Protection of ischemic hearts perfused with an anion exchange inhibitor, DIDS, is associated with beneficial changes in substrate metabolism

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Abstract

Objective: Metabolic interventions that promote glucose use during ischemia have been shown to protect the myocardium and improve functional recovery on reperfusion. In this study we evaluated if cardioprotection can be accomplished by inhibiting fatty acid uptake, which would be expected to increase glycolytic metabolism. Methods: Diisothiocyanostilbene sulfonic acid (DIDS), commonly used to inhibit Band-3 mediated anion exchanger, and has also been demonstrated to inhibit fatty acid transport in adipocytes, was used to inhibit fatty acid uptake prior to ischemia. Isolated rat hearts were perfused with buffer containing 5 mM glucose, 70 mU/l insulin, 0.4 mM palmitate, and 0.4 mM albumin, paced at 300 beats/min, and subjected to 50 min of low-flow ischemia followed by 60 min of reperfusion. Results: Ischemic injury, as assessed by creatine kinase release, was diminished in hearts perfused with DIDS (334 ± 672 in DIDS vs. 565 ± 314 IU/g dry wt in controls, P < 0.04). Increases in LVEDP during ischemia were attenuated (8 ± 6 15 ± 18 mmHg in DIDS vs. 15 ± 18 mmHg in controls, P < 0.03) and the % recovery of LV function with reperfusion was enhanced in DIDS-treated hearts (78 ± 10% of baseline in DIDS vs. 62 ± 19% of baseline in controls, P < 0.04). These beneficial effects of DIDS were associated with increased glucose metabolism and ATP content during ischemia and reperfusion. Furthermore, treatment with DIDS lowered the accumulation of long chain acyl carnitines. Conclusions: This study demonstrates that DIDS protects ischemic myocardium, and is associated with inhibition of fatty acid uptake, improved glucose metabolism, and enhanced functional recovery on reperfusion. The data presented here suggest a potential role for therapeutic agents that lower fatty acid uptake as a metabolic adjunct in the treatment of myocardial ischemia. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Recanalization therapies are effective in restoring blood flow to ischemic myocardium and thereby salvaging jeopardized tissue. However, adjunctive therapy based on metabolic modification may be additionally beneficial in the management of myocardial ischemia. In fact, recent reviews and editorials have called for a re-exploration of the use of metabolic strategies for myocardial preservation in patients with ischemic heart disease. Recent clinical studies have demonstrated that infusion of glucose–insulin–potassium, which decreases plasma fatty acid levels and thereby increases glucose use, is beneficial in patients suffering from myocardial infarction [1,2].

Previous experimental studies have demonstrated that failure of energy production appears to be critical in ischemic myocardium [3–8]. With ischemia, production of adenosine triphosphate (ATP) increasingly depends on glycolytic flux, which eventually ceases because of feedback inhibition by hydrogen ions, lactate, and fatty acid metabolites [3,4,7,9–11]. Several studies have shown that sustenance of glycolytic metabolism during ischemia can...
preserve metabolism [4,12–17], delay contracture — a hallmark of glycolytic failure and irreversible cell damage, and improve salvage with reperfusion.

Enhancement of glycolysis in the setting of low-flow ischemia can be accomplished by many approaches. In addition to enhancement of glucose metabolism directly with high glucose, stimulation of adenosine A₁ receptors [18,19], certain agents such as dichloroacetate or ranolazine [4,13,14,20] and ischemic preconditioning [4,21] may elicit beneficial effects in a low-flow ischemia model, in part, by sustaining glycolysis.

