Second window of preconditioning normalizes palmitate use for oxidation and improves function during low-flow ischaemia

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Aims

Although a major mechanism for cardioprotection is altered metabolism, little is known regarding metabolic changes in ischaemic preconditioning and subsequent ischaemia. Our objective was to examine the effects of the second window of preconditioning (SWOP), the delayed phase of preconditioning against infarction and stunning, on long-chain free fatty acid (LCFA) oxidation during ischaemia in chronically instrumented, conscious pigs.

Methods and results

We studied three groups: (i) normal baseline perfusion (n = 5); (ii) coronary artery stenosis (CAS; n = 5); (iii) CAS 24 h following 2 × 10 min coronary occlusions and 10 min reperfusion (n = 7). Ischaemia was induced by a left anterior descending (LAD) stenosis (40% flow reduction) for 90 min, dropping systolic wall thickening by 72%. LCFA oxidation was assessed following LAD infusion of 13C palmitate, i.e. during control or stenosis, by in vitro nuclear magnetic resonance of the sampled myocardium. Stenosis reduced subendocardial blood flow subendocardially, but not subepicardially, yet induced transmural reductions in LCFA oxidation and increased non-oxidative glycolysis. During stenosis, preconditioned hearts showed normalized contributions of LCFA to oxidative ATP synthesis, despite increased lactate accumulation. SWOP induced a shift towards LCFA oxidation during stenosis, despite increased malonyl-CoA, and marked protection of contractile function with a significant improvement in systolic wall thickening.

Conclusion

Thus, the second window of preconditioning normalized oxidative metabolism of LCFA during subsequent ischaemia despite elevated non-oxidative glycolysis and malonyl-CoA and was linked to protection of regional contractile function resulting in improved mechanical performance. Interestingly, the metabolic responses occurred transmurally while ischaemia was restricted solely to the subendocardium.

Keywords

Mitochondria • Coronary stenosis • Long-chain fatty acids • β-Oxidation • Ischaemic preconditioning

1. Introduction

Alteration of metabolic substrate utilization has been demonstrated to be an effective strategy for countering the detrimental effects of myocardial ischaemia.1–3 The goal of these therapies is to increase the efficiency of energy production and substrate utilization when the heart is in an oxygen-deficient state; specifically, to shift away from the greater oxidative demands of ATP synthesis from fatty acid utilization to that of glucose oxidation. During reperfusion after ischaemia, pharmacological interventions that inhibit myocardial fatty acid oxidation4–6 or directly activate carbohydrate oxidation7–13 serve to increase left ventricular (LV) function. However, almost all of the prior work on metabolic control of the ischaemic heart has been conducted in anaesthetized animals or isolated hearts,1–6,8–25 while the effects of anaesthesia on cardiac function and its regulation are well recognized.26 One of the unique features of the current investigation is the model of the chronically instrumented pig with coronary stenosis, which eliminates the variable of the depressant effects of anaesthesia and recent surgery on the heart. Additionally, coronary stenosis is much more common than total occlusion and reperfusion in patients with coronary artery disease.
Ischaemic preconditioning is a very powerful innate mechanism which protects the heart from the detrimental effects of ischaemia. Two distinct phases of ischaemic preconditioning have been described: classical, early preconditioning or the first window of preconditioning and late or second window of preconditioning (SWOP), each with distinct mechanisms. The protective effects of early preconditioning last for only 1–2 h, whereas the SWOP develops 12–24 h after initial stimulus and lasts for 3–4 days. Interestingly, despite investigative efforts to elucidate the underlying mechanisms of preconditioning that improve cell survival, relatively few studies have examined potentially cardioprotective metabolic mechanisms in the preconditioned myocardium and most of these studies examined the metabolic alterations during the early or first window of protection during sustained ischaemia. These previous studies demonstrated that during the subsequent ischaemic insult, the first window of protection provided retention of high-energy phosphates, reduced glycolysis, attenuated intracellular acidosis, and reduced accumulation of long and short-chain acyl carnitine. Additional metabolic alterations induced by the first window of protection include enhanced glucose uptake and translocation of GLUT4 to the plasma membrane. Much less is known regarding metabolic mechanisms associated with delayed preconditioning, i.e. second window. Furthermore, metabolic mechanisms associated with preconditioning during myocardial ischaemia induced by a moderate reduction in coronary blood flow have not been investigated. We and others have shown that during moderate myocardial ischaemia, in spite of lactate production, long-chain fatty acid (LCFA) oxidation continues to supply most of the energy for residual aerobic ATP formation. Considering that LCFA utilization is critical for energy synthesis, the goal of the current study was to determine the effects of the second-window protection on both the contributions of LCFA to oxidative metabolism and cardiac function, during myocardial ischaemia induced with a 40% reduction in coronary blood flow for 90 min in the chronically instrumented, conscious pig. The conscious pig model utilized in this investigation does not develop myocardial necrosis in control animals, and infarction was not used as an endpoint in this study. Importantly, this unique model permitted the examination of regional myocardial metabolism, both in subendocardial and subepicardial layers of the heart, along with regional myocardial function. Utilizing the model of coronary stenosis, rather than complete coronary artery occlusion (CAO) to induce myocardial ischaemia, permitted identification of the concomitant changes in regional myocardial function without and with preconditioning in the presence of myocardial ischaemia, whereas prior studies only examined function during reperfusion.

