CALL FOR PAPERS | Mitochondria in Cardiovascular Physiology and Disease

Enhanced resistance to permeability transition in interfibrillar cardiac mitochondria in dogs: effects of aging and long-term aldosterone infusion

Girma Asemu,1 Kelly A. O’Connell,1 James W. Cox,1 Erinne R. Dabkowski,1 Wenhong Xu,1 Rogerio F. Ribeiro, Jr,1 Kadambari C. Shekar,1 Peter A. Hecker,1 Sharad Rastogi,2 Hani N. Sabbah,2 Charles L. Hoppel,3 and William C. Stanley1

1Division of Cardiology, Department of Medicine, University of Maryland, Baltimore, Maryland; 2Department of Medicine, Division of Cardiovascular Medicine, Henry Ford Hospital, Detroit, Michigan; and 3Departments of Pharmacology and Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio

Submitted 11 September 2012; accepted in final form 27 November 2012

Asemu G, O’Connell KA, Cox JW, Dabkowski ER, Xu W, Ribeiro RF Jr, Shekar KC, Hecker PA, Rastogi S, Sabbah HN, Hoppel CL, Stanley WC. Enhanced resistance to permeability transition in interfibrillar cardiac mitochondria in dogs: effects of aging and long-term aldosterone infusion. Am J Physiol Heart Circ Physiol 304: H514–H528, 2013. First published December 15, 2012; doi:10.1152/ajpheart.00674.2012.—Functional differences between subsarcolemmal and interfibrillar cardiac mitochondria (SSM and IFM) have been observed with aging and pathological conditions in rodents. Results are contradictory, and there is little information from large animal models. We assessed the respiratory function and resistance to mitochondrial permeability transition (MPT) in SSM and IFM from healthy young (1 yr) and old (8 yr) female beagles and in old beagles with hypertension and left ventricular (LV) wall thickening induced by 16 wk of aldosterone infusion. MPT was assessed in SSM and IFM by Ca2+ retention and swelling. Healthy young and old beagles had similar mitochondrial structure, respiratory function, and Ca2+-induced MPT within SSM and IFM subpopulations. On the other hand, oxidative capacity and resistance to Ca2+-induced MPT were significantly greater in IFM compared with SSM in all groups. Old beagles treated with aldosterone had greater LV wall thickness and worse diastolic filling but normal LV chamber volume and systolic function. Treatment with aldosterone did not alter mitochondrial respiratory function but accelerated Ca2+-induced MPT in SSM, but not IFM, compared with healthy old and young beagles. In conclusion, in a large animal model, oxidative capacity and resistance to MPT were greater in IFM than in SSM. Furthermore, aldosterone infusion increased susceptibility to MPT in SSM, but not IFM. Together this suggests that SSM are less resilient to acute stress than IFM in the healthy heart and are more susceptible to the development of pathology with chronic stress.

Address for reprint requests and other correspondence: W. C. Stanley, Discipline of Physiology, Univ. of Sydney, Anderson Stuart Bldg. (F13), Sydney, NSW 2006, Australia (e-mail: wstanley@usyd.edu.au).

