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Dipropionylcysteine Ethyl Ester Compensates for Loss of Citric Acid Cycle Intermediates During Post Ischemia Reperfusion in the Pig Heart

Takhar Kasumov,

Department Nutrition, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

Naveen Sharma,

Department Nutrition, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

Hazel Huang,

Department of Physiology & Biophysics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

Rajan S. Kombu,

Department Nutrition, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

Andrea Cendrowski,

Department Nutrition, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

William C. Stanley, and

Department Nutrition, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA. Department of Physiology & Biophysics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

Henri Brunengraber

Department Nutrition, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

Takhar Kasumov: kasumot@ccf.org

Abstract

Purpose—During reperfusion, following myocardial ischemia, uncompensated loss of citric acid cycle (CAC) intermediates may impair CAC flux and energy transduction. Propionate has an anaplerotic effect when converted to the CAC intermediate succinyl-CoA, and may improve contractile recovery during reperfusion. Antioxidant therapy with N-acetylcysteine decreases reperfusion injury. To synergize the antioxidant effects of cysteine with the anaplerotic effects of propionate, we synthesized a novel bi-functional compound, N,S-dipropionyl cysteine ethyl ester (DPNCE) and tested its anaplerotic and anti-oxidative capacity in anesthetized pigs.

Correspondence to: Takhar Kasumov, kasumot@ccf.org.

Present Address: T. Kasumov, Department of Gastroenterology & Hepatology and Pathobiology, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue/NE 4-206, Cleveland, OH 44195, USA

Present Address: W. C. Stanley, Division of Cardiology, University of Maryland School of Medicine, HSF-II, Room S022, 20 Penn St, Baltimore, MD 21201, USA

Methods—Ischemia was induced by a 70% reduction in left anterior descending coronary artery flow for one hour, followed by 1 h of reperfusion. After 30 min of ischemia and throughout reperfusion animals were treated with saline or intravenous DPNCE ($1.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $n=8/\text{group}$). Arterial concentrations and myocardial propionate, cysteine, free fatty acids, glucose and lactate uptakes, cardiac mechanical functions, myocardial content of CAC intermediates and oxidative stress were assessed.

Results—Ischemia resulted in reduction in myocardial tissue concentration of CAC intermediates. DPNCE treatment elevated arterial propionate and cysteine concentrations and myocardial propionate uptake, and increased myocardial concentrations of citrate, succinate, fumarate, and malate compared to saline treated animals. DPNCE treatment did not affect blood pressure or myocardial contractile function, but increased arterial free fatty acid concentration and myocardial fatty acid uptake. Arterial cysteine concentration was elevated by DPNCE, but there was negligible myocardial cysteine uptake, and no change in markers of oxidative stress.

Conclusion—DPNCE elevated arterial cysteine and propionate, and increased myocardial concentration of CAC intermediates, but did not affect mechanical function or oxidative stress.

Keywords

Anaplerosis; Citric acid cycle; Heart; Ischemia; Reperfusion; Propionate

Introduction

Post-ischemic reperfusion injury is associated with oxidative stress that results in damage to cell membranes and key proteins involved in cardiac contraction and metabolism [1]. Recovery of ischemic myocardium requires adequate NADH generation from metabolism of carbon fuels in the citric acid cycle (CAC) and production of ATP by oxidative phosphorylation. This process, in addition to the constant supply of acetyl-CoA and oxygen, also relies on steady state concentrations of CAC intermediates which carry the carbons from acetyl-CoA as they are oxidized in the mitochondria [2]. Under normal conditions there is a small release of CAC intermediates, referred to as “cataplerosis”, that is balanced by the entry of CAC intermediates, termed “anaplerosis”. Oxygen deprivation augments cataplerosis, as seen by succinate release into extracellular space [3], suggesting hypoxia induced membrane damage. If the rate of anaplerosis is insufficient to compensate for cataplerosis to maintain an adequate rate of acetyl-CoA oxidation, NADH generation, and ATP production, then myocardial contractile function and cell survival would be compromised.

It has been proposed that myocardial ischemia can be treated by infusing anaplerotic substrates, like glutamate [4,5], pyruvate [6–8] and propionyl-CoA precursors [9,10], to prevent depletion of CAC intermediates and maintain NADH generation, oxidative phosphorylation and contractile function. The beneficial cardiac effect of propionate was demonstrated by Russell et al. [10], who observed that cardiac work cannot be maintained by perfusing the heart with acetoacetate, a fuel that is metabolized only to acetyl-CoA. However, the addition of anaplerotic substrates such as propionyl-carnitine enhanced the rate of acetoacetate oxidation and prevented or reversed the decline of cardiac function. The cardioprotective effect of propionate and its derivatives are attributed to anaplerosis from the carboxylation of propionyl-CoA to form the CAC intermediate succinyl-CoA [2].

