blood flow slightly increased (from 14 ± 8 to 24 ± 6 mL/min/100 g; $P < 0.05$) in the nisoldipine-treated animals, resulting in an increase in CP to 91 ± 24% of baseline and preventing further decreases in energy charge, as observed in the solvent-treated animals. After 2 hr of reperfusion in neither group return of contractile function of the post-ischaemic myocardium was observed. Post-ischaemic blood flow in the nisoldipine-treated pigs increased from 24 ± 6 mL/min/100 g to 76 ± 14 mL/min/100 g and from 19 ± 6 mL/min/100 g to 41 ± 6 mL/min/100 g in the solvent-treated animals ($P < 0.05$) after 2 hr of reperfusion. Myocardial work was significantly higher in the nisoldipine-treated animals (111 ± 15 mmHg.L/min as compared to 69 ± 14 mmHg.L/min in the solvent-treated pigs after 2 hr of ischaemia). The energy charge of the post-ischaemic myocardium was similar for both groups (0.84 ± 0.02 for the nisoldipine-treated and 0.83 ± 0.03 for the solvent-treated animals). The rate of sarcoplasmic reticular Ca^{2+} uptake of the non-ischaemic segment of the nisoldipine-treated animals was 61% higher ($P < 0.05$) than that of the solvent-treated animals. In the post-ischaemic myocardium similar rates of Ca^{2+} uptake were found in both groups, but the activities were markedly lower as compared to the non-ischaemic myocardium. It is concluded that nisoldipine increases blood flow during reperfusion, which may have been caused by coronary vasodilatation. However, attenuation of the"no-reflow" phenomenon also contributed, since more rapid rephosphorylation of ADP leading to an increase in CP during ischaemia may have preserved jeopardized cells. Moreover, nisoldipine increases the sarcoplasmic reticular Ca^{2+} pump activity independent of ischaemia, which may have contributed in reducing the Ca^{2+} overload.

The rapid and uncontrolled increase in cytosolic Ca^{2+} that occurs upon reperfusion of ischaemic myocardial tissue is believed to play a central role in the subsequent loss in cell viability. The Ca^{2+} overload triggers a chain of destructive events. Activation of Ca^{2+}-dependent reactions such as ATPases, proteases and phospholipases result in energy waste and membrane disruption. The cytosolic free Ca^{2+} concentration can increase by entrance via the Na^+:Ca^{2+} antiporter [1], via receptor- and voltage-operated Ca^{2+} channels and, as the cell membrane disintegrates via passive diffusion [2]. The loss in ability of the sarcolemma and sarcoplasmic reticulum (SR§) to remove Ca^{2+} from the cytosol could also contribute to cytosolic Ca^{2+} overload. Ischaemia causes a reduction in active Ca^{2+} transport rate of sarcoplasmic reticulum [3] and sarcolemma [4]. Ca^{2+} entry blockers are specific blockers of the voltage-operated Ca^{2+} channels [5] and therefore have the potential to protect the myocardium against Ca^{2+} overload [6]. Moreover, some dihydropyridines (nitrendipine and felodipine) have been shown to directly stimulate the *in vitro* Ca^{2+} uptake rate of canine sarcoplasmic reticulum, which may present another potential action site to prevent myocardial Ca^{2+} overload [7].

The activity of the sarcoplasmic reticulum Ca^{2+} pump is modulated by phospholamban phosphorylation [8, 9]. Experiments conducted with intact hearts indicate that cyclic AMP- and Ca^{2+}-induced phosphorylation of phospholamban is involved in the β-adrenergic activation of the calcium pump of the SR [10]. We obtained evidence that the ischaemia-induced inactivation of the calcium pump

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§ Abbreviations: SR, sarcoplasmic reticulum; CP, creatine phosphate; ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate; HPLC, high performance liquid chromatography; SDS, sodium dodecylsulphate; LADCA, left anterior descending coronary artery; $LVdP/dt_{max}$, maximum rate of rise of left ventricular blood pressure; *EDT*, end-diastolic wall thickness; *EST*, end-systolic wall thickness; *SWT*, systolic wall thickening; *PSWT*, post-systolic wall thickening; max*T*, maximal wall thickness.

is a consequence of the altered characteristics of phospholamban and may be of value as an early marker of long-term recovery of function following reperfusion of ischaemic myocardium [3].

In addition to their vasodilatory potential, leading to increases in blood flow to the area at risk and sparing of energy, Ca^{2+} entry blockers can also preserve myocardium by avoiding the loss of adenosine precursors [11], protect the vascular endothelial cells independent of vasodilatation [12], beneficially interact with the sarcolemma membrane [13] and mitochondria [14] of the myocardial cell. By protecting the myocardium via these mechanisms, Ca^{2+} antagonists may also indirectly prevent the reduction in the Ca^{2+} uptake and phospholamban phosphorylation of sarcoplasmic reticulum of post-ischaemic myocardium.

