Basic & Clinical Pharmacology & Toxicology

Basic & Clinical Pharmacology & Toxicology, 2014, **115**, 438–447 Doi: 10.1111/bcpt.12255

Reperfusion Therapy with Low-Dose Insulin or Insulin-Like Growth Factor 2; Myocardial Function and Infarct Size in a Porcine Model of Ischaemia and Reperfusion

Pirjo-Riitta Salminen[1,2], Geir Olav Dahle[1,2], Christian Arvei Moen[2], Anita Wergeland[3], Anne Kristin Jonassen[3], Rune Haaverstad[1,2], Knut Matre[2] and Ketil Grong[2]

[1]Section of Cardiothoracic Surgery, Department of Heart Disease, Haukeland University Hospital, Bergen, Norway, [2]Department of Clinical Science, University of Bergen, Bergen, Norway and [3]Department of Biomedicine, Faculty of Medicine and Dentistry, University of Bergen, Bergen, Norway

(Received 16 October 2013; Accepted 9 April 2014)

Abstract: In an open-chest porcine model, we examined whether myocardial pharmacological conditioning at the time of reperfusion with low-dose insulin or insulin-like growth factor 2 (IGF2), not affecting serum glucose levels, could reduce infarct size and improve functional recovery. Two groups of anaesthetized pigs with either 60 or 40 min. of left anterior descending artery occlusion (total n = 42) were randomized to receive either 0.9% saline, insulin or IGF2 infusion for 15 min., starting 5 min. before a 180-min. reperfusion period. Repeated fluorescent microsphere injections were used to confirm ischaemia and reperfusion. Area at risk and infarct size was determined with Evans blue and triphenyltetrazolium chloride staining. Local myocardial function was evaluated with multi-layer radial tissue Doppler strain and speckle-tracking strain from epicardial echocardiography. Western blotting and TUNEL staining were performed to explore apoptosis. Infarct size did not differ between treatment groups and was 56.7 ± 6.8%, 49.7 ± 9.6%, 56.2 ± 8.0% of area at risk for control, insulin and IGF2 group, respectively, in the 60-min. occlusion series. Corresponding values were 45.6 ± 6.0%, 48.4 ± 7.2% and 34.1 ± 5.8% after 40-min. occlusion. Global and local cardiac function did not differ between treatment groups. No differences related to treatment could be found in myocardial tissue cleaved caspase-3 content or the degree of TUNEL staining. Reperfusion therapy with low-dose insulin or with IGF2 neither reduced infarct size nor improved function in reperfused myocardium in this *in vivo* porcine model.

In experimental studies, administration of different pharmacological agents during myocardial reperfusion has demonstrated promising results decreasing infarct size; clinically results are variable [1]. Both necrosis and apoptosis play a part in myocardial cell death during ischaemia–reperfusion [2], and apoptotic cell death could primarily be a consequence of reperfusion [3,4]. Inhibition of apoptosis decreases infarct size and improves regional contractile function [5].

Experimentally, glucose–insulin–potassium (GIK) infusion and low-dose insulin alleviates myocardial damage after myocardial ischaemia–reperfusion [6,7]. However, in patients with suspected acute coronary syndrome, pre-hospital GIK infusion did not reduce progression to myocardial infarction and 30-day mortality [8]. Nevertheless, in a cohort of patients evaluated with technetium Tc 99m sestamibi imaging at 30 days after infarction, the infarct size was reduced for those who received GIK treatment. In isolated rat hearts, insulin administered for a short time period at reperfusion reduces apoptosis and myocardial infarct size by stimulating the phosphatidylinositol 3′-kinase (PI3-kinase)–Akt-dependent reperfusion injury salvage kinases (RISK) pathway [9].

Author for correspondence: Pirjo-Riitta Salminen, Section of Cardiothoracic Surgery, Department of Heart Disease, Haukeland University Hospital, Jonas Lies Vei 65, NO-5021 Bergen, Norway (fax +47-55975150, e-mail piri@helse-bergen.no).

This study examined whether RISK pathway up-regulation with low-dose insulin or IGF2, not affecting serum glucose levels and metabolism, administered in the early reperfusion period, could reduce reperfusion injury in an *in vivo* pig model. Myocardial cell death and infarction develops as a wave front from the subendocardium towards the subepicardium [10]. Therefore, myocardial infarct size and apoptotic activity were studied together with global and regional myocardial function and perfusion with focus on transmural differences between myocardial wall layers.

Materials and Methods

Anaesthesia. Experiments were performed in accordance with the European Communities Council Directive of 2010 (63/EU) and approved by the Norwegian State Commission for Laboratory Animals (Project 20113923). Sixty-seven Norwegian Landrace pigs of either gender, weighing 43 ± 4 kg (S.D.), were fasted overnight and premedicated with i.m. ketamine (20 mg/kg), diazepam (10 mg) and atropine (1 mg). Mask ventilation with 3% isoflurane allowed cannulation of two ear veins for induction of anaesthesia with loading doses of fentanyl (0.02 mg/kg), midazolam (0.3 mg/kg), vecuronium (0.2 mg/kg) and pentobarbital (15 mg/kg), followed by continuous infusion of fentanyl (0.02 mg/kg/hr), midazolam (0.3 mg/kg/hr), vecuronium (0.1 mg/kg/hr) and pentobarbital (4 mg/kg/hr) [11]. Ventilation (Julian, Dräger, Lübeck, Germany) with nitrous oxide (56–58%) and oxygen was commenced on mask and continued through a tracheotomy. For 30 min., 15 ml/kg/hr glucose 5% was given as fluid

substitution followed by Ringer's acetate for the rest of the experiment, both solutions with 20 mM KCl added.

