Nuclear and Mitochondrial Interaction Involving mt-Nd2 Leads to Increased Mitochondrial Reactive Oxygen Species Production

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NADH dehydrogenase subunit 2, encoded by the mtDNA, has been associated with resistance to autoimmune type 1 diabetes (T1D) in a case control study. Recently, we confirmed a role for the mouse ortholog of the protective allele (mt-Nd2) in resistance to T1D using genetic analysis of outcrossoes between T1D-resistant ALR and T1D-susceptible NOD mice. We sought to determine the mechanism of disease protection by elucidating whether mt-Nd2 affects basal mitochondrial function or mitochondrial function in the presence of oxidative stress. Two lines of reciprocal conplastic mouse strains were generated: one with ALR nuclear DNA and NOD mtDNA (ALR.mtNOD) and the reciprocal with NOD nuclear DNA and ALR mtDNA (NOD.mtALR). Basal mitochondrial respiration, transmembrane potential, and electron transport system enzymatic activities showed no difference among the strains. However, ALR.mtNOD mitochondria supported by either complex I or complex II substrates produced significantly more reactive oxygen species when compared with both parental strains, NOD.mtALR or C57BL/6 controls. Nitric oxide inhibited respiration to a similar extent for mitochondria from the five strains due to competitive antagonism with molecular oxygen at complex IV. Superoxide and hydrogen peroxide generated by xanthine oxidase did not significantly decrease complex I function. The protein nitrating agents peroxynitrite or nitrogen dioxide radicals significantly decreased complex I function but with no significant difference among the five strains. In summary, mt-Nd2 does not confer elevated resistance to oxidative stress; however, it plays a critical role in the control of the mitochondrial reactive oxygen species production.

Indeed, a cytosine to adenine transversion (C5178A) resulting in a leucine to methionine substitution in the human NADH dehydrogenase subunit 2 gene (mt-ND2) encoded in the mtDNA has been associated with increased longevity (1, 2) as well as reductions in atherosclerosis (3), blood pressure (4), myocardial infarction (5), and T1D incidence (6). The adenine-containing allele, mt-ND2 (adenine containing NADH dehydrogenase subunit 2 allele), was also associated with reduced islet autoimmunity as represented by significantly lower titers of autoantibodies against glutamic acid decarboxylase, insulin, and protein-tyrosine phosphatase, receptor type

N (Ptprn or IA-2) (6).

Insulin synthetic and secretory capacities of pancreatic β cells are highly dependent upon communication between the nucleus and the mitochondria (7) and are reliant upon mitochondrial ATP generation (8). Although mitochondria are critical for the life and function of β cells, there is strong evidence that mitochondria play a central role in apoptotic death of the β cell during autoimmune T1D (9–16). Therefore, sequence variation in the mtDNA may have profound effects on the β cell, both in life and death.

The function of ND2 is not clearly understood. Studies of Escherichia coli complex I have established that the bacterial ortholog of ND2, NuoN, folds into one of the many α-helices in the membrane arm of complex I (17, 18). The current understanding of the mechanism of complex I suggests that ND2 is involved in proton translocation across the inner mitochondrial membrane (17, 19–21), and its protein sequence shows similarity to an antiporter that is critical in pH regulation (20). ND2 is a hydrophobic subunit of complex I that has been conserved through the course of evolution from bacteria to mice to humans (22).

Recently, our group has confirmed a role for NADH dehydrogenase subunit 2 gene (mt-Nd2) in resistance to diabetes using crosses of the T1D-prone non-obese diabetic (NOD) mouse strain to the alloxan-resistant (ALR) mouse strain (23). Reciprocal backcross populations were generated with identical nuclear DNA but either ALR or NOD mtDNA. Spontaneous

Single nucleotide polymorphisms in the mtDNA have been associated with degenerative diseases and various cancers. Yet sequence changes may also result in resistance to disease.

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3 The abbreviations used are: T1D, type 1 diabetes; ALR, alloxan-resistant mouse strain; NOD, non-obese diabetic mouse strain; ROS, reactive oxygen species; B6, C57BL/6J; NO, nitric oxide; CS, conplastic mouse strain; ETS, electron transport system; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; ETFDH, electron transfer flavoprotein dehydrogenase; ALS, alloxan-susceptible.

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T1D incidence was 4-fold lower in the backcross population with ALR mtDNA than in the backcross population with NOD mtDNA (23). In both man and mouse the "A"-containing allele, mt-ND2a or mt-ND2b, respectively, resulted in a leucine to methionine amino acid substitution and contributed to T1D resistance.

ALR mice, which encode mt-Nd2a, resist both chemically induced and autoimmune T1D. Along with mt-ND2a, loci contributing to T1D resistance have been mapped to chromosomes 3, 8, and 17 (24). ALR mice were bred specifically for their resistance to T1D, which selectively destroys β cells through the generation of hydroxyl radicals (25). Biochemical analyses have revealed that in comparison with the NOD or co-selected alloxan-susceptible (ALS) strain, ALR mice contain elevated reduced to oxidized GSH in the liver and islets, as well as elevated activity of superoxide dismutase in the liver and islets, and elevated activity of glutathione peroxidase and glutathione reductase in islets (26, 27). The destruction of β cells during T1D pathogenesis has been characterized by increased reactive oxygen species (ROS) production and attenuated antioxidant defenses, including decreased levels of GSH and gradually reduced catalase, superoxide dismutase, and thioredoxin transcripts (28–30). Therefore, it has been suggested that increased redox potential in β cells may inhibit T1D by protecting β cells from apoptosis (27, 31, 32). Indeed, GSH, superoxide dismutase, and thioredoxin have been shown to block the actions of signal-regulating kinase, AP-1, and NF-κB, thereby inhibiting apoptosis (33–35).