The present study was conducted to investigate the effects of nisoldipine, given 30 min prior to and throughout reperfusion, on the Ca^{2+} transport activity and *in vitro* ^{32}P incorporation into phospholamban of SR, isolated from large transmural myocardial segments of pigs subjected to 1 hr of ischaemia followed by 2 hr of reperfusion. In view of the relative lack of data on the effects of nisoldipine on energy metabolism in an *in vivo* model, other biochemical parameters such as the energy charge and levels of creatine phosphate (CP) were measured in small transmural myocardial biopsies collected during ischaemia and reperfusion. Additionally, the effects of the drug on systemic haemodynamics, regional contractile function and coronary blood flow were investigated.

MATERIALS AND METHODS

General. After an overnight fast cross-bred Landrace × Yorkshire pigs of either sex (N = 18, 25–42 kg) were sedated with 120 mg azaperone (Janssen Pharmaceutica, Beerse, Belgium) i.m., anaesthetized with 150 mg metomidate (Janssen Pharmaceutica), i.v. and intubated for artificial ventilation with a mixture of oxygen and nitrous oxide (1:2). Respiratory rate and tidal volume were adjusted to keep arterial blood gases within the normal range: pH between 7.37 and 7.49; pCO_2 between 34 and 48 mmHg; pO_2 between 120 and 180 mmHg. A catheter was positioned into the superior caval vein for the administration of 160 mg/kg α-chloralose (Merck, Darmstadt, F.R.G.) followed by an infusion of 5 mg/kg/hr pentobarbitone sodium (Sanofi, Paris, France) and for the administration of the muscle relaxant pancuronium bromide (4 mg) prior to thoracotomy. Haemaccel (Behringwerke A.G., Marburg, F.R.G.) was administered to replace the blood, withdrawn during sampling (see below). An 8F micromanometer-tipped catheter (Millar, Houston, TX, U.S.A.) was inserted into the left ventricle by way of the left carotid artery, for the measurement of left ventricular blood pressure and its first derivative ($LVdP/dt$). Catheters were also inserted into the aorta, for measurement of central aortic blood pressure (50 AD pressure transducer, Spectramed, Bilthoven, The Netherlands), the collection of blood samples for the determination of blood gases and for the withdrawal of reference samples necessary for calibration of the radioactive microsphere flow measurements. After thoracotomy, an electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta for the measurement of aortic blood flow. The left anterior descending coronary artery (LADCA) was dissected free just distal from its first diagonal branch and an inflatable balloon (R. E. Jones, Silver Spring, MD, U.S.A.) was placed around the LADCA and connected to a 1 mL syringe (Hamilton Bonaduz, Bonaduz, Switzerland) driven by a micrometer (Hamilton Co., Reno, NV, U.S.A.). The vein accompanying the LADCA was cannulated for the withdrawal of blood samples for the determination of coronary venous blood gases.

Regional blood flow. To determine regional blood flows the left atrial appendage was catheterized for the injection of a batch of $1–2 \times 10^6$ radioactive microspheres, 15 ± 1 (SD) μm in diameter (NEN Company, Dreieich, F.R.G.), labelled with either ^{95}Nb, ^{103}Ru, ^{113}Sn, ^{46}Sc or ^{141}Ce. Full details of the procedures and the calculation of flow data using the reference sample technique have been reported earlier [15, 16].

Regional myocardial function. Regional myocardial function was estimated from recordings of myocardial wall thickness obtained with a 5 MHz ultrasonic transducer (Krautkamer-Branson, Lewistown, PA, U.S.A.) sutured onto a part of the epicardial surface perfused by the LADCA. From the tracings end-diastolic (EDT), end-systolic (EST) and maximal post-systolic ($\max T$) wall thickness were measured. Systolic wall thickening ($SWT,\%$) was calculated as $100 \times (EST - EDT)/EDT$, while post-systolic wall thickening ($PSWT, \%$) was defined as $100 \times (\max T - EST)/EDT$.

Experimental protocol. After systemic haemodynamics had been stable for at least 30 min following completion of the instrumentation, baseline values of systemic haemodynamics, regional myocardial function and arterial and coronary venous blood gases were obtained while a batch of microspheres was injected for the measurement of distribution of myocardial blood flow. Furthermore, transmural needle biopsies (processed as described below) were taken from the myocardium nourished by the distal part of the LADCA and from a segment of the anterior wall of the heart, which was not supplied by the LADCA. In all pigs the flow in the LADCA was then gradually reduced by slowly inflating the balloon, until complete loss of regional contractile function. After 30 min of ischaemia, the measurements of systemic haemodynamics and regional blood flows were repeated and a transmural biopsy was taken from the ischaemic area. In nine animals an infusion of nisoldipine (0.1 μg/kg/min) was started and in the other animals solvent was administered at a similar infusion rate (1 mL/min). If ventricular fibrillation occurred the animal was promptly (within 30 sec) defibrillated with DC-countershock. After 60 min, when all measurements had been repeated and biopsies were obtained from both the ischaemic and the non-ischaemic area, the balloon was deflated. Two hours later, the last