Animal preparation. The right femoral artery and vein were cannulated for blood sampling. Urine was drained with a bladder catheter and rectal temperature monitored. Midline sternotomy and pericardiotomy exposed the heart. Heparin 125 IU/kg was administered, and central venous and pulmonary artery pressures (SensoNor, Horten, Norway) and cardiac output (Vigilance-2; Edwards Lifesciences Inc., Irvine, CA, USA) were assessed with a Swan-Ganz continuous cardiac output catheter (139H-7,5F; Edwards Lifesciences Inc.) advanced through the mammary vein. Central aortic and left ventricular pressures were monitored with pressure-tip catheters (Millar MPC-500, Houston, TX, USA) advanced through the left internal mammarian artery and the apex of the heart. Variables were continuously sampled and digitized (Ponemah ACQ7700; DSI, St. Paul, MN, USA). The left atrium was cannulated for microsphere injections and for infusions of physiological saline, insulin or IGF2. The left anterior descending coronary artery (LAD) was dissected free in the middle segment allowing placement of a loose silk ligature and a vascular clamp. After instrumentation (1½-2 hr), a new bolus of heparin 125 IU/kg was given and animals were allowed to stabilize for 10 min. before measuring baseline variables.

Experimental protocols. Separately in two consecutive experimental series, animals were block-randomized in blocks of three with seven animals allocated into each treatment group. Two experimental series with either 60 min. (Occl-60) or 40 (Occl-40) min. of LAD occlusion, followed by 180-min. reperfusion were performed. Infusion of either saline, insulin 2 mU/kg or IGF2 2 μg/kg dissolved in saline was commenced via the left atrial catheter 5 min. before start of reperfusion and continued for 10 min. into the reperfusion period. In all animals, a total amount of 2 mg/kg lidocaine was given i.v. as small repeated boluses to handle the frequently observed arrhythmias during occlusion and early reperfusion. Ventricular fibrillation was electroconverted. No other anti-arrhythmic interventions were allowed. Additional heparin 125 IU/kg was administered at reperfusion, and after 3 hr, the animals were killed with intracardiac saturated KCl. The LAD ligature was tightened, the aorta partially cross-clamped and 2% Evans blue injected into the aortic root, staining the myocardium outside the area at risk (AAR). The hearts were quickly removed for tissue sampling and infarct quantification.

Myocardial tissue blood flow rate was estimated with 15-μm fluorescent microspheres (Dye-Trak "F"; Triton Technology Inc., San Diego, CA, USA) at baseline, 10 min. after start of occlusion, and 15 min. and 180 min. after start of reperfusion. Simultaneously, arterial blood gases (AVL Optil, AVL Scientific Corporation, Roswell, GA, USA) and s-glucose were registered. Serum troponin T was obtained at baseline and after three hours of reperfusion.

Fifteen animals were anaesthetized and instrumented but without occlusion of the coronary artery. Corresponding amounts of insulin (n = 5), IGF2 (n = 5) or saline (n = 5) were infused intra-atrially for 15 min. These hearts were then arrested and removed, and tissue samples were harvested and studied for phosphorylation of Akt.

Data acquisition and analysis. Haemodynamic variables were analysed for baseline, 10 min. after start of occlusion and 15, 30 and thereafter every 30 min. until 180 min. of reperfusion. Left ventricular peak systolic ($LVSP_{max}$) and end-diastolic (LVEDP) pressures, peak positive (LV-dP/dt_{max}) and peak negative (LV-dP/dt_{min}) of first derivate of left ventricular pressure together with cardiac index (CI) and mean aortic pressure (AOP_{mean}) were noted. Epicardial left ventricular short-axis and apical long-axis echocardiographic views (including the region of the myocardium rendered ischaemic) were recorded at baseline, and 15 min. and 180 min. after start of reperfusion (Vivid E9, GE Vingmed Ultrasound, Horten, Norway). The left ventricular anterior wall was analysed (EchoPac PC BT11, GE Vingmed Ultrasound) for peak systolic radial tissue Doppler strain (TDI) in the subendocardial, mid-myocardial and subepicardial wall layers and calculated as mean of three consecutive cardiac cycles. Short-axis and apical long-axis B-mode views were analysed for transmural peak systolic circumferential and longitudinal speckle-tracking echocardiography (STE) strain in six segments also as the mean of three consecutive cardiac cycles [12]. Strain values were obtained with spatial smoothing set to minimum. The diastolic and systolic wall thicknesses were measured at the start of the QRS complex and at aortic valve closure defined from pulsed wave Doppler recordings in the aortic annulus, and systolic wall thickening was calculated.

The hearts were removed and weighed. The left ventricle (LV) was cut into approximately 5-mm-thick slices. Each slice up to the level of the silk ligature on LAD was weighed separately, and the remaining LV was weighed en bloc. Two slices were used for tissue samples, and all the other slices including area at risk (AAR) were scanned (Epson ScanV500) on both sides, then incubated in 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) staining for 15 min. at 37°C and dipped in paraformaldehyde before a second scanning. Scans were analysed with computerized planimetry (Adobe Photoshop CS5 Extended, version 12.0, San Jose, CA, USA), and the extent of infarcted area (IA) (non-TTC-stained) *versus* AAR (Evans blue negative) and AAR *versus* total LV was quantified transmurally, but also separately in the subendocardial, mid-myocardial and subepicardial thirds of the LV wall.

Tissue samples from the subendocardial, mid-myocardial and subepicardial thirds of both the LV anterior and posterior wall were

