Na$^+$ Effects on Mitochondrial Respiration and Oxidative Phosphorylation in Diabetic Hearts

ANDRIY BABSKY,* NICOLAI DOLIBA,* NATALIYA DOLIBA,*,† ANDREY SAVCHENKO,*
SUZANNE WEHRLI,‡ AND MARY OSBAKKEN*,§,[1]

*Department of Biochemistry/Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104; †Covance, Princeton, New Jersey 08540; ‡Children's Hospital of Philadelphia; Philadelphia, Pennsylvania 19104; and §Aventis, Bridgewater, New Jersey

Intracellular Na$^+$ is approximately two times higher in diabetic cardiomyocytes than in control. We hypothesized that the increase in Na^+_i activates the mitochondrial membrane Na$^+$/Ca^{2+} exchanger, which leads to loss of intramitochondrial Ca^{2+}, with a subsequent alteration (generally depression) in bioenergetic function. To further evaluate this hypothesis, mitochondria were isolated from hearts of control and streptozotocin-induced (4 weeks) diabetic rats. Respiratory function and ATP synthesis were studied using routine polarography and ^{31}P-NMR methods, respectively. While addition of Na$^+$ (1–10 mM) decreased State 3 respiration and rate of oxidative phosphorylation in both diabetic and control mitochondria, the decreases were significantly greater for diabetic than for control. The Na$^+$ effect was reversed by providing different levels of extramitochondrial Ca^{2+} (larger Ca^{2+} levels were needed to reverse the Na$^+$ depressant effect in diabetes mellitus than in control) and by inhibiting the Na$^+$/Ca^{2+}exchanger function with diltiazem (a specific blocker of Na$^+$/Ca^{2+} exchange that prevents Ca^{2+} from leaving the mitochondrial matrix). On the other hand, the Na$^+$ depressant effect was enhanced by Ruthenium Red (RR, a blocker of mitochondrial Ca^{2+} uptake, which decreases intramitochondrial Ca^{2+}). The RR effect on Na$^+$ depression of mitochondrial bioenergetic function was larger in diabetic than control. These findings suggest that intramitochondrial Ca^{2+} levels could be lower in diabetic than control and that the Na$^+$ depressant effect has some relation to lowered intramitochondrial Ca^{2+}. Conjoint experiments with ^{31}P-NMR in isolated superfused mitochondria embedded in agarose beads showed that Na$^+$ (3–30 mM) led to significantly decreased ATP levels in diabetic rats, but produced smaller changes in control. These data support our hypothesis that in diabetic cardiomyocytes, increased Na$^+$ leads to abnormalities of oxidative processes and subsequent decrease in ATP levels, and that these changes are related to Na$^+$ induced depletion of intramitochondrial Ca^{2+}.

[Exp Biol Med Vol. 226(6):543–551, 2001]

Key words: diabetes; heart; mitochondria; sodium; calcium; oxidative phosphorylation

Introduction

Cytoplasmic Na$^+$ (Na^+_i) is elevated in diabetic cardiomyocytes (1–5). This increase is partially explained by a decrease of sarcolemmal Na$^+$-K$^+$ pump function (1, 2, 4, 5) by an increase of sarcolemmal Na$^+$-H$^+$ exchanger function (6) and, possibly, by changes in sarcolemmal membrane Na$^+$-Ca^{2+} exchanger function.

Na$^+$-induced abnormalities in energetics have been demonstrated in diabetes mellitus (DM) heart and isolated cardiomyocytes (5, 7). The role of mitochondria in these changes is still unclear. In DM heart mitochondria, we and others have shown that parameters of respiration and oxidative phosphorylation are decreased when α-ketoglutarate (KG) or pyruvate (plus malate) are used as substrate (7–10). At present, it is unclear whether changes in bioenergetics contribute to altered Na$^+$ transport, or whether altered Na$^+$ transport leads to progressively depressed mitochondrial bioenergetic function (or both), possibly by inducing changes mitochondrial matrix Ca^{2+} levels.

Mitochondrial Ca^{2+} uptake capacity is significantly depressed in diabetic cardiomyocytes compared with control (11). Cox and Matlib (12) have shown that activation of the mitochondrial Na$^+$-Ca^{2+} exchanger by extramitochondrial Na$^+$ leads to Ca^{2+} efflux and subsequent decrease in matrix-free Ca^{2+}. The role of mitochondrial Ca^{2+} is important in the regulation of several matrix dehydrogenases (DH; including pyruvate DH, isocitrate DH, and α-ketoglutarate DH) involved in oxidative ATP synthesis (13–15). It is possible that the decrease of mitochondrial Ca^{2+} can result in decreased activity of these Ca^{2+}-sensitive dehydrogenases, which can lead to a decrease in turnover of the Krebs cycle intermediates, resulting in a decreased rate of oxidative phosphorylation.

The goal of the work presented in this paper was to further evaluate the role of Na^+_i in regulation of mitochondrial respiration and oxidative phosphorylation in diabetic, in contrast to control, hearts. We found that Na^+_i does regulate mitochondrial ATP levels.

This work was supported by the NIH (grant no.R01-HL39208 to M.O.) and by the American Diabetic Association (grant to N.D.).

[1] To whom requests for reprints should be addressed at Biochemistry/Biophysics Department, 207 Anatomy/Chemistry Building, University of Pennsylvania, 37th Street and Hamilton Walk, Philadelphia, PA 19104. E-mail: mary.osbakken@aventis.com

Received September 20, 2000.
Accepted February 1, 2001.

0037-9727/01/2266-0543$15.00

Materials and Methods

Streptozotocin- (STZ) Induced Diabetes Mellitus. An animal model of DM was made using STZ to damage pancreatic cells. Sprague-Dawley (SD) male rats were injected intraperitoneally with STZ (65 mg/kg body wt dissolved in citrate buffer) (16). Blood glucose was measured from the tail vein blood using the blood glucose monitor, Accu-Chek bG (Bio-Dynamics-Boehringer, Indianapolis, IN). The diabetic state was maintained for 4 weeks prior to mitochondria isolation. In control (CON) and DM rats, blood glucose was 7.12 ± 0.10 mM and 20.7 ± 0.59 mM, respectively.

Polarographic Experiments. *Heart mitochondria preparation for polarographic studies.* For polarographic experiments, mitochondria were isolated from one or two male rat hearts using a method of differential centrifugation previously described (10). Briefly, hearts were quickly excised and chilled. Blood was washed from the heart through the aorta and pulmonary artery with cold buffer No. 1 containing (in millimoles): sucrose, 300; N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), 10 (pH 7.4); ethylene glycol-bis(β-aminoethyl ether)-2N,N,N′,N′-tetraacetic acid) (EGTA), 5 or 10; and bovine serum albumin (BSA), 0.25% at a temperature of 0° to 4°C.

Hearts were initially homogenized in buffer No. 1 (8 ml of buffer per 1 g of tissue) with a Teflon pestle containing a metal core. The primary homogenate was homogenized again with a routine Glass/Teflon Potter-Elvehjem homogenator. Nuclei and other cellular debris were sedimented at 420 to 500*g* for 8 min. The supernatant was centrifuged at 8000*g* for 15 min. The mitochondria pellet was resuspended and re-centrifuged in buffer No. 2 containing (in millimoles): KCl, 180; HEPES, 10 (pH 7.4); and EGTA, 0.05 at a temperature of 0° to 4°C. Finally, the mitochondrial pellet was diluted in buffer No. 2 to a concentration of 6 to 7 mg of protein per 1 ml, and was kept in the tube on ice until polarographic measurements were made.

