Domoic Acid Impairment of Cardiac Energetics

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Excitatory mediated neuronal injury has been shown to involve a complex cascade of events. However, the associated cardiac damage reported in humans and marine animals following exposure to excitotoxins has not been well characterized. We hypothesized that the excitotoxin domoic acid can traverse cardiac cell membranes and elicit a deleterious effect on cardiac mitochondrial energetics. Domoic acid (0.05–0.25 μM; 10 min) treatment of isolated rat cardiac mitochondria produced a marked decrease of both mitochondrial flavin adenine dinucleotide (FAD) and nicotinamide adenine linked respiratory control indices (p < 0.001). Enzymatic assays of the mitochondrial electron transport chain (complexes I–V) and the mitochondrial matrix marker enzyme citrate synthase, showed marked concentration-dependent impairment in activity and integrity following exposure to domoic acid (p < 0.01). Similar mitochondrial effects were seen following exposure to the glutamic acid analog, kainic acid (0.5–2 μM). Domoic acid (0.05–10 μM; 40 min) was shown by competitive enzyme-linked immunosorbent assay to traverse the cellular membrane of H9c2 rat cardiac myoblasts. Exposure of intact H9c2 cells to domoic acid (10 μM; 24 h) impaired complex II–III activity but did not compromise cellular viability as assessed using cell quantification or lactate dehydrogenase leakage assays. Assessment of reactive oxygen species (superoxide and hydrogen peroxide) production in both isolated cardiac mitochondria and H9c2 cardiomyocytes failed to show any significant differences following exposure to domoic acid (0.05–5 μM). This is the first study to demonstrate a direct effect of domoic acid on cardiac mitochondrial energetics. However, the absence of substantial damage to intact cardiomyocytes raises questions regarding direct toxicological effects on cardiac energetics or viability under conditions of natural domoic acid exposure.

Key Words: domoic acid; mitochondria; cardiac energetics; excitotoxins.

Cardiovascular pathology has been observed in both animals and humans after domoic acid intoxication. Domoic acid and kainic acid (KA) are structural analogs of glutamic acid and have been shown to mediate neuronal excitotoxicity via activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and KA receptors (Hampson et al., 1992). Domoic acid is present in many algae and marine diatoms (e.g., Pseudo-nitzschia multiseries), and has been implicated in both human and animal poisonings following the ingestion of contaminated shellfish. Although domoic acid and KA are most commonly associated with excitotoxic neuronal damage and lasting neurological impairments (Hunsberger et al., 2005; Liang et al., 2000; Scallet et al., 2004), a number of studies have also provided evidence of adverse cardiac symptoms and lesions after domoic acid intoxication (Kreuder et al., 2005; Silvagni et al., 2005). Following documented cases of human intoxication there have been reports of cardiac arrhythmias unrelated to any diagnosed primary cardiac disease, tachycardia and one death due to acute myocardial infarction three months after ingesting contaminated mussels. Hemodynamic instability with peripheral vasodilation, unstable blood pressure and hypotension were also reported in exposed individuals (Perl et al., 1990; Teitelbaum et al., 1990). Cardiac lesions within a Californian otter population, including myocardial pallor and multifocal myocardial necrosis combined with myocardial hemorrhage and fibrinous epicarditis, have been associated with domoic acid exposure. Inflammatory cells were also observed in both the atrial and ventricular myocardium from these animals (Kreuder et al., 2005).

Specific mechanisms underlying domoic acid exposure and cardiomyopathy have however, yet to be defined. Early studies indicated the presence of functional glutamate receptors on cultured cardiomyocytes (Winter and Baker, 1995). More recently, the presence of classical ionotropic glutamate receptor subunits (NR1, KA2, and GluR3 mRNAs, and GluR1 GluR2/3, GluR4, GluR5/6/7, KA2, and NR1 proteins) have been demonstrated within cardiomyocytes, intrinsic cardiac ganglia, nerve fibers, and specific components of the conducting systems in rat, monkey and human heart (Gill et al., 2007; Leung et al., 2002; Mueller et al., 2003). Given the location of these glutamate receptor subunits, it has been suggested that...
they participate in the regulation of impulse conduction in the heart and may in part explain the adverse cardiovascular effects reported following domoic acid intoxication (Gill et al., 2007). Within neuronal tissues, domoic acid directly activates both AMPA and KA receptors and provokes the release of endogenous glutamate. Prolonged stimulation of ionotropic glutamate receptors in turn drives a pathophysiological increase in intracellular Ca²⁺. Sequestration of Ca²⁺ by mitochondria leads to generation of reactive oxygen species (ROS) which cause cellular structural damage, reduced oxidative phosphorylation coupling, and loss of mitochondrial respiratory chain enzyme activities (Brown and Borutaite 2002; Kindler et al., 2003; Radi et al., 1994, 2002). The mitochondrial involvement in excitotoxin-mediated neuronal injury has been studied extensively (Giordano et al., 2007; Rego and Oliveira 2003).

