CALL FOR PAPERS | Mitochondria in Cardiovascular Physiology and Disease

Construction of two novel reciprocal conplastic rat strains and characterization of cardiac mitochondria

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Mitochondrial respiration is not only a source of energy (ATP) but also a major source of reactive oxygen species (ROS) with 0.2% of oxygen consumed, being normally converted into superoxide in a quiescent state (1, 40). ROS cause increased oxidative stress, which is strongly implicated in pathological signaling, leading to elevated BP or hypertension (6, 11, 30, 41, 43). Because several of the subunits of proteins participating in mitochondrial respiration for the generation of both ATP and ROS are encoded by the mitochondrial genome, it is possible that functional variants within the mitochondrial DNA (mtDNA) could serve as genetic determinants of ARC and/or BP, which in turn could affect life span. While there are reports of associations of the mitochondrial genome with exercise capacity and other cardiovascular phenotypes (21, 28, 29, 31, 42), inferences on cause-effect relationships cannot be drawn based on these association studies. To extend such observations beyond mere associations, we generated novel reciprocal conplastic strains using two of the most widely used selectively bred models of cardiovascular disease: the Dahl salt-sensitive (S) rat and the spontaneously hypertensive rat (SHR). The choice of S and SHR was based on three primary factors: 1) the divergence in BP as well as ARC between these two strains; 2) both S and SHR have highly permissive nuclear genomes for the development of high BP, which, in theory, would allow for the experimental detection of any subtle contributions of the reciprocally substituted mitochondrial genomes; and 3) our previous report (24) of complete mitochondrial genome sequencing data wherein 12 nonsynonymous variations were detected within genes coding for subunits of proteins essential for the electron transport chain, for mitochondrial ROS production, and within the D-loop region.

MATERIALS AND METHODS

All animal experiments were conducted as per preapproved protocols by the Institutional Animal Care and Use Committee of the University of Toledo College of Medicine and Life Sciences. The inbred S rat was developed in house at our Institution. The SHR (SHR/Hsd) was originally obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained in our colony.

Conplastic strain derivation. Two reciprocal conplastic strains of S and SHR were generated by taking advantage of the maternal inheritance of mitochondrial genomes. A single SHR female rat was crossed with a male S rat. The resultant F1 female offsprings were backcrossed with male S rats. This backcross procedure was repeated 9–12 additional times to generate S.SHRmt conplastic strains. Similarly, a single S female rat was bred with a male SHR. The F1 female offsprings were backcrossed with male SHR. This backcross proce-
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H24 CONPLASTIC STRAINS AND CARDIAC MITOCHONDRIA
to assess the nuclear genomes of S.SHRmt and SHR.Smt strains along with previous report (24). A total of 162 microsatellite markers were used and administered 2% NaCl containing diet as described in a previous report (25).

The isolation of DNA and genotyping. DNA was extracted from tail biopsies of S.SHRmt and SHR.Smt and sequenced as detailed in a previous report (25).

Survival study. Control rats (Dahl S rat and SHR) were housed two to a cage such that two different strains were in each cage. At 40–42 days of age, the rats were weaned onto a low-salt (0.3% NaCl) Harlan Teklad 7034 diet. Conplastic strains along with their respective control rats (Dahl S rat and SHR) were housed two to a cage such that two different strains were in each cage. At 40–42 days of age, the rats were switched to a 2% NaCl diet (Harlan Teklad, TD 94217) and maintained on this diet for the duration of the experiment. During days 25–28 on the 2% NaCl diet, each rat had its systolic BP measured by using the backcross 13 animals.

BP measurements. The experimental design for BP measurement by the tail-cuff method was as described previously (25). Briefly, at 30 days of age, rats were weaned onto a low-salt (0.3% NaCl) Harlan Teklad 7034 diet. Conplastic strains along with their respective control rats (Dahl S rat and SHR) were housed two to a cage such that two different strains were in each cage. At 40–42 days of age, the rats were switched to a 2% NaCl diet (Harlan Teklad, TD 94217) and maintained on this diet for the duration of the experiment. During days 25–28 on the 2% NaCl diet, each rat had its systolic BP measured by two blinded operators. During BP measurements, rats were restrained and warming to 28°C. The operators’ readings for each rat were averaged and recorded as that animal’s systolic BP. BP data were also monitored in fresh assay media without DCFH-DA. Oxidant generation was measured at the excitation wavelength of 488 nm and emission wavelength of 525 nm using Molecular device fluorescence microplate reader for 30 min. The amount of oxidant generation was quantified by using the dichlorofluorescein acid method from Pierce with bovine serum albumin as standard.