Measurement of oxygen consumption. Mitochondria respiration and oxidative phosphorylation were studied with polarographic methods using a Clark oxygen electrode (YSI 5300 Biological Oxygen Monitor, YSI Inc., Yellow Springs, OH). State 3 (oxygen consumption stimulated by ADP), State 4 (oxygen consumption after cessation of ADP phosphorylation), respiratory control index (RCI; ratio State 3 and State 4), and rate of oxidative phosphorylation (ROP; State 3 x ADP/O) were measured. The incubation medium (buffer No. 3) for this procedure contained (in millimoles): KCl, 180; $KHPO_4$, 1; and HEPES, 5 (pH 7.4). Because Cox and Matlib (12, 17) demonstrated that 0.2 to 1 mM α-ketoglutarate (but not 10 mM) was the optimal substrate concentration's range to reveal the Na^+ effect on State 3 and NADH formation in heart mitochondria, we decided to use 1 mM α-ketoglutarate as oxidative substrate for our polarographic experiments. Different concentrations of NaCl (in millimoles: 1.0, 3.0, 5.0, and 10) were added to the polarographic cuvette (volume of 1.5 ml) to evaluate the effects of Na^+ on mitochondrial function.

To check that mitochondrial function for NMR experiments was comparable with that of mitochondria used for routine polarography, mitochondrial respiratory function was determined prior to NMR studies using buffer No. 4 containing (in millimoles): sucrose, 250; KCl, 50; HEPES, 5 (pH 7.4); and ethylenediaminetetraacetic acid (EDTA), 0.5.

For polarography experiments, the concentration of mitochondria in the cuvette was 1.13 ± 0.04 mg of protein. In some experiments, 1 μM Ruthenium Red (RR), a blocker of mitochondrial Ca^{2+} uptake, or 250 μM diltiazem (DLTZ), a blocker of Na^+/Ca^{2+} exchange, was added to the cuvette before the addition of NaCl. In another set of experiments to evaluate the effect of Ca^{2+}on the Na^+ effect, two different concentrations of Ca^{2+} (0.035 and 0.114 μM) were added to the cuvette before the addition of NaCl. In another set of experiments to evaluate the pH dependence of Na^+ effect, buffer No. 3 with different pHs (adjusted with KOH and/or HCl to 6.7, 7.0, 7.2, and 7.4) were used.

NMR Experiments. *Heart mitochondria preparation for NMR studies.* Mitochondria were isolated from four hearts for each experiment. The mitochondria isolation techniques for NMR studies were similar to those described above for polarographic studies with the following differences. After final centrifugation, three-fourths of the mitochondria pellet was suspended in 0.8 ml of buffer No. 5 containing (in millimoles): sucrose, 150; KCl, 50; KH_2PO_4, 1; HEPES, 20 or 30; $MgSO_4$, 2; EDTA, 0.5; creatine, 2.5; KG, 5; ADP, 0.6; and ATP, 2. The remainder (approximately one-fourth) of the mitochondria pellet was suspended in buffer No. 2 (0.2 ml) for polarographic measurement of mitochondrial function. After completion of polarographic measurements, all isolated mitochondria not used in polarography were combined with the other mitochondria and were embedded in agarose beads.

Incorporation of mitochondria into agarose beads for NMR experiments. To maintain mitochondria in a stable environment during NMR experiments, they were placed in agarose beads using methods previously described (18, 19). In brief, the mitochondria suspension was added to 1.5 ml of gelling agarose (6%; Sigma type VII: low gelling temperature of 70°C) in buffer No. 5 (37°C) and stirred. The mitochondria/agarose suspension was immediately added to 50 ml of mineral oil (37°C) and was stirred continuously with a magnetic stirrer for 5 min, while decreasing the water bath temperature to 10°C. The interaction of the oil and agarose caused the formation of the beads with the following characteristics: bead size ~800 to 1000 μm; 2000 to 2500 beads/cm^3; 6 to 7 mg of mitochondrial protein per 1 ml; and the average volume of mitochondria in the beads was 84 to 120 μl (18). The oil was removed and beads were rewashed with 200 ml of buffer No. 5, now containing 0.25% BSA.

Perfusion apparatus for NMR experiments. Beads containing mitochondria were placed in a 10-mm diameter glass NMR tube and maintained in place by a filter (100-µm pore size) (17) and continuously superfused at 2.7 ml/min (28°C). The superfusion medium (buffer No. 3 without ATP and BSA) was gassed with 95% O_2/5% CO_2.

^{31}P-NMR. The NMR measurements were performed on a Bruker AM-400 spectrometer (Billerica, MA) at 162 MHz using 60° pulses and a 0.62-sec repetition time with 1000 accumulations over 10 min (18, 19).

Protein Measurement. Mitochondrial protein was measured prior to polarography with the Bio-Rad Protein Assay (Bio-Rad Assay, Hercules, CA) method (rapid determination of protein) to estimate the amount of protein to add to the polarography cuvette (~1 mg of protein/cuvette). To accurately measure mitochondrial protein used for each set of polarographic studies, mitochondrial protein was measured with the Lowry method (19) after each polarographic experiment. Rates of respiration and oxidative phosphorylation were calculated per 1 mg of protein (Lowry method).

To determine protein for each NMR experiment, mitochondria in agarose beads were homogenized in 10 ml of 0.1 N NaOH, and protein concentration was measured with the Lowry method.

Statistical Analysis. The parameters of mitochondrial respiration and high-energy phosphate content were analyzed by two-way analysis of variance (ANOVA). Then, *post hoc* comparisons among the experimental groups were performed using least significant difference (LSD) tests. All values are reported as means ± SEM.

Results

Polarographic Experiments. *Na^+ effects on mitochondrial respiratory function in control and diabetic heart mitochondria.* We hypothesized that increased cytosolic sodium is involved in regulation of mitochondrial oxidative phosphorylation (State 3, RCI, and ROP). To investigate this hypothesis, different concentrations of NaCl were added to mitochondria in a polarographic cuvette, while respiratory function was monitored. Of note, baseline respiratory parameters were lower in DM than CON (Table I). State 3 and ROP were the most sensitive parameters of the Na^+ effect. Na^+ in concentrations greater than 3 mM significantly decreased mitochondrial respiratory function in both CON and DM ($P < 0.01$ compared with baseline; Table I). Maximum effects of Na^+ were found at 5 and 10 mM NaCl. The effects of increasing Na^+ concentrations in DM mitochondria were greater than those found in CON (Fig. 1, State 3 data are presented).

When comparisons between CON (Na^+ 5 mM) and DM (Na^+ 10 mM) (Na^+ concentrations, which could exist *in vivo*) were done, we found that State 3 and ROP were significantly lower in DM than CON (see Table I).