Whether mitochondrial damage underlies cardiac injury following exposure to domoic acid is unknown. In the present study we assessed whether domoic acid can traverse cardiac cell membranes and exert damaging effects on cardiac mitochondrial energetics or drive the generation of free radicals. We employed an embryonic rat heart–derived H9c2 (2-1) cell line and isolated adult rat cardiac mitochondria to assess intracellular levels and potential cardiotoxic actions of domoic acid and KA. In vitro toxicity assays were used to measure membrane lactate dehydrogenase (LDH) leakage, cell survival, mitochondrial function and oxidative stress.

**MATERIALS AND METHODS**

**Materials.** Chemicals used in the following protocols were obtained from BDH (Palmerston North, NZ), Sigma-Aldrich (Castrol Hill, NSW, Australia), or Roche Diagnostics (Auckland, NZ) unless otherwise specified. Domoic acid and KA were purchased from Tocris Pty Ltd (Bristol, UK). The fluorescent probes dihydroethidine (DHE) and 2,7'-dichlorodihydrofluorescin diacetate (H₂DCFDA) were purchased from Molecular Probes (Invitrogen Detection Technologies, Eugene, OR).

**Cell culture.** The embryonic rat heart–derived cell line H9c2 was obtained from the American Type Culture Collection (Manassas, VA) and cultured in 15mM Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, MO) supplemented with 0.365 g/l glutamine, 1.5 g/l sodium bicarbonate, and 1.35 g/l glucose, 10% fetal calf serum, and 1% streptomycin (Sigma-Aldrich) in 10% CO₂, at 37°C.

**Isolation of cardiac mitochondria.** All procedures described in this study were carried out in accordance with the “Guidelines on the Care and Use of Laboratory Animals” set out by the University of Otago Animal Ethics Committee and the “Guide for Care and Use of Laboratory Animals” (NIH Publication No. 85-23, 1996). Male Sprague-Dawley rats (250–280 g) were obtained from the University of Otago Animal Facility and housed under controlled light/dark cycles.

A midline thoracotomy was performed under ether anesthesia and the cardiac ventricles were immersed in ice-cold isolation medium containing (in mM) 225 mannitol, 75 sucrose, 10 Tris, 0.1 phenylmethylsulfonyl fluoride, and 2 ethylene glycol-bis-(β-aminoethylether)-N,N′,N′,N′-tetraacetic acid (pH 7.2). Ventricles were minced and homogenized using four strokes of a glass-tetlon Potter-Elvehjem homogenizer (clearance 50 μm) and mitochondria isolated using differential centrifugation as previously described (Sammut et al., 2001). Protein concentrations for all samples were assessed using a microplate assay kit based on the Lowry method in accordance with the manufacturer’s instructions (Bio-Rad, UK).

**In vitro treatment of isolated cardiac mitochondria with domoic acid and KA.** Mitochondrial isolates (20 mg of mitochondrial protein/ml) were incubated in the presence of either domoic acid (0.05–0.25μM), KA (0.5–2μM), or phosphate buffered saline (PBS; vehicle control) for 10 min prior to testing. The concentrations for domoic acid and KA were chosen on the basis of previous work conducted using hippocampal brain slices (Kerr et al., 2002). Final KA concentrations were selected on the basis of pilot studies which showed that low micromolar concentration were ineffective. Treated mitochondrial samples were either assessed immediately for oxidative phosphorylation or stored at −80°C for future mitochondrial enzyme kinetic analysis.

**Mitochondrial respiratory function.** Mitochondrial oxygen consumption was measured polarographically using a Clark-type oxygen electrode (World Precision Instruments, Sarasota, FL) as previously described (Sammut et al., 2001). Oxidative phosphorylation was assessed by recording the rate of oxygen consumption during state 4 respiration (substrate driven rate alone) and state 3 respiration (substrate driven rate in the presence of adenosine diphosphate [ADP]). All experiments were carried out at 32°C using a standard respiratory medium saturated with oxygen, containing (in mM) 100 KCl, 0.05 ethylendiaminetetraacetic acid (dipotassium salt), 75 mannitol, 25 sucrose, 10 Tris-HCl, and 10 KH₂PO₄ (pH 7.4). Mitochondrial respiration was initiated by the addition of cardiomycin (0.5 mg of mitochondrial protein) in the presence of essentially fat-free bovine serum albumin (0.2 mg) to make up a final chamber volume of 250 μl. Following equilibration, at which point a steady endogenous rate of respiration had been reached, state 4 respiration was initiated by either 7mM succinate in the presence of 50μM rotenone (flavin adenine dinucleotide (FAD)-linked respiration) or 7mM glutamate and 7mM malate (NAD⁺[nicotinamide adenine dinucleotide]-linked respiration). State 3 respiration was initiated by the addition of ADP (200 nmol). Mitochondrial respiratory control indices (RCIs) were calculated as the ratio of state 3/state 4 respiration.