Heart muscle mitochondria are divided into two spatially distinct subpopulations: subsarcolemmal mitochondria (SSM) located in the outer region of the cell and interfibrillar mitochondria (IFM) found between the myofibrils. Studies in rat myocardium found that IFM have a ~40% greater maximal rate of respiration per milligram mitochondrial protein than SSM in normal rats (33, 34) or rats with infarct-induced heart failure (30, 38). Furthermore, studies in rat heart found that IFM are more resistant to stress-induced MPT than SSM, as reflected in greater Ca2+ retention capacity and Ca2+-induced release of cytochrome c (15, 20, 35). This is not a consistent finding, as we found similar Ca2+ retention capacities in IFM and SSM in mitochondria from healthy hamsters and in normal rats or rats with infarct-induced heart failure (8, 9, 30). In contrast to those of rats and healthy hamsters, IFM from cardiomyopathic hamsters have lower respiration rates and a greater susceptibility to Ca2+-induced MPT than SSM (8, 16). Furthermore, recent studies in diabetic mice suggest that IFM have greater proteomic alterations and pathology than SSM (2, 7, 56). On the other hand, aging in rats has a greater impact on IFM than SSM, as seen in a decline in the ability of IFM to resist Ca2+-induced MPT, whereas this parameter does not change with age in SSM (15). There is little information regarding MPT in either young or old large animals. Young dogs with advanced heart failure with contractile dysfunction and left ventricular (LV) chamber enlargement have a greater susceptibility to MPT in permeabilized cardiomyocytes (50), but the effects of early stage heart failure associated with LV hypertrophy (LVH) is not known. Furthermore, differ-
ences between IFM and SSM resistance to MPT with age and heart failure have not been assessed.

The goals of the present investigation were to assess Ca\(^{2+}\)-induced MPT in IFM and SSM in healthy young and old dogs (1 and 8 yr old), and in old dogs with hypertension and LV wall thickening induced by 16 wk of aldosterone infusion. This resulted in LV wall thickening and impaired diastolic filling but did not increase end-diastolic volume or pressure or impair contractile function. We hypothesized that IFM would have a greater resistance to Ca\(^{2+}\)-induced MPT than SSM in young animals and that IFM would have progressively increased susceptibility to MPT with age and with aldosterone infusion in older dogs. We studied females because they comprise ~60% of heart failure patients with preserved ejection fraction (4, 31). The size and structure of cardiac mitochondria change with advanced heart failure (44, 51); thus we assessed mitochondrial size and complexity in IFM and SSM using flow cytometry (6, 7, 36). In addition, we isolated mitochondria from the right ventricle (RV) and LV, as there may be different responses to aging and aldosterone infusion between the two chambers.

**METHODS**

**Experimental Design**

All procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23) and were approved by the University of Maryland Institutional Animal Care and Use Committee. Three groups of female beagle dogs were studied: young untreated dogs (1 yr old; n = 8), old untreated dogs (8-yr-old retired breeders; n = 8), and old dogs treated with an infusion of aldosterone for 16 wk to induce hypertension (n = 7). All dogs were purchased from a commercial vendor (Covance Research Products, Cumberland, VA) and were ovariectomized by the vendor 2 to 6 wk before delivery. All of the terminal procedures and mitochondrial isolations were completed over a 5-wk period and performed by the same personnel. The personnel performing the mitochondria isolation and measurements were blinded to the group assignment.

Dogs were housed two per pen and provided with food (Pedigree, Harlan, Frederick, MD) and water ad libitum. Following acclimatization to our facilities for 3 to 5 days, arterial blood pressure was measured in the right forelimb 7 to 2 days before the terminal procedure. In the aldosterone-treated dogs, blood was drawn before initiation of treatment and at 8 and 15 wk of infusion. All blood samples were taken between 11:00 and 14:00 in the nonfasted state.

**Noninvasive Blood Pressure Monitoring**

Arterial blood pressure was measured in conscious dogs using an oscillometric automated blood pressure cuff (PetMAP) on the tail. The coccgeal artery was occluded by placing the cuff 1 cm distal to the base of the tail with arrow positioned along the ventral midline with the dog laying on its side. The values obtained for systolic and diastolic pressure and heart rate were taken from the average of five consecutive recordings, and mean arterial pressure was calculated as diastolic pressure + (pulse pressure/3).