Reperfusion after myocardial ischemia causes oxidative stress induced injury and leads to irreversible myocardial damage. A novel approach to cardioprotection is to synergize the anti-oxidant capacity of cysteine with the anaplerotic effects of propionate. Antioxidants are an established approach to prevent reperfusion injury, including several thiol (–SH) containing compounds (dimethylthiourea [11] and mercaptopropionyl glycine [12]). These agents exert

their protective effect through a number of reductive mechanisms, including reduction of oxygen radicals and protection of thiol groups of proteins, and key regions of cellular membranes. Several reports have noted the cardio protective effects of N-acetylcysteine (NAC) [13,14].

To synergize the antioxidant effects of cysteine with the anaplerotic effects of propionate, we designed and synthesized a novel bi-functional NAC analog, N,S-dipropionyl cysteine ethyl ester (DPNCE) (Fig. 1), and evaluated its cardioprotective and metabolic effect in an in vivo pig model of ischemia/reperfusion. DPNCE also serves as a sodium free pro-drug that is rapidly converted to propionate and cysteine in the plasma, and thus avoids the problems of sodium overload encountered with a high dose of sodium propionate. We assessed the effect of DPNCE administration on cardiac mechanical function and in myocardial content of CAC intermediates during a 70% reduction in coronary blood flow and a subsequent period of reperfusion. We hypothesized that treatment with intravenous DPNCE would increase the plasma concentration and myocardial uptake of both cysteine and propionate, and increase the cardiac content of CAC intermediates via propionate carboxylation to succinyl-CoA (Fig. 2), and together this would decrease indices of oxidative stress and improve cardiac contractile function during ischemia and reperfusion.

Methods

Materials

Chemicals and biochemicals were obtained from Sigma-Aldrich. [2,2,3,3,3-²H₅]propionate, [U-¹³C₄] succinic acid, and [U-¹³C₆]citric acid were purchased from Isotec (Miamisburg, OH). [U-¹³C₃]malonyl-CoA was prepared as described previously [15]. Briefly, malonic acid first was activated with thiophenol and then corresponding tioester was transesterified with free CoA. The labeled malonyl-CoA was purified by HPLC and its water solution was standardized by UV spectroscopy. 3-hydroxy-[2,2,3,4,4-²H₅]glutarate was prepared by (i) isotopic exchange of β-ketoglutarate in 40% NaO²H in ²H₂O (Isotec), (ii) reduction of β-keto-[²H₄]-glutarate with NaB²H₄, and (iii) acid extraction of product.

Synthesis of DPNCE

N,S-dipropionylcysteine ethyl ester was prepared in one step by acylation of the thiol and amino groups of L-cysteine ethyl ester with propionic anhydride in the presence of pyridine and N,N-dimethyl pyridine as a catalyst. Into a three necked 2 l round flask loaded with L-cysteine ethyl ester (34.5 g, 0.18 mol), N,N-dimethylpyridine (0.1 g, a catalytic amount) and freshly distilled pyridine (60.0 ml, 0.36 mol, 2 eq) in 700 ml of anhydrous methylene chloride, propionic anhydride (46.2 ml, 2 eq.) was slowly added under N₂ gas with continuous mechanical stirring. After the complete addition, the reaction mixture was stirred for 4 h at 65°C and then one hour at room temperature. The reaction mixture washed consequently three times with 1 N HCl, 10% NaOH, and water. The organic layer was dried over sodium sulfate, and the product was isolated as a white solid compound after evaporation of the solvent. N,S-dipropionylcysteine ethyl ester was dried over P₂O₅ in vacuum. M.p. 43–45°C (ethyl acetate). The yield was 89%.

¹H NMR (CDCl₃, δ, ppm): 1.18 (m, 6H, 2CH₃CH₂C(O)), 1.3 (t, 3H, J=7.1, CH₃CH₂O), 2.25 (quadruplet, 2H, J=7.0, CH₃CH₂C(O)), 2.61 (quadruplet, 2H, J=7.0, CH₃CH₂C(O)), 3.38 (d, 2H, J=6.8, CH₂S), 4.21 (quadruplet, 2H, J=7.1, CH₃CH₂), 4.82 (dd, 1H, J₁=6.8, J₂=6.5, CH₂CHNH), 6.23 (d, 1H, J=6.5, NH).

The purity (higher than 99%) and molecular weight of this compound was confirmed by GC-MS in positive ammonia chemical ionization mode using mass-to-charge ratio *m/z*=262 (M +H⁺).