Na^+ effects on mitochondrial function in diabetic heart compared with control: overall ANOVA analysis. The overall two-way ANOVA showed a significant disease (diabetic) effect with respect to State 3, State 4, ROP, ADP/O ratio, and RCI (Table II). The ANOVA also showed a significant sodium effect with respect to State 3, State 4, ROP, but not with respect to ADP/O ratio and RCI. The results of *post hoc* comparisons for CON and DM groups are presented following text.

RR effects on Na^+-induced changes in mitochondrial respiratory function. To evaluate the possible role of mitochondrial Ca^{2+}-uptake and/or intramitochondrial Ca^{2+} levels on Na^+-induced effects on State 3, RR, which blocks Ca^{2+} uptake into mitochondria, was added to the polarographic cuvette before addition of Na^+.

RR alone did not change mitochondria respiratory function. For example, State 3 (in nanograms of atoms of oxygen per minute per milligram of protein) without RR was 184.5 ± 16.6; and with RR it was 184.8 ± 16.9. However, when RR was used in conjunction with Na^+, Na^+ caused larger depression in mitochondria respiratory function (State 3) in both CON and DM (Fig. 2). For example, in CON mitochondria, the decrease of State 3 after addition 10

Table I. Effect of Na^+ on Respiratory Function in Control and Diabetic Mitochondria (Means ± SE)

Parameters	N	NaCl (mM)	State 3	State 4	RCI	ADP/O	ROP
CON	8	0	183.1 ± 10.2	58.7 ± 4.83	3.12 ± 0.17	2.50 ± 0.07	8.08 ± 0.45
DM	8	0	148.7 ± 12.7^{a}	46.9 ± 4.59	3.17 ± 0.27	2.52 ± 0.08	6.26 ± 0.75
CON	5	1	155.4 ± 21.9	58.5 ± 3.69	2.66 ± 0.37	2.52 ± 0.12	7.20 ± 0.59
DM	5	1	102.4 ± 18.1^{b}	46.6 ± 6.60	2.20 ± 0.39	2.30 ± 0.10	4.30 ± 0.88^{a}
CON	5	3	144.0 ± 19.3	52.9 ± 4.80	2.72 ± 0.36	2.47 ± 0.09	6.47 ± 0.83
DM	5	3	87.6 ± 14.4^{a,b}	41.9 ± 6.78	2.09 ± 0.34	2.29 ± 0.09	3.94 ± 1.00
CON	5	5	123.6 ± 2.32^{c}	46.1 ± 3.63	2.68 ± 0.21	2.43 ± 0.05	5.07 ± 0.10^{c}
DM	5	5	81.5 ± 11.6^{d,e}	39.7 ± 5.78	2.05 ± 0.29^{b}	2.22 ± 0.08^{b}	2.74 ± 0.53^{a,b}
CON	4	10	101.2 ± 7.17^{c}	37.4 ± 3.03^{f}	2.71 ± 0.19	2.44 ± 0.10	3.87 ± 0.53^{c}
DM	4	10	73.4 ± 8.95^{a,e,g}	34.7 ± 5.21	2.11 ± 0.26^{b}	2.31 ± 0.10	2.64 ± 0.81^{a,e,h}

Note. State 3, oxygen consumption stimulated by ADP (200 µM) in ng-atoms O/min/mg protein; State 4, oxygen consumption after cessation of ADP phosphorylation in ng-atoms O/min/mg protein; RCI, Respiratory Control Index = State 3/State 4; ADP/O, efficiency of oxidative phosphorylation in nmoles ADP/ng-atoms O; ROP, Rate of Oxidative Phosphorylation in nmoles ADP/min/mg protein. Significance: CON versus DM: ^{a}, $P < 0.05$, ^{d}, $P < 0.01$; CON: Na^+ versus baseline (0 mM Na^+): ^{f}, $P < 0.05$, ^{c}, $P < 0.01$; DM: Na^+ versus baseline (0 mM Na^+): ^{b}, $P < 0.05$, ^{e}, $P < 0.01$; CON 5 mM versus DM 10 mM: ^{g}, $P < 0.0006$, ^{h}, $P < 0.001$.

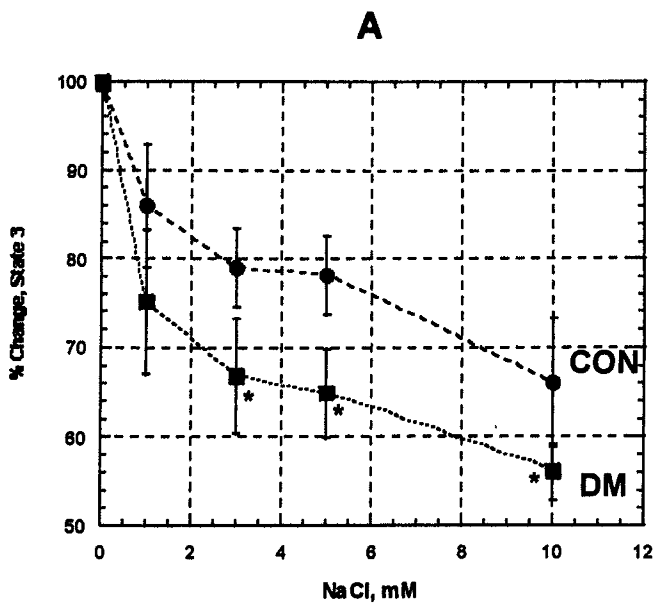

Figure 1. Influence of different concentrations of Na^+ on ADP stimulated mitochondrial oxygen consumption (State 3) in CON and DM heart mitochondria (means ± SE, $n = 5$). *, $P < 0.05$ CON versus DM. Baseline State 3 (without Na^+) is assumed to be 100%.

mM Na^+ without RR was −32.3% ($P < 0.05$) and with RR it was −42.1% ($P < 0.01$). In DM, this effect was −43.3% ($P < 0.05$) and −61.1% ($P < 0.01$), respectively (Fig. 2). It appears the RR influence on the Na^+ effect is greater in DM than in CON.

Ca^{2+} effects on Na^+-induced changes in mitochondrial respiratory function. To further demonstrate that Na^+ effects can be related to intramitochondrial Ca^{2+} levels, $CaCl_2$ (0.035 and 0.114 μM) was added to the buffer before adding 3 mM NaCl. In this set of experiments, because we were looking for Na^+-induced regulatory mechanisms (which very likely would occur at low Na^+ levels), we used a Na^+ concentration (3 mM) that we have shown earlier to produce a metabolic effect, but that is not the maximal effect (Table I). The lower Ca^{2+} concentration (0.035 μM) abolished the Na^+ effect on State 3 and ROP in CON mi- tochondria, but not in DM mitochondria (Fig. 3). The higher Ca^{2+} concentration (0.114 μM) abolished the Na^+ effect in both CON and DM mitochondria. In fact, Ca^{2+} in this concentration actually stimulated respiration in both CON and DM.