**Echocardiography**

LV function was evaluated by echocardiography (MyLab 30CV, Esaote North America, Indianapolis, IN) using a 3.5–1.6-MHz probe. The echocardiographic examination was performed 7 to 3 days before the terminal study in all dogs. For the old dogs treated with aldosterone, measurements were also made 7 to 2 days before the pump instrumentation and at 15 wk of aldosterone infusion. The exam was performed with the dog laying on the table in a right lateral decubitus position. The area was clipped free of hair, and two-dimensional and M-mode echocardiography was performed with the probe on the left side. Images were obtained from a left parasternal approach at the mid-papillary muscle level and mitral valve (46). No anesthesia or physical restraint was used. M-mode frames were recorded from the parasternal short axis, and pulsed-wave Doppler measurements were recorded from the apical view. Anterior and posterior LV wall thicknesses were obtained at end-diastole, and absolute wall thickness was calculated as the sum of anterior and posterior wall thicknesses, and relative wall thickness as absolute wall thickness/end diastolic LV diameter. End-systolic and -diastolic volumes (ESV and EDV) were calculated as the LV diameter\(^2\) × 1.047 (46). Ejection fraction was calculated as (EDV - ESV)/EDV × 100. Transmitral Doppler index peak rapid filling velocity (E) and peak atrial filling velocity (A) were measured, and the E-to-A (E/A) ratio was calculated.

Venous blood was sampled in conscious dogs from a superficial forelimb 7 to 2 days before the terminal procedure. In the aldosterone-treated dogs, blood was drawn before initiation of treatment and at 8 and 15 wk of infusion. All blood samples were taken between 11:00 and 14:00 in the nonfasted state.

**Infusion Pump Implantation**

Aldosterone was continuously infused with a battery-powered programmable infusion pump (iPRECIO model MK02-V2, Data Sciences International, St. Paul, MN) that delivered aldosterone into the jugular vein. \(\Delta\)-Aldosterone (Sigma-Aldrich) was infused into the jugular vein at a dose of 30 µg·kg\(^{-1}\)·day\(^{-1}\) in a solution of 15% ethanol, 50% DMSO, and 35% water at a concentration of 10 mg aldosterone/ml. Eight dogs initiated treatment with aldosterone, but one dog was discontinued because of infection at the site of pump implantation; thus data are reported for seven animals. The young and old control animals were not instrumented. The dose of aldosterone has been chosen based on our preliminary data showing that it increased blood pressure. Pump implantation was performed under general anesthesia [propofol (4 to 6 mg/kg) plus isofluran (1.5–3.0%) to effect] with local infiltration with bupivacaine (~2 ml of 0.25%). The right external jugular vein was exposed, and the pump cannula was inserted and advanced 3–5 cm proximally. The vein was ligated distal to the point of insertion, and the catheter was secured to the vessel with suture ties. A subcutaneous pocket for the pump was created lateral to the incision and the pump sutured in place and the incision closed. Dogs were infused at ~30 µl/day, with the exact rate adjusted to the body mass of the dog at the time of implantation to deliver 30 µg·kg\(^{-1}\)·day\(^{-1}\). The pump reservoir was 900 µl and was refilled percutaneously every 20–30 days through an injection port on the pump. The pump reservoir was evacuated before refilling to ensure the pump had properly discharged its contents and was then refilled using a 26-gauge needle. This procedure was done in conscious animals with no evidence of discomfort. After 16 wk, these dogs underwent a terminal procedure that was identical to the young and old animals, as detailed below.

**Terminal Procedure to Assess Cardiac Function**

The terminal procedure was performed to assess LV pressure and harvest the heart for mitochondrial studies. General anesthesia was induced as described above, and a 5-Fr manometer-tip catheter (Millar Instruments, Houston, TX) was inserted into the left carotid artery and advanced into the LV. Baseline LV pressure was recorded, anesthesia was increased to 5% for 1 min, and a left side thoracotomy was rapidly performed. The animal was euthanized by severing the superior vena cava, and the heart was rapidly excised to obtain myocardial tissue samples. The total time from incision to removal of myocardial sample was <90 s. Myocardium for mitochondrial isolation was taken from the anterior free wall of the LV and RV (3 and 1.5 g, respectively) and placed in ice-cold buffer. Tissue from the lateral LV free wall was fixed in embedding medium (Tissue-Tek OCT Compound, Sakura) for subsequent histological analysis and frozen at −80°C.
The residual LV and RV tissue was carefully dissected and weighed for assessment of LV and RV mass.