Animal experiments

Studies were performed using an anesthetized open-chest pig model in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 85-23). The protocol was approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. Animals were instrumented as previously described in detail [16,17]. Briefly, domestic male pigs were sedated with 6 mg/kg of telazol, anesthetized by mask with isoflurane (5%), intubated via a tracheotomy, and ventilated with O₂ to maintain blood gases in the normal range (PO₂ > 100 mmHg), PCO₂ 35–45 mmHg, and pH 7.35–7.45. Anesthesia was maintained with isoflurane (1.0–1.5%). The right femoral artery and vein were catheterized. A midline sternotomy was performed to expose the heart and three left side ribs were partially resected. Heparin was infused into the femoral vein to prevent clotting (200 U kg⁻¹ bolus, followed by 100 U kg⁻¹ hr⁻¹, i.v.). Left anterior descending (LAD) coronary artery flow was controlled by an extracorporeal perfusion circuit via a roller pump with blood from the femoral artery, as previously described [16,17]. Arterial blood samples were obtained from a constant flow (10 ml/min) withdrawal loop from the LAD perfusion circuit so that sampling would not disturb coronary artery blood flow. The anterior interventricular vein was catheterized for coronary venous blood sampling. A high fidelity pressure transducer catheter was used to monitor left ventricular pressure (Millar Instruments), and myocardial segmental shortening in the anterior LV free wall was measured by sonomicrometry (Triton Technologies, San Diego, CA) in duplicate using two pairs of piezoelectric crystals placed at midmyocardial depth, as previously described [16,17]. Heart rate, left ventricular pressure, and segment length were continuously monitored using an online data acquisition system. Contractile function in the LAD perfusion territory was assessed from the left ventricular pressure—segment length loop area, as previously described [16].

Protocols

Dose escalation study: A dose escalation study was performed on one anesthetized pig (29.5 kg) in order to determine the cardiovascular safety and optimal dose of DPNCE to be used in the myocardial infarction studies. DPNCE was infused intravenously at 0.4, 1.6, and 3.2 mg kg⁻¹.min⁻¹ for 30 min at each dose. After baseline sampling, arterial and venous bloods were collected at 10, 20, and 30 min of each dose level, and were analyzed for propionate and cysteine concentrations. Heart rate and LV blood pressure were monitored throughout the infusion.

Myocardial ischemia/reperfusion studies: Two groups of overnight-fasted domestic pigs (Saline treatment: 31.5 ± 4.1 kg, *n*=8), and (DPNCE treatment, 30.5 ± 1.9 kg, *n*=8) were instrumented as described above. Blood flow in the LAD territory was adjusted to give an interventricular venous O₂ saturation of 35–45%. Physiological steady state was achieved following the surgical procedure, as indicated by constant blood gas measurements, heart rate and LV pressure, and segment length systolic shortening. Two baseline blood samples were taken from the LAD coronary artery and the interventricular vein at 10 and 5 min prior to ischemia. Ischemia was induced by a 70% reduction in left anterior descending coronary artery flow for one hour, followed by 1 h of reperfusion. After 30 min of flow reduction animals were infused intravenously with either normal saline (3 ml/min), or with DPNCE (1.5 mg·kg⁻¹.min⁻¹) continuously throughout reperfusion. Arterial and interventricular blood samples were taken simultaneously at 20, 25, 50, 55, 90, 95, 115 and 120 min following the initiation of ischemia (Fig. 3), and were analyzed for the concentration of oxygen, lactate, and glucose in blood, and propionate, cysteine and free fatty acids in plasma. After the last blood sample a large (~3 g) transmural biopsy was taken from the anterior LV free wall for the analysis of citric acid cycle intermediates, malonyl-CoA and oxidative stress markers (glutathione and lipid peroxidation products). Tissue samples were immediately freeze-clamped on aluminum blocks precooled in liquid nitrogen and stored at -80°C until analyses.

Analytical procedures: Arterial and venous pH, PCO₂, and PO₂ were determined on a blood gas analyzer (NOVA Profile Stat 3, NOVA Biomedical Watham, MA), and hemoglobin concentration and saturation were measured on a hemoximeter (Avoximeter, San Antonio, TX). Blood samples for glucose and lactate were deproteinized in ice-cold 1 M perchloric acid (1:2 v/v) and analyzed for glucose and lactate using enzymatic spectrophotometric assays on a 96-well plate reader. Plasma free fatty acids (FFA) were measured by using a commercially available enzymatic spectrophotometric kit (Wako Chemicals; Richmond, VA).