An alternative approach to achieving the switch to glycolysis is by inhibiting fatty acid uptake [4,22–25]. Decreasing fatty acid uptake limits accumulation of intermediates, such as citrate, ATP, and NADH, that inhibit glucose use [4]. Thus, theoretically, blockade of fatty acid uptake may enhance myocardial glucose use via the Randle cycle and thereby protect ischemic myocardium. Diisothiocyanostilbene sulfonic acid (DIDS) has been commonly used to inhibit Band-3 mediated anion channel. However, DIDS has also been shown to inhibit fatty acid uptake in cell culture and to characterize fatty acid binding sites in fatty acid transport proteins [26–28]. Since studies demonstrating DIDS as an inhibitor of fatty acid transporter were performed using adipocytes, it is unclear if DIDS would inhibit fatty acid uptake in isolated perfused rat hearts. Therefore, our goal in this study was to investigate if DIDS would inhibit myocardial fatty acid uptake in isolated perfused hearts, limit ischemic damage and enhance the benefits of reperfusion.

2. Methods

All studies were performed with the approval of the Institutional Animal Care and Use Committee at Columbia University, New York. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, 1996).

All chemicals of the highest purity were obtained from commercial sources and were used without further purification except bovine serum albumin (BSA). Fatty acid free BSA (Amersham) was dialyzed for 48 h to remove low molecular weight impurities. Furthermore, BSA preparations were tested to rule out any endotoxin contamination. Total long chain fatty acid content of these BSA solutions was measured by gas chromatography and determined to be less than 0.02 mM.

2.1. Isolated perfused heart preparation

Experiments were performed using an isovolumic isolated heart preparation as published earlier [13,14,21] and modified for the use in rat hearts. Rats were anesthetized using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). After deep anesthesia was achieved, hearts were rapidly excised, placed into iced saline, and retrogradely perfused at 37°C in a non-recirculating mode through the aorta at a rate of 12.5 ml/min. Hearts were perfused with modified Krebs–Henseleit buffer containing (in mM) NaCl 118, KCl 4.7, CaCl₂ 1.2, MgCl₂ 1.2, NaHCO₃ 25, glucose 5, palmitate 0.4, BSA 0.4, and 70 mU/l insulin. The concentrations of palmitate and albumin used in this study were based on concentrations of fatty acids and albumin present in rat plasma. The perfusate was equilibrated with a mixture of 95% O₂–5% CO₂, which maintained perfusate Po₂ >600 mmHg. All hearts were paced at 300 beats per min. Left ventricular developed pressure (LVPD) and left ventricular end diastolic pressure (LVEDP) were measured using a latex balloon in the left ventricle. LVPD, heart rate, and coronary perfusion pressure were monitored continuously on a four-channel Gould recorder.

2.2. Palmitate uptake in the presence of DIDS

To determine the ability of DIDS to inhibit palmitate uptake, in a separate set of experiments, hearts were perfused with DIDS (10 μM) under normoxic conditions. In order to accurately monitor fatty acid uptake, after equilibration, hearts were subjected to perfusion with recirculating buffer (100 ml). The difference between the concentration of palmitate in the perfusate before and after recirculation yields the amount of palmitate used by the heart. A similar set of studies was performed to measure palmitate uptake during reperfusion.

2.3. Measurement of glucose oxidation

To determine if DIDS alters glucose oxidation, [¹⁴C]glucose oxidation rates were measured in hearts perfused with DIDS. The radiotracer technique used in this study is similar to that published by Lopaschuk et al. [9,10,29,30]. Briefly, hearts were perfused for 60 min with [U-¹⁴C]glucose (specific activity 440 000 dpm/ml) in the recirculating mode and the perfusate analyzed for glucose oxidation product ¹⁴CO₂ as published in the literature [9,10,29,30].

2.4. Ischemia/reperfusion protocol

Cardiac function was monitored throughout the protocol. All hearts were paced at 300 beats/min with the use of pacing electrodes placed on to the right atrium. Two groups of hearts were subjected to 50 min of low flow ischemia (flow reduced from 12.5 to 0.7 ml/min) and 60 min of reperfusion. Perfusion temperature was maintained at 37°C at all times during the protocol (i.e. during baseline, ischemia, and reperfusion). After an equilibration period of 30 min, the control group (CON, n=9) of hearts were perfused with modified Krebs–Henseleit buffer throughout ischemia and reperfusion. In the second group
of hearts, after the equilibration period of 30 min, hearts treated with DIDS \((n=6)\) were perfused with modified Krebs–Henseleit buffer containing 10 \(\mu\)M DIDS starting 10 min prior to ischemia and continuing throughout ischemia and reperfusion.