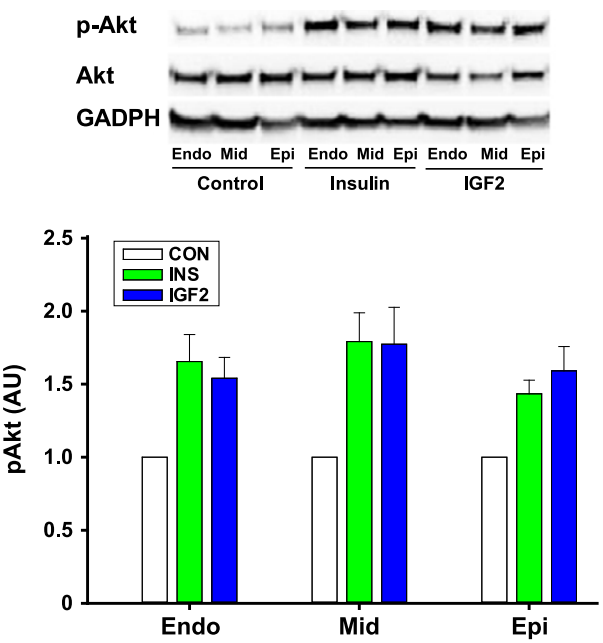

Fig. 1. A representative immunoblot (upper part) of phosphorylated Akt (p-Akt), total Akt and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in left ventricular myocardium from animals without LAD occlusion with 15 min. infusion of saline (CON), insulin (INS) or IGF2. Densitometric analysis of samples from subendocardial (Endo), mid-myocardial (Mid) and subepicardial (Epi) wall layers (control n = 5, insulin n = 5, IGF2 n = 5), normalized to an internal control present on each blot. pAkt as a fraction of total Akt in the same sample. IGF2 = insulin-like growth factor 2.

isolated, snap-frozen in liquid nitrogen and stored at −80°C. Corresponding tissue samples were immersed in paraformaldehyde, washed in ethanol, embedded in paraffin and sectioned for histology. Tissue samples from the three myocardial wall layers were also analysed for tissue blood flow rate and water content.

Cleaved caspase-3 (22 kDa) (BD-Pharmingen, San Diego, CA, USA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were determined in the three wall layers of the LV anterior wall. Homogenization, protein quantification, sample preparation (40 μg/lane) and electrophoresis were performed as previously described Jonassen *et al.* [9]. Densitometric analyses were performed using Quantity One software (Bio-Rad, Hercules, CA, USA). The densitometric readings from each sample were normalized to a control sample included on all blots.

Terminal deoxynucleotidyl transferase-mediated 2-deoxyuridine 5-triphosphate nick end labelling (TUNEL) assay was performed to detect and quantify apoptosis. The number of apoptotic nuclei in the region within the AAR close to intact myocardium and in the non-ischaemic posterior wall was counted. For each section, coded for the investigator, quantification of the number of apoptotic nuclei in an area of 1 million of square pixels ($1Mpx^2$) was performed.

Statistical analysis. Statistical analyses were performed (SPSS 20 Inc, Chigaco, IL, USA) and values reported as mean ± S.E.M. unless otherwise noted. The two study series (Occl-60 and Occl-40) were analysed separately. Baseline variables and variables recorded 10 min. after LAD occlusion were compared between groups by one-way analysis of variance. Haemodynamic variables, blood flow rate and strain after reperfusion with or without baseline included were analysed by two-way RM-ANOVA with time as within-factor and treatment (control/insulin/IGF2) as grouping factor. Wall layers in the same heart were the related (within) factor for tissue caspase-3, the number of apoptotic cells (after logarithmic transformation) and layer-specific infarct size. If Mauchly's test for sphericity was violated ($p < 0.05$), the Greenhouse–Geisser adjustment of degrees of freedom was selected for evaluation of main effects. A significant interaction effect called for new ANOVAS for simple main effects. Cell means were finally compared with Newman–Keuls multiple contrast tests when appropriate. A p value <0.05 was considered statistically significant.

Results

Before intervention.

Ten animals (six in Occl-60 and four in Occl-40 series) developed irreversible ventricular fibrillation within 20–30 min. after LAD occlusion and were replaced. In the animals without LAD occlusion, there was an increase in phosphorylated Akt relative to total Akt with infusion of insulin or IGF2 ($p < 0.05$ for all wall layers) compared to the controls with saline infusion (fig. 1).

There were no differences in arterial blood gases or serum electrolytes either between groups or between series. Serum glucose was equal between groups (a grand mean 6.4 ± 0.3 mM at baseline and 5.9 ± 0.2 mM after 10 min. of LAD occlusion). Troponin T at baseline was 34.0 ± 11.9 ng/l in the Occl-60 series and 25.8 ± 4.0 ng/l in Occl-40 series. The haemodynamic variables at baseline and at 10 min. after LAD occlusion did not differ significantly between groups in either series, except for a slightly increased baseline CI in the Occl-40 insulin group (fig. 2). At baseline, regional myocardial function in the anterior LV wall did not differ between treatment groups as judged by the three-layer radial peak systolic TDI strain, circumferential and longitudinal STE strain and wall thickening (figs. 3 and 4, table 1). Three-layer TDI strain demonstrated the typical transmural heterogeneity in non-ischaemic myocardium with lower radial strain values in outer compared with inner wall layers. Myocardial tissue blood flow rate was similar for all groups at baseline and demonstrated severe and transmural ischaemia affecting all wall layers in the anterior wall (AAR) during LAD occlusion (fig. 4). In the Occl-60 series, the non-ischaemic posterior wall tissue blood flow rate was 0.90 ± 0.05, 0.85 ± 0.04 and 0.85 ± 0.05 ml/min per g subendocardially, mid-myocardially and subepicardially, respectively. Corresponding values in the

Table 1.

End-diastolic wall thickness in the ischaemic (Ant_{ED}-) and non-ischaemic ($Post_{ED}$-) region before, and 15 and 180 min. after reperfusion following 60 and 40 min. of LAD occlusion. Mean ± S.E.M. for seven animals in each group.