2. Methods

Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, revised 1996). This study has been approved by the University of Medicine and Dentistry Institutional Animal Care and Use Committee, protocol 10004.

2.1 In vivo studies

Seventeen domestic swine, weighing 34.7 ± 0.9 kg, were anaesthetized with isoflurane (0.5 to 1.5 vol%) and instrumented through a left thoracotomy using aseptic technique as described previously. Briefly, a miniature solid-state pressure gauge was implanted in the LV to measure LV pressure and dP/dt. The proximal left anterior descending coronary artery (LAD) was dissected immediately distal to the bifurcation of the left main coronary artery and a catheter was placed as described previously. A proximal LAD hydraulic occluder was implanted on the LAD distal to the intracoronary catheter to induce coronary artery stenosis (CAS). An LAD flow probe was placed 2 cm distal to the proximal hydraulic occluder. A second hydraulic occluder was implanted on the distal LAD to induce CAO and coronary artery reperfusion (CAR). Ultrasound crystals were implanted transmurally to measure systolic wall thickening in the potentially ischaemic (distal LAD) and non-ischaemic zones. Regional systolic wall thickening was calculated as the difference between end-systolic thickness (EST) and end-diastolic thickness (EDT). Per cent systolic wall thickening was calculated as 100 × (EST − EDT)/EDT. All pigs received pre-operative intercostal nerve blocks (bupivacaine), and buprenorphine was administered as a pre- and post-operative analgesic at 0.02 mg/kg im twice a day for 3 days. The experiments were conducted 5–7 days after surgery. Haemodynamics were measured and recorded, and radioactive microspheres used to assess tissue blood flow, as described previously.

2.2 Experimental protocols

The conscious pigs were divided into three experimental groups: Group 1 (n = 5), control without CAS; Group 2 (n = 5), control with CAS; and Group 3 (n = 7), SWOP with CAS (Figure 1). SWOP was induced in Group 3 pigs with a preconditioning stimulus (two episodes of 10 min, CAO/10 min CAR) followed by 24 h CAR. The distal LAD occluder was used to induce SWOP because a CAO of the proximal LAD occluder would have invariably induced cardiac fibrillation. In Group 2 and 3 pigs, the LAD stenosis was created by inflating the proximal LAD occluder to reduce coronary blood flow by ~40%. The degree of coronary blood flow reduction was continuously monitored and sustained for 90 min. Arterial blood was sampled during coronary stenosis for analysis of LCFA content. For both stenosis groups, infusion of labelled substrates was initiated at 50 min into the experimental protocol. All pigs were fasted overnight prior to the experimental procedure.