haemodynamic measurements were taken and biopsies were collected. The heart was then excised and immediately cooled on ice and transmural myocardial samples (5–7 g) were obtained from the post-ischaemic segment and from the posterior wall of the heart (non-ischaemic segment). The segments were homogenized for isolation of SR membrane vesicles (see below). Furthermore, the radioactive label present in the pellet after the first centrifugation step (10,000 *g*, 20 min) for SR isolation was counted in order to obtain myocardial blood flow data.

Adenine nucleotides, creatine phosphate and creatine. The transmural myocardial biopsies, taken with a Tru-Cut needle (Travenol Laboratories Inc., Deerfield, IL, U.S.A.) from the ischaemic area and the adjacent non-ischaemic area were dipped into 0.9% NaCl of 0° to remove adherent blood, and immediately (within 10 sec) frozen in liquid nitrogen. The biopsies were stored at −80° until analysis. The biopsies (5–20 mg) were homogenized in 0.5 mL 0.42 M $HClO_4$ at liquid nitrogen temperature with the Braun micro-dismembrator (B. Braun, Melsungen, F.R.G.), thawed, shaken and centrifuged. After neutralization of the supernatant the adenine nucleotides, CP and creatine were separated and the concentrations estimated with an isocratic ionpairing high performance liquid chromatography (HPLC) [17], except that 175 mM potassium phosphate, 2.3 mM tetrabutylammonium hydrogensulfate, 2.5% acetonitrile, pH 6.25 was used as a running buffer. With this HPLC method we also checked the purity of the [γ-^{32}P]ATP used in the phospholamban phosphorylation assays.

Isolation of sarcoplasmic reticulum. The transmural myocardial tissue samples were homogenized three times during 10 sec in 4 volumes of 10 mM $NaHCO_3$, pH 7.0 at 0° with a Polytron PT 10 (Kinematica GmbH, Luzern, Switzerland). The SR was isolated as described [3]. Immediately after isolation, the SR suspension was frozen in liquid nitrogen and stored at −80° until analysis of Ca^{2+} uptake and phospholamban phosphorylation activities.

Phosphorylation of phospholamban and Ca^{2+} uptake activity. The cyclic AMP-dependent phosphorylation of SR vesicles (5–10 μg protein) was determined as described [3] with 5 μM cyclic AMP to activate the endogenous protein kinase and 300 units/mL exogenous catalytic subunit of cyclic AMP-dependent protein kinase (Sigma Chemical Co, St Louis, MO, U.S.A.). The samples were preincubated for 2 min at 25° and the phosphorylation reaction was started by the addition of 200 μM (final concentration) [γ-^{32}P]ATP (150 TBq/mol). After 5 min the reaction was stopped with a mixture of sodium dodecylsulphate (SDS), β-mercaptethanol and glycerol [3, 18]. The samples were then heated at 95° to dissociate phospholamban into monomers, and subjected to SDS–polyacrylamide gel electrophoresis [18]. Phosphorylated phospholamban was located on dried gels by autoradiography and ^{32}P content in the pieces, excised from the gel, was estimated by liquid scintillation counting. Ca^{2+} uptake activity was measured by incubation of SR with 50 μM ^{45}Ca in the presence of 1 mM ATP [3]. After the incubation the ^{45}Ca-containing SR vesicles were filtered through Millipore filters (0.45 μm). The ^{45}Ca content of the SR vesicles remaining on the filters was estimated by liquid scintillation counting [3]. Ca^{2+} uptake activity in blank reactions, obtained by omitting ATP from the reaction mixture, were subtracted.

Protein. The protein content of SR was estimated with the method of Lowry *et al.* [19]. In the needle biopsies, protein was estimated by a procedure other than in the crude pellet obtained after centrifugation of the homogenate acidified with $HClO_4$. After dissolution in 0.1 M KOH, protein was estimated with the Coomassie Brilliant Blue assay [20] obtained from Bio-Rad (Bio-Rad Laboratories, Munich, F.R.G.). For both methods bovine serum albumin was used as the standard.

Drugs. Nisoldipine (Bayer A.G., Wuppertal, F.R.G.) was dissolved in a mixture of polyethylene glycol 400, glycerol and distilled water. The nisoldipine solution (0.1 mg/mL) was diluted with 0.9% (w/v) NaCl immediately before use. Preparation of the solution and administration of nisoldipine occurred while the drug was protected from light.

Statistical analysis. All data have been presented as means ± SE. The significance of the changes produced by the LADCA flow reduction in the animals was evaluated by Duncan's new multiple range test once an analysis of variance had revealed that the samples represented different populations. The significance of the nisoldipine-induced changes was determined by comparing these changes with those observed in the solvent-treated animals at corresponding times. Statistical significance was accepted for $P < 0.05$.