Effect of DLTZ on Na^+-induced changes in State 3. The depletion of intramitochondrial Ca^{2+} can result from mitochondrial Na^+-Ca^{2+} exchanger activation by extramitochondrial Na^+. To estimate the possible role of mitochondrial Na^+-Ca^{2+} exchange on respiratory function, DLTZ, a specific inhibitor of the mitochondrial Na^+-Ca^{2+} exchanger (which maintains the intramitochondrial Ca^{2+} levels), was used in conjunction with exogenous Na^+. (DLTZ, a well-known inhibitor of L-type Ca^{2+} channels in sarcolemma, can be used as specific blocker of Na^+-Ca^{2+} exchange with K_m 250 μm in isolated mitochondria [12]). The *post hoc* LSD test showed a significant effect of DLTZ (250 μM) with respect to State 3. DLTZ abolished the Na^+- (5 mM) induced decrease of State 3 in both CON ($P < 0.015$) and DM ($P < 0.023$) mitochondria (Fig. 4). Of note, DLTZ alone did not change State 3 compared with baseline.

pH effects on Na^+-initiated changes in mitochondrial respiratory. The pH experiments were done to determine the optimum pH to evaluate the Na^+ effect (polarography and ^{31}P NMR), and to evaluate effects of H^+ change on Na^+-induced changes in metabolism (Na^+-H^+ exchanger). We exposed isolated mitochondria to different H^+ levels by changing pH in the polarographic cuvette. In CON, the largest effects of Na^+ were found at buffer pH 7.4; State 3 decreased 13.8% ($P < 0.05$); ROP decreased 14.1% ($P < 0.05$; Table III). Increasing pH above 7.4 did not lead to significant increases in the Na^+ effect. On the other hand, decreasing buffer pH (7.2, 7.0, and 6.7) led to decreased Na^+ effect on State 3 and ROP in both DM and CON. However, in DM mitochondria, pH needed to be decreased to lower levels to abolish the Na^+ effect; i.e., lowering pH to 7.0 did not abolish the Na^+-induced changes in State 3, which is in contrast to what we found in CON (Table III). At pH 7.0, the effect without Na^+ minus the effect with Na^+ was significantly different in DM than in CON (Table III). This may be because the baseline intracellular pH (and thus, intramitochondrial pH) is lower in DM than CON. (Note: As measured with a pH electrode, the addition of mitochondria to buffer caused pH to decrease from 0.1 to 0.15 units: for example buffer pH 7.4 was decreased to 7.30 to 7.25. Thus, the actual pHs of the buffer/mitochondria combination were similar to those found in intact cells).

NMR Experiments. *Greater sensitivity of diabetic heart mitochondria to increasing extramitochondrial Na^+.* In support of the polarographic data, we used ^{31}P NMR to study the influence of different concentrations of NaCl on mitochondria ATP levels (Table IV). (For NMR experiments, only well-functioning mitochondria with RCI of 7.10 ± 0.53 in polarographic buffer No. 4 were used). Exposure of DM mitochondria to Na^+ (5–30 mM) led to greater decreases in βATP and greater increases in P_i than that found in CON. In this case, βATP/P_i ratio was significantly lower in DM mitochondria (mostly due to decrease of βATP) after perfusion with 3, 5, 10, and 30 mM NaCl (Fig. 5A). DLTZ, an inhibitor of mitochondrial Na^+-Ca^{2+} exchange, abolished the Na^+ (5–30 mM) initiated decrease of ATP in DM mitochondria and reduced the increase of $P_{i,}$ with resultant values of βATP/P_i similar in both CON and DM mitochondria (Fig. 5B).

Discussion

Others have demonstrated that Na^+ transport mechanisms such as sarcolemmal Na^+-K^+ ATPase, Na^+-Ca^{2+} exchange, Ca^{2+}-pump, and Ca^{2+}-binding activities are decreased in diabetic cardiomyopathy (1, 21–26). Experimental data collected in our laboratory with ^{23}Na NMR also suggest that abnormalities in Na^+ transport mechanisms may be involved in the DM disease process (4). We and

Table II. Result of F Test for Overall Two-Way Analysis of Variance (ANOVA) Comparison of Diabetic and Control Mitochondria and Different NaCl Concentrations on Parameters of Oxidative Phosphorylation

Effect	State 3	State 4	RCI	ADP/O	ROP
Disease	20.4* ($P < 0.000035$)	9.21* ($P < 0.0041$)	13.9* ($P < 0.0006$)	5.64* ($P < 0.0223$)	24.6* ($P < 0.000012$)
Sodium	11.6* ($P < 0.000001$)	3.97* ($P < 0.0084$)	2.54 ($P < 0.055$)	1.48 ($P < 0.226$)	13.0* ($P < 0.000001$)

Note. Disease, Diabetes Mellitus; Sodium, NaCl (in mM): 0, 1, 3, 5, and 10. For definitions of other abbreviations, see Table I.

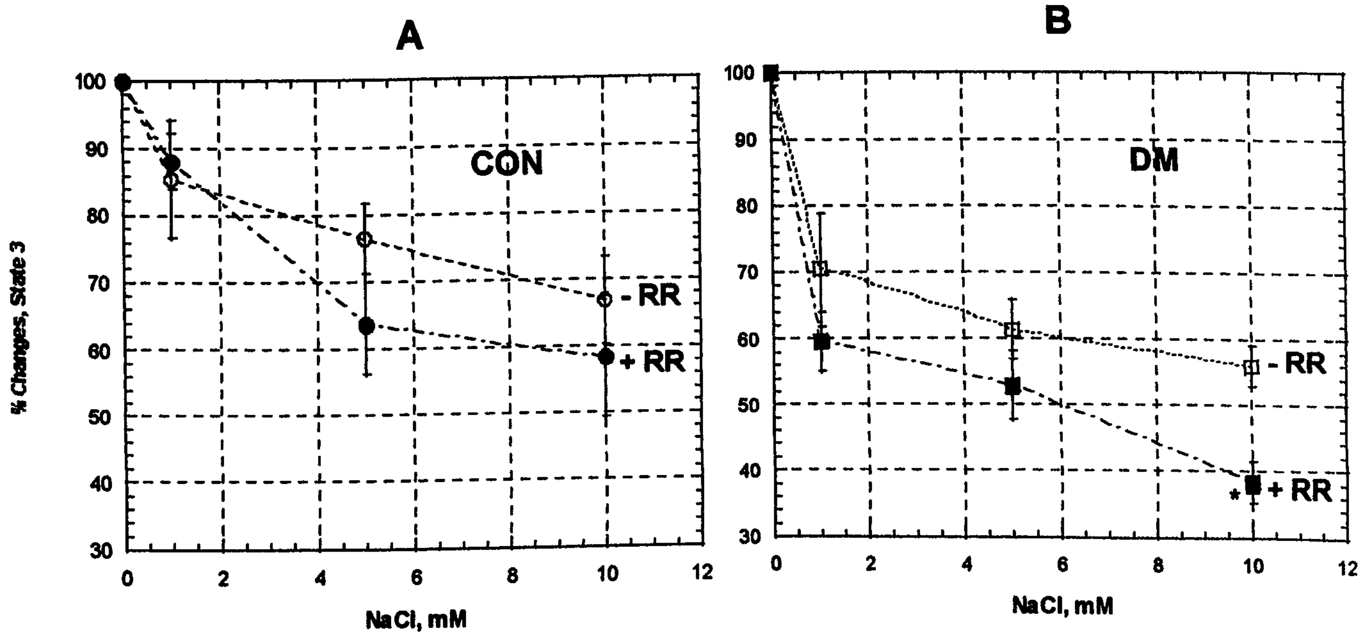

Figure 2. Effect of RR (1 μM) on Na^+-initiated changes in CON (A) and DM (B) mitochondria (means ± SE, $n = 5$). *, $P < 0.05$ with RR versus without RR. Baseline State 3 (without Na^+) is assumed to be 100%.

others have found that Na_i^+ is significantly increased (at least 1.5–2 times) in DM cardiomyocytes (1, 3, 5, 22, 23). The changes in myocardial contractile function (depression of contractile force and slower rate of relaxation [27]) may be related to changes in sodium transport and Na^+_i.