Mitochondria are both generators (36) and important targets of ROS (37). In this context, it has been proposed that mt-Nd2a imparts upon the mitochondria an increased resistance to oxidative stress (6) as many of the diseases against which mt-ND2a imparts resistance, such as aging and T1D, have been associated with ROS damage. In support of the hypothesis that mt-Nd2a results in elevated resistance to oxidative stress, Turko et al. (38) have reported that complex I enzymatic activity of ALR was not affected by free radicals, although the same treatments reduced complex I activity in ALS by ~50%.

The aim of this study was to determine the effect of mt-Nd2a on basal mitochondrial function as well as on mitochondrial function in the presence of oxidative stress to further characterize its role in the protection against T1D. To study the specific role of mt-Nd2a, two lines of reciprocal conplastic mouse strains (CS) were developed, one with ALR nuclear DNA and NOD mtDNA (ALR.mtNOD) or with NOD nuclear DNA and ALR mtDNA (NOD.mtALR). By combining mt-Nd2a with NOD nuclear DNA, the effects of this allele could be considered separately from the protective effects of the nuclear genome of ALR. Here we assess basal mitochondrial functions and determine the resistance of mitochondria to free radicals. We find that mt-Nd2a does not confer elevated resistance to oxidative stress. However, mt-Nd2a suppresses mitochondrial ROS production.

**EXPERIMENTAL PROCEDURES**

*Mice—ALR/LtJ, NOD/LtJ, and C57BL/6* (B6) mice were bred and maintained in the animal research facility at the Rangos Research Center, Pittsburgh, PA. Conplastic strains of mice, NOD/LtJ-mtALR/LtJ-Mx (NOD.mtALR) and ALR/LtJ-mtNOD/LtJ-Dva/Mx (ALR.mtNOD), were generated as described below. All mice were bred and maintained in a specific pathogen-free vivarium and allowed free access to food (autoclaved diet NIH-31, 6% fat, PMI, St. Louis, MO) and acidified drinking water. All procedures involving animals were approved by the Children’s Hospital of Pittsburgh and were in compliance with “Principles of Laboratory Animal Care” and the current laws of the United States.

**Reagents—**All reagents were obtained from Sigma unless otherwise noted.

*Generation of Reciprocal Conplastic Strains of Mice—*Lines of reciprocal CS mice were generated to determine the role of mt-ND2a in resistance to T1D and for any possible effects on mitochondrial function. Strains with ALR nuclear DNA and NOD mtDNA (ALR.mtNOD) or with NOD nuclear DNA and ALR mtDNA (NOD.mtALR) were generated as described previously (39) with minor modifications. Because mtDNA is inherited exclusively from the egg, only female breeders with the appropriate mtDNA were employed. The generation of ALR.mtNOD CS utilized an F1 outcross of ALR males to NOD females resulting in F1 progeny with NOD mtDNA. Females of this outcross were then backcrossed to ALR males for 10 generations, allowing for continued inheritance of the NOD mtDNA. Conversely, to generate NOD.mtALR CS, NOD males were outcrossed to ALR females resulting in F1 progeny with ALR mtDNA. At each generation females were backcrossed to NOD males until the 10th backcross generation and then intercrossed. Single nucleotide polymorphism typing was conducted to determine the mt-Nd2 allele in the CS as described (23). To preclude nuclear DNA contamination in the CS mice, genotyping was performed by PCR amplification of 94 polymorphic microsatellite primers (Invitrogen) covering all 19 autosomes (24) (supplemental Table).

*Liver Mitochondrial Isolation—*Livers were removed and homogenized in ice-cold isolation buffer (IB) I (225 mM mannitol, 75 mM sucrose, 10 mM HEPES potassium salt, 0.10% bovine serum albumin, fatty acid-free, and 1 mM EDTA, pH 7.4). The homogenate was centrifuged at 1,300 g for 10 min. The supernatant was transferred into new tubes, diluted with IB I, and centrifuged at 10,000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in IB II (225 mM mannitol, 75 mM sucrose, 10 mM HEPES potassium salt, and 100 µM EDTA, pH 7.4) and spun at 10,000 × g for 10 min. The resulting pellet was resuspended in ~100 µl of IB II.

*Brain Mitochondrial Isolation—*Brains were removed, homogenized in ice-cold 12% Percoll in IB I, layered on top of a gradient of 24 and 42% Percoll, and centrifuged at 27,000 × g for 10 min. The mitochondrial fraction was removed with a syringe, diluted with IB I, and centrifuged at 10,000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in ~100 µl of IB II. Protein concentration of both liver and brain mitochondria was determined using the BCA protein assay (Pierce).

*Mitochondrial Respiration—*Mitochondria (1.6 mg/ml) were incubated in media containing 125 mM KCl, 2 mM K2HPO4, 5...
mm MgCl₂, 10 mm HEPES, and 10 μM EGTA, pH 7.1 (IM). To
assay respiration through complex I, the assay medium was
supplemented with 5 mm L-glutamate and 5 mm L-malate. To
assay respiration through complex II, the assay medium was
supplemented with 5 mm succinate. State 3 respiration was
stimulated by the addition of 1.81 mm NADH. The respiratory
control ratio was calculated by dividing state 3 respiration rates
by state 4 respiration rates. Mitochondrial respiration was
determined using a Clark-type oxygen electrode (Hansatech
Instruments Ltd., Norfolk, UK). Assays were performed at
37 °C with constant stirring.