Tissue concentration of malonyl-CoA was analyzed by GC-MS using our previously described method. Briefly, malonyl-CoA was analyzed after spiking with [U-¹³C₃] malonyl-CoA, alkaline hydrolysis as TBDMS derivatives of free acids. Under ammonia-positive chemical ionization, ions monitored were 333.2 (M+H⁺) and 336.2 (M+H⁺+3) for unlabeled and labeled malonyl-CoA, respectively. The concentration of CAC intermediates were assayed using TBDMS derivatization with some procedure modification [18,19]. For quantification tissue samples were spiked with following standards: [U-¹³C₆]citrate for citrate, [U-¹³C₄] succinate for succinate and fumarate, and 3-hydroxy-[2,2,3,4,4-²H₅]glutarate for malate. Under electron ionization, ions signals monitored were 459, 465 for citrate, 287, 291 for fumarate, 289, 293 for succinate, 419 for malate, and 438 for 3-hydroxy-[2,2,3,4,4-²H₅]glutarate.

Plasma propionate concentrations were analyzed after spiking samples with [2,2,3,3,3-²H₅] propionate internal standard and derivatization with pentafluorobenzylbromide by ammonia negative chemical ionization GC-MS [20]. The concentration of propionate was calculated from the signals at mass-to-charge ratio (m/z) 73 (unlabeled propionate) and 78 (internal standard, [2,2,3,3,3-²H₅]propionate) using a calibration curve.

Plasma cysteine was analyzed using a chloroformate derivatization technique [21]. Briefly, EDTA-treated blood samples were centrifuged to obtain EDTA-stabilized plasma. After treatment with dithioerythritol solution containing [3,3-²H₂]cysteine (internal standard), samples were incubated at room temperature for 20 min to reduce cysteine disulfide bonds. Samples then were deproteinized and the supernatant was derivatized with ethyl chloroformate and pyridine in toluene. Ethylformate derivatives of cysteine were analyzed by GC-MS in positive chemical ionization mode using ammonia as an ionization gas. Ion pair 311 (M +NH₄⁺) and 313 (M+2+NH₄⁺) were used for cysteine and [3,3-²H₂] cysteine analysis, respectively. The concentration of cysteine was calculated using a calibration curve, run in parallel with plasma samples.

The cardiac tissue oxidative stress markers GSH and DTT-reducible “bound” forms of glutathione, i.e., GSSR, were analyzed using an electrospray-ionization API 4000 Q Trap mass spectrometer (Applied Biosystems, Foster City, CA) coupled to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) [22]. Powdered frozen heart tissue (50–100 mg) was spiked with 10 µL of *homo*-glutathione (20 µg/mL) internal standard and extracted with 4 volume of ice cold 50 mM iodoacetamide solution in 10 mM ammonium bicarbonate using a Polytron homogenizer. The proteins were precipitated by adding 3 volumes of acetonitrile and centrifugation. To derivatize reduced glutathione, the supernatant was incubated with 0.1 mL of 50 mM iodoacetate for 45 min. Solvents were evaporated to dryness under nitrogen at 50° C for 20 min in a TurboVap LV (Caliper LifeSciences, Inc, USA). The residue was reconstituted in 100 µL of formic acid in water (0.1%):acetonitrile (99:1). Five µL was injected onto HPLC-MS system.

For the analysis of total glutathione 0.2 mL of DTT (0.1 mM) was added tissue extract to reduce oxidized “bound” glutathione (GSSR). After 15 min, 0.2 mL of iodoacetonitrile was added and samples were analyzed as above.

The auto sampler cooler of the HPLC system was maintained at 6°C. The analysis was performed on a Hypersil Gold C18 column (2.1×150 mm, 5 µm particle size; Thermo Electron Corp.) maintained at 25°C. Mobile phase A was formic acid in water (0.1%):acetonitrile (99:1, v/v) and mobile phase B was formic acid in acetonitrile (0.1%). The HPLC flow rate was 0.2 mL/min. For elution the mobile phase A was initially held at 100% for 5 min. By 1 min the aqueous phase was brought down to 20% and held for 1 min. Finally the gradient was brought back to 100% A by 1 min and was held for 9 min for equilibration.

The turbo ion spray source was maintained at 500°C under nitrogen nebulization at a pressure of 30 p.s.i. The heater gas (nitrogen) was at a pressure of 60 p.s.i. The curtain gas pressure was 35 p.s.i. and the collision-activated dissociation gas pressure was held at high; turbo ion-spray voltage was 5,500 V. Declustering potential was 65 V; entrance potential was 10 V; collision cell exit potential was 12 V; and channel electron multiplier was 5,500 V. The MRM pairs monitored were 366.20→237.20 for carboxymethyl-GSH (reduced glutathione), 347.20→218.10 for cyanomethyl-GSH (“bound” glutathione) with a dwell time of 100 msec. Analyst software (version 1.4.1; Applied Biosystems) was used for data registration and calibration.

Tissue lipid peroxidation was determined using a fluorometric assay of thiobarbituric acid (TBA)-reactive material. This assay is based on measurement of fluorescent red 1:2 adduct which is formed between malonodialdehyde (MDA) and TBA at 532 nm. Because of nonspecificity of TBA adduct, the absorbance measured, in addition to MDA also represents all other TBA-reactive aldehydes. To prevent oxidation during the assay samples were treated with butylated hydroxytoluene (BHT) in the beginning of the assay.