Other studies in animal models of DM reveal that myocardial dysfunction is associated with depressed cardiomyocyte bioenergetics, as evidenced by changes in mitochondrial function (State 3, ROP, and ATP levels) (7–10, 28).

The goal of the present group of studies was to show that increased Na^+_i is a cause of depressed mitochondrial bioenergetics. Our first set of experiments, done with mitochondria from CON rats, was designed to simulate the increased intracellular Na^+ found in the diabetic condition (5, 29). In this case, exposure of heart mitochondria from control rats to increasing levels of Na^+ caused decreases in State 3 and ROP; thus suggesting that changes in cellular Na^+ levels can be involved in depression of bioenergetic function in cardiomyopathies such as those associated with DM.

To further evaluate the Na^+ effect in a DM model, we also exposed DM mitochondria to varying levels of Na^+. We found that DM mitochondria were much more sensitive to extramitochondrial Na^+ levels than CON, suggesting that the DM condition (and long-term exposure to increased Na^+_i) causes residual changes (possibly with upregulation and/or downregulation of various mitochondrial membrane and transport systems) in mitochondrial function. We went on to further explore the potential etiology of these effects.

Since we knew from the literature and from some our earlier studies that Na^+ and Ca^{2+} transport (and their potential effects on metabolism) are intimately related (17, 29), we completed a number of experiments to evaluate the potential role of intramitochondrial Ca^{2+} on the Na^+ effects in both CON and DM mitochondria. We found that when Ca^{2+} uptake was blocked by RR, the Na^+ (10 mM) depressant effect on bioenergetics was increased in both CON and DM, but that in DM mitochondria, the effect of RR was significantly greater. These data suggest that baseline Ca^{2+} is lower in DM than CON. Further support of this hypothesis was found when the addition of Ca^{2+} to the mitochondrial cuvette caused an amelioration of the Na^+ depressant effects on State 3 and ROP. Of note, higher concentrations of Ca^{2+} were needed to reverse the effects of Na^+ in DM compared with CON.

These results are consistent with those of others, which show that in DM the energy-dependent intramitochondrial Ca^{2+} uptake is inhibited, resulting in decreased intramito-

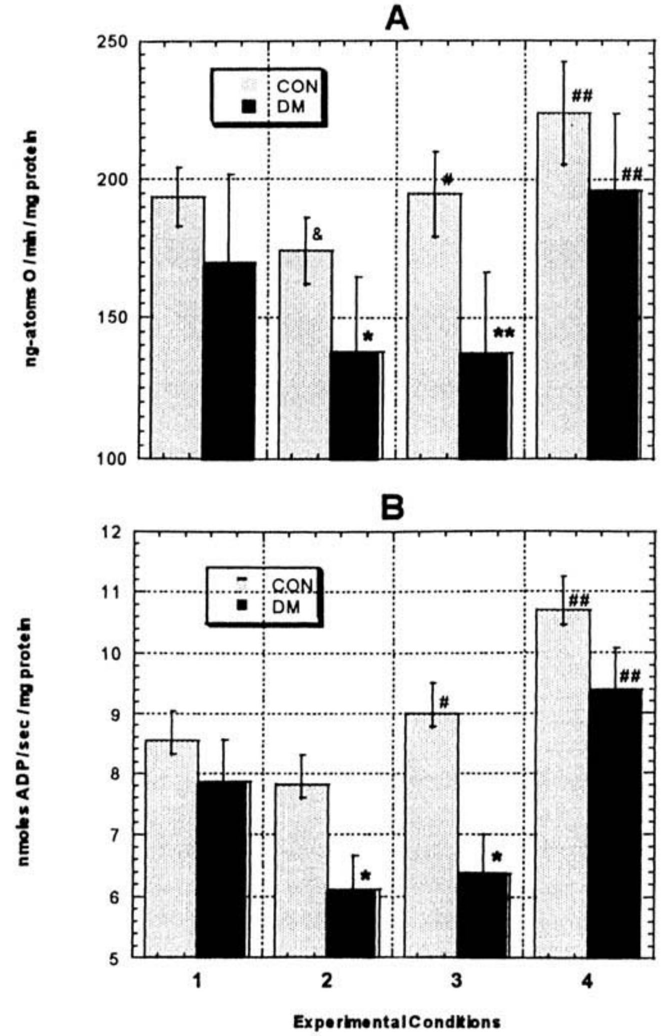

Figure 3. Effect of Ca^{2+} on Na^+-initiated decrease in State 3 (A) and on ROP (B) in CON and DM mitochondria (means ± SE, $n = 5$). 1, Baseline (NaCl, 0 mM); 2, NaCl, 3 mM; 3, NaCl, 3 mM + $CaCl_2$, 0.035 μM; 4, NaCl, 3 mM + $CaCl_2$, 0.114 μM. Significance: CON versus DM: *, $P < 0.03$, **, $P < 0.004$; Na^+ with Ca^{2+} versus Na^+ without Ca^{2+}: #, $P < 0.05$, ##, $P < 0.01$; Na^+ (3 mM NaCl) versus baseline (0 mM NaCl): &, $P < 0.05$.

chondrial Ca^{2+} (8, 11, 26). Ca^{2+} regulation of bioenergetic functions can be realized in the physiological range of Ca^{2+} (0.1–1 μM). In our experiments, the absence of a clear saturation effect of Na^+-induced decrease of State 3 could be explained by a decrease of mitochondrial Ca^{2+} to a range lower than that found in physiological conditions, which in turn could lead to depletion of bioenergetic processes and nonreversible decrease of membrane potential and ATP synthesis.

The level of intramitochondrial Ca^{2+} is also under control of the mitochondrial Na^+-Ca^{2+} exchanger, which mediates the efflux of Ca^{2+} in exchange for the influx of Na^+ (30, 31). It is possible that the increase Na^+_i in diabetic cardiomyocytes leads to activation of the Na^+-Ca^{2+} exchanger, which mediates mitochondrial Ca^{2+} release from mitochondria (1–3, 22, 23). DLTZ, an inhibitor of the Na^+-Ca^{2+} exchanger, abolished the Na^+ effect on bioenergetics in CON and DM, further confirming the intimate relationship of Na^+ and Ca^{2+} on bioenergetics.