Individual Mitochondrial Electron Transport Chain
Complex Enzymatic Activity Assays—For each assay, mitochondria
samples were subjected to membrane disruption by freeze-
thawing. All assays were run at 30 °C.

The activity of complex I (NADH:ubiquinone oxidoreduc-
tase) was determined by monitoring the oxidation of NADH at
340 nm. The assay medium contained potassium phosphate (25
mm, pH 7.2 at 20 °C), 5 mm MgCl₂, 2.5 mg/ml bovine serum
albumin (fraction V), and 2 mm KCN. A base line was estab-
lished for 1 min after the addition of 0.13 mm NADH, 65 μM
ubiquinone₁, and 2 μg/ml antimycin A. The reaction was initi-
ated by the addition of mitochondria (200 μg/ml), and the rate of
oxidation of NADH was recorded for 3 min. Rotenone (2
μg/ml) was then added, and the rate of change in absorbance
was measured for an additional 3 min. Complex I activity was
determined by subtracting the rotenone insensitive activity
from the total activity.

The activity of complex II (succinate:ubiquinone oxi-
do-reductase) was determined by monitoring the reduction of 2,6-
dichloroindophenolate at 600 nm. The assay medium
contained potassium phosphate (25 mm, pH 7.2 at 20 °C), 5
mm MgCl₂, and 20 mm sodium succinate. Mitochondria (40
μg/ml) were incubated in the assay medium at 30 °C for 10
min. A base line was recorded for 1 min after the addition of
2 μg/ml antimycin A, 2 μg/ml rotenone, 2 mm KCN, and 50
μM 2,6-dichloroindophenolate. The reaction was initiated
by the addition of 65 μM ubiquinone₁, and the rate of reduction
of 2,6-dichloroindophenolate was recorded for 3 min (40).

The activity of complex III (cytochrome c reductase) was
determined by monitoring the reduction of ferricytochrome c
at 550 nm. The assay medium contained potassium phosphate
(25 mm, pH 7.2 at 20 °C), 5 mm MgCl₂, 2.5 mg/ml bovine serum
albumin (fraction V), and 2 mm KCN. KCN was included in the
assay media to prevent the reoxidation of the product, ferricy-

Toxochrome c, by cytochrome c oxidase. Nonenzymatic
activity was recorded for 1 min after the addition of 15 μM ferricyto-
chrone c, 2 μg/ml rotenone, 0.6 mm dodecyl-β-d-maltoside,
and 35 μM ubiquinol. Ubiqinol was prepared by dissolving 8
μg of ubiquinone in 1 ml of ethanol; the solution was adjusted
to pH 2 with 6 M HCl. Ubiqinone was reduced using excess
sodium borohydride. Ubiqinol was extracted into 2:1 (v/v)
diethyl ether/cyclohexane, evaporated under nitrogen gas,
dissolved in 1 ml of ethanol, and acidified to pH 2 with 6 M HCl.
The complex III activity assay was initiated by the addition of
mitochondria (100 μg/ml), and the rate of reduction of ferricy-
chrone c to ferrocytochrome c was recorded for 1 min. The
activity quickly became nonlinear, and the rate was calculated
based on the linear first 30 s. In replicate wells, 2 μg/ml antimi-
cin A was added, and the complex III specific activity was cal-
culated by subtracting the antimycin A insensitive activity from
the total activity (40).

Complex IV (cytochrome c oxidase) activity was determined
by monitoring the oxidation of ferrocytochrome c at 550 nm.
The assay medium contained 10 mm Tris-HCl and 120 mm KCl,
pH 7.0. The nonenzymatic rate was recorded for 1 min after the
addition of 2 μg/ml antimycin A, 0.45 mm dodecyl-β-d-malto-
side, and mitochondria (2.5 μg/ml). The reaction was initiated
by the addition of 11 μM ferrocytochrome c, and the rate of
oxidation of ferrocytochrome c to ferrocytochrome c was mea-
ured for 3 min. The activity quickly became nonlinear, and the
rate was calculated based on the linear first 30 s. In replicate
wells, 2 μg/ml KCN was added, and the complex IV specific
activity was calculated by subtracting the KCN insensitive
activity from the total activity. Ferrocytochrome c was prepared
by reducing ferrocytochrome c with 0.5 mm dithiothreitol (40).

Mitochondrial Membrane Potential and ROS Production—
Membrane potential and free radical production were mea-
sured by fluorescence using a Shimadzu RF-5301 spectrofluor-
imeter (Kyoto, Japan) as described previously (41). All assays
were performed with 350 μM of mitochondrial protein sus-
pended in IM plus 5 mm L-glutamate and 5 mm L-malate or 5
mm succinate with constant stirring at 37 °C. ROS production
was measured using 2 μM fluorescent Amplex Red dye (Molec-
ular Probes, Eugene, OR) in the presence of 1 unit/ml horserad-
ish peroxidase. The excitation wavelength was 560 nm (slit 1.5
nm), and the emission wavelength was 590 nm (slit 3 nm).
Mitochondrial transmembrane potential (Δψₘₚ) was measured
using the fluorescence quenching of the cationic dye safranin O
(2.5 μM). The excitation wavelength was 495 nm (slit 3 nm), and
the emission wavelength was 586 nm (slit 10 nm).