Perfusate and coronary venous effluent samples were analyzed for \(\text{PO}_3\) with an Instrumentation Laboratories IL 213 pH-blood gas analyzer. Myocardial oxygen consumption was determined as previously published \([13,14,21]\). Samples were also analyzed for creatine kinase and lactate.

2.5. Tissue lipid content

To determine the effect of DIDS on changes in fatty acids, triacylglycerols, and phospholipids, tissue levels of each of these compounds were assayed in fast frozen myocardial samples homogenized in methanol–water (5:2) and extracted in chloroform and methanol. The organic phase fractions were evaporated under nitrogen and subjected to thin-layer chromatography in petroleum ether, diethyl ether, and acetic acid (97:52:3). Plates were scraped at locations indicated by cochromatography of known standards.

2.6. Long chain acylcarnitine (LCA)

Previous studies have suggested a link between accumulation of LCA and depressed contractile function. To evaluate the effect of DIDS on intermediates of cellular fatty acid metabolism, we analyzed tissue content of LCA after baseline, ischemia, and reperfusion. Briefly, hearts were frozen rapidly in Wollenberger clamps precooled in liquid nitrogen, homogenized, extracted in perchloric acid, and centrifuged. The pellet fraction containing LCA was separated. Carnitine was measured radioisotopically after hydrolysis with KOH to convert LCA to free carnitine \([10,31–33]\).

2.7. High energy phosphate content

To determine if DIDS maintained high energy phosphate content during ischemia, tissue levels of adenosine triphosphate (ATP) and phosphocreatine (PCr) were determined using high pressure liquid chromatography (HPLC) on neutralized perchlorate extracts of frozen myocardium obtained after baseline, ischemic, and reperfusion conditions \([34]\). For the separation of the nucleotides, a Model RP-318 Bio-Rad Hi-Pore Reverse Phase column (250\(\times\)4.6 mm) (Bio-Rad Laboratories, CA) was used. A Kratos model 757 variable wavelength UV/VIS spectrophotometer was used for the detection of PCr and ATP. All compounds were quantified by integration of peaks of unknowns and comparing them to the peaks of known concentrations of standards. Measurements are expressed as \(\mu\)mol/g left ventricular weight.

2.8. Statistical analysis

Values are expressed as means±S.D. Significance of the differences was determined using the one-way analysis of variance for repeated measurements with additional post-hoc tests for differences. \(P\)-values less than 0.05 were considered statistically significant.

3. Results

3.1. Mechanical function

To determine if the fatty acid uptake inhibition by DIDS would protect ischemic myocardium, and improve functional recovery on reperfusion, hearts treated with DIDS were subjected to ischemia and reperfusion and compared with results obtained in control hearts. LVDP was similar in both groups under baseline conditions (Table 1). Reduction of perfusate flow resulted in cessation of LVDP in both groups. Reperfusion resulted in a greater LVDP recovery in DIDS-treated hearts compared to controls (LVDP recovery was 78±10% of baseline in DIDS-treated hearts compared with 62±19% of baseline in controls, \(P<0.04\)).

LVEDP was similar in both groups at the start of ischemia. During ischemia, the rise in LVEDP, i.e. contracture, was attenuated in the hearts treated with DIDS compared to controls \((P=0.03)\). Reperfusion resulted in much higher LVEDP in controls compared to hearts treated with DIDS.

Myocardial oxygen consumption was similar in both groups under baseline and reperfusion conditions, and was unaffected by DIDS perfusion (Table 1).

3.2. Palmitate uptake in the presence of DIDS

To determine if DIDS lowers fatty acid uptake under baseline and reperfusion conditions, hearts were perfused...
in the presence or absence of DIDS in the re-circulating mode. Palmitate concentration was measured in the perfusate prior to the start, and at the end of recirculation of the buffer. Fig. 1 demonstrates a reduction in myocardial palmitate uptake by 48 and 46% in DIDS perfused hearts under baseline and reperfusion conditions, respectively.