Intracoronary delivery of 13C-enriched palmitate, at a final blood concentration of 0.4 mM, was performed to measure LCFA oxidation by the myocardium. Exogenous, 2.0 mM [2,4,6,8,10,12,14,16-13C8] palmitate/albumin complex (3:1 molar ratio) in saline was infused into the left anterior coronary artery during the final 40 min of each protocol. An infusion pump was used to deliver the labelled palmitate at 5 mL/min per 25 mL/min of measured flow through the LAD, so that the infusion was always 20% of coronary blood flow for 40 min. This time period was conservative in the intent to establish steady-state isotope enrichment. Regional myocardial blood flow measurement was performed using a stable-isotope-labelled microsphere technique (BioPal, Worcester, MA, USA), was performed at baseline, and again during coronary constriction with substrate infusion (20 min infusion). At the end of each protocol, during continued infusion, the animals were anaesthetized rapidly with sodium pentobarbital (120 mg/kg, left atrial bolus) and a section of the anterior LV wall distal to the distal LAD occluder was excised, divided rapidly into subendocardial and subepicardial segments, and freeze-clamped in liquid nitrogen, all in <45 s. We have previously shown that the effects of transmural sectioning on the excised tissue before freezing does not introduce any significant degree of incidental ischaemia that may influence the measurements.

2.3 In vitro nuclear magnetic resonance spectroscopy and tissue chemistry

Tissue metabolites were extracted from frozen ventricle with the use of 7% perchloric acid and assayed according to published methods. Myocardial triglyceride content was extracted and quantified as described previously. In vitro nuclear magnetic resonance (NMR) data were collected on a 9.4 T NMR system (Bruker Instruments). The fractional 13C enrichment acetyl-CoA produced from [2,4,6,8,10,12,14,16-13C8] palmitate (Fe) was determined from the relative signal intensities and multiplet peaks of
2.4 Statistical analysis

All data are expressed as mean ± SEM. One-way ANOVA among the groups with the Tukey test for post hoc comparison of means was employed for comparing multiple groups. Paired Student’s t-test was used to compare ischaemic vs. non-ischaemic values as well as subendocardium vs. subepicardium data taken from the same heart. A value of $P \leq 0.05$ was taken as the level for significance.

3. Results

3.1 Haemodynamics and tissue blood flow and regional myocardial function

Haemodynamic measurements for all groups are shown in Table 1. Measured values did not change from baseline for the control group throughout the experimental protocol, including during...
substrate infusion. Neither the administration of substrates nor the infusion itself appeared to affect global haemodynamics or regional blood flow for any group.

Coronary blood flow was reduced similarly from baseline during coronary stenosis, by experimental design, for stenosis (−40 ± 0.2%) and the SWOP + stenosis (−39 ± 1.1%) groups. Regional myocardial tissue blood flow (using labelled microspheres) as a per cent change from baseline in the ischaemic zone is shown in Figure 2. Coronary stenosis resulted in significant (P < 0.05) reductions in ischaemic zone subendocardial blood flow for all groups. Coronary stenosis did not result in a significant change in subepicardial blood flow for any group.

Absolute values for ischaemic and non-ischaemic regional systolic wall thickening are shown in Table 1. Baseline systolic wall thickening was similar for all groups prior to stenosis. Wall thickening was reduced from baseline less (P < 0.05) during coronary stenosis in SWOP + stenosis (−53 ± 2.1%) compared with the stenosis group (−71 ± 1.4%) indicating preservation of regional function.

### 3.2 LCFA oxidation

Despite non-oxidative glycolytic metabolism in the ischaemic myocardium, exogenous LCFA oxidation was retained during stenosis but at a reduced level (Figure 3). During hypoperfusion in the stenosis group, the fraction of acetyl-CoA produced from $^{13}$C palmitate was reduced from normal levels, accounting for only 17% of acetyl-CoA produced from $^{13}$C palmitate was reduced (Figure 3). Despite non-oxidative glycolytic metabolism in the ischaemic myocardium and subendocardial blood flow for any group. Black bar indicates subendocardium (n = 5); white bar indicates subepicardium (n = 5). Baseline subendocardial blood flow for stenosis and SWOP + stenosis groups were $1.27 ± 0.13$ and $1.54 ± 0.20$ mL/min/g, respectively. Baseline subepicardial blood flow for stenosis and SWOP + stenosis groups were $1.21 ± 0.08$ and $1.11 ± 0.06$ mL/min/g, respectively. *P < 0.05: per cent change subendocardial vs. subepicardial. †P < 0.05 vs. baseline.