Free Radical Treatment for Mitochondrial Respiration—Mito-
chondrial respiration was assayed as described above.
Approximately 30 s after the addition of ADP, 15 μM nitric
oxide (NO) was added using the NO donor diethylamine
NONOate (Cayman Chemical, Ann Arbor, MI). Percent oxy-
gen consumption was calculated by comparing chamber oxy-
gen content at the time of NO addition to the chamber oxy-
gen content 1.5 min after the addition of NO. Calculations were
based on the percent of control oxygen consumption. To test
whether the effect of NO was reversible, bovine hemoglobin
(1.32 mg/ml) was added after 5 min of incubation with 15 μM
NO.

Free Radical Treatment for Complex I Enzymatic Activity
Assay—Mitochondria were treated with either a 3 mm bolus of
NO donated by diethylamine NONOate or a 1 μM steady-state
level of NO donated by DET- NONOate (Cayman Chemical,
Ann Arbor, MI) for 2 h. Mitochondria were treated with hydrogen
peroxide and superoxide generated by 100 μM xanthine and
20 milliunits of xanthine oxidase and incubated for 2 h. Control
samples were treated with xanthine without xanthine oxidase.
Peroxynitrite or heme peroxidase-dependent reactions were
employed to facilitate protein nitration. Mitochondria were
treated with 0.25, 0.50, or 1.00 mm peroxynitrite (Upstate Bio-
technology, Inc., Lake Placid, NY) and incubated for 1 h. Con-
trols were treated with the equivalent concentration of

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mt-Nd2 Affects ROS Production

**TABLE 1**

Comparison of the individual enzymatic activities of ETS complexes I–IV for ALR, NOD, B6, NOD.mtALR, and ALR.mtNOD

Complex I was assayed as the rotenone-sensitive activity following the oxidation of NADH. Complex II was assayed following the reduction of 2,6-dichloroindophenolate (DCIP). Complex III was assayed as the antimycin A-sensitive activity following the reduction of cytochrome c (Cyt c) over 10 s. Complex IV was assayed as the KCN-sensitive activity following the oxidation of cytochrome c over 30 s. For details see "Experimental Procedures." Values are reported as mean ± S.D. For each strain, n ≥ 3, with mitochondria from each mouse run in triplicate.

| Strain       | Complex I (nmol of NADH oxidized) | Complex II (nmol of DCIP reduced) | Complex III (nmol of Cyt c reduced) | Complex IV (nmol of Cyt c oxidized) |
|--------------|-----------------------------------|-----------------------------------|-------------------------------------|-------------------------------------|
|              | min × mg protein                  | min × mg protein                  | min × mg protein                    | min × mg protein                    |
| ALR          | 35.83 ± 6.41                      | 62.76 ± 9.10                      | 125.94 ± 12.73                      | 282.14 ± 65.58                      |
| NOD          | 36.10 ± 6.24                      | 62.52 ± 13.26                     | 124.70 ± 8.96                       | 256.76 ± 48.14                      |
| B6           | 34.70 ± 6.04                      | 62.54 ± 7.33                      | 121.51 ± 9.25                       | 285.65 ± 34.59                      |
| NOD.mtALR    | 35.41 ± 3.15                      | 64.13 ± 10.69                     | 126.12 ± 4.62                       | 263.53 ± 18.56                      |
| ALR.mtNOD    | 35.78 ± 4.13                      | 64.45 ± 8.68                      | 128.19 ± 15.09                      | 273.42 ± 37.78                      |

**TABLE 2**

Comparison of complex I- or complex II-supported respiration for mitochondria from ALR, NOD, B6, NOD.mtALR, and ALR.mtNOD

Respiration was assayed using a Clark oxygen-type electrode using the complex I substrates glutamate and malate or the complex II substrate succinate. State 3 respiration was stimulated by the addition of 1.81 mM ADP. Respiratory control ratio was calculated by dividing state 3 by state 4 respiration rates. For more details see "Experimental Procedures." Values are reported as mean ± S.D. For each strain, n ≥ 6 with mitochondria from each mouse run in triplicate.

| Strain       | Complex I substrates | Complex II substrates |
|--------------|----------------------|-----------------------|
|              | State 4, nmol O₂     | State 3, nmol O₂       |
|              | mg × ml              | mg × ml               |
| ALR          | 38.34 ± 11.28        | 138.14 ± 13.09        |
| NOD          | 39.45 ± 11.48        | 137.64 ± 23.49        |
| B6           | 31.70 ± 8.52         | 136.54 ± 12.23        |
| NOD.mtALR    | 38.32 ± 13.02        | 140.68 ± 23.59        |
| ALR.mtNOD    | 32.47 ± 9.23         | 136.46 ± 37.31        |

Free Radical Treatment for Complex IV Enzymatic Activity Assay—Mitochondria were incubated with 15 μM NO donated by diethylamine NONOate and incubated for 1 h. Complex IV enzymatic activity was assayed as described above following this incubation period.

Statistical Analysis—All values reported are of at least n = 3. Significance was determined by one-way analysis of variance using Graphpad Prism 4 for Windows (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at p < 0.05.