	Baseline	15′ rep.	180′ rep.	RM-ANOVA	
60′ LAD-occl:					
Ant_{ED}-CON (mm)	6.86 ± 0.22	15.00 ± 0.95	12.81 ± 1.07	$p_w < 0.001$	
Ant_{ED}-INS (mm)	7.05 ± 0.29	12.71 ± 1.29	11.95 ± 1.34	$p_g = 0.66$	Baseline ≠ 15′ ≠ 180′
Ant_{ED}-IGF2 (mm)	7.52 ± 0.38	13.71 ± 0.98	12.24 ± 1.00	$p_i = 0.37$	
$Post_{ED}$-CON (mm)	7.19 ± 0.23	8.19 ± 0.22	8.19 ± 0.24	$p_w < 0.001$	
$Post_{ED}$-INS (mm)	7.33 ± 0.26	8.76 ± 0.21	8.71 ± 0.34	$p_g = 0.20$	Baseline ≠ 15′ = 180′
$Post_{ED}$-IGF2 (mm)	7.95 ± 0.25	8.81 ± 0.33	8.38 ± 0.25	$p_i = 0.19$	
40′ LAD-occl:					
Ant_{ED}-CON (mm)	6.71 ± 0.26	13.67 ± 0.69	12.10 ± 0.51	$p_w < 0.001$	
Ant_{ED}-INS (mm)	6.90 ± 0.29	12.05 ± 1.40	11.48 ± 1.15	$p_g = 0.68$	Baseline ≠ 15′ ≠ 180′
Ant_{ED}-IGF2 (mm)	7.00 ± 0.13	11.81 ± 0.87	11.57 ± 0.94	$p_i = 0.43$	
$Post_{ED}$-CON (mm)	7.05 ± 0.26	7.76 ± 0.38	7.62 ± 0.31	$p_w = 0.005$	
$Post_{ED}$-INS (mm)	6.90 ± 0.27	7.74 ± 0.29	7.81 ± 0.46	$p_g = 0.81$	Baseline ≠ 15′ = 180′
$Post_{ED}$-IGF2 (mm)	7.43 ± 0.27	8.00 ± 0.31	7.67 ± 0.44	$p_i = 0.78$	

Ant_{ED} and $Post_{ED}$ = anterior and posterior left ventricular end-diastolic; CON, INS and IGF2 = control, insulin and insulin-like growth factor 2 group, respectively; p_w, p_g and p_i = probability for significance for the within factor (time), grouping factor and interaction between time and group, respectively.

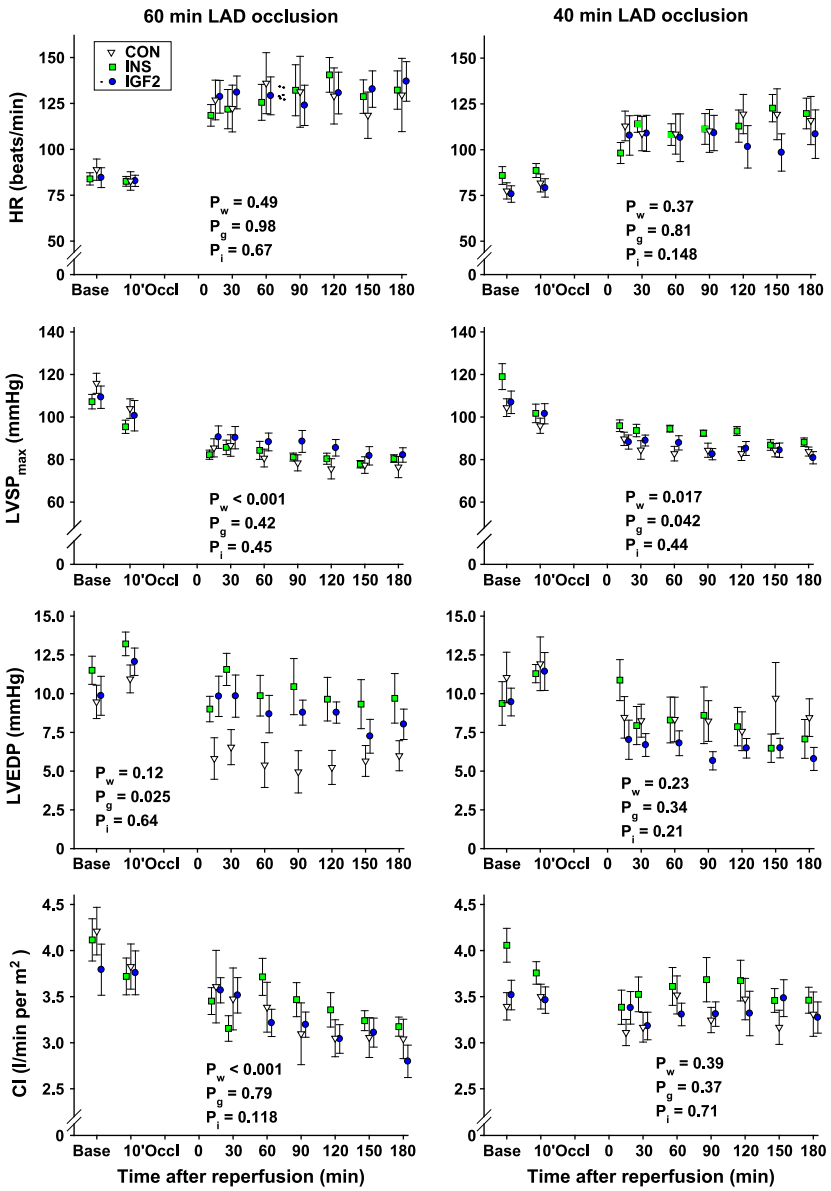

Fig. 2. Haemodynamic variables in pigs with 60 and 40 min. of LAD occlusion and 180 min. of reperfusion. HR = heart rate; LVSP$_{max}$ = left ventricular peak systolic pressure; LVEDP = left ventricular end-diastolic pressure; CI = cardiac index. CON = saline; INS = insulin; IGF2 = insulin-like growth factor 2. Mean values (±S.E.M.) for baseline (base), 10 min. into the occlusion period (10'Occl) and after reperfusion. p_w, p_g and p_i = probabilities for within, group and interaction effect from RM-ANOVA for the reperfusion period.

Occl-40 series were 0.97 ± 0.04, 0.87 ± 0.04 and 0.92 ± 0.03 ml/min per g.

After reperfusion.