Mitochondria Isolation and Measurements

Cardiac SSM and IFM were isolated using a protocol modified from Palmer et al. (33) and Rosca et al. (43). Briefly, an ~3-g transmural section of LV and 1.5-g section of RV anterior free wall tissue were homogenized in a solution containing 100 mM KCl, 50 mM MOPS, 5.0 mM MgSO4, 1.0 mM EGTA, 1.0 mM ATP, and 2 mg/ml BSA (pH 7.4) without addition of collagenase, in contrast to our previous study in dogs where the minced muscle was treated with collagenase (43). IFM were released by treating the resuspended pellet with trypsin (5 mg/ml wet mass) in a similar solution as described above but without BSA, followed by mechanical homogenization (20, 43). Respiration was measured using glutamate + malate (20 and 10 mM), pyruvate + malate (20 and 10 mM), palmitoylcarnitine (40 μM), and succinate (20 mM + 7.5 μM rotenone) as substrates. State 4 was measured before and after addition of oligomycin (5 μg/ml), and the respiratory control ratio (RCR) was calculated as state 3/state 4 without oligomycin.

Ca2⁺-induced MPT was evaluated in SSM and IFM from LV myocardium using two previously described methods (20–22). First, mitochondrial swelling was evaluated from the change in absorbance at 540 nm using a 96-well plate reader. Plates were read at 37°C, and 250 μg/ml mitochondrial protein was added to each well in a calcium-free buffer consisting of 100 mM KCl, 10 mM MOPS, 1 mM EGTA, and 2 mg/ml BSA (pH 7.4) with glutamate + malate as the substrate (5 and 2.55 mM, respectively) and read for 20 min with readings taken every 7 s. Second, the capacity for Ca2⁺ uptake was evaluated in isolated mitochondria by following the extramitochondrial [Ca2⁺] with a progressive exposure to Ca2⁺. Briefly, using a 96-well fluorometric plate reader at 37°C, 250 μg/ml of mitochondrial protein was suspended in the same calcium-free buffer as previously mentioned with glutamate + malate as the substrate (5 and 2.55 mM). A bolus injection of 20 nmol free Ca2⁺ was injected every 3 min, and extramitochondrial [Ca2⁺] was recorded every 2 s using 750 nM Calcium Green-5N.

Mitochondrial size and membrane potential were measured as previously described (6, 36). Briefly, isolated SSM and IFM were stained with MitoTracker Green FM (Molecular Probes) and assessed using a flow cytometer (FACScan, BD Biosciences). The arithmetic mean output from the forward scatter detector was used as an index of mitochondrial size. For membrane potential, mitochondria were incubated with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carboxyanine iodide (JC-1) (Molecular Probes, Carlsbad, CA) at a final concentration of 0.3 μM. The shift to orange is due to dye aggregates forming upon polarization, causing shifts in emitted light from 530 nm (green) to 590 nm (orange).

Hydrogen peroxide production in isolated LV mitochondrial subpopulations was determined using the oxidation of the fluorogenic indicator amplex red (Invitrogen) in the presence of horseradish peroxidase. The concentrations of horseradish peroxidase and amplex red in the incubation were 0.1 unit/ml and 50 μM, respectively, and detection of fluorescence was assessed on a Molecular Devices Flex Station 3 fluorescence plate reader (Molecular Devices, Sunnyvale, CA) with 530-nm excitation and 590-nm emission wavelengths. Standard curves were obtained by adding known amounts of H2O2 to the assay medium in the presence of the substrates amplex red and horseradish peroxidase. H2O2 production was initiated in mitochondria using glutamate + malate and succinate + rotenone as substrates.