RESULTS

Single Nucleotide Polymorphism Typing for Conplastic Strains—Pyrosequencing was performed for the A4738C single nucleotide polymorphism to confirm the presence of the mt-Nd2 allele in both CS. The pyrograms from ALR (supplemental Fig. A) and NOD.mtALR (supplemental Fig. C) were equivalent and demonstrated the presence of the mt-Nd2a allele. NOD (supplemental Fig. B) and ALR.mtNOD (supplemental Fig. D) both contained the mt-Nd2b allele. Typing with a panel of microsatellite markers that discriminate ALR from NOD DNA (24) was performed from N1 to N7 to confirm the elimination of degraded peroxynitrite. Mitochondria were treated with 200 μg/ml glucose and 60 μg/ml or 40 ng/ml glucose oxidase (to generate H₂O₂) in the presence of 0.5 mM sodium nitrate and 100 mM myeloperoxidase and incubated for 1 h. In the presence of hydrogen peroxide, myeloperoxidase has been shown previously to oxidize nitrite to the nitrogen dioxide radical, which is capable of nitrating phenolic protein residues (42). Controls were treated with glucose, sodium nitrate, and myeloperoxidase without glucose oxidase. Complex I enzymatic activity was assayed as described above following incubations with each free radical generator.

Basal Mitochondrial Enzymatic Activities, Respiration, and Transmembrane Potential—To discern effects of the two mt-Nd2 alleles on basal mitochondrial function, the mitochondrial ETS was assayed. As shown in Table 1, no differences were measured in enzymatic activities of complexes I–IV when comparing ALR, NOD, B6, NOD.mtALR, and ALR.mtNOD. Basal mitochondrial respiration supported by complex I or complex II substrates was also assayed (Table 2). State 4 respiration was measured after the addition of mitochondria to the chamber, and state 3 respiration was measured after the addition of ADP. Among the five strains, no differences were detected in either state 4 respiration, state 3 respiration, or the respiratory control ratio (rate of state 3 respiration divided by rate of state 4 respiration) while respiring via complex I or complex II (Table 2). No differences in Δψm values supported by complex I or II substrates among the five strains were observed (data not shown).

Mitochondrial ROS Production—An assay that measured changes in fluorescence resulting from oxidation of Amplex Red was used to test the effect of mt-Nd2a on mitochondrial ROS production. ROS production supported by complex I substrates glutamate and malate did not differ among ALR, NOD, B6, and NOD.mtALR. However, mitochondria isolated from ALR.mtNOD produced ~30% more ROS than each of the other four strains (Fig. 1A, black bars). Similar results were obtained for ROS production supported by the complex II substrate succinate, and ALR.mtNOD produced ~30% more ROS than the other four strains (Fig. 1B, black bars).
respiring on glutamate and malate (Fig. 1)

B. ALR.mtNOD mitochondria respiring on glutamate and malate

FIGURE 1. Basal and uncoupled mitochondrial ROS production. ROS production was detected by following the change in fluorescence because of the oxidation of Amplex Red (see “Experimental Procedures”). Mitochondria were uncoupled using 400 nM FCCP. ROS production was measured for mitochondria respiring on both the complex I substrates glutamate and malate (A) the complex II substrate succinate (B). Values are reported as means ± S.D. For basal ROS production (black bars): ALR, n = 10; NOD, n = 12; B6, n = 4; ALR.mtNOD, n = 13; and NOD.mtALR, n = 7. a versus b, p < 0.01. For uncoupled ROS production (white bars), n = 3 for all strains. c versus d, p < 0.05.

To determine whether the observed increase in ROS production in ALR.mtNOD mitochondria was dependent upon the proton motive force (Δp), mitochondrial ROS production was assayed in the presence of 400 nM FCCP. FCCP completely uncouples mitochondrial O2 consumption from ATP production and dissipates Δp, as it is an efficient proton transporter across the inner mitochondrial membrane. FCCP decreased mitochondrial ROS production from all strains by ~50% when respiring on glutamate and malate (Fig. 1A, white bars) and 60% on succinate (Fig. 1B, white bars). However, uncoupled ALR.mtNOD mitochondria respiring on glutamate and malate or on succinate still produced ~30% more ROS than ALR, NOD, NOD.mtALR, or B6 mitochondria.

Of physiological importance, ROS production was also assayed in the presence of ADP (1.81 mM). Although overall ROS production was decreased by the reduction in Δψm, due to ATP production, ALR.mtNOD mitochondria still produced 30% more ROS than the other four strains. This increase in ROS production was witnessed when respiration was supported by either complex I or complex II substrates (data not shown).

To determine whether increased H2O2 production was present globally in ALR.mtNOD, brain mitochondrial ROS production was assessed. As has been reported previously, when brain mitochondria respire on the complex I substrates glutamate and malate, there is little to no free radical production (43). Therefore, there were no differences in basal complex I substrate-supported H2O2 production among ALR, NOD, ALR.mtNOD, and NOD.mtALR (Fig. 2). However, while respiring on the complex II substrate succinate, ALR.mtNOD mitochondria produced ~30% more H2O2 than ALR, NOD, and NOD.mtALR (Fig. 2).

Effect of NO on Mitochondrial Respiration—To determine the effect of mt-Nd2a on free radical-induced mitochondrial dysfunction, respiring mitochondria were treated with 15 μM NO 30 s after the addition of ADP. Comparisons were based on the percent of control oxygen consumption 1.5 min after the addition of NO. While respiring through complex I or complex II (Table 3), mitochondria from ALR, NOD, B6, ALR.mtNOD, and NOD.mtALR were inhibited equally by NO. These results suggest that mt-Nd2a does not alter the susceptibility of respiring mitochondria to NO-induced inhibition.

Effect of Free Radicals on Complex I Enzymatic Activity—To test the theory that the mt-Nd2a allele confers resistance against free radical-induced mitochondrial dysfunction, mitochondrial ROS production was witnessed when respiration was supported by either complex I or complex II substrates (data not shown).