With depletion of intramitochondrial Ca^{2+}, bioenergetic functions (State 3, ROP, and substrate oxidation) have been found to be significantly reduced (7–10, 28). These abnormalities could be the result of altered dehydrogenase activity; e.g., Ca^{2+}-dependent DH such as α-ketoglutarate DH, pyruvate DH, or isocitrate DH could be depressed due to decrease in Ca^{2+}. The decrease of enzyme activity can lead to a decrease in turnover of the Krebs cycle intermediates, resulting in a decreased rate of oxidative phosphorylation. The decrease of the Ca^{2+}-dependent Krebs cycle DH activity could also lead to an increased NAD^+/NADH ratio, thus decreasing electron flow through the mitochondrial respiratory chain, and decreasing the electrochemical proton gradient on the mitochondrial membrane. These changes subsequently could lead to decreased ATP synthesis. Our ^{31}P NMR data support this hypothesis; i.e., exposure of mitochondria to increasing levels of exogenous Na^+ was associated with decrease of ATP levels. These decreases were significantly greater in DM mitochondria compared with CON. The energetic deficit may be a causal factor in the development of contractile dysfunction and may be linked to the lower levels of Ca^{2+} in mitochondria from DM rats.

Because the Na^+ effect may be related to H^+ levels and may be at least partially dependent on Na^+-H^+ exchanger activity, in one set of experiments we exposed mitochondria to different H^+ levels (by changing pH) to evaluate the interaction of Na^+ with H^+ ions. We found in both DM and CON that external buffer pH 7.4 (the plasma pH that is generally considered to be the most physiologic) allowed the largest Na^+ effect. Higher pHs did not significantly change the Na^+ depressant effect, while lower pHs (7.2, 7.0, and 6.7) decreased the Na^+ depressant effect on State 3 and ROP. However, DM mitochondria were less sensitive to changes in external buffer pH, as demonstrated by persistence of Na^+ effects down to pH 7.0. This may be due to the fact that intracellular pH is generally lower in DM compared with CON cardiomyocytes due to downregulation of the Na^+-H^+ exchanger (32). The pH effect may be related to a number of physiological processes, including pH dependence of the mitochondrial Na^+-Ca^{2+} exchanger and the Na^+-H^+ exchanger. In addition, the pH effect may be related to the opening of the permeability transition pore (PTP). The mitochondrial permeability transition can be caused by a loss of Ca^{2+} (for example, due to Na^+-induced activation of the Na^+-Ca^{2+} exchanger), and an entry of protons, giving rise to a greater decrease of the transmembrane potential and ΔpH (33). The maximum opening probability is observed at pH close to 7.3 (34, 35). It is interesting that our data show that the maximum effect of Na^+ depression on State 3 was found at pH 7.27 ± 0.02 (Table II), a pH close to the maximum opening probability for the PTP. At pH 7.0 and lower, the opening of the PTP is inhibited (34). This may explain why the Na^+ inhibitory effect on State 3 does not occur at lower pHs (7.0 and 6.7).

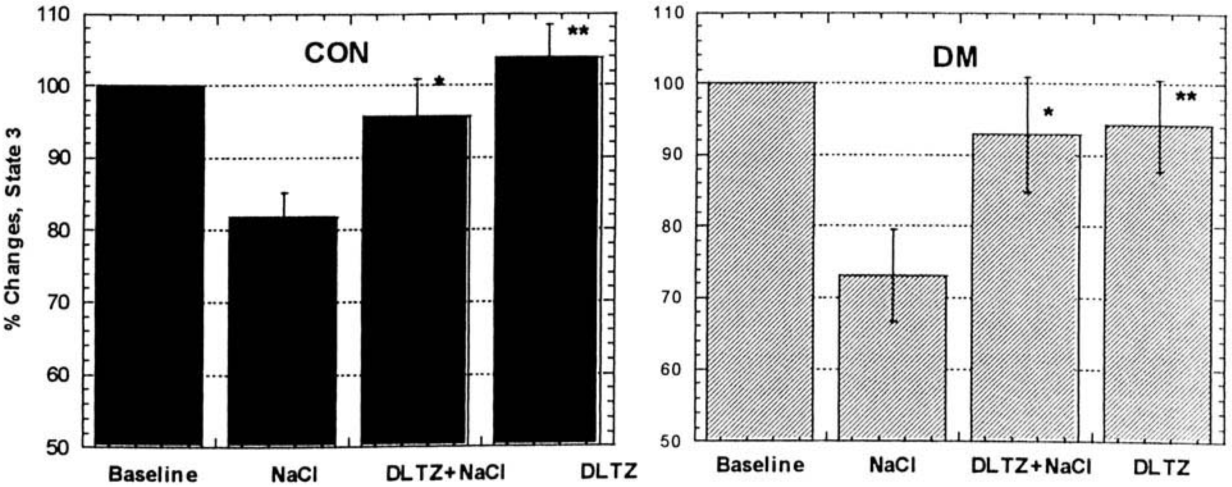

Figure 4. Influence of DLTZ on Na$^+$ induced decrease of State 3 respiration in CON and DM mitochondria (means ± SE, $n = 7$ [CON] and 3 [DM]). Baseline State 3 (without Na$^+$) is assumed to be 100%. Concentrations: NaCl, 5 mM; DLTZ, 250 µM. Significance: NaCl versus NaCl + DLTZ: *, $P < 0.015$ (CON), *, $P < 0.025$ (DM); NaCl versus DLTZ: **, $P < 0.0003$ (CON), **, $P < 0.023$ (DM).

Table III. Effect of Na$^+$ and pH on Respiratory Function in Control (CON) and Diabetic (DM) Heart Mitochondria (Means ± SE, $N = 4$)

pH	CON/DM	NaCl (mM)	State 3	State 3* (value of Na$^+$ effect)	State 4	RCI	ADP/O	ROP	ROP* (value of Na$^+$ effect)
6.7	CON	0	262.7 ± 16.2	−2.43 ± 4.94	47.3 ± 5.83	5.68 ± 0.43	2.58 ± 0.15	10.5 ± 0.29	0.22 ± 0.10
	CON	5	265.2 ± 19.4		41.0 ± 4.17	6.47 ± 0.29	2.52 ± 0.15	10.3 ± 0.21	
	DM	0	211.2 ± 15.7	−1.45 ± 6.66	37.8 ± 5.74	6.04 ± 0.47	2.65 ± 0.12	9.78 ± 0.79	0.05 ± 0.64
	DM	5	212.6 ± 10.2		36.6 ± 5.32	6.13 ± 0.75	2.74 ± 0.09	9.73 ± 0.43	
7.0	CON	0	240.2 ± 7.08	3.75 ± 5.45	55.7 ± 7.81	4.45 ± 0.55	2.53 ± 0.14	9.78 ± 0.30	0.12 ± 0.09
	CON	5	236.4 ± 10.3		52.5 ± 8.25	4.87 ± 0.68	2.57 ± 0.11	9.66 ± 0.33	
	DM	0	210.4 ± 14.9	21.4 ± 4.31^a	52.5 ± 8.12	4.40 ± 0.53	2.67 ± 0.06	9.63 ± 0.72	1.24 ± 0.46^a
	DM	5	189.0 ± 11.4		47.5 ± 8.13	4.48 ± 0.65	2.64 ± 0.18	8.39 ± 0.68	
7.4	CON	0	189.1 ± 5.20	26.1 ± 5.52	70.7 ± 8.45	2.81 ± 0.38	2.39 ± 0.18	7.45 ± 0.50	1.06 ± 0.14
	CON	5	163.0 ± 5.17^b		66.8 ± 10.2	2.61 ± 0.40	2.42 ± 0.18	6.40 ± 0.55	
	DM	0	169.9 ± 11.9	42.3 ± 6.07^a	76.7 ± 13.7	2.48 ± 0.31	2.35 ± 0.13	6.68 ± 0.68	1.49 ± 0.36
	DM	5	127.7 ± 13.3^c		63.4 ± 12.9	2.33 ± 0.39	2.29 ± 0.15	5.19 ± 0.90	

Note. State 3* and ROP*, value of the Na$^+$ effect: subtraction of values without Na$^+$ minus with Na$^+$. For definitions of other abbreviations, see Table I. Significance: with Na$^+$ versus without Na$^+$: ^{c}, $P < 0.05$; CON (without Na$^+$ minus with Na$^+$) versus DM (without Na$^+$ minus with Na$^+$): ^{a}, $P < 0.05$. ^{b}, "d" in Table I.