3.3. Glucose oxidation measurements

To determine if DIDS inhibition of fatty acid uptake would shift the heart to use more glucose, hearts were perfused with $^{14}$C-labeled glucose and the $^{14}$CO$_2$ release was measured. Under baseline conditions, glucose oxidation rates were significantly higher in DIDS-treated hearts compared to control hearts (oxidation rates in nmol/min per g dry wt were 758$\pm$391 in DIDS-treated vs. 319$\pm$89.5 in control hearts, $P<0.01$, $n=6$ in each group). Similar increases in glucose oxidation were also observed during reperfusion in DIDS perfused hearts with oxidation rates being 562$\pm$129 in DIDS versus 365$\pm$93 nmol/min per g dry wt in control hearts, $P<0.03$, $n=6$ in each group. These data are indicative of either increased exogenous glucose use or redirection of glucose to the oxidative pathway in DIDS perfused hearts.

3.4. Tissue levels of fatty acids and triglycerides in ischemic hearts

Since it is difficult to measure fatty acid uptake under ischemic conditions due to low levels of perfusate flow, we measured tissue levels of fatty acids and triglycerides to reflect changes in fatty acid metabolism. DIDS-treated ischemic hearts had significantly lower levels of fatty acids compared to untreated ischemic hearts (total fatty acids content were 401$\pm$36 nmol/g wet wt. in DIDS-treated ischemic vs. 598$\pm$21 nmol/g wet wt. in untreated ischemic hearts, $P<0.03$). Similarly, the triglyceride content was significantly lower in DIDS-treated ischemic hearts (triglyceride content was 1.64$\pm$0.18 $\mu$mol/g wet wt. in DIDS hearts vs. 2.85$\pm$0.29 $\mu$mol/g wet wt. in untreated ischemic hearts, $P<0.04$).

3.5. Creatine kinase release during reperfusion

Creatine kinase release, a marker of ischemic injury, was measured during reperfusion in both groups of hearts. Hearts perfused with DIDS prior to ischemia demonstrated a 45% reduction in creatine kinase release compared to control hearts (Fig. 2) ($P<0.03$). However, if DIDS was present only during reperfusion, the reduction in creatine kinase release was less than 5%, suggesting that the protective properties of DIDS occur prior to and during ischemia.

3.6. High energy phosphates

As shown in Figs. 3 and 4, baseline levels of ATP and PCr were similar in controls and DIDS-treated hearts. At the end of ischemia, PCr levels were markedly lower in both groups of hearts. End-ischemic ATP levels were modestly higher in hearts perfused with DIDS compared to the controls. ATP and PCr levels during reperfusion were greater in DIDS hearts compared to controls ($P<0.03$).

3.7. Lactate efflux during ischemia

Lactate efflux is a measure of anaerobic glycolysis in ischemic myocardium. As shown in Fig. 5, lactate efflux was markedly increased in both control and DIDS-treated hearts at the onset of ischemia. However, after about 15 min of ischemia, lactate efflux was significantly reduced in control hearts while lactate efflux was maintained at a higher level in hearts treated with DIDS. Total lactate

![Fig. 1. Total palmitate utilization, expressed as nmol/min per g dry wt., in control ($n=6$), and DIDS ($n=6$) hearts during perfusion prior to ischemia and on reperfusion after ischemia. *$P<0.03$ versus control.](image)

![Fig. 2. Release of creatine kinase (CK) during reperfusion in control ($n=9$) and DIDS ($n=6$) hearts. Hearts treated with DIDS had diminished CK release indicative of the metabolic protection with this agent. *$P<0.03$ versus control hearts.](image)
Fig. 3. PCr content, expressed as μmol/g dry wt., in control (n=6) and DIDS (n=6) perfused hearts at baseline, at the end of ischemia and at the end of reperfusion. DIDS was successful in maintaining higher levels of PCr during reperfusion. *P<0.05 versus control hearts.