Calculations: The external mechanical power generated by the myocardium in the LAD perfusion bed was calculated as the product of the pressure-segment length area and heart rate, as previously described [16,17]. MVO_2 , uptakes of glucose, lactate, glucose, free fatty acids, and propionate were calculated as the products of the arterial-venous concentration difference and LAD blood flow. The LAD blood flow was calculated as the perfusion pump rate divided by the mass of LAD tissue. The relative contribution to the CAC flux from anaplerosis from propionate was calculated by dividing the myocardial uptake of propionate by the CAC flux as estimated from the MVO_2 , as previously described [23].

Statistical analysis—Data are presented as the mean ± SEM. Metabolite concentrations are presented as an average of triplet assays. An average of duplicate GC-MS and LC-MS injections, which differed by <2%, were used for mass-spectrometric analysis. Treatment effects for blood metabolites were evaluated using a 2-way ANOVA for repeated measures. Differences in tissue metabolite were assessed using a 1-way ANOVA. Post hoc analysis was performed using the Bonferroni test (Graph Pad Prism Software, version 3).

Results

Dose escalation study

DPNCE was infused intravenously in a pig at 0.4, 1.6, and 3.2 mg·kg⁻¹·min⁻¹ in 30 min steps. Heart rate and left ventricular pressure did not change over the course of the infusion. Only trace amounts of unhydrolyzed DPNCE and a small fraction of N-propionylcysteine, a product of partial DPNCE hydrolysis, were found in the arterial plasma (data not presented), indicating that the thiol ester was completely hydrolyzed, presumably by plasma esterases. The concentrations of arterial propionate and cysteine were increased in a dose dependent manner (Fig. 4). The preinfusion concentration of propionate was low (0.04 mM) and increased ~20-fold to 0.74 mM at a dose of 3.2 mg·kg⁻¹·min⁻¹ (Fig. 4a). Cysteine concentration was 0.25 mM prior to infusion and increased 3-fold at the highest dose of DPNCE (Fig. 4b). Since 1.6

mg·kg⁻¹·min⁻¹ infusion of DPNCE resulted in ~0.28 mM arterial propionate and 0.4–0.5 mM cysteine concentrations (twice baseline cysteine levels), we concluded that a 1.5 mg·kg⁻¹·min⁻¹ dose would elicit an acceptable concentration of propionate and cysteine for the subsequent studies with ischemia/reperfusion. We considered 0.28 mM arterial propionate concentration acceptable based on our previous studies which have shown that anaplerosis from 0.25 mM arterial propionate in pig accounted for ~8% of myocardial CAC flux [23], compared to 5% anaplerosis from all sources at zero propionate concentration in perfused rat heart [9]. We also postulated that doubling the levels of arterial cysteine (a limiting substrate for glutathione synthesis) would be sufficient to stimulate the glutathione synthesis.

Myocardial ischemia/reperfusion studies

Cardiovascular parameters—Myocardial ischemia and reperfusion decreased regional contractile function, with no differences between Saline and DPNCE groups at any time point (Table 1). In addition, there were no significant changes over the course of the experiment or between groups in the heart rate, peak left ventricular (LV) systolic pressure, peak dP/dt (Fig. 5), or LV end-diastolic pressure (data not shown). Baseline myocardial oxygen uptake was similar in the two groups and decreased to a similar extent during ischemia to 40% of baseline values, and return to 80% of baseline values at the end of reperfusion (Fig. 5). Taken together, functional data suggest that DPNCE did not affect cardiac mechanical function.

Metabolites concentrations and fluxes—The baseline arterial and venous plasma cysteine concentrations (Fig. 6) were similar in both groups (~0.2 mM) and were constant throughout the study for the Saline group. In the DPNCE group the arterial and venous cysteine concentrations increased, reaching ~0.5 mM at the end of reperfusion. No significant differences between arterial and venous cysteine concentrations were observed, indicating that the heart did not take up any measurable amount of cysteine despite an elevation in arterial concentration.