The structural resemblance of domoic acid and KA to glutamate suggested that these compounds may be capable of acting as substrates or inhibitors within the mitochondrial electron transport chain. This was assessed by replacing known substrates with non-rate limiting concentrations of either domoic acid (40μM) or KA (400μM) in the presence and absence of malate, a known transporter within the electron transport chain. State 3 respiration was initiated by the addition of ADP (200 nmol). The ability of domoic acid to inhibit the mitochondrial electron transport chain directly was also investigated by adding domoic acid 30 s prior to the addition of the 5mM glutamate and 5mM malate. State 4 and state 3 rates and RCIs were compared.

**Mitochondrial enzyme assays.** Isolated mitochondrial samples, treated with either domoic acid or KA, were freeze-thawed (× 3) to ensure complete mitochondrial lysis and diluted to a final concentration of 1 mg mitochondrial protein/ml in mitochondrial isolation media. Mitochondrial complex I assay (NADH-ubiquinone oxidoreductase; EC 1.6.9.3), mitochondrial complex II–III assay (succinate-ubiquinone/ubiquinol-cytochrome c reductase; EC 1.8.3.1), mitochondrial complex IV assay (cytochrome c oxidase; EC 1.9.3.1), mitochondrial complex V assay (ATPase; EC 3.6.1.3) the mitochondrial integrity marker, citrate synthase activity (EC 4.1.3.7), and aconitate activity (citrate [iso]citrato) hydro-lyase; EC 4.2.1.3), a marker of oxidative stress, were evaluated. Mitochondrial enzyme activities were assessed using a SpectraMax-Plus 96-well spectrophotometer (Molecular Devices, Crawley, UK) at 30°C as previously described (Sammut et al., 2001). Optical path-lengths were corrected for microplate use and results expressed as nmol/min/mg protein for complex I, II–III, V, citrate synthase, and aconitate as the first order rate constant k/min/mg protein for complex IV.

**ROS assays.** Mitochondrial impairment associated with free radical production was assessed following exposure to domoic acid. Superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) generation was measured in isolated cardiac mitochondria and H9c2 cells using the fluorescent leuco dyes, DHE, and H₂DCFDA, respectively. Isolated mitochondria (20–30 mg/ml) were loaded (30 min) in the dark with DHE (3μM) or H₂DCFDA (1μM). Mitochondria were washed in mitochondrial isolation media, pelleted (11,500 × g; 12 min) and resuspended in 250 μl of PBS. ROS readings were...
recorded in domoic acid (0.05–0.25 μM) treated and untreated mitochondria in the absence and presence of various substrates and inhibitors alone and in combination. Glutamate and malate were employed as substrates to fuel electron transfer through complex I, transferring electrons from NADH to ubiquinone; succinate was used to drive electron flow through complex II, reducing FAD to FADH$_2$. This assay also employed the complex I inhibitor, rotenone, the complex III inhibitor, antimycin A, and the complex IV inhibitor, cyanide. The use of these inhibitors has been shown to reduce the flow of electrons through the respiratory chain at specific points, resulting in a reduction of the upstream components and favoring the escape of electrons and the formation of ROS (Adam-Vizi, 2005). Final concentrations of 0.2 mg mitochondrial protein/ml, 10mM succinate, 10mM glutamate, 10mM malate, 50μM rotenone, 4μM cyanide, and 0.5μM antimycin A, were obtained in a total volume of 200 μl in each well of a 96-well quartz microplate (Hellma, Germany). DHE fluorescent endpoint readings were taken at 0, 10, 20, and 30 min after the addition of mitochondria on a Gemini EM fluorometric reader (Molecular Devices, Crawley, UK) at an optimized $\lambda_{ex}$ = 520 nm/$\lambda_{em}$ = 590 nm (37°C). H$_2$DCFDA fluorescent endpoint readings were taken at 0, 10, 20, and 40 min at $\lambda_{ex}$ = 495 nm/$\lambda_{em}$ = 525 nm (37°C).

Subconfluent H9c2 cells were seeded in 12 well tissue culture plates at 5000 cells/cm$^2$ in 2 ml of supplemented DMEM. Cells reached 80% confluence in 48 h and the media was replaced. Cells were treated with varying concentrations of domoic acid (0.05–5 μM) or KA (50 μM) for 40 min, then washed in PBS. Fresh media containing DHE (10 μM) or H$_2$DCFDA (10 μM) was added to each well and fluorescent endpoint readings at 0, 10, 20 and 30 min were taken on a Gemini EM fluorometric microplate reader optimized at $\lambda_{ex}$ = $\lambda_{em}$ = 518 nm/$\lambda_{em}$ = 605 nm and at $\lambda_{ex}$ = 495 nm/$\lambda_{em}$ = 525 nm (37°C) respectively.