FIGURE 2. Complex I or complex II supported ROS production for brain mitochondria from ALR, NOD, NOD.mtALR, and ALR.mtNOD. Brain mitochondria were isolated as described under “Experimental Procedures,” and H2O2 production was assayed by monitoring the increase in fluorescence because of the oxidation of Amplex Red. Glutamate and malate were used as the complex I substrates, and succinate was used as the complex II substrate. Values are reported as mean ± S.D. For each strain, n ≥ 3 with mitochondria from each mouse run in triplicate.* p < 0.05.

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FIGURE 2. Complex I or complex II supported ROS production for brain mitochondria from ALR, NOD, NOD.mtALR, and ALR.mtNOD. Brain mitochondria were isolated as described under “Experimental Procedures,” and H2O2 production was assayed by monitoring the increase in fluorescence because of the oxidation of Amplex Red. Glutamate and malate were used as the complex I substrates, and succinate was used as the complex II substrate. Values are reported as mean ± S.D. For each strain, n ≥ 3 with mitochondria from each mouse run in triplicate.* p < 0.05.
The effects of hydrogen peroxide and superoxide were studied, using xanthine and xanthine oxidase to generate a steady-state level of these ROS. The function of xanthine oxidase was tested by spectrofluorometrically following the oxidation of Amplex Red with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Using 20 millimolars/ml xanthine oxidase, complex I activity was not significantly decreased from control activity in mitochondria from any of the mouse strains (Table 4). The effect of hydrogen peroxide alone on complex I activity was also studied. No difference in complex I activity was seen when using either 0.25 or 0.50 millimolar H₂O₂ (data not shown).

Given a previous report suggesting resistance of ALR to protein nitration (38), we also assayed complex I function under conditions known to nitrate phenolic protein residues using varying concentrations of peroxynitrite or heme peroxidase-dependent reactions. As shown in Table 5, 0.25, 0.50, and 1.00 millimolar peroxynitrite significantly inhibited mitochondrial complex I activity from each mouse strain by 12, 27.5, and 46%, respectively. Each peroxynitrite concentration produced a significant decrease in complex I activity relative to the other concentrations. However, there was no difference among the individual complex I activities of the five mouse strains for any of the peroxynitrite concentrations. Sodium nitrite and myeloperoxidase in the presence of glucose and glucose oxidase (to generate H₂O₂) were used to generate nitrogen dioxide radicals known to nitrate phenolic protein residues (42). The function of glucose oxidase was tested as described above for xanthine oxidase. With 40 ng/ml glucose oxidase, complex I activity was not decreased. With 60 micrograms/ml glucose oxidase, complex I activity was significantly decreased from control activity by ~24%; however, there was no difference in activity loss among the five mouse strains (Table 4).

### Table 3

**Effect of 15 μM NO on mitochondrial respiration**

Respiration was assayed as described under “Experimental Procedures.” NO (15 μM) was added 30 s after the addition of 1.81 millimolar ADP. Values represent percent of O₂ consumption, which was calculated by comparing the concentration of oxygen in the chamber at the time of NO addition to the concentration of oxygen in the chamber 1.5 min after NO addition. Calculations were based on the percent of control oxygen consumption. Values are reported as mean ± S.D. For each strain, n ≥ 3 with mitochondria from each mouse run in triplicate.

| Strain          | Complex I | Complex II |
|-----------------|-----------|------------|
| ALR             | 57.52 ± 5.67 | 57.12 ± 9.70 |
| NOD             | 57.32 ± 1.55 | 54.89 ± 8.79 |
| B6              | 55.50 ± 10.83 | 54.83 ± 8.27 |
| NOD.mtALR       | 55.17 ± 6.12 | 58.92 ± 4.93 |
| ALR.mtNOD       | 54.12 ± 5.82 | 53.87 ± 6.42 |

### Table 4

**Effect of free radicals on mitochondrial complex I enzymatic activity**

Complex I activity was assayed as described under “Experimental Procedures.” The percent of control complex I activity is displayed after the following: a 2-h incubation with 3 millimolar NO; a 2-h incubation with a steady-state level of 1 millimolar NO; a 2-h incubation with 20 millimolars/ml xanthine oxidase, or a 1-h incubation with 0.5 millimolar sodium nitrite, 100 millimolar myeloperoxidase, and 200 micrograms/ml glucose in the presence of 60 micrograms/ml or 40 milligrams/ml glucose oxidase. Values are reported as mean ± S.D. For each strain, n = 6 with mitochondria from each mouse run in triplicate.

| ROS generator | ALR | NOD | B6 | NOD.mtNOD | NOD.mtALR |
|---------------|-----|-----|----|-----------|-----------|
| NO 3 millimolar | 99.7 ± 10.61 | 97.0 ± 5.75 | 100.34 ± 10.44 | 104.42 ± 10.66 | 99.24 ± 10.64 |
| Xanthine oxidase 20 millimolars/ml | 102.84 ± 11.15 | 100.61 ± 15.56 | 102.34 ± 18.52 | 100.92 ± 12.79 | 97.73 ± 12.49 |
| Glucose oxidase 40 micrograms/ml | 96.87 ± 7.06 | 97.28 ± 11.30 | 90.30 ± 14.04 | 95.55 ± 15.13 | 96.81 ± 5.32 |
| Glucose oxidase 60 micrograms/ml | 77.61 ± 4.60 | 77.93 ± 10.27 | 77.19 ± 6.88 | 73.19 ± 4.69 | 76.18 ± 8.87 |

*P < 0.05 compared with the 40 ng/ml group.