The activity of the citric acid cycle enzyme citrate synthase was measured in myocardial homogenates from frozen LV and RV samples at 37°C using a previously described spectrophotometric method (53). Histological analysis of LV samples for extracellular fibrosis, myocyte cross-sectional area, and capillary density were assessed as previously described (45).

Statistical Analysis

Values are shown as means ± SE. SSM and IFM values were compared using a two-way repeated-measures ANOVA with a Bonferroni post hoc test. A one-way ANOVA was used for single parameters taken at the terminal time point. The time course for tail-cuff blood pressure in the aldosterone-treated dogs was compared with baseline using a repeated-measures one-way ANOVA. Echocardiographic data from baseline were compared with 15-wk values with a paired t-test. A P value of <0.05 was considered significant. Statistical comparisons were not made between the LV and RV.

RESULTS

Effects of Aldosterone Infusion

Mean arterial pressure significantly increased compared with baseline, with a peak increase observed between 4–8 wk of infusion of aldosterone, followed by a gradual decline back to near baseline values by 15 wk (Fig. 1). No difference was observed in baseline serum aldosterone concentrations among the three groups (290 ± 21, 235 ± 28, and 272 ± 37 pg/ml for the Young, Old, and Old + Aldo groups, respectively). Serum aldosterone concentration significantly increased at 8 wk of aldosterone infusion to 423 ± 54 pg/ml (P < 0.001 vs. baseline values) but decreased to 79 ± 21 pg/ml at 16 wk infusion

<table>
<thead>
<tr>
<th>Table 1. Body and heart mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
</tr>
<tr>
<td>Age at termination, yr</td>
</tr>
<tr>
<td>Initial body mass, kg</td>
</tr>
<tr>
<td>Terminal body mass, kg</td>
</tr>
<tr>
<td>LV mass, g</td>
</tr>
<tr>
<td>RV mass, g</td>
</tr>
<tr>
<td>LV mass + RV mass, g</td>
</tr>
<tr>
<td>LV mass/terminal body mass, g/kg</td>
</tr>
<tr>
<td>RV mass/body mass</td>
</tr>
</tbody>
</table>

Values are means ± SE. LV, left ventricular; RV, right ventricular. *P < 0.0001 compared with Young; †P < 0.005 compared with initial body mass within Old + aldosterone (Aldo) group by paired t-test.
This surprising finding suggests that chronic aldosterone infusion causes adaptations that increase the clearance of aldosterone from the circulation.

Body mass was similar among the three groups (Table 1). Body mass in the Old + Aldo group increased by 1.6 ± 0.4 kg from baseline to 16 wk of aldosterone infusion (P < 0.005) (Table 1). LV, RV, and LV + RV mass were not significantly
different among groups, though there was a trend for a greater LV mass in the Old + Aldo.

Tail-cuff measurements acquired in conscious unrestrained dogs 3 to 7 days before the terminal study show similar heart rate and blood pressure among groups (Table 2). Blood pressure in the untreated old and young dogs (Table 2) was not different from measurements taken at the same time point (4 to 10 days after arrival in our facility) in the group of old dogs that subsequently underwent infusion pump implantation and aldosterone infusion (systolic pressure 167 ± 5 mmHg, diastolic pressure 88 ± 5 mmHg, mean pressure 114 ± 4 mmHg, and a heart rate of 113 ± 9 beats/min). Invasive assessment of LV pressure in anesthetized animals showed no difference in heart rate, LV Peak systolic pressure, and maximum and minimum first derivative of LV pressure. LV end diastolic pressure was significantly higher in the young dogs than in both the Old and Old + Aldo groups, though all dogs were in the normal healthy range (Table 2). Echocardiographic measurements showed no difference in LV chamber size and ejection fraction among groups, but increased LV wall thickness after 15 wk of aldosterone infusion (Fig. 2 and Table 2). Diastolic dysfunction was detected via Doppler echocardiography as seen in a decrease in the peak E/A ratio in Old + Aldo dogs, however, LV end diastolic pressure was normal (Fig. 2 and Table 2). Histological assessment of LV myocardium found no increase in extracellular fibrosis or myocyte cross-sectional area with aging or infusion of aldosterone (Table 3). Furthermore, capillary density was similar among the three