### 3.3 Non-oxidative metabolism

Increased non-oxidative, glycolytic metabolism in the ischaemic myocardium is confirmed by elevated lactate and alanine content, both of which are metabolites of the glycolytic end product, pyruvate (Table 2). Somewhat unexpected was the finding that the subepicardium and subendocardium obtained similar contributions from LCFA oxidation, despite preservation of essentially normal blood flow rates in the subepicardial layer (16 ± 10% reduction from baseline) compared with a more dramatic reduction in subendocardial blood flow (53 ± 7% reduction from baseline). These data indicate that the reduction in LCFA oxidation in the mitochondria during coronary stenosis was independent of a regional, transmural gradient of myocardial blood flow.

In contrast, SWOP normalized the contribution of palmitate to mitochondrial oxidation in the ischaemic myocardium to 26 ± 3 and 22 ± 3% of acetyl-CoA in the ischaemic subepicardium and subendocardium, respectively. This contribution of palmitate to oxidative metabolism during SWOP + stenosis was significantly (P < 0.05) greater than the respective values in the stenosis group (Figure 3).
3.4 Tissue malonyl-CoA and acetyl-CoA content

Tissue malonyl-CoA content from subendocardial samples is shown in Table 3. Subendocardial malonyl-CoA content was similar in SWOP + stenosis (0.95 ± 0.07 nmol/g) and control (0.99 ± 0.07), but lowered (P < 0.05) in the stenosis (0.32 ± 0.05) group. Tissue acetyl-CoA was similar between stenosis and SWOP + stenosis groups, with each group significantly (P < 0.05) less than control.

Subendocardial malonyl-CoA to acetyl-CoA ratios are shown in Table 3. SWOP resulted in a significant (P < 0.05) increase in malonyl-CoA to acetyl-CoA ratio during coronary stenosis compared with both control and stenosis groups.

4. Discussion

The current study tested the hypothesis that second-window protection would result in reduced LCFA oxidation with improved regional myocardial function and mechanical performance, during coronary stenosis. Surprisingly, the metabolic changes induced by the second window of preconditioning run counter to these notions, as we now demonstrate that SWOP elevated the contribution of LCFA to oxidative metabolism during stenosis, to similar levels in the non-ischaemic, normal myocardium, but still improved regional function and mechanical performance despite ischaemia. The changes induced by the SWOP indicate normalization in the reliance on LCFA oxidation, despite levels of non-oxidative glycolysis similar to the ischaemic myocardium, during persistent coronary stenosis and reduced regional blood flow.

We have previously shown during coronary stenosis in pigs that the capacity of mitochondria for oxidation of fuels through β-oxidation and the citric acid cycle actually exceeds the regulated entry of LCFA across the mitochondrial membrane during the hypoxia induced by a 40% reduction in regional blood flow, as implemented similarly in the current work. In the prior study, the hypoperfused myocardium preferentially oxidized short-chain fatty acids (SCFA) over endogenous LCFA compared with normal hearts under baseline conditions. Although an indirect assessment, the results of the previous study suggest a regulatory mechanism at the level of LCFA transport into the mitochondria via the carnitine palmitoyltransferase 1 (CPT-1). Since SCFA do not require CPT, this may explain the increased percentage of SCFA oxidized by the hypoperfused myocardium. Importantly, our previous work demonstrated that LCFA oxidation appears to be inhibited during hypoperfusion by factors other than the availability of residual tissue oxygenation, as the SCFA bypassed the mechanism for restricting LCFA oxidation and were amply oxidized in mitochondria despite the 40% reduction in coronary blood flow. The findings of the current study, which directly monitored LCFA contributions to mitochondrial oxidation, are consistent with these previous results. While hypoperfusion reduced the contribution of LCFA to oxidative metabolism, preconditioning revealed an improved capacity for LCFA oxidation during similar reductions in coronary blood flow. It is of interest to note that the tissue lactate levels in the SWOP + stenosis group, although not significantly different, were elevated relative to the stenosis group, a finding which would be consistent with inhibition of the pyruvate dehydrogenase complex secondary to an increase in fatty acid oxidation, i.e. the Randle cycle effect.