RESULTS

Ventricular arrhythmias

In both groups seven out of nine animals (78%) encountered an episode of ventricular fibrillation during the ischaemic period. All animals were defibrillated within 25 sec and resumed prefibrillation values. Ventricular fibrillation was not observed during reperfusion in any animal.

Systemic haemodynamics

During the first 30 min of LADCA flow reduction in the solvent-treated animals mean arterial blood pressure, cardiac output, stroke volume and $LVdP/dt_{max}$ had decreased ($P < 0.05$) by 23, 30, 36 and 32%, respectively, left ventricular end diastolic pressure increased from 8 to 13 mmHg ($P < 0.05$), while heart rate was not affected (Table 1). During the 2 hr of reperfusion there was a further decline in mean arterial blood pressure (to 59% of baseline), $LVdP/dt_{max}$ (to 54% of baseline), cardiac output (to 52% of baseline) and stroke volume (to 56% of baseline), while left ventricular end diastolic pressure further increased to 14 ± 2 mmHg. Vasoconstriction (increase in systemic vascular resistance to 35% above baseline) prevented a more severe fall in mean arterial blood pressure (Table 1). During ischaemia there were similar decreases in mean arterial blood pressure, cardiac output, stroke volume and $LVdP/dt_{max}$ in the nisoldipine-treated animals (Table 1). During reperfusion cardiac output

Table 1. Systemic haemodynamics in solvent-treated and nisoldipine-treated open-chest anaesthetized pigs at baseline (BL), 30 and 60 min of ischaemia (I) and 120 min of reperfusion (R). Nisoldipine (0.1 μg/kg/min) was started at 30 min of ischaemia and lasted throughout reperfusion

		BL	30 I	60 I	120 R
MAP	SOL	97 ± 5	75 ± 5*	72 ± 3*	57 ± 4*
	NIS	90 ± 4	66 ± 5*	65 ± 3*	65 ± 6*‡§
CO	SOL	2.3 ± 0.1	1.6 ± 0.1*	1.6 ± 0.1*	1.2 ± 0.2*
	NIS	2.5 ± 0.2	1.8 ± 0.1*	1.8 ± 0.1*	1.6 ± 0.2*§
HR	SOL	94 ± 5	98 ± 4	95 ± 7	88 ± 8
	NIS	95 ± 4	100 ± 6	97 ± 4	103 ± 8
$LVdP/dt_{max}$	SOL	2590 ± 130	1760 ± 120*	1850 ± 140*	1390 ± 230*
	NIS	2390 ± 160	1690 ± 90*	1550 ± 120*	1900 ± 240*†§
LVEDP	SOL	8 ± 1	13 ± 1*	12 ± 1*	14 ± 2*
	NIS	10 ± 1	15 ± 1*	14 ± 1*	13 ± 1*§
SV	SOL	25 ± 2	17 ± 2*	18 ± 2*	14 ± 2*
	NIS	27 ± 2	18 ± 2*	19 ± 1*	16 ± 2*
SVR	SOL	43 ± 3	48 ± 4	46 ± 3	58 ± 10
	NIS	38 ± 2	40 ± 3	37 ± 2	40 ± 4
MW	SOL	225 ± 16	121 ± 16	118 ± 14*	69 ± 14*
	NIS	222 ± 18	130 ± 13	124 ± 11*	111 ± 15*‡§

Data are presented as means ± SE; N = 9 for both groups; SOL = solvent-treated, NIS = nisoldipine-treated; *MAP* = mean arterial blood pressure (mmHg); *CO* = cardiac output (L/min); *HR* = heart rate (beats/min); $LVdP/dt_{max}$ = maximum rise in left ventricular pressure (mmHg/sec); *LVEDP* = left ventricular end diastolic blood pressure (mmHg); *SV* = stroke volume (mL); *SVR* = systemic vascular resistance (mmHg/(L/min)); *MW* = myocardial work (mmHg.L/min).

* $P < 0.05$ versus baseline;

† nisoldipine-induced changes versus baseline are significantly different from changes in the solvent-treated animals;

‡ nisoldipine-induced changed versus 30 min of ischaemia are significantly different from changes in the solvent-treated animals;

§ nisoldipine-induced changes versus 60 min of ischaemia are significantly different from changes in the solvent-treated animals.

further declined; the decrease versus baseline was, however, significantly smaller than in the solvent-treated animals (Table 1). Mean arterial blood pressure did not further decrease and $LVdP/dt_{max}$ even increased ($P < 0.05$ versus changes in solvent-treated animals). Left ventricular end diastolic pressure decreased from 14 ± 1 to 13 ± 1 mmHg, which was significantly different from the increase, observed in the solvent-treated animals. Heart rate was not affected and stroke volume decreased similarly as in the solvent-treated animals.