Plasma propionate concentrations (Fig. 6) were low at the baseline in both groups (0.023±0.001 and 0.017±0.001 mM in the coronary artery and vein, respectively). In the Saline group these values did not change significantly throughout the ischemia and the reperfusion periods. However, after 20 min of DPNCE infusion propionate concentration in the artery and vein increased to 0.234±0.002 and 0.167±0.008, respectively, and remained stable throughout reperfusion. Propionate uptake in the LAD bed was not detected in the Saline group, but in the DPNCE group was 0.022±0.005 and 0.041±0.008 μmol·g⁻¹·min⁻¹, with an extraction fraction of 29% and 45%, during ischemia and reperfusion respectively. An estimate of baseline CAC flux, calculated based on myocardial oxygen uptake, were very similar in both groups (0.969±0.078 and 0.934±0.060 μmol·g⁻¹·min⁻¹ for Saline and DPNCE groups respectively). Using propionate uptake by myocardium as an estimate of the rate of propionate conversion to the CAC intermediate succinyl-CoA, the rate of anaplerosis from propionate as a fraction of CAC flux was calculated as previously described [23]. In the DPNCE group the anaplerosis from exogenous propionate uptake accounted for 5.0±0.6% and 6.7±0.2% of CAC flux during ischemia and reperfusion, respectively. Anaplerosis from propionate during DPNCE infusion was comparable to the values previously observed with a similar arterial propionate concentration obtained during sodium propionate infusion under normal aerobic conditions (8.0±0.5%) [23]. Since there was no detectable propionate uptake at baseline and throughout the ischemia/reperfusion protocol in the Saline group, there was negligible anaplerosis from endogenous propionate.

Table 2 presents myocardial tissue concentrations of CAC intermediates in Saline and DPNCE treated pigs. Compared to Saline treatment, DPNCE increased the tissue content of fumarate, succinate and malate, indicating a clear anaplerotic effect (Table 2). There was no difference

in citrate content between the Saline and DPNCE groups. Figure 7 shows the tissue content of CAC intermediates expressed as a percent of previously published values from normal healthy untreated pigs [18]. Compared to normal animals, ischemia/reperfusion decreased the tissue contents of citrate, succinate, and fumarate by 30–50% in the Saline group, and treatment with DPNCE clearly prevented the decrease in succinate and fumarate, and increased the content of malate above normal values (Fig. 7).

The baseline arterial lactate, fatty acid, and glucose concentrations were similar in both groups (Table 3). Arterial lactate concentration for the Saline group did not change significantly throughout the study, while it increased with DPNCE treatment. Ischemia/reperfusion did not significantly affect the arterial concentration of fatty acids in the Saline group, but in the DPNCE group fatty acid concentration increased progressively throughout the study (Table 3).

Ischemia resulted in a similar switch from lactate uptake to lactate production in both groups, which reverted back to lactate uptake during reperfusion, with no significant differences between treatment groups (Fig. 8). Glucose uptake was not different between groups and was not significantly affected by ischemia or reperfusion (Table 4). Fatty acid uptake decreased during ischemia uptake in both groups ($p < 0.05$) which remained below baseline values during after reperfusion in the Saline group, but returned within baseline levels in the DPNCE group, presumably due to propionate uptake. No difference was observed in the tissue contents of malonyl-CoA (6.15 ± 1.05 versus 8.05 ± 1.51 nmol/g wet wt in the Saline and DPNCE groups), a key regulator of carnitine palmitoyltransferase-1 and myocardial fatty acid oxidation.

Treatment with DPNCE did not affect markers of oxidative stress, as seen in the similar values for TBA-reactive dialdehydes and the GSH/GSSR ratios for both groups (Table 5). Note that GSSR includes GSSG and other heterogeneous pools of oxidized “bound” glutathione and therefore it is more precise index of oxidative stress than GSH/GSSG [22].

Discussion

This initial investigation of the novel bi-functional NAC analog DPNCE demonstrates that it is an effective precursor to cysteine and propionate in vivo, and significantly elevates myocardial content of CAC intermediates following ischemia/reperfusion. The immediate disappearance of DPNCE from the blood and the appearance of cysteine and propionate in plasma support our hypothesis that this bi-functional compound is a bioavailable prodrug of cysteine and propionate. The pig heart extracted a substantial portion of arterial propionate in a single pass through the coronary vasculature, which boosted anaplerotic flux into the CAC as demonstrated by a significant increase in the tissue content of CAC intermediates. Anaplerosis from propionate contributed ~5% and ~7% of the estimated CAC flux during ischemia and reperfusion, respectively, which is similar to the value we previously reported in non-ischemic pigs [23]. However, despite the seemingly favorable metabolic effects and an elevation in plasma cysteine with DPNCE treatment, there was no improvement in cardiac contractile function.