**Assessment of cell viability and integrity.** Cell viability was assessed in H9c2 cells as previously described (Mickuviene et al., 2004). Briefly, 24-well plates were seeded with $1 \times 10^4$ cells/well and cultured until they reached 80% confluence prior to treatment with domoic acid (0.05–10 μM; 24 h). Cells were fixed by addition of 96% ethanol for 10 min and 1 ml of 0.05% crystal violet in 20% ethanol, 20% ethanol was added to the wells and allowed to stain for 30 min. Cells were washed gently in PBS (× 4) and the residual dye within the cellular layer was dissolved in 2 ml of 0.1% acetic acid solution in 50% ethanol (3 h). Optical density at 585 nm was recorded on a SpectraMax spectrophotometer and normalized against control untreated (100% viable) cells (Mickuviene et al., 2004). A positive control standard curve was also established using 2–10% DMSO treatment.

LDH leakage was assessed as a marker of loss of cellular integrity following exposure of H9c2 cardiomyocyte cells to domoic acid (10 μM; 24 h). LDH activity in cell medium was assessed using a commercially available kit (Sigma, NSW, Australia) adapted to a 96-well format as previously described (Adlam et al., 2005). Two hundred and fifty microliters of reaction mixture containing 0.139mM NADH and 4.63mM pyruvate was added to each microplate well, and the reaction was initiated by the addition of 40 μl of cell medium. LDH activity was based on the conversion of NADH to NAD$^+$ and was calculated as the difference between the natural logarithms of the absorbance at 340 nm ($\lambda_{340}$) at 25°C for three time points; 1, 2, and 3 min. Results were expressed as a percentage of activity in cell lysed with 1% triton X 100.

![FIG. 1](https://academic.oup.com/toxsci/article-abstract/105/2/395/1650068) Effects of domoic acid (□ 0.05–0.25 μM; A and C) and KA (□ 0.5–2 μM; B and D) treatment (10 min) versus vehicle (■ PBS) on isolated cardiac mitochondrial NAD$^+$-linked state 4 respiration rates and RCIs. Each value represents the mean ± SEM of six to eight separate experiments. *$p < 0.05$, ***$p < 0.001$ versus vehicle controls.
Preparation of H9c2 homogenates for mitochondrial enzyme analysis.

Cells were seeded at $5 \times 10^5$ cells/cm$^2$ so as to reach 80% confluence in 48 h prior to incubation in the presence of PBS or 10μM domoic acid (24 h; 37°C; 10% CO$_2$). Untreated cells served as controls ($n = 5$).

Following the 24-h incubation period, cells were trypsinized and washed three times by suspending in PBS and centrifuging (126 × g; 4 min; 4°C) before finally being resuspended in 1 ml of PBS and pelleted down (126 × g; 4 min; 4°C). Recovered cells (resuspended in 100 μl of mitochondrial isolation media) were freeze/thawed in liquid nitrogen ($\times 3$) in order to ensure complete cellular and mitochondrial lysis. Cellular protein concentrations were assayed using the microplate adaptation of the method of Lowry outlined above. Cellular lysates were diluted in mitochondrial isolation buffer to 1 mg/ml final protein concentration and mitochondrial complex enzyme assays conducted as described above.

Examination of the ability of domoic acid to traverse the cell membrane. H9c2 cells seeded at $5 \times 10^5$ cells/cm$^2$ ($n = 5$) were treated with domoic acid (0.05–10μM) or PBS vehicle (control) for 40 min at 37°C. Cells were trypsinized and washed four times by suspending in PBS and centrifuging (126 × g; 4 min; 4°C). The cell pellet was resuspended in 100 μl of 1% sodium dodecyl sulfate in 1M Tris/HCl and 0.1% complete protease inhibitor. To separate out the cytosolic fraction, cells were repeatedly freeze/thawed in liquid nitrogen to completely lyse the cells, then subjected to a final centrifugation at 30,000 × g for 15 min at 4°C.

Domoic acid in the cytosolic fraction was quantified by competitive enzyme-linked immunosorbent assay (ELISA) kits (Biosense Laboratories, Bergen, Norway) according to the manufacturer’s instructions, as previously described (Hesp et al., 2005). In brief, diluted samples, standards and controls were incubated (1 h at room temperature) in the dark, in domoic acid-coated 96 well plates in the presence of anti-domoic acid antibodies conjugated to horseradish peroxidase. Following incubation, plates were washed using 10mM PBS with 0.05% Tween 20 and incubated for 15 min at room temperature in the dark with TMB peroxidase substrate. The reaction was stopped using 0.3M H$_2$SO$_4$ and absorbance at 450nm read after 5 min using a 96-well SpectraMax plate reader. All samples were compared with an internally generated standard curve (10–300 pg/ml domoic acid) run on each ELISA plate. Cellular domoic acid (DOM) concentrations were expressed as ng DOM/2 × 10$^6$ cells.