Regional myocardial perfusion and myocardial oxygen consumption

The microsphere data revealed that inflation of the balloon had caused similar decreases in transmural myocardial blood flow in the solvent-treated (from 82 ± 5 mL/min/100 g to 19 ± 9 mL/min/100 g) and the nisoldipine-treated animals (from 87 ± 12 to 14 ± 8 mL/min/100 g, Fig. 1) during the first 30 min of ischaemia. There were no further changes in the solvent-treated animals during the following 30 min of LADCA stenosis (LADCA blood flow was 19 ± 6 mL/min/100 g at 30 min of ischaemia). In the nisoldipine-treated animals myocardial blood flow increased from 14 ± 8 mL/min/100 g to 24 ± 6 mL/min/100 g. After 2 hr of reperfusion transmural myocardial blood flow of the LADCA-perfused area had increased to 41 ± 6 mL/min/100 g and 76 ± 14 mL/min/100 g in the solvent-treated and the nisoldipine-treated animals, respectively ($P < 0.05$ versus 60 min of ischaemia; Fig. 1).

In both the solvent- and the nisoldipine-treated animals transmural blood flow to the non-ischaemic area decreased slightly during ischaemia, secondary to the fall in arterial blood pressure. However, during reperfusion, vasodilatation prevented perfusion decreasing parallel to the fall in perfusion pressure in the nisoldipine-treated but not in the solvent-treated pigs.

Baseline values of myocardial oxygen consumption of the LADCA-perfused myocardium were 2.9 ± 0.3 mL O_2/min/g and 3.5 ± 0.5 mL O_2/min/g in the solvent-treated and the nisoldipine-treated animals, respectively. After 2 hr of reperfusion these respective values were 0.56 ± 0.06 and 1.18 ± 0.30 mL O_2/min/g ($P > 0.05$).

Regional myocardial wall function

Reduction of coronary blood flow totally abolished systolic wall thickening of the segment perfused by the LADCA in both groups (baseline values in the nisoldipine-treated and the solvent-treated animals were 31 ± 4% and 33 ± 4%, respectively), which did not change in either group neither during the remainder of the ischaemic episode nor during the reperfusion period. *PSWT* of the ischaemic myocardium after 30 and 60 min of ischaemia was 11 ± 1% and 8 ± 1% in the solvent-treated, and 10 ± 2% and 10 ± 2% in the nisoldipine-treated animals, respectively.

High energy phosphates

During ischaemia and reperfusion there were no

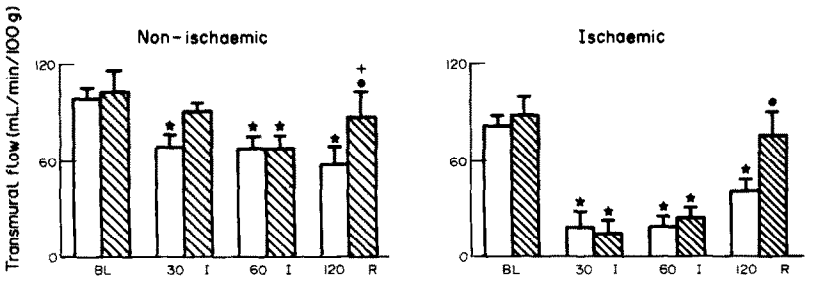

Fig. 1. Transmural myocardial blood flow (mL/min/100 g) of non-ischaemic and ischaemic myocardium in solvent-treated (open bars, N = 9) and nisoldipine-treated (hatched bars, N = 9) pigs at baseline (BL), after 30 and 60 min of ischaemia (I), and 120 min of reperfusion (R). (*) $P < 0.05$ versus baseline, (+) changes versus baseline in the nisoldipine-treated animals are significantly different from the changes versus baseline in the solvent-treated animals; (•) changes versus 60 min of ischaemia in the nisoldipine-treated animals are significantly different from the changes versus 60 min of ischaemia in the solvent-treated animals.