Fig. 4. ATP content, expressed as μmol/g dry wt., in control (n=6) and DIDS (n=6) perfused hearts at baseline and at the end of ischemia. DIDS was successful in maintaining higher levels of ATP during ischemia and reperfusion. *P<0.05 versus controls.

Fig. 5. Time course of lactate in the effluent, expressed as μmol/min per g wet wt., during ischemia in control (■) (n=9) and in DIDS (○) (n=6) perfused hearts. Lactate release was maintained in DIDS perfused hearts (*P<0.05 vs. control hearts). Continued lactate production during ischemia is beneficial in that it represents continued anaerobic metabolism.

release during ischemia was significantly higher in DIDS hearts than in control hearts (P<0.05).

3.8. Long chain acylcarnitine (LCA)

To evaluate if the cardioprotection afforded by DIDS was associated with reduction in LCA levels, we measured LCA levels in both groups of hearts. Compared with controls, DIDS lowered LCA levels under baseline, ischemic and reperfusion conditions (Fig. 6).

4. Discussion

The data presented here demonstrate a novel role for DIDS in lowering fatty acid uptake, increasing glucose use, and protecting ischemic myocardium. Furthermore, it was also demonstrated that the cardioprotection afforded by DIDS perfusion is associated with lowering fatty acid uptake and LCA levels and associated with increases in lactate efflux and ATP. These data also provide evidence that altering fatty acid uptake can contribute to metabolic protection during low-flow ischemia.

4.1. DIDS and fatty acid uptake

Studies in heart and skeletal muscle have demonstrated the presence of three putative fatty acid transporters [35]. The plasma membrane fatty acid binding protein (FABPpm), glycosylated fatty acid translocase (FAT) — identified as the rat homolog of human CD36, and fatty acid transport protein (FATP) have been identified and their roles in long chain fatty acid uptake were determined [35]. Luiken et al. [35] demonstrated that palmitate uptake by vesicles from rat hearts correlated well with the
expression of FABPpm and FAT/CD36 and that inhibition of these transporters significantly lowered palmitate uptake. In earlier studies it has been shown that DIDS binds irreversibly to FAT/CD36 and inhibits fatty acid uptake [27,28]. It is likely that the decreased palmitate uptake observed in this study in hearts perfused with DIDS could be due to the binding of DIDS to FAT/CD36.

4.2. Inhibition of fatty acid uptake and cardioprotection

Switching of substrate utilization from fatty acid to glucose confers direct benefit to ischemic myocardium. Reduction in fatty acid uptake and oxidation by inhibiting CPT-I using etomoxir has been shown to relieve fatty acid inhibition of PDH and increase glucose use [4,36]. Similarly, the use of carnitine or propionyl carnitine has been demonstrated to protect ischemic myocardium by increasing glucose metabolism and decreasing tissue long chain acyl carnitine content [9,11,30,37].

In this study, we demonstrated that inhibiting fatty acid uptake with DIDS prior to ischemia, increased glucose use, maintained lactate release and ATP during ischemia, lowered creatine kinase release, and improved left ventricular function on reperfusion.

4.3. DIDS and lactate efflux during ischemia

Since the availability of oxygen during flow regulated ischemia is reduced, the ability of the myocardium to produce energy from oxidation of fatty acids and carbohydrates is greatly reduced [4,6–8]. Anaerobic glycolysis becomes an important energy source in ischemic myocardium.

Although glycolytic flux, as measured by lactate efflux, increases at the onset of ischemia, the increase is short lived. The flux after 50 min of ischemia was still greater than the initial flux, although it was lower than the maximal flux in the early phase (Fig. 5). This decrease in glycolytic flux is likely due to feedback inhibition of accumulating products such as NADH, lactate and protons [8]. In this study, glucose oxidation, lactate production and ATP levels were higher in hearts perfused with DIDS compared to controls. Furthermore, inhibition of fatty acid uptake and increased lactate production during ischemia are consistent with DIDS switching the substrate utilization towards glucose.