In the Occl-60 series, there were no differences between treatment groups regarding LVSP$_{max}$, heart rate (HR) and CI early after reperfusion (fig. 2, left). LVSP$_{max}$ and CI decreased gradually over time in all groups from 15 to 180 min. of reperfusion ($p_w < 0.001$) with no difference between interventions. LVEDP was low ($p_g < 0.009$) in control animals compared with both intervention groups. HR, LV-dP/dt$_{max}$, LV-dP/dt$_{min}$ and AOP$_{mean}$ did not change. In the Occl-40 series, haemodynamic and global cardiac variables did not differ between groups, LVSP$_{max}$ decreased slightly ($p_w = 0.017$) over time, and the other haemodynamic variables were unchanged (fig. 2, right).

At early reperfusion, radial TDI strain in the subendocardium was close to zero or negative in all groups and remained unchanged 3 hr after reperfusion (fig. 3). In the mid-myocardial and subepicardial layers, the TDI strain declined further from early to late reperfusion in the Occl-60 animals, whereas in Occl-40 series, there was no difference from early to late reperfusion. Peak systolic circumferential STE strain in the anteroseptal segment (within the AAR) in Occl-60 series was reduced (less negative) at early reperfusion and was unchanged after 180-min. reperfusion with no differences

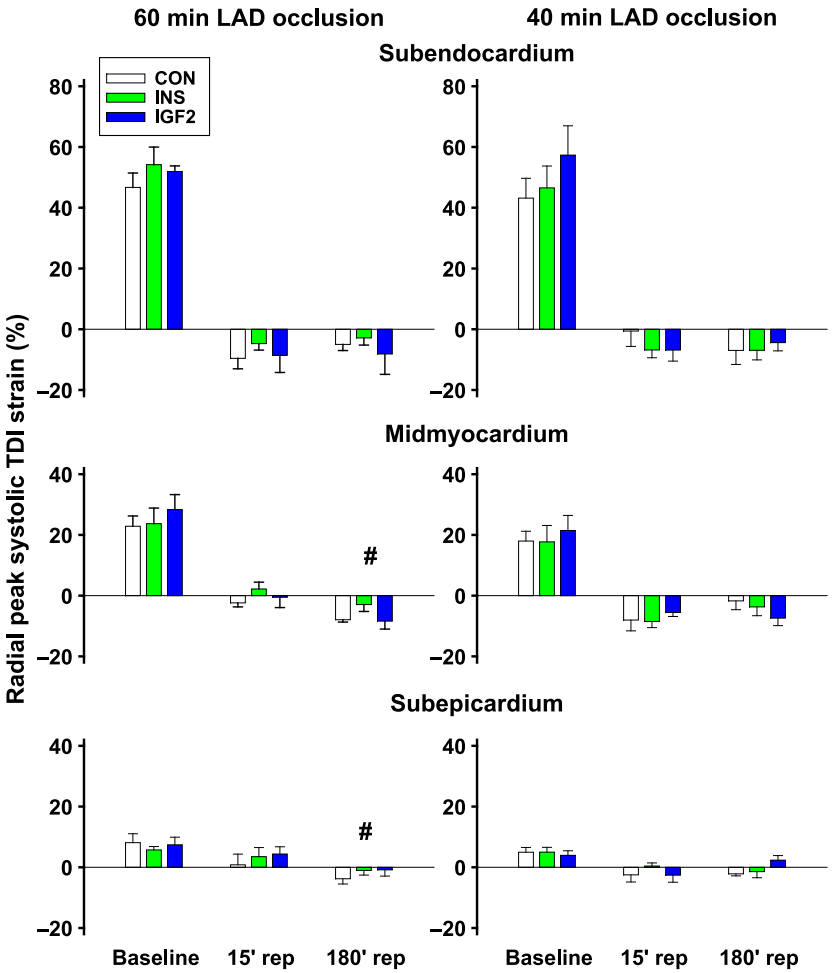

Fig. 3. Radial peak systolic strain by tissue Doppler imaging in the area at risk of the left ventricular anterior wall at baseline and at 15 and 180 min. of reperfusion (rep) after LAD occlusion. Bars are mean + S.E.M. (n = 7). CON = saline; INS = insulin; IGF2 = insulin-like growth factor 2. #$p < 0.05$ *versus* 15′ reperfusion (pooled means).

between treatment groups (fig. 4). Similar results were also obtained in Occl-40 series. Longitudinal STE strain was low and unchanged in the apicoanterior segment (within the AAR) in all groups in both series, both at early and late reperfusion. Left ventricular anterior wall thickening decreased in all groups from baseline (grand mean 49.2 ± 1.9% in Occl-60 series and 51.2 ± 2.5% in Occl-40 series) to nearly zero or to negative values both at early and late reperfusion.

Tissue blood flow, wall thickness and oedema.

Successful reperfusion and hyperaemia was observed macroscopically in the AAR region immediately after releasing the vessel clamp on the LAD. This was also confirmed with the third microsphere injection 15 min. after reperfusion in all groups and wall layers at early reperfusion compared with the corresponding baseline values (fig. 5). At late reperfusion, tissue blood flow rate was decreased to levels significantly lower than at baseline ($p_w < 0.01$) with no differences between treatment groups. After LAD occlusion and reperfusion, flow rates in the non-ischaemic posterior wall did not change. Tissue water content increased in the anterior wall of AAR compared with the posterior non-ischaemic myocardium. End-diastolic wall thickness doubled (table 1) at early reperfusion compared with baseline and decreased slightly at 180 min. after reperfusion in all groups. Tissue oedema (% increase in water content in the anterior *versus* corresponding posterior LV wall layer) was increased in the control and insulin groups compared with IGF2 the Occl-60 series in the subendocardial (3.3 ± 0.4% and 3.5 ± 0.3% *versus* 1.9 ± 0.3%) and in the mid-myocardial (4.1 ± 0.4% and 3.1 ± 0.5% *versus* 2.8 ± 0.6%) wall layers ($p < 0.05$ for all comparisons). In the subepicardium, there was no significant oedema in the IGF2 group, compared with 3.6 ± 0.5% and 3.9 ± 0.4% in control and insulin groups. In Occl-40 series, there was a significant tissue oedema in the anterior wall, but with no differences between groups and wall layers (grand mean 3.4 ± 0.2%).