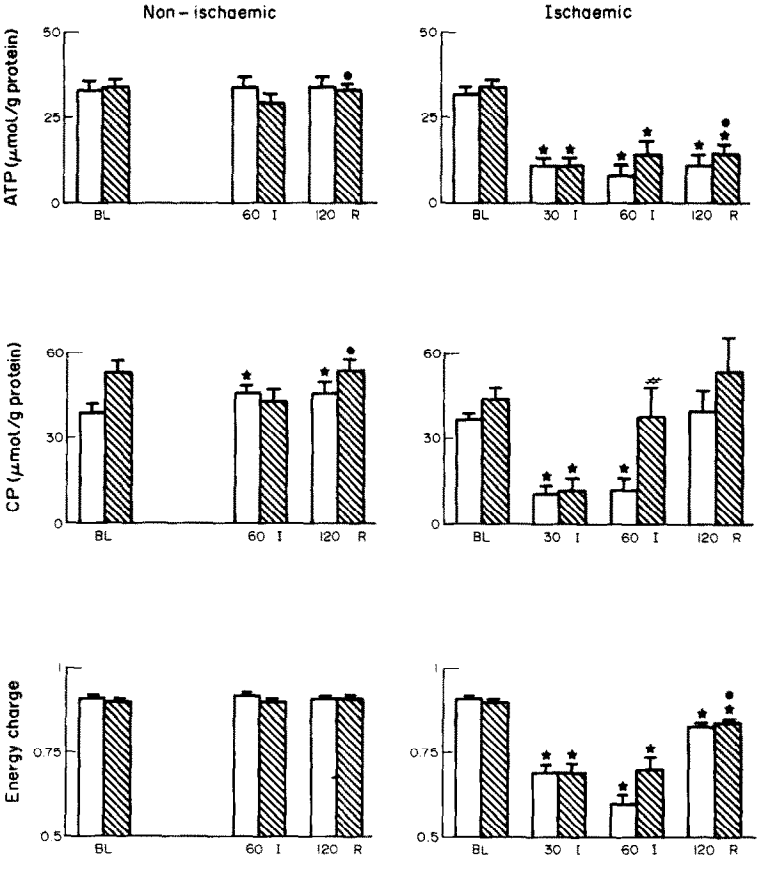

Fig. 2. ATP and CP (μmol/g protein) and energy charge [(ATP + ½ADP)/(ATP + ADP + AMP)] of the ischaemic and non-ischaemic myocardium of solvent-treated (open bars, N = 9) and nisoldipine-treated pigs (hatched bars, N = 9) at baseline (BL), at 30 min (only for the ischaemic segment) and 60 min of ischaemia (I) and after 120 min of reperfusion (R). (*)$P < 0.05$ versus baseline; (#) changes versus 30 min of ischaemia in the nisoldipine-treated animals are significantly different from changes versus 30 min of ischaemia in the solvent-treated animals; (•) changes versus 60 min of ischaemia in the nisoldipine-treated animals are significantly different from the changes versus 60 min of ischaemia in the solvent-treated animals.

changes in the levels of ATP, adenine nucleotides and the energy charge in the non-ischaemic myocardium of both the solvent- and the nisoldipine-treated animals (Figs 2 and 3). The concentrations of CP and total creatine decreased in the nisoldipine-treated animals while there was a slight increase in the solvent-treated animals ($P < 0.05$; Figs 2 and 3). After 2 hr of reperfusion the differences between

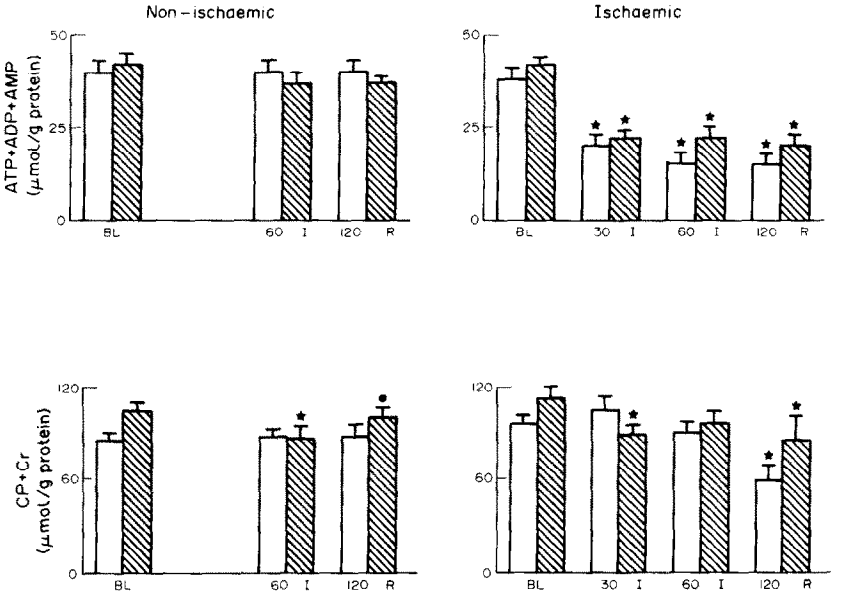

Fig. 3. Total adenine nucleotides (ATP + ADP + AMP) and total creatine (CP + creatine) of the non-ischaemic and ischaemic myocardium in solvent-treated (open bars, N = 9) and nisoldipine-treated (hatched bars, N = 9) pigs at baseline (BL), at 30 min (only for the ischaemic segment) and 60 min of ischaemia (I) and after 120 min of reperfusion (R). (*) $P < 0.05$ versus baseline; (•) changes versus 60 min of ischaemia in the nisoldipine-treated animals are significantly different from the changes versus 60 min of ischaemia in the solvent-treated animals.

groups in concentrations of CP and total creatine were no longer present.