Table IV. ^{31}P-NMR Data: Effect of Extramitochondrial Na$^+$ on P_i, βATP, and γATP/βADP in Control (CON) and Diabetic (DM) Heart Mitochondria (Means ± SE, $n = 4$)

NaCl (mM)	P_i		βATP		γATP/βADP	
	CON	DM	CON	DM	CON	DM
1	99.6 ± 12.7	115 ± 10.9	97.8 ± 7.52	87.6 ± 3.07	107 ± 6.24	76.0 ± 4.67
3	104 ± 5.48	115 ± 5.68	103 ± 10.6	80.4 ± 3.17	109 ± 7.39	75.9 ± 6.53^a
5	102 ± 4.95	130 ± 7.49^a	103 ± 6.13	69.9 ± 7.07^a	100 ± 8.77	77.4 ± 10.2
10	111 ± 8.45	126 ± 10.1	102 ± 6.34	63.9 ± 5.69^{aa}	105 ± 4.97	59.3 ± 9.50^{aa}
30	118 ± 6.30	123 ± 8.81	87.4 ± 4.08	49.3 ± 5.46^{aa}	88.9 ± 6.14	52.3 ± 10.1^a

Note. Each P_i and βATP value in this table is the percent change from baseline (without Na$^+$) of peak areas (normalized using an MDP standard). Significance: DM versus CON: ^{a}, $P < 0.05$, ^{aa}, $P \leq 0.01$.

In summary, altered bioenergetics found in DM cardiomyopathy may be associated with Na$^+$ and Ca^{2+} abnormalities: both higher baseline cytoplasmic Na^+_i and lower mitochondrial Ca^{2+} may contribute to altered bioenergetics found in the cardiomyopathic myocytes. The effects of Na$^+$ on bioenergetics may be mediated through effects on Ca^{2+}; i.e., due to activation of mitochondrial Na$^+$-Ca^{2+} exchange by Na$^+$, and energy-dependent decreased Ca^{2+} uptake, leading to decreased mitochondrial matrix Ca^{2+}, which in turn prevents optimum activity of mitochondrial Ca^{2+}-dependent

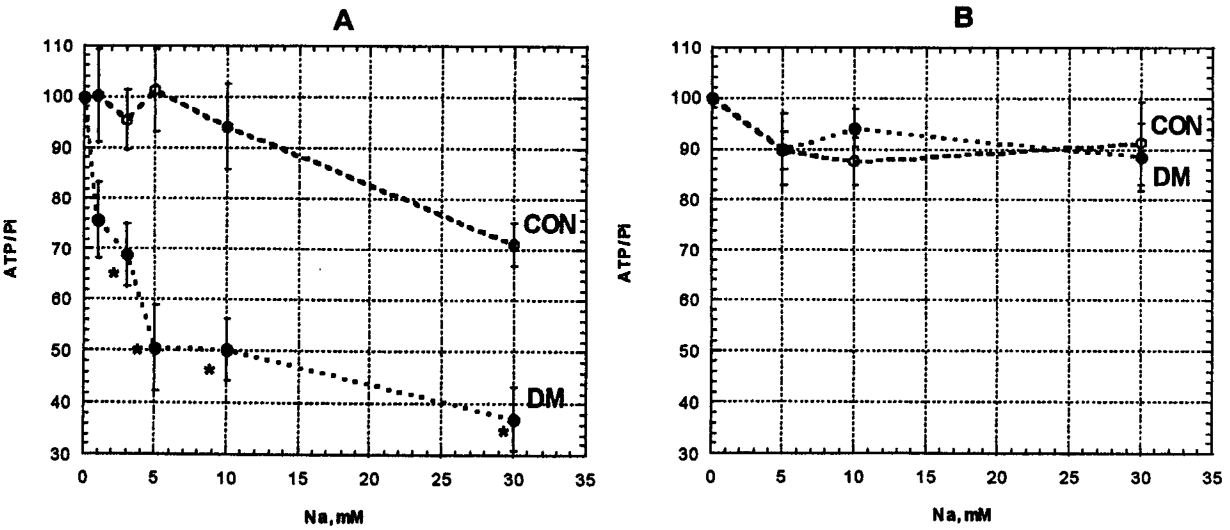

Figure 5. ^{31}P-NMR data. The effect of extramitochondrial Na^+ and DLTZ on βATP and P_i ratio in CON and DM heart mitochondria (means ± SE, $n = 4$). (A) Without DLTZ. (B) With 250 μM DLTZ. Each data point is the mean of the ratio of βATP and P_i peak area. Baseline (without Na^+) is assumed to be 100%. Significance: DM versus CON: *, $P < 0.05$.

dehydrogenases. It is possible that the changes (increased Na^+ and decrease Ca^{2+}) found in DM mitochondria may act similar to the ischemic preconditioning effects found in isolated perfused control hearts, in that they may allow DM hearts to withstand ischemia better than non-preconditioned control hearts (36).

Our data suggest that while localized changes in bioenergetic function may be a cause of ion transport abnormalities, it is just as likely that abnormalities of cytoplasmic ion content and mitochondrial ion transport mechanisms may contribute to metabolic (bioenergetic and respiratory function) abnormalities. Results of the present study suggest that increased Na^+ concentration in diseased cardiomyocytes may be a factor leading to chronically decreased myocardial bioenergetics and to development of diabetic cardiomyopathy.