<table>
<thead>
<tr>
<th>Young</th>
<th>Old</th>
<th>Old + Aldo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate synthase activity, μmol·g⁻¹·min⁻¹</td>
<td>135 ± 10</td>
<td>98 ± 8*</td>
</tr>
<tr>
<td>LV</td>
<td>105 ± 6</td>
<td>92 ± 9</td>
</tr>
<tr>
<td>RV</td>
<td>529 ± 17</td>
<td>529 ± 16</td>
</tr>
<tr>
<td>Histological analysis of LV myocardium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocyte cross-sectional area, μm²</td>
<td>9.4 ± 0.5</td>
<td>9.4 ± 0.7</td>
</tr>
<tr>
<td>Capillary density, capillaries/mm²</td>
<td>2,410 ± 132</td>
<td>2,067 ± 94</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 compared with Young.
groups. Taken together, whereas there was echocardiographic evidence for a modest decline in LV diastolic filling and wall thickening, there was no evidence for frank diastolic dysfunction or heart failure in the Old + Aldo group.

Mitochondrial Parameters

Mitochondrial yield and size. The yields for SSM and IFM in young dogs was similar to values previously reported for LV myocardium from normal female mongrel dogs ~1.5 yr of age (43). Total LV mitochondrial yield was significantly decreased in Old + Aldo group compared with Old and Young groups (Fig. 3). Mitochondrial IFM yield was also lower in the Old + Aldo group compared with the Old group, and there was a significant decrease in mitochondrial SSM yield in Old + Aldo compared with the Young group (Fig. 3). In the RV, total mitochondrial yield was not significantly different among groups, except for a decrease in IFM yield in the Old + Aldo group compared with the Old group (Fig. 4). Myocardial activity of the Krebs cycle enzyme citrate synthase showed a significant decrease in activity in old dogs compared with young dogs with no effect of aldosterone treatment, whereas in the RV there were no differences among groups (Table 3).

Mitochondrial morphology was assessed by flow cytometry and showed a main effect for larger mitochondrial size in SSM than IFM in both the LV and RV (Figs. 3 and 4). In the LV, SSM were larger that IFM in the Old and Old + Aldo groups but not in the Young group (Fig. 3). The Old + Aldo group had larger SSM compared with the Young and Old groups, suggesting that aldosterone infusion caused selective mitochondrial enlargement in this subpopulation (Figs. 3 and 4). Mitochondrial internal complexity was greater in SSM than IFM and was significantly increased in SSM in the Old + Aldo group compared with the Old and Young groups in both the LV and RV. Mitochondrial membrane potential was lower in IFM than SSM in the Old and Old + Aldo groups in both the LV and RV and was significantly increased in SSM in the Old + Aldo dogs compared with the Young groups (Figs. 3 and 4).

Mitochondrial respiration. State-3 respiration with glutamate + malate, pyruvate + malate, palmitoylcarnitine, and succinate + rotenone was not affected either by age or treatment in IFM or SSM in the LV or RV (Figs. 5 and 6). There was a significantly higher state-3 respiration rate in IFM than SSM with all substrates in the LV (Fig. 5). A similar effect was observed in the RV for glutamate + malate and palmitoylcarnitine, but not for pyruvate + malate or succinate + rotenone (Fig. 6).

The RCR was not different between SSM and IFM, except for a small but significantly greater RCR for IFM with succinate + rotenone as a main effect (Fig. 5, right). The RCR was lower in the Old + Aldo group compared with the Old group with pyruvate + malate and succinate + rotenone and was strongly trending lower with palmitoylcarnitine (P < 0.051), suggesting that aldosterone treatment lowered mitochondrial responsiveness to ADP-stimulated of respiration (Fig. 5).