In the LADCA-perfused myocardium of the solvent- and the nisoldipine-treated animals 30 min of flow reduction caused decreases in ATP (by 66 and 68%, respectively), CP (by 70 and 73%, respectively) and total adenine nucleotides (47 and 48%, respectively), while the energy charge decreased from 0.90 ± 0.01 and 0.91 ± 0.01 in the nisoldipine- and solvent-treated animals, respectively, to 0.69 ± 0.04 in both groups. During the following 30 min of ischaemia in the solvent- but not in the nisoldipine-treated animals the level of ATP and the energy charge further decreased. Furthermore, the increase in CP to 92 ± 24% of baseline observed in the nisoldipine-treated animals was absent in the solvent-treated pigs. The total creatine pool showed no changes in the solvent-treated animals during ischaemia, while a slight but significant decrease was observed in the nisoldipine-treated animals after 30 min of ischaemia. After 2 hr of reperfusion no recovery of the levels of ATP and total adenine nucleotides was observed in either group, while those of CP were at baseline and the energy charge had recovered similarly in both groups to 0.83 ± 0.03. The level of total creatine remained 25 and 35% below baseline in the nisoldipine- and the solvent-treated animals, respectively.

Ca^{2+} *uptake and phospholamban phosphorylation*

Ca^{2+} uptake and *in vitro* phosphorylation of phospholamban could only be determined at the end of reperfusion because of the amount of myocardium (5 g), needed for the isolation of sufficient amounts of SR membrane vesicles. The rate of Ca^{2+} uptake of the non-ischaemic segment of the nisoldipine-treated animals was 61% higher ($P < 0.05$) than that of the solvent-treated animals, and phospholamban phosphorylation, on the other hand, was 33% lower ($P < 0.05$). (Table 2) The rates of Ca^{2+} uptake of SR vesicles isolated from post-ischaemic segments were not different: 531 ± 94 nmol/min/mg and 727 ± 212 nmol/min/mg in the solvent-treated and the nisoldipine-treated animals, respectively. The *in vitro* ^{32}P incorporation remained 43% lower in the nisoldipine-treated (765 ± 96 pmol/mg) compared to the solvent-treated animals (1336 ± 263 pmol/mg).

DISCUSSION

The present study demonstrates that nisoldipine increased post-ischaemic myocardial blood flow. This could be due to a direct coronary vasodilatory action of the drug, since vascular resistance of the non-ischaemic myocardium decreased (from 0.91 ± 0.09 mmHg/(mL/min/100 g) to 0.74 ± 0.10 mmHg/(mL/min/100 g) ($P < 0.05$ versus baseline) in the nisoldipine-treated animals, whereas there was no change in the solvent-treated animals (baseline 1.02 mmHg/(mL/min/100 g)). However, the higher myocardial oxygen demand reflected by the higher double-product (heart rate times systolic arterial pressure) in the nisoldipine-treated animals ($94 \times 10^2 \pm 11 \times 10^2$ mmHg.beats/min versus $71 \times 10^2 \pm 11 \times 10^2$ mmHg.beats/min in the solvent-treated pigs) also contributes to the

Table 2. Effect of nisoldipine or its solvent on Ca^{2+} uptake and *in vitro* ^{32}P incorporation into phospholamban of sarcoplasmic reticulum isolated from post-ischaemic myocardium in open-chest anaesthetized pigs

		Ca^{2+}-uptake (nmol/min/mg)	^{32}P incorporation (pmol/mg)
Non-ischaemic myocardium	SOL	830 ± 117	1605 ± 170
	NIS	1335 ± 107*	1083 ± 48*
Post-ischaemic myocardium	SOL	531 ± 94	1336 ± 263
	NIS	727 ± 212	765 ± 96*

Data are presented as means ± SE; SOL = solvent-treated (N = 7); NIS = nisoldipine-treated (N = 9).

* $P < 0.05$ versus solvent-treated animals.

vasodilatation of the coronary bed of the non-ischaemic myocardium.

The failure of tissue to reperfuse after a transient ischaemic period has been called the no-reflow phenomenon. The higher blood flow in the post-ischaemic myocardium in the nisoldipine-treated animals could also be secondary to attenuation of this phenomenon by nisoldipine. The mechanisms by which nisoldipine can preserve cardiomyocytes and endothelial cells are diverse. Takahashi and Kako [21] demonstrated that nisoldipine was capable of suppressing the ischaemia-induced increase in phospholipid breakdown of canine cardiac sarcolemma. It has also been shown that nisoldipine decreased transcoronary extravasation [12] and the authors postulated that secondary to a reduction in Ca^{2+} uptake during reperfusion, nisoldipine prevented endothelial deformation and formation of interendothelial gaps. We found that nisoldipine caused a significant increase in CP already during ischaemia. The further decreases in the level of ATP and the energy charge, as observed in the saline-treated animals during the last 30 min of ischaemia, were prevented in the nisoldipine-treated pigs, albeit that these differences were not significant. Nevertheless, since the increase in CP must be a consequence of a more rapid rephosphorylation of ADP, the energy metabolism in the nisoldipine-treated animals during ischaemia must have been more favourable. This might have led to preservation of jeopardized cells contributing to the maintenance of the microvascular integrity.