Statistics. Statistical analysis was performed using SigmaStat v2.03 (SPSS, Inc., Chicago, IL) by a one-way ANOVA. Bonferroni pair-wise tests were used for post hoc comparisons between control and treatment groups. Results are expressed as the mean ± SEM, with a p value of < 0.05 being considered significant. Inverse log correlations were run if equal variance failed, and the one-way ANOVA and Bonferroni pair-wise analysis repeated.

RESULTS

Effects of Domoic Acid on Isolated Cardiac Mitochondrial Respiration

Domoic acid treatment (0.1μM) of isolated mitochondria uncoupled the respiratory chain resulting in a significant increase in NAD$^+$-linked state 4 respiration as compared with control ($p < 0.05$; Fig. 1A). However, higher concentrations of domoic acid (0.25μM), directly inhibited complex I (Fig. 3A)

**FIG. 2.** Effects of domoic acid (black bars): 0.05–0.25μM; A and C) and KA (black bars): 0.5–2μM; B and D) treatment (10 min) versus vehicle (grey bars: PBS), on isolated cardiac mitochondrial FAD-linked state 4 respiration rates and RCIs. Each value represents the mean ± SEM of six to eight separate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus vehicle controls.
causing a concomitant decrease in state 4 respiration (Fig. 1A). A similar increase was seen for FAD-linked state 4 respiration, significant only at 0.25 μM (p < 0.05; Fig. 2A). Neither domoic acid nor KA inhibited state 3 respiration (results not shown). Assessment of overall NAD⁺-linked respiration following domoic acid (0.05–0.25 μM) treatment revealed a significant concentration-dependent decrease in RCIs (F = 17.395; p < 0.001; Fig. 1C). Similar effects were noted with FAD-linked respiration (p < 0.001; Fig. 2C). KA (0.5–2 μM) treatment produced similar, but less pronounced effects on FAD-linked and NAD⁺-linked respiration (Figs. 1D and 2D, respectively) in isolated cardiac mitochondria (p < 0.001; Fig. 2D).

**Domoic Acid Reduces Respiratory Chain Enzyme Activities and Compromises Mitochondrial Membrane Integrity in Isolated Cardiac Mitochondria**

To further delineate the extent of mitochondrial damage, enzyme complexes I (Fig. 3A), II–III (Fig. 3B), IV (Fig. 3C), and V (Fig. 3D) were assessed. The effects of domoic acid (0.05–0.25 μM) versus vehicle (PBS) treatment (10 min) on isolated cardiac mitochondrial enzymes are shown in Fig. 3. Each value represents the mean ± SEM of six to eight separate experiments. *p < 0.05, **p < 0.001 versus vehicle controls. TNB = 5-thio-2-nitrobenzoic acid, cyt c ox = oxidized cytochrome c and cyt c red = reduced cytochrome c.
were assessed. Complex I, complex II–III, and complex V showed significant concentration-dependent decreases in activity in the presence of domoic acid at all concentrations measured (0.05–0.25 μM \( p < 0.001 \); Figs. 3A, 3B, and 3D). Complex IV activity was shown to be reduced significantly following treatment with 0.25 μM domoic acid only \( (p < 0.01; \text{Fig. 3C}) \). Unlike domoic acid, KA failed to alter the level of complex I or complex IV activity (Figs. 4A and 4C). Assessment of complex II–III activity did however show a significant decrease at all three concentrations of KA compared with control \( (p < 0.05; \text{Fig. 4B}) \). Assessment of ATPase (complex V) activity also revealed a modest decrease in activity at all three concentrations compared with control \( (p < 0.001; \text{Fig. 4D}) \).

Assessment of the mitochondrial integrity marker, citrate synthase, showed a significant decrease in activity for all three concentrations (0.05–0.25 μM) of domoic acid compared with vehicle controls \( (p < 0.001; \text{Fig. 3E}) \). Similarly, exposure to all three concentrations of KA (0.5–2 μM) also resulted in a significant decrease in activity \( (p < 0.001; \text{Fig. 4E}) \).

**FIG. 4.** Effects of KA (■ 0.5–2 μM) versus vehicle (□ PBS) treatment (10 min) on isolated cardiac mitochondrial enzymes. Kinetic data obtained is shown for complex I (A), complex II–III (B), complex IV (C), complex V (D), citrate synthase (E), and aconitase (F). Each value represents the mean ± SEM of six to eight separate experiments. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \) versus vehicle controls. TNB = 5-thio-2-nitrobenzoic acid, cyt c ox = oxidized cytochrome c and cyt c red = reduced cytochrome c.
experiments exposure of freeze fractured mitochondrial homogenates to domoic acid (up to 1 μM) failed to inhibit citrate synthase activity (data not shown). Assessment of mitochondrial aconitase activity, a marker of oxidative injury, failed to reveal any significant change in activity of isolated cardiac mitochondria following exposure to either domoic acid (0.05–0.25 μM) or KA (0.5–2 μM) (Figs. 3F and 4F, respectively).