Assessment of ARC. ARC was determined in 10-wk-old rats using a standard ramped treadmill test described by Henderson et al. (14). The equipment used for treadmill running was from Columbus Instruments (Model Exer-4; Columbus, OH). The rats were euthanized after completion of the ARC experiment for collection of heart samples. These samples were used for all biochemical and protein expression studies.

Survival study. S, S.SHRmt, SHR, and SHR.Smt rats (backcross-10) were raised and administered 2% NaCl containing diet as described under the BP measurements section. These rats were continued on the 2% NaCl diet until their natural death.

Isolation of heart mitochondria. Heart mitochondria were isolated by using differential centrifugation method (7). Briefly, heart homogenate was obtained using isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% fatty acid-free BSA, and 10 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 1,000 g for 10 min, and the supernatant was centrifuged again at 12,000 g for 10 min. Mitochondrial pellet was washed twice, centrifuged at 12,000 g, and resuspended in isolation buffer without EGTA. All centrifugation steps were carried out at 4°C. Protein content was quantified by using the bicinchoninic acid method from Pierce with bovine serum albumin as standard.

Determination of oxidant production. Oxidant generation was monitored in mitochondria isolated from the hearts of S, SHR, and conplastic strains by using the redox-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA) according to previously described methods (19, 27). In brief, isolated mitochondria (~0.2 mg protein) were incubated in the assay media consisting of (in mM) 137 KCl, 2.5 MgCl2, 2 K2HPO4, 10 Tris-HCl (pH 7.4), 5 glutamate, and 5 malate and 5 mM DCFH-DA at 37°C for 10 min to allow DCFH-DA to cross the mitochondrial membrane. The solution was then centrifuged at 12,000 g for 10 min, and the supernatant was discarded. The pellets were resuspended in fresh assay media without DCFH-DA. Oxidant generation was measured at the excitation wavelength of 488 nm and emission wavelength of 525 nm using Molecular device fluorescence microplate reader for 30 min. The amount of oxidant generation was expressed as DCF formed per minute per milligram of protein.

Mitochondrial permeability transition pore assay. The opening of the permeability transition pore causes mitochondrial swelling and is continuously assayed as a decrease in light scattering of a mitochondrial suspension. Mitochondrial permeability transition pore activity

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Dahl S, Dahl salt-sensitive rat (S); SHR, spontaneously hypertensive rat; mt, mitochondrial.
was measured using previously described methods (8, 23). In brief, isolated mitochondrial pellet was suspended at the concentration of 1 mg protein/ml in 200 μl of assay buffer containing (in mM) 225 mannitol, 75 sucrose, 5 glutamate, 5 malate, and 10 HEPES (pH 7.4). The samples were continuously monitored at optical density of 540 nm for 30 min with or without the addition of 15 μmol/mg protein of Ca²⁺. The rate of fall in absorbance was recorded using a microplate reader (Molecular Devices).

Analysis of mitochondrial respiration and mtDNA copy number. Mitochondrial respiratory chain activities of complex I, II, IV, and V were assessed in mitochondria isolated from heart samples of all groups of rats. Activities were measured using kits from Mitoscience-Abcam according to the manufacturers’ instructions. mtDNA copy number was measured by real-time PCR (Bio-Rad) method using SYBR green. Total DNA was isolated from heart tissue (n = 4). mtDNA copy number was assessed by amplification of the mitochondrial D-Loop versus the nuclear Gapdh gene. The difference in CT between D-loop and Gapdh values was used as the measure of relative abundance of the mitochondrial genome. Fold change was calculated and expressed by using the 2−ΔΔCT method (37).

Protein content of mitochondrial respiratory subunits. Immuno blotting was performed to measure the mitochondrial respiratory protein levels in isolated mitochondrial pellet from hearts of S, S.SHRMt, SHR, and SHR.SMt rats. Samples were homogenized in ice-cold RIPA lysis buffer with protease inhibitor cocktail (Pierce). Thirty microgram of proteins were boiled with Laemmli loading buffer for 5 min at 95°C. Protein samples were resolved using 10% NuPAGE Bis-Tris gel (Invitrogen) at room temperature and transferred on to polyvinylidene difluoride membrane (Millipore). Mitochondrial protein levels were quantitated by Western blot analysis using mouse monoclonal antibodies purchased from Mitoscience-Abcam for the following proteins: β-subunit of the F1F0 ATP synthase (Atp5a), mitochondrial cytochrome-c oxidase subunit 1 (Cox1), mitochondrial cytochrome c oxidase subunit 3 (Cox3), cytochrome c oxidase subunit 4 (Cox4), core 2 subunit of ubiquinol-ferricytochrome c oxidoreductase (Uqcrf2), 39-kDa subunit of NADH:ubiquinone oxidoreductase (Ndufa9), and flavor protein subunit of succinate dehydrogenase (Sdha). Heart homogenates were used for protein Western blot analysis for nuclear encoded transcription factor proliferator-activated receptor coactivator-1α (Pgc-1α; Abcam) and peroxisome proliferator-activated receptor-γ (Ppar-γ; Santa Cruz). Membranes were blocked with 5% fat-free milk and incubated with primary and secondary antibodies before visualization by the chemiluminescence method (Pierce). ImageJ was used to quantify the protein expression level, and values were expressed as arbitrary densitometry units.