Reversibility of NO-induced Respiratory Dysfunction—Given that NO had no effect on the activity of complex I in any of the mouse strains assayed, it was hypothesized that NO reversibly inhibited mitochondrial respiration by acting as a competitive antagonist of molecular oxygen at complex IV. To test whether the NO-induced inhibition was reversible, 15 micromolar NO was used to inhibit mitochondrial respiration as described above, and following 5 min of inhibition with NO, 1.32 milligrams/ml Hb was added to bind NO. After the addition of Hb, state 3 respiration resumed and was shown to be not significantly different from the state 3 respiratory rate before the addition of NO (Table 6). Control traces of buffer with and without Hb showed that Hb alone did not alter the oxygen content in the chamber (data not shown). When Hb was added to mitochondria in state 3 respiration, there was no effect on the rate of oxygen consumption (data not shown).

NO, Competitive Antagonism at Complex IV—To confirm that NO was in fact acting as a competitive antagonist of complex IV, mitochondria were incubated with 15 micromolar NO for 1 h. Complex IV enzymatic activity was then assayed. For mitochondria from each of the strains, NO inhibited complex IV activity by ~95% compared with controls without nitric oxide (Table 7). However, there was no difference in inhibition among the individual strains.

**DISCUSSION**

The mitochondrial gene NADH dehydrogenase subunit 2 has been associated with resistance to T1D in both humans (6) and mice (23). The protective alleles in both human and mouse differ from the more common allele by a C to A transversion resulting in a leucine to methionine amino acid substitution. In this study, we sought to explore how mt-Nd2 alters mitochondrial function to better understand how this allele provides resistance to T1D. We used isolated mitochondria from T1D-resistant ALR mice encoding mt-Nd2a and T1D-prone NOD mice with mt-Nd2d (cytosine containing NADH dehydrogenase subunit 2 allele) (23). To further investigate the effects of the allelic variation of mt-Nd2, we generated two lines of CS as follows: one with ALR nuclear DNA and NOD mtDNA (ALR.mtNOD) and one with NOD nuclear DNA and ALR mtDNA (NOD.mtALR). B6 mice, encoding mt-Nd2d, were used as controls in all experiments.

We first analyzed several parameters of basal mitochondrial function from each of the five strains and only detected a difference when analyzing mitochondrial ROS production. Mitochondria from ALR.mtNOD were shown to produce significantly more ROS than ALR, NOD, B6, and NOD.mtALR.
mt-Nd2 Affects ROS Production

Mitochondria (Fig. 1). The elevated ROS production by liver mitochondria from ALR.mt<sup>NOD</sup> occurred in both the presence and absence of ADP. ROS production by liver mitochondria after dissipating Δp with FCCP was decreased by ~50% on complex I substrates and 60% on complex II substrates, yet followed a similar trend compared with basal mitochondrial ROS production; uncoupled ALR.mt<sup>NOD</sup> mitochondria produced significantly more ROS on either complex I (Fig. 1A, \textit{white bars}) or complex II (Fig. 1B, \textit{white bars}) compared with uncoupled mitochondria from ALR, NOD, B6, or NOD.mt<sup>ALR</sup>. Mitochondrial transmembrane potential did not differ among the five strains; these results indicate that the increased ROS production in ALR.mt<sup>NOD</sup> mitochondria is not dependent upon Δp, as the increased ROS production is still present after dissipation of Δp with FCCP.

To determine whether the elevated ROS signal in ALR.mt<sup>NOD</sup> mitochondria was present globally, we also assayed ROS production in isolated brain mitochondria. As expected, given the well-characterized low to absent ROS production using complex I substrates and 60% on complex II substrates, yet after dissipating and absence of ADP. ROS production by liver mitochondria as mean

\[ \text{Procedure.} \]

Values represent percent of control complex I activity and are reported as means ± S.D. For each strain, \( n = 3 \) with mitochondria from each mouse run in triplicate. \( ^{a,b,c} \), \( p \leq 0.05 \).

**TABLE 5**

Effect of peroxynitrite on complex I activity

Mitochondria (200 μg/ml) were treated with 0.25, 0.50, or 1.00 mM peroxynitrite for 1 h. Complex I enzymatic activity was assayed as described under “Experimental Procedures.” Values represent percent of control complex I activity and are reported as mean ± S.D. For each strain \( n = 3 \) with mitochondria from each mouse run in triplicate. \( ^{a,b,c} \), \( p \leq 0.05 \).

| Strain   | 0.25 mM | 0.50 mM | 1.00 mM |
|----------|---------|---------|---------|
| ALR      | 87.81 ± 5.68<sup>a</sup> | 73.76 ± 6.36<sup>b</sup> | 55.27 ± 5.19<sup>c</sup> |
| NOD      | 90.49 ± 5.49<sup>a</sup> | 72.10 ± 5.65<sup>b</sup> | 52.67 ± 8.06<sup>c</sup> |
| B6       | 88.01 ± 5.90<sup>a</sup> | 73.52 ± 5.62<sup>b</sup> | 52.23 ± 6.45<sup>c</sup> |
| NOD.mt<sup>ALR</sup> | 89.46 ± 4.41<sup>a</sup> | 72.27 ± 5.34<sup>b</sup> | 55.77 ± 4.65<sup>c</sup> |
| ALR.mt<sup>NOD</sup> | 85.30 ± 4.71<sup>a</sup> | 70.91 ± 3.77<sup>b</sup> | 54.75 ± 6.31<sup>c</sup> |