**Domoic Acid Exposure Does Not Result in Free Radical Generation in Isolated Cardiac Mitochondria**

Mitochondrial impairment associated with free radical (O$_2^−$ and H$_2$O$_2$) production was assessed following exposure to domoic acid. Coincubation of the free radical-sensitive fluorescent probe (DHE) with domoic acid (0.05–0.25 μM) in the absence of mitochondria, failed to stimulate O$_2^−$ production (Fig. 5A). Significant time-dependent increases in superoxide production were seen in succinate-driven mitochondria (Fig. 5C), and this was greatly enhanced in the presence of the complex III inhibitor antimycin A (Fig. 5D), confirming that DHE acts as a fluorescent indicator of superoxide production within mitochondria. However, domoic acid treatment of mitochondria in the absence and presence of various substrates and inhibitors failed to stimulate additional O$_2^−$ production (Figs. 5B–D). Domoic acid (0.05–0.25 μM) did significantly block the succinate-driven increase in O$_2^−$ production in the presence and absence of antimycin A at 30 min as compared with control ($p < 0.01$; Fig. 5D).

A small yet significant increase in H$_2$O$_2$ production in the absence of mitochondria at all three time points (10, 20, and 40 min: $p < 0.001$; Fig. 6A) was produced following coincubation of fluorescent dye and domoic acid (0.05–0.25 μM) alone. As previously reported, respiring cells normally produce a basal level of free radicals (Boveris and Chance, 1973; Skulachev, 1996). Domoic acid (0.05–0.25 μM)

![Figure 5](https://academic.oup.com/toxsci/article-abstract/105/2/395/1650068/401)
treatment failed to increase H$_2$O$_2$ production in the presence of various substrates and inhibitors (Figs. 6B–D).

**Domoic Acid Does Not Act as a Substrate or an Inhibitor of the Krebs Cycle**

Oxidative phosphorylation was assessed in the presence of domoic acid (40 $\mu$M) alone (in place of glutamate), or coadministered with malate (5mM). Respiratory data presented in Table 1 shows that domoic acid, alone or in the presence of malate, failed to drive mitochondrial respiration above basal levels of oxygen consumption. Similar findings were obtained following administration of KA (400 $\mu$M). Acute domoic acid administration with glutamate/malate also failed to alter mitochondrial respiration, suggesting it did not act as an inhibitor.

**Domoic Acid Traverses the Cellular Membrane**

H9c2 cardiac myoblasts were incubated with domoic acid (0.05–10 $\mu$M; 40 min) and subsequently washed, lysed and centrifuged to separate the cytosolic fraction. Domoic acid was shown to be present within the cytosol of the rat H9c2 cardiac myoblasts by a domoic acid-specific ELISA at all concentrations of domoic acid administered ($p < 0.001$) (Fig. 7).

**TABLE 1**

Assessment of the Ability of Domoic Acid (40 $\mu$M) and KA (400 $\mu$M) to Act as a Mitochondrial Substrate or Inhibitor during NAD$^+$-Linked Respiration in Isolated Cardiac Mitochondria

<table>
<thead>
<tr>
<th></th>
<th>RCI</th>
<th>State 4 rate (ng O/min/mg protein)</th>
<th>State 3 rate (ng O/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate/malate</td>
<td>6.89±0.54</td>
<td>25.20±2.61</td>
<td>202.0±17.34</td>
</tr>
<tr>
<td>Glutamate/malate/DOM</td>
<td>5.60±0.38</td>
<td>31.35±2.80</td>
<td>174.0±16.25</td>
</tr>
<tr>
<td>Malate</td>
<td>2.54±0.28</td>
<td>15.27±1.53</td>
<td>38.40±5.61</td>
</tr>
<tr>
<td>Malate/DOM</td>
<td>3.01±0.38</td>
<td>13.23±2.82</td>
<td>36.70±4.12</td>
</tr>
<tr>
<td>DOM</td>
<td>0.84±0.15</td>
<td>1.83±0.38</td>
<td>1.34±0.20</td>
</tr>
<tr>
<td>KA</td>
<td>0.95±0.17</td>
<td>1.40±0.73</td>
<td>1.86±0.93</td>
</tr>
</tbody>
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*Note.* Each value represents the mean ± SEM of eight separate experiments.
Domoic Acid Does Not Alter Cellular Viability

Cell viability and cellular integrity were quantified using the relative absorbance of crystal violet dye uptake and LDH leakage assays respectively. Exposure to a known cytotoxic agent DMSO (2–10%) produced a concentration-dependent decrease in crystal violet staining \( (p < 0.001; \text{Fig. 9A}) \) validating the assay.