Recently, Gross *et al.* [22] investigated the effect of the dihydropyridine amlodipine on subendocardial segment length shortening, regional blood flow and myocardial high energy phosphate levels in coronary ligated (45 min of total occlusion) dogs followed by 2 hr of reperfusion). LADCA blood flow during ischaemia and systemic haemodynamics in that study were similar as those in the present study. However, at variance with the present study, Gross *et al.* [22] observed a marked and sustained improvement in systolic wall function at the end of the reperfusion period. The authors postulated that a positive inotropic action, as also observed for the Ca^{2+} antagonist felodipine [23], might have reversed myocardial stunning, thereby attenuating the rebound in CP.

In the present study the higher post-ischaemic blood flow in the nisoldipine-treated animals was not accompanied by a return of systolic contractile function during early reperfusion. The explanation for this can be four-fold. Firstly, a negative inotropic action of the drug prevented return of function. This is highly unlikely as in pigs this dose of nisoldipine does not cause negative inotropy [24]. Secondly, the myocardial tissue is irreversibly injured. Garcia-Dorado *et al.* [25] have shown (by tetrazolium-staining) that in pigs 30–40% of the myocardium at risk was still viable after 1 hr of total coronary artery occlusion. In the present study we reduced coronary blood flow to 15–20% of baseline during the 60 min of ischaemia and a significant fraction of the affected myocardium must therefore ultimately (even after days) recover in function. Thirdly, the low energy charge of the post-ischaemic myocardium prevented contractile function. However, it has repeatedly been demonstrated that enhanced recovery of function occurs while the ATP-levels are still low [26–28]. Finally, Krause *et al.* [29] have suggested that the inability of the stunned myocardium to function normally is due to a slight reduction of the activity of the Ca^{2+} pump.

Ca^{2+}-uptake in the non-ischaemic myocardium of the nisoldipine-treated pigs was higher than that of the solvent-treated animals. Two possible mechanisms of action can be forwarded: (i) a direct stimulation of the pump by nisoldipine is involved since it has been demonstrated that dihydropyridines stimulate Ca^{2+} uptake of canine cardiac sarcoplasmic reticulum [7] and interestingly also of porcine cardiac sarcolemma [30]. However, this mechanism of action may be expected to be masked by the SR isolation procedure, during which drugs are removed from their target proteins or phospholipids. On the other hand, the lipophilic nature of the dihydropyridines [31] may have retarded their removal from the phospholipid bilayer; (ii) the activation of the Ca^{2+} pump is secondary to a β-adrenoceptor-mediated mechanism. In the nisoldipine-treated animals the $LVdP/dt_{max}$ was significantly increased and there was a tendency for the heart rate to increase, both suggesting a higher level of β-adrenoceptor activity. The nisoldipine-induced decrease in *in vitro* ^{32}P incorporation of phospholamban, found in the non-ischaemic myocardium, is in agreement with an increased β-adrenoceptor activity causing an *in vivo* phosphorylation of the protein, although rapid

dephosphorylation of sarcolemmal phospholamban has been shown to occur during the homogenization and fractionation of the myocardial tissue needed for isolation of the membrane vesicles [18]. Therefore, to definitely prove that the increase of Ca^{2+} uptake rate and decrease of *in vitro* ^{32}P incorporation in myocardium by nisoldipine is due to increased β-adrenoceptor activity, the degree of phosphorylation of phospholamban should be measured *in vivo*. We have no explanation for the lack of a significant increase of Ca^{2+} uptake by nisoldipine in the ischaemic myocardium.

A lower *in vitro* ^{32}P incorporation into phospholamban in ischaemic myocardium has been associated with a modification of the structure or membrane component of the protein [3, 18, 32]. Evidence has been obtained that the reduction in sarcoplasmic reticulum Ca^{2+} pump is causally related with the modified properties of phospholamban [3]. We found a reduced Ca^{2+} uptake and *in vitro* ^{32}P incorporation into phospholamban of the ischaemic segments of both the solvent- and the nisoldipine-treated animals. However, the effects of nisoldipine on Ca^{2+} uptake and *in vitro* ^{32}P-incorporation into phospholamban observed in the ischaemic myocardium tend to be similar to those in the non-ischaemic myocardium. Therefore, the interpretation of the nisoldipine-induced effects on SR function during ischaemia-reperfusion are complicated by a possible direct or β-adrenoceptor-mediated action of the drug.

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