**TABLE 6**

Reversibility of NO induced respiratory dysfunction

Oxygen consumption was measured using a Clark oxygen-type electrode, and state 3 was induced by the addition of 1.81 mM ADP (see “Experimental Procedures”). NO (15 μM) was added 30 s after the addition of ADP to inhibit mitochondrial respiration. After 5 min of incubation with NO, Hb (1.32 mg/ml) was added to bind NO. Percent of control values represent the comparison of the state 3 respiration rate after the addition of Hb to the initial state 3 respiration rate before the addition of NO. Values are reported as means ± S.D. For each strain, \( n = 3 \) with mitochondria from each strain run in triplicate.

| Strain    | Complex I substrates | Complex II substrates |
|-----------|----------------------|-----------------------|
|           | State 3 (pre-NO)     | State 3 (post-NO)     | State 3 (pre-NO) | State 3 (post-NO) |
| ALR       | 121.65 ± 20.72       | 113.50 ± 26.24        | 188.48 ± 26.70  | 177.33 ± 32.53  |
| NOD       | 119.69 ± 7.16        | 115.76 ± 10.23        | 184.44 ± 18.59  | 169.70 ± 20.80  |
| B6        | 131.02 ± 9.99        | 122.74 ± 14.38        | 176.98 ± 14.87  | 165.56 ± 7.78   |
| NOD.mt<sup>ALR</sup> | 131.78 ± 11.09      | 123.92 ± 10.37        | 194.94 ± 9.89   | 188.01 ± 3.70   |
| ALR.mt<sup>NOD</sup> | 129.80 ± 15.97      | 122.89 ± 9.82         | 201.72 ± 13.84  | 188.60 ± 7.16   |
mt-Nd2 Affects ROS Production

**TABLE 7**

Effect of 15 μM NO on complex IV activity

| Strain       | Percent of control complex IV activity |
|--------------|----------------------------------------|
| ALR          | 95.18 ± 2.09                           |
| NOD          | 94.08 ± 1.70                           |
| B6           | 95.18 ± 2.07                           |
| NOD.mtNOD    | 93.46 ± 1.85                           |
| ALR.mtNOD    | 94.76 ± 1.03                           |

as a competitive antagonist with molecular oxygen to inhibit the mitochondrial respiration, as demonstrated previously (47).

In contrast to the proposed mechanism of elevated resistance to oxidative stress by Uchigata et al. (6), we suggest that allelic variants of mt-Nd2 play a critical role in the production of ROS by the mitochondrial ETS. Our data show that ALR.mtNOD mitochondria produce ~30% more ROS than both parental strains or the reciprocal CS, indicating that an interaction is occurring between a protein encoded by the nuclear DNA of ALR and the protein product of mt-Nd2. Furthermore, based on elevated ROS production supported by either complex I or complex II substrates (Fig. 1), it is likely that an altered interaction between complexes I and III results in increased ROS. It is unlikely that the increased complex II-supported ROS production in ALR.mtNOD mitochondria is solely due ROS production from complex I redox centers, because the increase is present after the addition of the uncoupler FCCP, which prevents reverse electron flow back to complex I (Fig. 1).

This mechanism may also account for the results obtained in the initial backcross study implicating the combination of mt-Nd2 and ALR nuclear genes in resistance against T1D (23). In that study, reciprocal backcross mice were generated: (NOD/LtDv × ALR/Lt)F1 (♀) × NOD/LtDv (♂) and (ALR/Lt (♀) × NOD/LtDv (♂))F1 (♀) × NOD/LtDv (♂). The former backcross population encoded mt-Nd2 and developed spontaneous T1D at a 4-fold higher rate than the latter population encoding mt-Nd2 (♂) (23). mt-Nd2 combined with ALR nuclear DNA on chromosome 3, 8, or 17 in this backcross may have resulted in an elevated ROS production leading to increased free radical defenses. Support for this hypothesis has been provided by previous studies that have associated very low β cell antioxidant defenses with increased susceptibility and increased islet antioxidants with resistance against NOD-derived immune effectors (27, 32, 48–51, 53–57).

A potential candidate gene is Ndufb7, which is contained within the Idd22 confidence interval on chromosome 8 previously reported to contribute to the resistance of ALR to T1D (24). The phosphorylation of this complex I subunit by pyruvate dehydrogenase kinase has been reported to lead to an increased mitochondrial superoxide production (58). Likewise, the suppressor of superoxide production locus (Suspx, encoded on chromosome 3), which has been shown to account for resistance against T1D in ALR, contains electron transfer flavoprotein dehydrogenase (Etfdh), a protein that resides in the inner mitochondrial membrane. Electrons can be transferred by ETFDH to ubiquinone entering the mitochondrial respiratory chain (59). Deficiency in ETFDH leads to accumulation of glutaric acid resulting in increased oxidative stress (52). We have measured a decrease in Etfdh expression in ALR compared with NOD (data not shown). Sequence variation of Etfdh in ALR leading to reduced levels of ETFDH in the mitochondrial inner membrane could lead to increased mitochondrial ROS production in combination with mt-Nd2 that is eliminated when combined with mt-Nd2♂.

In summary, the two alleles of mt-Nd2 studied here do not alter basal mitochondrial function, and only exhibit differences in mitochondrial ROS production when mt-Nd2♂ is combined with specific ALR-derived nuclear factors. As measures of mitochondrial function were equal after incubation with different radical species when comparing the five strains, it is unlikely that mt-Nd2♂ itself scavenges ROS. Rather, these findings suggest that an elevation in ROS production resulting from a structural change in the ETS when mt-Nd2♂ is combined with ALR nuclear DNA is responsible for a decrease in the incidence of T1D. Further, the presence of mt-Nd2♂ results in a lower ROS production and subsequent protection from the occurrence of T1D.

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