---

1. Ramasamy R, Schaefer S. Inhibition of Na^+-H^+ exchanger protects diabetic and non-diabetic hearts from ischemic injury: insight into altered susceptibility of diabetic hearts to ischemic injury. J Mol Cell Cardiol **31**:785–797, 1999.
2. Kjeldsen K, Brandgaard H, Sidenius P, Larsen JS, Norgaard A. Diabetes decreases Na^+-K^+ pump concentration in skeletal muscles, heart ventricular muscle, and peripheral nerves of rat. Diabetes **36**:842–848, 1987.
3. Lagadic-Gossmann D, Feuvray D. Intracellular sodium activity of papillary muscle from diabetic rat heart. Exp Physiol **76**:147–149, 1991.
4. Babsky A, Doliba Nic, Doliba Nat, Wehrli S, Osbakken M. Metabolic control of sodium transport in diabetic hearts. (abstract) FASEB J **12**(Part 1):A74, 1998.
5. Doliba NatM, Babsky AM, Wehrli SL, Doliba NM, Ivanics TM, Friedman MF, Osbakken MD. Metabolic control of sodium transport in streptozotocin-induced diabetic rat heart. Biokhimia **65**(4):502–508, 2000.
6. Williams B, Howard R. Glucose-induced changes in Na^+-H^+ antiport activity and gene expression in cultured vascular smooth muscle cells: Role of protein kinase C. J Clinic Invest **93**:2623–2631, 1994.
7. Kuo T, Moore K, Giacomelli F, Wiener J. Defective oxidative metabolism of heart mitochondria from genetically diabetic mice. Diabetes **32**:781–787, 1983.
8. Flarsheim C, Grupp I, Matlib M. Mitochondrial dysfunction accompanies diastolic dysfunction in diabetic rat heart. Am J Physiol **271**(2):H192–H202, 1996.
9. Taegtmeyer H, Zirafi C, Nguyen B. Function and metabolism of the heart in diabetes: A fresh look at an old problem. J Appl Cardiol **2**(1):37–48, 1987.
10. Doliba Nic, Sweet I, Babsky A, Doliba Nat, Forster RE, Osbakken M. Simultaneous measurement of oxygen consumption and $^{13}C^{16}O_2$ production from ^{13}C-pyruvate in diabetic rat heart mitochondria. In: Harrison DK and Delpy DT, Eds. Oxygen Transport to Tissue XIX. New York: Plenum Press, pp269–275, 1997.
11. Pierce G, Dhalla N. Heart mitochondrial function in chronic experimental diabetes in rats. Can J Cardiol **1**:48, 1985.
12. Cox D, Matlib M. A role for the mitochondrial Na^+-Ca^{2+}-exchanger in the regulation of oxidative phosphorylation in isolated heart mitochondria. J Biol Chem **268**:938–947, 1993.
13. DiLisa F, Fan C, Gambassi G, Hogue B, Kudryashova I, Hansford R. Altered pyruvate dehydrogenase and mitochondrial free Ca^{2+} in hearts of cardiomyopathic hamsters. Am J Physiol **264**(6 Pt. 2):H2188–H2197, 1993.
14. McCormack J. Characterization of the effects of Ca^{2+} on the intramitochondrial Ca^{2+}-sensitive enzymes from rat liver and within intact rat liver mitochondria. Biochem J **231**:581–595, 1995.
15. Hansford R, Zorov D. Role of mitochondrial calcium transport in the control of substrate oxidation. Mol Cell Biochem **184**:359–369, 1998.
16. Friedman M, Ramirez I, Edens NK, Granneman J. Food intake in diabetic rats: Isolation of primary metabolic effects of fat feeding. Am J Physiol **249**(18):R44–R51, 1985.
17. Cox D, Matlib M. Modulation of intramitochondrial free Ca^{2+} concentration by antagonists of Na^+-Ca^{2+} exchange. Trends Plant Sci **14**:408–413, 1993.
18. Osbakken M, Ivanics T, Zhang D, Mitra R, Blum H. Isolated cardiomyocytes in conjunction with NMR spectroscopy techniques to study metabolism and ion flux. J Biol Chem **267**:15340–15347, 1992.
19. Doliba Nic, Wehrli S, Babsky AM, Doliba Nat, Osbakken MD. Encapsulation and perfusion of mitochondria in agarose beads for functional studies with ^{31}P-NMR spectroscopy. Magnetic Resonance Med **39**(5):679–684, 1998.
20. Lowry O, Rosenbrough N, Farr A, Randall R. Protein measurement with the Folin phenol reagent. J Biol Chem **193**(1):265–275, 1951.

21. Kent R, Rozich J, McCollam P, McDermott D, Thacker U, Menick D, McDermott, Cooper G. Rapid expression of the Na^+-Ca^{2+} exchanger in response to cardiac pressure overload. Am J Physiol **265**(3 Pt. 2):H1024–H1029, 1993.
22. Regan T, Beyer-Mears A, Torres R, Fusilli L. Myocardial inositol and sodium in diabetes. Int J Cardiol **37**:309–316, 1992.
23. Warley A. Changes in sodium concentration in cardiac myocytes from diabetic rats. Scanning Microsc **5**:239–244, 1991.
24. Allo SN, Lincoln T, Wilson G, Green F, Watanabe A, Schaffer SW. Non-insulin-dependent diabetes-induced defects in cardiac cellular calcium regulation. Am J Physiol **260**(6 Pt. 1):C1165–C1171, 1991.
25. Pierce GN, Ramjiawan B, Dhalla N, Ferrari R. Na^+-H^+ exchange in cardiac sarcolemmal vesicles isolated from diabetic rats. Am J Physiol **258**(1 Pt. 2):H255–H261, 1990.
26. Makino N, Dhalla K, Elibman V, Dhalla N. Sarcolemmal Ca^{2+} transport in streptozotocin-induced diabetic cardiomyopathy in rats. Am J Physiol **253**:E202–E207, 1987.
27. Jackson CV, Mcgrath GM, Tahiliani AG, Vadlamudi RV, McNeill JH. A functional and ultrastructural diabetic rat myocerdium: Manifestation of a cardiomyopathy. Diabetes **34**:876–887, 1985.
28. Mokhtar N, Lavoie J, Rouessan-Migneron S, Nadean A. Physical training reverses defect in mitochondrial energy production in heart of chronically diabetic rats. Diabetes **42**:682–687, 1993.
29. Babsky A, Doliba Nic, Doliba Nat, Wehrli S, Osbakken M. Can increased intracellular sodium be a cause, not a consequence, of bioenergetic abnormalities in diabetic cardiomyocytes? (abstract) Circulation **98**(Suppl):1253–1254, 1998.
30. Li H, Moreno-Sanchez R, Rottenberg H. Alcohol inhibits the activation of NAD-linked dehydrogenases by calcium in brain and heart mitochondria. Biochim Biophys Acta **1236**(2):306–316, 1995.
31. Crompton M, Capano M, Carafoli E. The sodium-induced efflux of calcium from heart mitochondria. Eur J Biochem **69**:453–462, 1976.
32. Khandoudi N, Laville M, Bril A. Protective effect of the sodium/hydrogen exchange inhibitors during global low-flow ischemia. J Cardiovasc Pharmacol **28**(4):540–546, 1996.
33. Gunter TE, Rizutto R, Pozzan T, Simpson AW. A role for calcium influx in the regulation of mitochondrial calcium in endothelial cells. J Biol Chem **271**:10753–10759, 1996.
34. Jouaville LS, Ichas F, Mazat J-P. Modulation of cell calcium signals by mitochondria. Mol Cell Biochem **184**:371–376, 1998.
35. Nicolli A, Petronilli V, Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by matrix pH: Evidance that the pore open-closed probability is regulated by reversible histidine protonation. Biochem **32**:4461–4465, 1993.
36. Ramasamy R, Liu H, Anderson S, Lundmark J, Schaefer S. Ischemic preconditioning stimulates sodium and proton transport in isolated rat hearts. J Clin Invest **96**:1446–1472, 1995.