Although DPNCE substantially increased arterial cysteine concentration, the myocardium did not extract a measurable amount of cysteine. As predicted, from the lack of uptake of the plasma cysteine in this study, DPNCE did not affect markers of the oxidative stress during ischemia and reperfusion. These results are in contrast to previous studies that demonstrated the anti-oxidative effects with another cysteine derivative, NAC, in post-ischemic myocardium [13, 14]. However, this discrepancy could be related to the slow turnover rate of the myocardium glutathione pool. Although there are no data on glutathione kinetics in pig heart, the available data on pig erythrocytes glutathione fractional synthesis (~60%/day) [24] may suggest that 1.5

h experiment with DPNCE perfusion may not be sufficient to observe the changes in glutathione production from cysteine. This is also supported with another study [25] confirming that NAC treatment did not alter total glutathione concentrations in dog hearts, which went through 90 min of proximal left descending artery occlusion followed by reperfusion with the administration of NAC beginning 30 min post occlusion and continuing for 3 h after reperfusion. Another explanation for unchanged myocardial glutathione levels in this study could be that one hour with a 70% flow reduction did not cause sufficient oxidative stress during reperfusion.

Previous studies found that treatment with propionate can improve cardiac metabolic and contractile function with ischemia and/or reperfusion [26,27], an effect which may be attributed to increased anaplerosis. Our earlier work showed that propionate is readily converted to the CAC intermediate succinyl-CoA in pig myocardium and perfused rat hearts and thus is a very effective anaplerotic substrate in the heart [23,28]. In this study we have demonstrated that ischemia results in reduction of CAC intermediates concentrations in myocardium, which was abolished by DPNCE infusion. In addition DPNCE infusion significantly increased myocardial malate content compared to both ischemic and non-ischemic pig hearts, indicating that the cardiac malate pool is the major “sink” for propionate induced anaplerosis. This is in agreement with our previous study on anaplerosis from propionate on perfused rat heart showing that propionate perfusion increases total content of CAC intermediates, and that about 80% of this increase is accounted for by the accumulation of malate [9]. However, in our previous study on the quantitative assessment of anaplerosis from propionate in normal pig heart in vivo we did not observe any changes in the myocardial concentrations of succinate, fumarate and citrate, the three CAC intermediates measured in that study [23]. It is most likely that propionate infusion would increase myocardial malate content, which was not measured.

However, despite the substantial relative anaplerosis from propionate, DPNCE infusion did not significantly improve the absolute total CAC flux, estimated based on substrate utilization (Table 4) and myocardial oxygen uptake (Fig. 5d). In addition, in the present study the increase in the myocardial concentration of CAC intermediates did not have any effect on contractile function during DPNCE treatment, suggesting that limited anaplerotic flux does not impair mitochondrial function during short-term ischemia/reperfusion. This also was evident from our functional data which demonstrates that ischemic myocardium had a relatively high contractile function despite the reduction of a 70% in myocardial blood flow. It is possible that the duration and extend of ischemia in our study was not sufficient to induce substantial oxidative stress and impairment of mitochondrial metabolic function. The experimental preparation used in this study is similar to the clinical conditions of acute coronary syndrome or unstable angina, and it is not a model of acute myocardial infarction. Further studies are warranted in models of acute myocardial infarction with prolonged ischemia (1–2 h) followed by reperfusion to stimulated oxidative stress and anaplerosis.

In conclusion, an intravenous infusion of DPNCE, a novel synthetic bi-functional compound, increased arterial concentration of propionate, cysteine and myocardial tissue content of CAC intermediates in swine myocardium, but did not affect markers of oxidative stress or cardiac mechanical function. Additional studies are needed with more severe ischemia and extended treatment during reperfusion to fully evaluate this approach to treating myocardial reperfusion injury.

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Fig. 1.
Structure of N,S-Dipropionylcysteine ethyl ester (DPNCE)

Fig. 2.
Proposed metabolism of DPNCE in myocardium

Fig. 3. Study protocol. Blood flow in the LAD was reduced by 70% from 0 to 60 min, and the flow was restored from 90 to 120 min. DPNCE or saline was infused from 30 to 120 min

Fig. 4.

Arterial blood concentrations of propionate and cysteine in one anesthetized pig treated with a progressive intravenous infusion of DPNCE in three 30 min steps. The *inserts* show the calculated A-V difference of propionate and cysteine

Fig. 5. Cardiovascular parameters during the ischemia/reperfusion study: Left ventricular (LV) peak systolic pressure (a), Peak first derivative of LV pressure with time (dP/dt) (b), heart rate (c), and myocardial oxygen consumption (MVO_2) plotted as a function of time. There were no statistical differences between treatment groups for any of the parameters. * $P < 0.05$ compared to both baseline and reperfusion periods

Fig. 6. Propionate and cysteine concentrations in the coronary arterial blood and interventricular venous blood. Throughout the period of DPNCE infusion the propionate and cysteine concentrations were significantly elevated above the baseline values ($P < 0.05$), while there were no significant changes in saline treated animals

Fig. 7.