Assessment of crystal violet staining following cellular exposure to domoic acid \((0.05–10 \mu M; 24 \text{ h})\) failed to reveal any alteration in cell viability \( (\text{Fig. 9B}) \). In addition, domoic acid \((10 \mu M; 24 \text{ h})\) did not induce LDH leakage from cardiac myoblasts as compared with both untreated and PBS treated cardiac myoblasts \( (\text{Fig. 9C}) \). Triton X 100 \((1\%)\) served as a positive control inducing maximal LDH leakage.

DISCUSSION

This is the first study to investigate the direct effect of domoic acid on isolated cardiac mitochondria and on cardiomyocyte viability. Treatment of isolated cardiac mitochondria with the glutamate analogs domoic acid and KA resulted in a concentration-dependent decrease in mitochondrial respiratory parameters. Domoic acid increased both NAD\(^+\)- and FAD-linked state 4 respiration rates suggesting an increased membrane proton leak. This may indicate a direct effect of domoic acid on the mitochondrial membrane, a disruption of the mitochondrial electron transport chain, or the induction of a membrane permeability transition \( (\text{Borutaite et al., 1995}) \). Decreased state 3 respiration rates following domoic acid exposure, coupled with the loss of matrix marker citrate synthase, also suggests the possibility of a permeability transition effect \( (\text{Sammut et al., 2001}) \). KA behaved similarly, producing significant decreases in mitochondrial function and integrity at roughly 10-fold higher concentrations than domoic acid.

In isolated mitochondria, complex I \((\text{NAD}^+\text{-linked})\) and complex II–III \((\text{FAD-linked})\) kinetic activities were reduced in a concentration-dependent manner following domoic acid exposure. This inhibition only affected NAD\(^+\)-linked state 4 respiration rates at the higher domoic acid concentrations \((0.25 \mu M)\), suggesting mitochondrial supercomplexes operate at a greater kinetic capacity than required to drive electron transport in intact mitochondria \( (\text{Sammut et al., 2001}) \). Consistent with the reduction in activity of complexes I and II–III, domoic acid also produced a concentration-dependent inhibition of the phosphorylating enzyme, complex V. Once again, the effect on state 3 respiration was only noted in the presence of high concentrations of domoic acid \((0.25 \mu M)\) and KA \((2\mu M)\), suggesting that they exert a direct effect on mitochondrial complexes. In addition, both KA and domoic acid treatment resulted in a concentration-dependent decrease in citrate synthase activity, a marker of membrane integrity, suggesting an increase in membrane permeability. This loss of integrity was less pronounced than the effects on the electron transport chain components noted above, confirming that the effects of domoic acid stemmed from a direct inhibition of these enzymes rather than the destruction of mitochondrial compartments. Although domoic and KA bear a structural resemblance to tricarboxylic substrates present within the Krebs’s cycle, our studies indicate that neither of these compounds directly drive or inhibit the citric acid cycle in...
isolated ventricular mitochondria. The lack of effect seen on citrate synthase activity in response to domoic acid treatment on crude preparations of enzymes from freeze fractured mitochondria in this study discounts direct inhibition by domoic acid on this matrix enzyme. We suggest that the reduction in citrate synthase activity reported in intact mitochondria (Fig. 3E) occurs as a consequence of a loss of the enzyme from the isolated mitochondrial matrix.

Under normal physiological conditions, O$_2$ is generated by the electron transport chain as a consequence of proton leak onto molecular oxygen (Boveris and Chance, 1973; Sammut et al., 2001). ROS and peroxynitrite compounds have been shown to inhibit a number of mitochondrial components; complexes I, II/III, IV, V, andaconitase (Brown and Borutaite, 2001, 2002). However, we found no evidence of domoic acid–induced O$_2$ or H$_2$O$_2$ production in either cardiac mitochondrial preparations or H9c2 cardiomyocytes. The absence of any detected increase in O$_2$ or H$_2$O$_2$ levels is consistent with the lack of damage to aconitase, a marker of oxidative stress. Consequently ROS do not appear to be implicated in the mitochondrial dysfunction seen in this study. Although we did not specifically assess nitric oxide (NO) synthase activity, the activity of NO-sensitive complex IV (Brown, 2001) was only reduced in this study in the presence of high concentrations of domoic acid. Therefore, it is doubtful that mitochondrial-generated NO plays a significant role in domoic acid–induced mitochondrial damage. Although, the H9c2 cardiomyocyte cell line employed in this study may not be fully representative of the myocardium as a whole, the lack of ROS involvement in this study is in agreement with previous ROS studies in domoic acid treated rat neonatal microglia (Mayer et al., 2001).