Importantly, the SWOP also produced a significant improvement in wall function during the coronary stenosis. Whereas numerous studies have demonstrated cardioprotection and reduced infarct size with the second window of preconditioning and, correspondingly, improved recovery of cardiac function during reperfusion following a complete coronary occlusion of sufficient duration to induce infarct, these studies could not demonstrate protection of regional function in the absence of infarction. This is likely because the prior studies utilized models involving complete coronary occlusion. However, Sun et al. demonstrated reduced stunning after

### Table 2 Metabolite content: lactate and alanine

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Subepicardium</th>
<th>Subendocardium</th>
<th>Subepicardium</th>
<th>Subendocardium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>5.0 ± 0.4</td>
<td>5.7 ± 1.1</td>
<td>4.4 ± 0.5</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.5 ± 2.2</td>
<td>17.1 ± 2.2</td>
<td>8.4 ± 1.4</td>
<td>9.9 ± 1.0</td>
</tr>
</tbody>
</table>

Values shown as μmol/g dry weight (mean ± SEM).

### Table 3 Subendocardial tissue malonyl-CoA content in control, stenosis, and SWOP + stenosis groups (n = 3 in each group)

<table>
<thead>
<tr>
<th>Malonyl-CoA</th>
<th>Acetyl-CoA</th>
<th>Malonyl-CoA:Acetyl-CoA Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.98 ± 0.21</td>
<td>12.2 ± 0.60</td>
</tr>
<tr>
<td>Stenosis</td>
<td>0.32 ± 0.04*</td>
<td>5.63 ± 0.62*</td>
</tr>
<tr>
<td>SWOP + stenosis</td>
<td>0.95 ± 0.06*</td>
<td>5.18 ± 0.97*</td>
</tr>
</tbody>
</table>

Values shown as nmol/g wet weight (mean ± SEM).

*P < 0.05 different from control.

**P < 0.05 different from stenosis.
Preconditioning raises LCFA use and ischaemic function

repetitive brief coronary occlusions. In the current investigation, we employed a model of sublethal coronary stenosis, which permitted the observation of protected regional function during ischaemia induced by coronary stenosis. These data suggest that mechanical performance is improved in the preconditioned heart, compared with the control myocardium subjected to ischaemia, since the blood flow reduction was similar in the two groups, yet regional contractile function was better after preconditioning. Whether this can be translated to an improvement in cardiac efficiency requires a measurement of venous oxygen, which was not possible in our study. It is likely that oxygen extraction was not affected by preconditioning, since ischaemia induces near maximal extraction of oxygen in the heart.

Interestingly, the reduction in LCFA oxidation in the mitochondria during coronary stenosis in the absence of preconditioning was independent of the regional, transmural gradient of myocardial blood flow. As expected, during coronary stenosis in the normal pig heart, the contribution of palmitate to mitochondrial oxidation declined. However, the reduced LCFA oxidation occurred transmurally, despite the lack of any decrease in blood flow within the subepicardium. This reduced oxidation of palmitate coincided with elevated production of end products of non-oxidative glycolysis, lactate and alanine, which also occurred transmurally despite no blood flow reduction to the subepicardium. Thus, the regional metabolic profile responded to coronary stenosis, even in the subepicardium where blood flow was not reduced.

The finding that LCFA oxidation was reduced similarly in both sub-endocardial and subepicardial tissue during coronary stenosis, despite maintenance of subepicardial blood flow, is consistent with our previous finding of transmural changes in SCFA oxidation. This disassociation between transmural blood flow and metabolism indicates that oxygen availability alone is not the limiting factor that produces the relative shifts in glycolytic activity and LCFA oxidation during coronary stenosis, with or without preconditioning. Transmural differences in blood flow were previously shown not to correlate with changes in creatine phosphate levels in the presence of coronary stenosis in open-chest dogs.