Myocardial content of citric acid cycle intermediates expressed as a percentage of normal (*non-ischemic*) myocardium, * $P < 0.05$ compared with normal myocardium; ** $P < 0.05$ compared with saline treatment (*ischemic reperfusion*); † $P < 0.05$ compared with normal myocardium and saline treatment

Fig. 8.
Net myocardial lactate uptake. * $P < 0.05$ compared to baseline and reperfusion samples within treatment group

Table 1

Anterior external wall power (expressed as a % of baseline)

	Ischemia (Pretreatment)	Ischemia ($\pm$ Treatment)	Early reperfusion	Late reperfusion
Saline Treatment	79.4 $\pm$ 8.6	51.5 $\pm$ 4.3	65.9 $\pm$ 4.1	67.0 $\pm$ 4.6
DPNCE Treatment	82.1 $\pm$ 11.4	46.4 $\pm$ 5.4	69.6 $\pm$ 5.3	64.9 $\pm$ 4.9

Pretreatment Ischemia values were taken at 20–25 min of ischemia, and Ischemia $\pm$ Treatment values at 50–55 min. Early reperfusion measurements were taken at 20–25 min and late at 55–60 min of reperfusion. All values were significantly different from the pre-ischemic baseline measurements ($p < 0.05$), and there were no difference between treatment groups

Table 2

Myocardial citric acid cycle intermediates concentrations after 1 h of reperfusion

	Citrate	Succinate	Fumarate	Malate
Non-ischemic Myocardium	430±43	207±52	50±5	112±13
Saline Treatment	308±41 *	118±27 *	15±3 *	92±11
DPNCE Treatment	339±26	210±39 **	49±5 **	205±13 ***

Values expressed as nmol/g wet wt

* $P<0.05$ compared to non-ischemic group** $P<0.05$ compared to saline treated group*** $P<0.05$ compared to non-ischemic and saline treated groups

Table 3

Arterial concentrations (mM) of glucose and lactate in whole blood, and plasma free fatty acids

	Baseline	Ischemia ($\pm$ Treatment)	Late reperfusion
Glucose			
Saline Treatment	3.50 $\pm$ 0.47	3.21 $\pm$ 0.42	2.97 $\pm$ 0.39
DPNCE Treatment	2.96 $\pm$ 0.39	2.60 $\pm$ 0.41	2.43 $\pm$ 0.63
Lactate			
Saline Treatment	1.63 $\pm$ 0.13	1.81 $\pm$ 0.38	1.96 $\pm$ 0.34
DPNCE Treatment	1.86 $\pm$ 0.12	2.41 $\pm$ 0.18 ^{**}	3.61 $\pm$ 0.34 ^{**}
Free Fatty Acids			
Saline Treatment	0.21 $\pm$ 0.02	0.18 $\pm$ 0.01	0.19 $\pm$ 0.01
DPNCE Treatment	0.26 $\pm$ 0.04	0.41 $\pm$ 0.06 [*]	0.52 $\pm$ 0.09 [*]

^{*} $P < 0.05$ compared with baseline

^{**} $P < 0.05$ indicates differences between saline treatment at same time point

Table 4

Rates of free fatty acid and glucose uptake ($\mu\text{mol g wet wt}^{-1} \text{ min}^{-1}$) in the left anterior descending coronary artery perfusion bed during conditions of normal flow, ischemia with treatment, and reperfusion

	Baseline	Ischemia ($\pm$ Treatment)	Late reperfusion
Glucose Uptake			
Saline Treatment	0.36 $\pm$ 0.16	0.12 $\pm$ 0.03	0.17 $\pm$ 0.08
DPNCE Treatment	0.22 $\pm$ 0.06	0.16 $\pm$ 0.04	0.43 $\pm$ 0.19
Free Fatty Acid Uptake			
Saline Treatment	0.022 $\pm$ 0.01	0.013 $\pm$ 0.019*	0.011 $\pm$ 0.007*
DPNCE Treatment	0.031 $\pm$ 0.01	0.018 $\pm$ 0.006*	0.023 $\pm$ 0.008

* $P < 0.05$ indicates significantly different from baseline values within treatment group

Table 5

Myocardial glutathione redox state and thiobarbituric acid reactive products (TAB-RP) in reperfused myocardium. Reduced glutathione (GSH) and oxidized forms of glutathione (GSSR, which includes GSSG and other heterogeneous pools of “bound” glutathione). Glutathione redox state was calculated from GSH and GSSR content. GSH and GSSR are expressed in $\mu\text{mol/g}$ wet wt, and TBA-RP in nmol/g wet wt

	[GSH]	[GSSR]	[GSH]/[GSSR]	[TBA-RP]
Saline treatment	0.90 $\pm$ 0.09	0.19 $\pm$ 0.03	5.0 $\pm$ 0.5	180 $\pm$ 20
DPNCE treatment	0.82 $\pm$ 0.11	0.23 $\pm$ 0.04	4.0 $\pm$ 0.4	232 $\pm$ 27