Apoptosis.

The myocardial content of cleaved caspase-3 was low in ischaemic/reperfused myocardium in the Occl-60 series

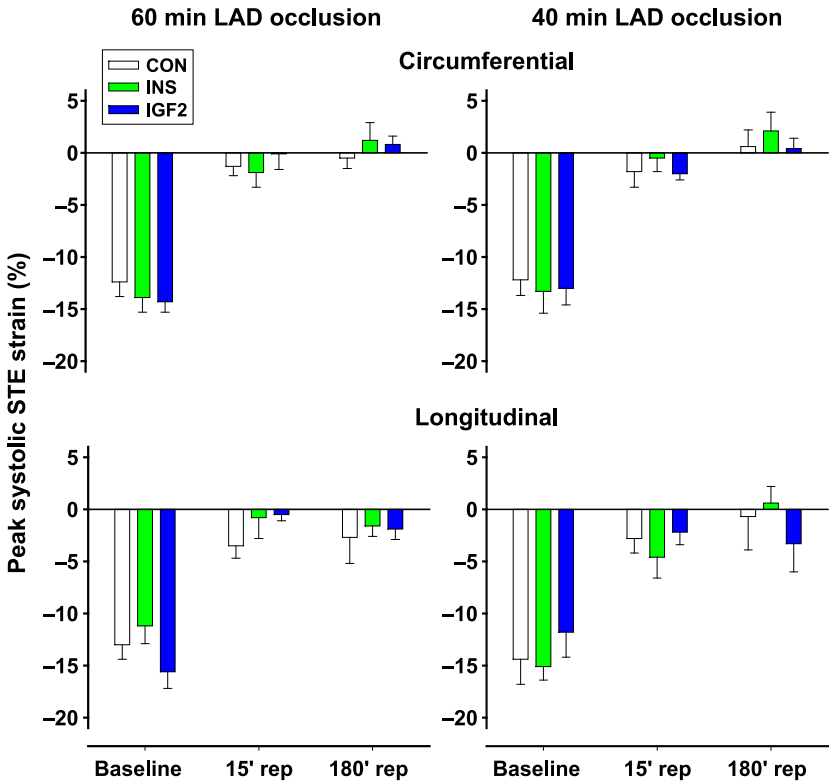

Fig. 4. Peak systolic strain by speckle-tracking echocardiography (STE) in circumferential and longitudinal direction at baseline and 15 and 180 min. after reperfusion. Abbreviations as in fig. 3.

compared with Occl-40 series. There was no significant difference between treatment groups or wall layers, neither in the Occl-60 nor in the Occl-40 series (fig. 6, top). In the Occl-60 series, there was a transmural gradient with decreasing density of TUNEL-stained nuclei from the subendocardium to the subepicardium ($p_w < 0.001$), but with no differences between treatment groups ($p_g = 0.44$) and no significant interaction effect ($p_i = 0.86$) (fig. 6, middle). In the Occl-40 series, no layer ($p_w = 0.21$) or group ($p_g = 0.29$) difference was found.

Infarct size.

Area at risk varied from 13.7% to 17.6% of left ventricular mass, with no differences between the treatment groups. In animals in Occl-60 series, myocardial infarct size (IA/AAR) was 56.7 ± 6.8%, 56.2 ± 8.0% and 49.7 ± 9.6% for the control, insulin and IGF2 group, respectively. Corresponding values were 45.6 ± 6.0%, 48.4 ± 7.2% and 34.1 ± 5.8% in the Occl-40 series. There were no significant differences between treatment groups in either series. Layer-specific analyses of the extent of infarction revealed no significant differences between treatment groups (fig. 5, bottom).

Glucose and troponin-T.

Serum glucose values remained unchanged at early reperfusion compared with baseline, but were slightly decreased after 180 min. of reperfusion in all groups, averaging 5.4 ± 0.4 and 6.1 ± 0.2 mM in Occl-60 and Occl-40 series, respectively. Troponin T was 1584 ± 601 ng/l, 2674 ± 444 ng/land 3156 ± 646 ngl for control, insulin and IGF2 group, respectively, in animals with 60-min. LAD occlusion and 1642 ± 159 ngl, 1807 ± 219 ng/l and 1432 ± 218 ng/l for control, insulin and IGF2 group, respectively, after 40 min. of LAD occlusion. There were no significant differences between treatment groups for troponin T levels.

Discussion

Several experimental studies have demonstrated a cardioprotective nature of insulin and insulin-like growth factors by stimulating the PI3-kinase–Akt pathway [6,7,9,13]. This pathway can modulate the apoptotic cascade, reduce lethal reperfusion injury and thus reduce infarct size after ischaemia–reperfusion. In the present study, reperfusion therapy with low-dose insulin or IGF2 did not reduce myocardial infarct size when measured after 3 hr of reperfusion, following 60 or 40 min. of LAD occlusion.

We aimed at stimulating the PI3-kinase–Akt pathway without altering the serum glucose levels. In animals without LAD occlusion, the infusion of low-dose insulin or IGF2 for 15 min. increased the levels of phosphorylated Akt relative to total Akt in the myocardium by 1.5 to 2, thus demonstrating an activation of PI3-kinase–Akt signalling pathway (fig. 1). In fasting pigs, a corresponding dose of intracoronary insulin (1.8-2.3 mU/kg) was administered without causing severe hypoglycaemia [14] and was shown to stimulate Akt phosphorylation [15].