Coronary stenosis in the absence of preconditioning in the current study was associated with a significant reduction in tissue malonyl-CoA content. This was not unexpected, as any reduced production of acetyl-CoA from fatty acid oxidation by mitochondria would then reduce the production of malonyl-CoA by acetyl-CoA carboxylase-2 (ACC-2). Ischaemia also increases AMPK-activated protein kinase activity, which can phosphorylate and inhibit ACC-2, thereby lowering malonyl-CoA levels during ischaemia. Malonyl-CoA plays a key role in control of fatty acid oxidation by inhibiting CPT-1 and mitochondrial oxidation of LCFA. However, the current data sets, showing reduced malonyl-CoA in the hypoperfused myocardium with low palmitate oxidation as well as increased malonyl-CoA in the preconditioned myocardium showing normalized palmitate oxidation during hypoperfusion, do suggest a complex relationship, or perhaps even compartmentation issues, when examined in light of the widely accepted notion of malonyl-CoA inhibition of CPT-1 activity. As mentioned, AMPK phosphorylates and inhibits ACC-2 activity, serving to decrease malonyl-CoA levels during ischaemia. Therefore, an unexpected result was that SWOP resulted in increases in both LCFA contributions to mitochondrial oxidation and tissue malonyl-CoA content during coronary stenosis when compared with during coronary stenosis in the absence of preconditioning. Indeed, malonyl-CoA content and the contribution of LCFA to oxidation produced in the preconditioned, ischaemic myocardium were both similar to levels observed in the non-ischaemic myocardium. These data are in conflict with the proposed inhibitory role of malonyl-CoA on CPT-1 transport and LCFA oxidation, but are not inconsistent with similar findings in the in vivo porcine heart.

Although the inverse association between malonyl-CoA content and LCFA oxidation is widely accepted, very rarely are any discrepancies to this theory mentioned. Studies in isolated mitochondria suggest that malonyl-CoA is a prime factor in reducing LCFA oxidation, but in some studies of intact hearts, this is not the case. For example, an increase in cardiac workload induced by aortic constriction and dobutamine infusion in open-chest pigs resulted in a 2.5-fold increase in LCFA oxidation and malonyl-CoA content. The authors of this study proposed a possible compartmentalization of malonyl-CoA that was masked by the total tissue content which was reported (i.e. malonyl-CoA inhibits CPT-1 on the cytosolic side of the enzyme and the increase in tissue malonyl-CoA in this study may have been a selective increase in malonyl-CoA inside the mitochondrial matrix). Whether the decrease in malonyl-CoA content during coronary stenosis in the absence of preconditioning in the current study resulted from a selective decrease in mitochondrial matrix malonyl-CoA is not known, since cellular malonyl-CoA distribution is difficult, if not impossible to measure. However, the increase in malonyl-CoA content in the preconditioned, ischaemic myocardium was clearly the product of the elevated LCFA oxidation in response to SWOP. In any case, the data on myocardial malonyl-CoA content alone measured in our current study do not offer particular insight into the mechanism by which SWOP normalized the contributions of palmitate to oxidative metabolism, despite the presence of coronary stenosis and regional hypoperfusion. Importantly though, the ratio malonyl-CoA to acetyl-CoA (Table 3), which serves as an index for ACC-2 activity, was significantly elevated in the presence of ischaemia plus SWOP when compared with control hearts. This difference, despite near identical proportions of LCFA entering β-oxidation in both groups (Table 3), suggests not only a different level of ACC-2 activity in response to SWOP but also a different mechanism for regulating the entry of fatty acids into the mitochondria through CPT-1 due to preconditioning and ischaemia.

### 5. Conclusions

In summary, SWOP improved function despite the presence of ischaemia and induced changes in LCFA regulatory mechanisms and oxidation during coronary stenosis. SWOP normalized contributions of LCFA to oxidative metabolism, despite the presence of both reduced regional blood flow and increased non-oxidative glycolysis. Additionally, the reduction in LCFA oxidation during coronary stenosis in the absence of SWOP was independent of the transmural gradient of myocardial blood flow and occurred despite a significant reduction in tissue malonyl-CoA content. These findings indicate that factors other than blood flow and tissue oxygenation regulate LCFA metabolism in the hypoperfused myocardium and that LCFA oxidation supports the enhanced function afforded by SWOP.

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