Statistical analyses. Statistical analyses were conducted by ANOVA using the SPSS software (SPSS, Chicago, IL). Data are presented as means ± SE. A P value of ≤0.05 was used as a threshold for statistical significance. Survival data were analyzed and plotted using the Kaplan-Meier plot.

RESULTS

Genomes of conplastic strains. The mitochondrial genotypes of the conplastic strains were verified by direct sequencing of the complete mitochondrial sequences as S in the SHR.SMt strain and SHR in the S.SHRMt strain, respectively. The mtDNA sequence of the SHR.SMt strain was identical to the mtDNA sequence of the S strain.
rat (Genbank accession number GU997608). Similarly, the mtDNA sequence of the S.SHR<sup>mt</sup> strain was identical to the reported mtDNA sequence of the SHR (Genbank accession number GU997610). The nuclear genomic DNA of both the conplastic strains were verified by whole genome genotyping conducted using 162 microsatellite markers (Table 1). The average distance between the markers was 14.54 Mb. At all the 162 locations examined, the nuclear genome of the S.SHR<sup>mt</sup> strain was represented by S alleles and the nuclear genome of the SHR.S<sup>mt</sup> strain was represented by SHR alleles (Table 1).

**Body weights and relative heart weights.** Body weights of S and SHR were significantly different (Fig. 1A). However, the body weights of the conplastic strains were not significantly different from their relative inbred strain comparisons (Fig. 1A). Relative heart weights were not different between any of the experimental groups (Fig. 1B).

**Intrinsic aerobic capacity.** Inbred SHR had a significantly higher running capacity than inbred S rats (Fig. 1B). The total distance run by SHR (267 ± 45 m) was significantly longer than the distance run by the S rat (126 ± 10 m) (Fig. 1C, P < 0.0006). The total duration for which SHR could run (16 ± 2.5 min) was also higher than that of the S rat (8 ± 0.6 min) (Fig. 1D, P < 0.003). Similarly, the velocity of running of the SHR (17.6 ± 1.3 m/min) was higher than the velocity of the S rats (13.7 ± 0.32 m/min) (Fig. 1E, P < 0.003). Substituting the mitochondrial genome from the SHR into the S rat (S.SHR<sup>mt</sup> conplastic strain) significantly improved the ARC of S rats (Fig. 1, C–E). The distance, duration, and velocity run by the S rats was significantly improved in the S.SHR<sup>mt</sup> conplastic rats by 10.22 ± 0.33 m (Fig. 1C, P < 0.0004), 3.6 min (Fig. 1D, P < 0.0001), and 1.8 m/min (Fig. 1E, P < 0.0002), respectively. Introducing the mitochondrial genome from the S rat into the SHR in the SHR.S<sup>mt</sup> conplastic strains did not, however, alter the ARC of the SHR (Fig. 1, C–E).

**Blood pressure.** Systolic BP of the S rat (228 ± 5 mmHg) was significantly higher than that of the SHR (178 ± 5 mmHg) (Fig. 2A, P < 0.001). Systolic BP of S.SHR<sup>mt</sup> (241 ± 3 mmHg) and SHR.S<sup>mt</sup> (175 ± 8 mmHg) were not statistically different from the systolic BP of S and SHR rats, respectively (Fig. 2A). Although the overall tail-cuff BP data were not significantly different between the inbred and respective conplastic strains, the BP data of S and S.SHR<sup>mt</sup> were further corroborated by radiotelemetry (Fig. 2B).

**Survival study.** The median survival of S rats was 87 days, which was significantly lower than the median survival of >620 days for the SHR (Fig. 3). The S.SHR<sup>mt</sup> conplastic strain had a median survival of 95 days, which was significantly higher than that of the S rat (Fig. 3, P < 0.0006). The reciprocal conplastic strain, SHR.S<sup>mt</sup>, had a median survival of >554 days, which was lower than the median survival of the SHR by 66 days, but the difference in median survival was not statistically significant (Fig. 3, P < 0.956).