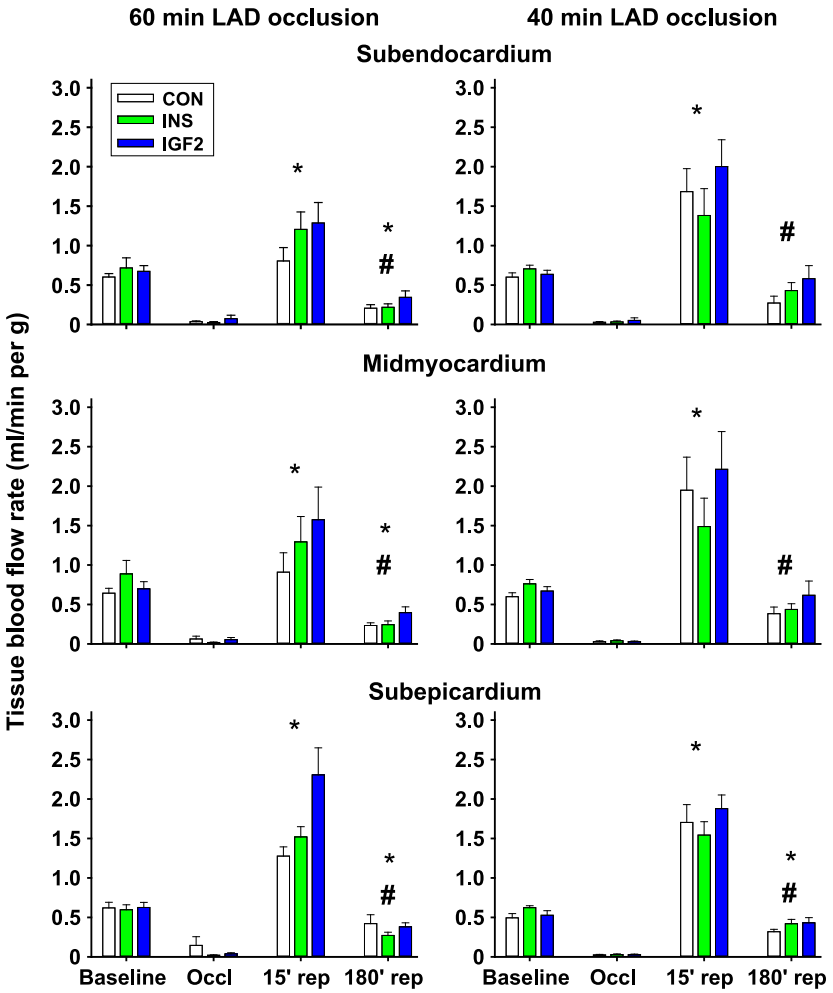

Fig. 5. Tissue blood flow rate in the left ventricular anterior wall in subendocardial, mid-myocardial and subepicardial wall layers after 60 (left) and 40 min. (right) of LAD occlusion followed by reperfusion. Abbreviations as in fig. 3. *Pooled mean different from pooled mean at baseline; #Pooled mean different from pooled mean at 15′ rep ($p < 0.01$ for all).

The amount of IGF2 administered, 2 μg/kg, was decided from our previous study demonstrating an anti-apoptotic effect when administered intracoronarily together with blood cardioplegia, resulting in a total amount of 0.252 μg/kg [16]. Timing of administration was based on studies in isolated rat heart models showing that a single dose of insulin must be administered at the time of reperfusion to reduce infarct size; a delay of 15 min. will abolish the effect [9]. Protocols with 60 and 40 min. of LAD occlusion followed by reperfusion were based on previous studies in pigs demonstrating a cut-off time of approximately 50 min. for an acute coronary occlusion before reperfusion must occur; prolonged occlusion time results in an irreversible ischaemic trauma making reperfusion interventions ineffective [10]. However, there are also comparable experiments in pigs reporting favourable effects of reperfusion interventions after even 75- and 90-min. coronary occlusion [13,17].

This study demonstrates no obvious signs of infarct size reduction when administering insulin or IGF2 at the onset of reperfusion, either after 60 or 40 min. of LAD occlusion despite the activation of RISK pathway. Exenatide, a glucagon-like peptide, reduced apoptosis and infarct size, and improved myocardial function in pigs when administered repeatedly for three days [17]. The prolonged time of elevated insulin levels and a long observation time before estimating infarct size and cardiac function could explain the difference compared with the present study. One single low dose of IGF1 administered as late as 2 hr after reperfusion in a chronic pig model reduced infarct size and improved cardiac function together with reduced apoptosis [13]. It is speculated whether IGF1 contributed to the stimulation of cardiomyogenesis and angiogenesis. In a canine study, the reduction in infarct size and improved cardiac function by administering low-dose insulin at reperfusion was comparable to GIK infusion for 4 hr starting 10 min. before reperfusion [7]. Compared with dogs, the collateral blood flow rate in the myocardium is low in pigs (fig. 5) [18]. Recently, it has been demonstrated in pigs that infarct size reduction after mechanical post-conditioning is independent of RISK phosphorylation [19] and the reperfusion itself can