4.4. Influence of long chain acylcarnitine

In addition to enhancement of glycolysis, prevention of myocardial accumulation of long-chain fatty acids may modify intermediary metabolism favorably by decreasing NADH/NAD⁺ (decreasing the lactate/pyruvate ratio, thereby increasing glucose oxidation); decreasing acetyl-CoA/CoASH with consequent decreases in citrate levels and deinhibition of PFK; deinhibiting pyruvate dehydrogenase and pyruvate carboxylase; and increasing the ratio of long-chain acyl-CoA to LCA, with diminution of LCA. Studies have shown that accumulation of LCA is toxic to myocardium [10,22,23,31–33] and that they induce arrhythmias, inhibit contractile function perhaps by altering the activity of ATP translocate [3,38] and decrease glycolytic flux [22,23,36]. Oxefenicine, originally developed as an anti hyperglycemic agent, increases myocardial glucose utilization and decreases accumulation of LCA [22]. In a rabbit model of diabetic cardiomyopathy, we demonstrated in isolated perfused hearts that decreasing the amount of fatty acid perfusing the heart was associated with diminished myocardial long-chain fatty acids and triacylglycerol and was accompanied by immediate improvement in contractile function [39], suggesting the utility of approaches that decrease deleterious levels of myocyte fatty acids in improving function. Previous studies by others have suggested the mechanism by which substances such as propionyl-carnitine or l-carnitine work may, in fact, be via enhancement of glucose metabolism [11,30]. Inhibition of fatty acid uptake, attenuation of LCA levels, increased lactate efflux, and maintenance of ATP by DIDS are consistent with the above findings.

4.5. Limitations

It was demonstrated in this study that protection afforded by DIDS was associated with beneficial changes in fatty acid metabolism. However, it should be noted that there are other effects of DIDS that may also contribute to the observed cardioprotection. DIDS is well known to inhibit the chloride–bicarbonate exchanger and thus influence pH recovery on reperfusion [40,41]. A study by Meiltz et al. demonstrated that inhibition of the anion exchanger with DIDS protects anoxic embryonic hearts against reperfusion-induced dysfunction [42]. Furthermore, Lui et al. showed that DIDS inhibits the cardiac sodium channel, which could also lead to cardioprotection [43]. In this study we did not measure intracellular pH or cardiac sodium channel activity in the presence of DIDS either during ischemia or on reperfusion. The goal was to demonstrate the property of DIDS in lowering fatty acid uptake and thereby increasing glucose use during ischemia. However, the influence of pH changes or inhibition of sodium channel as a factor in mediating beneficial properties of DIDS cannot be ruled out.

It should be noted that glucose oxidation does contribute to energy production during ischemia, albeit at a reduced level [44]. Hence, the potential for contribution of glucose oxidation towards energy production in ischemic DIDS hearts cannot be ruled out.

The data on cardioprotection by DIDS must be interpreted within the limitations of experimental design. We employed a model of severe low-flow ischemia to demonstrate cardioprotection by DIDS. In such a model of low-flow ischemia, several studies have demonstrated the

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importance of increasing glycolysis as a way of protecting these hearts. However, it should be noted that in a setting of global ischemia, increased glycolysis has been demonstrated to be detrimental (see review by Stanley et al. [4]). Hence, our data on cardioprotection by DIDS must be interpreted within the limitations of the low-flow ischemia model.

5. Conclusion

The data presented here demonstrate that hearts perfused with DIDS have decreased fatty acid uptake and increased glucose oxidation. When subjected to ischemia, hearts treated with DIDS maintain anaerobic metabolism and higher levels of high energy phosphates, and do not develop contracture. Ischemic injury is thus decreased and functional recovery enhanced with reperfusion in DIDS-treated hearts. The protection afforded by DIDS was also associated with lower LCA levels during ischemia. These findings suggest a potential role for agents that inhibit fatty acid uptake as a novel metabolic adjunct in the treatment of myocardial ischemia.

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