**Mitochondrial oxidant generation and mitochondrial swelling.** To determine whether the previously reported sequence variation in the mitochondrial genome was responsible for the differential intrinsic aerobic capacity and survival effects de-
scribed above, we studied the functionality of isolated mito-
chondria from the SHR conplastic and progenitor inbred
strains. Mitochondria from the S rats generated significantly
higher ROS compared with the SHR (Fig. 4, \( P < 0.001 \)). ROS
production of the S rats was significantly attenuated by intro-
gression of the SHR mitochondrial genome in S.SHR con-
plastic rats (Fig. 4, \( P < 0.015 \)). Replacing the SHR mtDNA
with that of the S in the SHR.Smt conplastic strain did not
change ROS production in the SHR (Fig. 4, \( P < 0.905 \)).
Superoxide dismutase activity was not different among any of
the experimental groups (data not shown). Because ROS are
known to affect the functionality of the mitochondrial perme-
ability transition pore, we evaluated the extent of mitochon-
drial swelling in the conplastic and progenitor strains. While
there were no differences in basal absorbance measured in the
absence of calcium, in the presence of exogenously added
calcium, increased mitochondrial swelling was observed in S
rats but not in the SHR (Fig. 5A, \( P < 0.001 \)). Despite the
decrease in ROS observed in the S.SHR conplastic strain
compared with the S rat, the extent of mitochondrial swelling
in response to calcium was not different between S.SHR and
S rats (Fig. 5A, \( P < 0.609 \)). Basal absorption and mitochon-
drial swelling in response to calcium was also unaffected in
the SHR by the introduction of the S mitochondrial genome into
the SHR in SHR.Smt conplastic strain. (Fig. 5A, \( P < 0.859 \)).
Tracings of absorption plotted against time with and without
the addition of calcium in all samples is shown in Fig. 5B.

Enzyme activities in isolated mitochondria. Analysis of
enzyme activities of respiratory chain complexes revealed
significantly lower activity of complex II in the SHR.Smt
conplastic strain compared with the SHR (Fig. 6, \( P < 0.05 \)). In
contrast, we found no strain differences in the activities of com-
plexes I, IV, and V between any of the strain comparisons (Fig. 6).

mtDNA copy number and respiratory enzyme levels.
The mitochondrial genome from SHR rat, when introduced into the S
rat, resulted in an increase in mtDNA copy number of the
S.SHR mt strain (Fig. 7, \( P < 0.003 \)). The mtDNA copy numbers of
the SHR and SHR.Smt conplastic strain were not significantly
different from each other (Fig. 7, \( P > 0.55 \)). In addition, we
quantitated several protein subunits involved in mitochondrial
respiration. When compared with levels in the S rats, succinate
dehydrogenase complex, subunit A (Sdha), and ATP synthase
\( \alpha \)-subunit gene (Atp-5a) were higher and mitochondrial cyto-
chrome c oxidase 1 was lower in the S.SHR mt strain (Fig. 8).
When compared with those of the SHR, none of the proteins were
altered in the SHR.Smt rats (Fig. 8). Uncropped Western films
were indistinguishable from each other between strains in the
regions cropped in Fig. 8.

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Fig. 4. Assessment of mitochondrial oxidant generation. Values are expressed
dichlorofluorescein (DCF) fluorescence per minute per milligram of mito-
chondrial protein. Values are means \( \pm SE \); \( n = 4 \)/group. Bars labeled with the
same letters were not significantly different from each other. Bars labeled with
different letters were significantly different from each other, \( P < 0.05 \).

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Fig. 5. Monitoring basal absorbance and mitochondrial swelling. Mitochondria (200 \( \mu \)g) isolated from hearts of experimental rats were incubated in the presence
or absence of \( Ca^{2+} \) (15 mmol/mg protein) in the assay buffer as described under MATERIALS AND METHODS. A: bars represent the difference in rate of fall in
absorbance/minute observed between the mitochondrial suspension with and without exogenously added \( Ca^{2+} \). Values are means \( \pm SE \); \( n = 4 \)/group. Bars
labeled with the same letters were not significantly different from each other. Bars labeled with different letters were significantly different from each other, \( P <
0.05 \). B: tracing of mitochondrial swelling monitored by fall in optical absorbance at 54 nm over time (\( n = 4 \) rats/group).
Protein content of other nuclear-encoded master regulators of mitochondrial biogenesis. To ascertain that the observed differences between the conplastic and progenitor strains were not due to major changes in the protein content of known regulators of mitochondrial biogenesis, we compared the levels of Ppar-γ and its transcriptional coactivator Pgc-1α. Levels of Pgc-1α were significantly different between S and SHR (P < 0.021), but these differences were not transferred by reciprocal exchange of mitochondrial genomes between S and SHR (Fig. 9). Levels of Ppar-γ were not significantly different between any of the strain comparisons (Fig. 9). Uncropped Western films were indistinguishable from each other between strains in the regions cropped in Fig. 9.