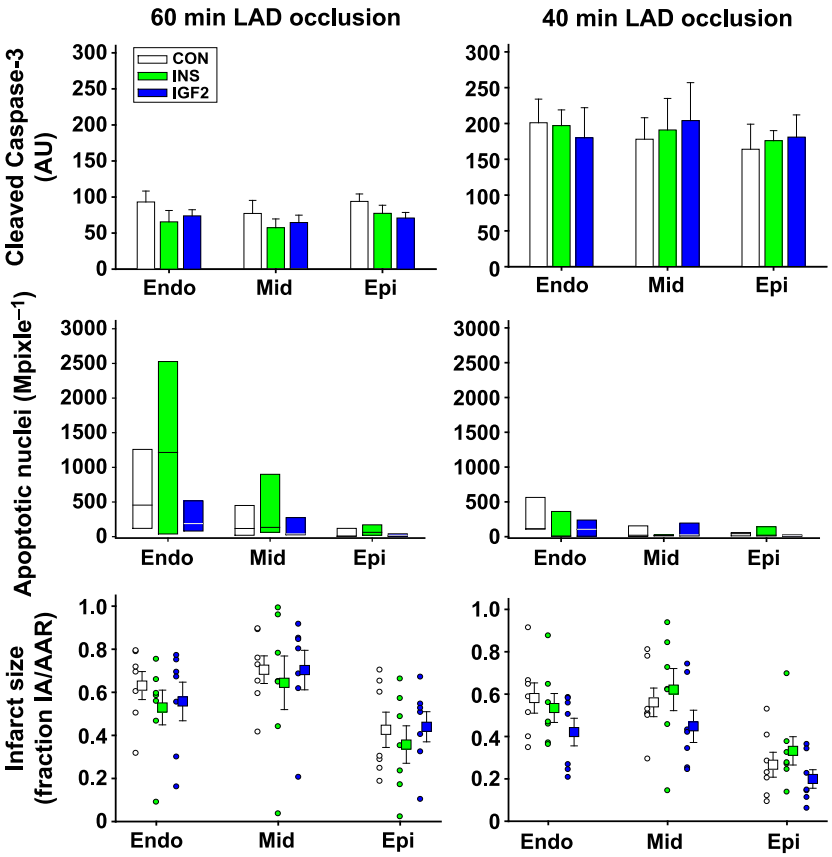

Fig. 6. Cleaved caspase-3 (~22 kDa), number of apoptotic nuclei (TUNEL staining) and infarct size in wall layers of reperfused myocardium in animals with 60 and 40 min. of LAD occlusion followed by 180-min. reperfusion. Bars are mean + S.E.M., box plots presents median with 25 and 75 percentiles, single experiments and mean (squares) ± S.E.M. AU = arbitrary units; IA/AAR = infarct area/area at risk; CON = saline; INS = insulin; IGF2 = insulin-like growth factor 2; Endo, Mid, Epi = subendocardial, mid-myocardial and subepicardial thirds of the left ventricular wall.

phosphorylate this pathway without resulting in a decreased infarct size.

The wavefront phenomenon with myocardial cell death extending both laterally and from the subendocardium to the subepicardium after an acute coronary occlusion has been described clinically and in different experimental animal models, also in pigs [10]. It is thus reasonable to assume that salvageable myocardium is predominantly found in the subepicardium of the area at risk. The myocardial wall was severely ischaemic after LAD occlusion, irrespective of wall layer (fig. 5), but with less extensive development of infarction in the subepicardium where infarct size (% of area at risk) averaged 40.8 ± 0.1% in the Occl-60 and 26.6 ± 0.1% in the Occl-40 series (fig. 6). However, we could not demonstrate any significant reduction in infarct size by insulin or IGF2 in any wall layer. Furthermore, the degree of apoptosis, here evaluated by tissue content of cleaved caspase-3 and TUNEL staining, was not affected by the interventions with insulin or IGF2.

The present study is an acute model with reperfusion for three hours after the ischaemic event. Previously, we have shown that IGF2 added to repeated cold blood cardioplegia improved contractility in the LV wall after 60 min. of cardiac arrest [16]. In the present study, no improvement of post-ischaemic function in reperfused myocardium was demonstrated, irrespective of the myocardial wall layer (figs. 3 and 4). The circumferential STE strain reflects function in mid-myocardial fibres oriented circumferentially, whereas the longitudinal STE strain predominantly describes subendocardium with longitudinal fibres [12]. Neither the radial multilayer TDI strain nor the circumferential or longitudinal STE strain showed functional differences between groups. The persistent reduction in myocardial function could partly be explained by myocardial stunning, lasting from several hours to days after severe ischaemia and reperfusion [20]. This would conceal any favourable effect of the anti-apoptotic interventions in the present study. Stunned myocardium is viable [21]. As the development of infarction in the AAR was not affected by insulin or by IGF2, one might not expect any functional differences between treatment groups, even with prolonged observation. However, a difference between animals with 40 and 60 min. of LAD occlusion could have been detected. This could explain why CI and radial TDI strain decreased slightly between 15 min. and 180 min. of reperfusion in the Occl-60 series, but remained unchanged in animals with 40 min. of LAD occlusion.

Previous studies have demonstrated a vasodilatory effect of insulin via activation of Akt, leading to phosphorylation of endothelial nitric oxide synthase (eNOS) [6]. This increases production of endogenous nitric oxide (NO) resulting in vasodilatation and increased myocardial perfusion. Coronary endothelial apoptosis and dysfunction was reduced and NO production increased in a canine model after treatment with GIK, but not with glucose–potassium alone [22]. In the present study, the hyperaemic response at reperfusion was not affected by insulin or IGF2 (fig. 5).

Limitations

The present *in vivo* pig model could be representative for a subgroup of previously healthy patients with an acute coronary occlusion and STEMI eligible for acute PCI. However, in many patients with STEMI, the acute coronary occlusion is a consequence of a long history of ischaemic heart disease with development of collateral circulation and thus a larger potential for myocardial salvage. In similar *in vivo* models, beneficial effects are demonstrated if the reperfused myocardium is exposed to the anti-apoptotic agent, being either insulin, IGF1 or IGF2, for a long time period after reperfusion [17,23]. In the present study, the amount of insulin or IGF2 may not be fully optimized to effectively activate anti-apoptotic pathways and thus reducing reperfusion injury and myocardial stunning. However, as intended, an additional effect related to glucose metabolism and potassium was not observed.

In conclusion, we could not demonstrate any signs of infarct size reduction or improved regional function when administering low-dose insulin or IGF2 at early reperfusion after 60 and 40 min. of LAD occlusion in an *in vivo* pig model. Neither insulin nor IGF2 reduced apoptotic activity measured as tissue content of activated caspase-3 or the number of apoptotic myocytes in reperfused myocardium.

Acknowledgements

We acknowledge the technical assistance from Lill-Harriet Andreassen, Cato Johnsen, Gry-Hilde Nilsen, Anne Aarsand, Inger Vikøyr, Ingrid Strand and the staff at the Vivarium, University of Bergen. P-R. Salminen and G.O. Dahle received research fellowships from the Western Norway Regional Health Authority. C.A. Moen was a research fellow financed by the University of Bergen. A. Wergeland was the recipient of a research fellowship from Norske Kvinners Sanitetsforening. A.K. Jonassen was funded by Bergen Medical Research Foundation. Financial support was obtained from the Bergen University Heart Fund and the Western Norway Regional Health Authority.

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