This study has shown that domoic acid, at concentrations up to 10μM, does not alter cellular viability in the H9c2 cardiomyocyte cell line. In addition, only mitochondrial complex II–III activity was affected by domoic acid in the intact cardiac cells. Previous reports by Dabbeni-Sala et al. (2001) have similarly described a kainate (0.05–1mM) mediated impairment of the catalytic portion of neuronal mitochondrial complex II, with a subsequent loss of succinate dehydrogenase activity. This effect was only observed in intact cells but not in isolated mitochondria leading the authors to suggest a receptor–mediated pathway of damage. The observation of the inhibitory effects of KA on isolated cardiac mitochondrial complexes II–II and IV may possibly be
attributed to the sensitivity of the kinetic assays employed in
the present study compared with the histochemical staining
methods previously used. Other groups working with domoic
acid, have also noted that this structural analog of kainate is
more potent, producing apoptosis in cultured cells at much
lower concentrations (0.2µM) (Pinto-Silva et al., 2008) than
KA (50µM) (Phillips et al., 2000). Similarly, the present study
shows (Figs. 1–4) clear concentration-dependent changes in
cardiac mitochondrial activity with 0.05µM domoic acid but
only limited inhibition with 2µM KA.

Domoic acid binds to both AMPA and KA receptors
(Hampson et al., 1992). Overstimulation of these ionotropic
 glutamate receptors ultimately leads to Ca2+ sequestration into
mitochondria, ROS generation and compromised cellular
viability (Budd and Nicholls, 1996a, b; Dykens, 1994; Luetjens
et al., 2000; Peng and Greenamyre, 1998; White and Reynolds,
1995). The H9c2 cardiac cell line expresses the NR1
N-methyl-D-aspartate (NMDA) receptor subunit (Leung et al.,
2002), however, there is no evidence of functional NMDA, AMPA,
or KA receptors within these cells. Hence, the ability of domoic
acid to exert cytotoxic effects through classical ionotropic
GluR mechanisms is unlikely.

We have shown that domoic acid traverses the cardiomyo-
cyte membrane and accumulates in the cytoplasm in a concen-
tration-dependent fashion. The mechanism of entry into the cell
is still unknown, however we speculate that domoic acid is
likely to enter through a specific transporter, rather than through
a physical insertion into the membrane. A physical disruption to
the membrane would result in LDH leakage, however this was
not observed. Hence we suspect that domoic acid may be
actively transported across the membrane. Several excitatory
amino acid transporter isoforms (EAAT-1, -2, and -3) have been
detected in cardiac tissues (King et al., 2004; Kugler, 2004;
Ralph et al., 2004). Although domoic acid is generally
regarded as a nontransportable EAAT substrate in neuronal and
glial cells (Danbolt, 2001; Ross et al., 2000), the possibility
exists that cardiac EAATs may play a role in actively
transporting this and other kainoid ligands, especially when
available in micromolar concentrations outside the cell.

Based on published data for the volume of H9c2 cells in
culture (Merten et al., 2006), our estimates indicate total
cytosolic DOM concentrations between 0.8 and 1µM after 24 h
incubation in 5–10µM DOM. The moderately high levels of
domoic acid detected in the cytosol, coupled with the lack of
cytotoxicity and mitochondrial dysfunction in the intact
cardiomyocytes, suggests that domoic acid may be buffered
within the cytoplasmic compartment and that mitochondria are
protected from damage.

The levels of domoic acid utilized within this study have
previously been shown to cause strong excitatory responses in
rat hippocampal brain slices (250–500nM) (Kerr et al., 2002)
and apoptosis in brain slices (10µM) (Erin and Billingsley,
2004) and human colorectal adenocarcinoma Caco-2 cells
(0.22µM) (Pinto-Silva et al., 2008). This current study used

**FIG. 9.** Cell viability was assessed following 24-h incubation of
embryonic rat H9c2 cardiomyocytes with DMSO (A) or domoic acid (B)
using crystal violet absorbance. LDH activity in the surrounding medium was
used as a marker of membrane disruption in the presence of domoic acid (C).
Each value represents the mean ± SEM of six to eight separate experiments.

***p < 0.001 versus vehicle controls.
a range of concentrations 50nM–10μM (3.1 mg/l) covering the calculated effective human ingested toxic dose of 66 μg/kg (Pinto-Silva et al., 2008). Recent studies by our group however indicate that exposure of \textit{ex vivo} intact hearts to domoic acid (5μM for 40 min) does not alter Langendorff cardiohemodynamics, suggesting that an alternative mechanism may be involved in the direct induction of cardiomyopathy by this excitotoxin (unpublished observations). In addition, our ongoing whole animal studies suggest that indirect effects associated with excitation and/or damage to central nervous system autonomic control centers play a significant role in driving cardiac damage during domoic acid exposure. Thus, seizure activity and “catecholamine storm” may provoke or exacerbate gross and ultrastructural myocardial damage, consistent with the pathology reported following domoic acid exposure \textit{in vivo} (Tramoundanas et al., 2006). The current study has demonstrated for the first time that domoic acid induces mitochondrial dysfunction in isolated cardiac mitochondria. However, its inability to compromise cardiomyocyte viability does not support the possibility of a direct cardiotoxic pathology in domoic acid poisoning cases.

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