DISCUSSION

Although studies on mitochondrial function in health and disease have provided evidence to associate variants of the mitochondrial genome with pathological states, demonstrating cause and effect relationships that go beyond mere associations is made feasible by characterizing conplastic strains (15, 34, 38, 48, 49), which are derived through custom breeding strategies to swap maternally inherited mitochondrial genomes between strains. In the present studies, we found that conplastic rat strains of S and SHR, with virtually identical nuclear genomes but with divergent mitochondrial genomes, demonstrated alterations in cardiac mitochondrial copy numbers and ROS production that were associated with significant differences in ARC and longevity, but not BP.

The S and SHR mitochondrial genomes differ by 106 variations (24). Because the mitochondrial genome is inherited as a single unit, our study points to all of the 106 variants of the mitochondrial genomes of S and SHR, either individually or collectively as haplotypes, as candidate genetic determinants for aerobic capacity and longevity. Out of these 106 total variants, 25 variations are within genes coding for ribosomal or transfer RNA, four of which are within the functionally important D-loop region of mtDNA, with two of them located in the mitochondrial transcription factor-1 binding site (13). Because significant differences in copy numbers were observed between the inbred S and S.SHRmt conplastic strains, it is possible that the variants within the SHR mitochondrial genome transcribing the D-loop region may influence binding of mitochondrial transcription factor 1 and account for increased mitochondrial copy numbers in S.SHRmt. The lack of difference in copy numbers between SHR and SHR.Smt indicates that the nuclear genomic effects of SHR could override the influence of S rat mitochondrial genomic effects on copy numbers.

Besides the variations within the genes coding for various forms of RNA, 12 nonsynonymous variants exist within Nd2, Cox2, Atp6, Nd4, Nd6, and Cytb. Out of these, nine variants are within the genes coding for the complex I protein subunits of the electron transport chain, Nd2, Nd4, and Nd6 genes. Since there was no difference in complex I activity between S and S.SHRmt, it is reasonable to conclude that without affecting the efficiency of the respiratory chain complex, the substitutions occurring as a result of these variants may contribute to the observed reduced ROS production in the S.SHRmt conplastic compared with S by
lowering the extent of electron leakage into the mitochondrial matrix. Complex II activity was, however, lower in the reciprocal SHR.Smt conplastic strain compared with S rats, but there were no statistically significant differences in ARC, BP, or longevity between these strains. This suggests that lower complex II activity of the S mitochondrial genome was, per se, insufficient to alter the extent of ARC, BP, and longevity of the SHR. These data point to a substantial nuclear genomic effect of the SHR that may not be permissive to accommodate the phenotypic alterations, if any, imparted by the lower complex II activity observed in the SHR.Smt compared with S rat.

In rat models that were selectively bred for divergent ARC, a clear dichotomy in emergence of risk for cardiovascular and metabolic diseases was previously observed (47). Recently, with the use of these rat models, intrinsic aerobic capacity was additionally demonstrated to set the divide for aging and longevity (20). Whether the mitochondrial genomes contributed to this divide is an important question that requires proof from conplastic strains. Our study, although with different inbred strains, provides this required evidence to suggest that variants of the mitochondrial genome, independent of the nuclear genome, contribute to both ARC and longevity but
may not contribute significantly to cardiovascular risk by way of altering BP. Although our results strongly suggest that ARC is directly related to mitochondrial copy numbers and inversely related to mitochondrial ROS production, the focus of our work was limited to the heart, primarily because of our motivation to assess the function of an organ related to BP control. Given this limitation, our data lend support to the view that the genetic determinants of the emergence of risk for cardiovascular diseases may largely reside within the nuclear genome, whereas genetic determinants of aerobic capacity and longevity are shared between both nuclear and mitochondrial genomes. The conplastic strains developed in this study will serve as important tools to further dissect the potential nuclear and mitochondrial genomic interactions that govern both the genetic and epigenetic control of aerobic capacity and longevity.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


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CONPLASTIC STRAINS AND CARDIAC MITOCHONDRIA