Fig. 4. Right ventricular (RV) mitochondrial yield and flow cytometry results. *P < 0.05, IFM compared with SSM; †P < 0.05 compared with Old within given mitochondrial subpopulation; #P < 0.05 compared with Young within given mitochondrial subpopulation.
LV state-4 respiration measured in the absence of oligomycin was higher in IFM than SSM with all substrates and was not different among the three groups with pyruvate + malate, palmitoylcarnitine, and succinate + rotenone as substrates (Fig. 7, left). With glutamate + malate as substrate, there was a higher state-3 rate in Young and Old + Aldo than in the Old group in IFM, but no differences among groups in SSM. State 4 was also measured with the addition of oligomycin to block ATP production by complex V and to thus provide a measure of proton leak across the inner mitochondrial membrane. This resulted in lower rates of respiration (Fig. 7, right) with persistently higher state-4 rates in IFM than SSM, but no difference among groups. In the RV, state 4 measured in the absence of oligomycin was higher in IFM than SSM with all substrates except pyruvate + malate (Fig. 8), and the Old + Aldo group had a higher state-4 rate than the Young and Old groups. State 4 was lowered by the addition of oligomycin, with higher rates in IFM with glutamate + malate and palmitoylcarnitine as substrates (Fig. 8 right). There were no differences among groups with the exception of higher rates in IFM with glutamate + malate in Old + Aldo in the IFM group.

Permeability transition. Two established methods were used to assess Ca\(^{2+}\)-induced MPT in LV mitochondria. First, MPT was assessed from mitochondrial swelling induced by high [Ca\(^{2+}\)] as reflected by the decrease in absorbance at 540 nm following the addition of a bolus of Ca\(^{2+}\) to isolated mitochondria. Measurement of initial baseline absorbance before the addition of Ca\(^{2+}\) showed greater absorbance in IFM than SSM.

---

Fig. 5. LV mitochondrial state-3 respiration in SSM (white bars) and IFM (black bars) (left) and the respiratory control ratio (state 3/state 4 without oligomycin) (right). *P < 0.05 IFM compared with SSM.
in all groups and higher absorbance in the Old + Aldo group in both SSM and IFM compared with the young and old groups (Fig. 9). There was a decrease in absorbance with addition of either 0.5 or 1.0 μmol Ca²⁺/mg protein in all groups, with a greater decline in IFM than SSM, and no differences among the three groups with SSM or IFM (Fig. 10).

Ca²⁺-induced MPT was also assessed from the measurement of the ability of isolated mitochondria to take up added Ca²⁺. IFM had significantly enhanced Ca²⁺ retention capacity compared with SSM, as reflected by significantly lower extramitochondrial [Ca²⁺] for a given cumulative Ca²⁺ load (Fig. 11). There was a significantly greater sensitivity to Ca²⁺-induced MPT in SSM from the Old + Aldo group compared with the Old and Young groups, as reflected by a higher extramitochondrial [Ca²⁺] for a given cumulative Ca²⁺ load (Fig. 11). There were no significant differences in this relationship in IFM among the three groups (Fig. 12). These results indicate that IFM were more resistant to Ca²⁺-induced MPT than SSM and that aldosterone infusion caused a significant increase in susceptibility to MPT only in the SSM.

Hydrogen peroxide production. The capacity for mitochondrial generation of H₂O₂ was assessed in SSM and IFM from LV myocardium using the amplex red assay. There were no differences among groups within mitochondrial subpopulations with either glutamate + malate or succinate in the presence of the rotenone to inhibit complex I (Table 4). The
maximal rate measured with succinate plus rotenone showed no difference between IFM and SSM. With glutamate + malate as substrates, the rate was lower in IFM than SSM as a main effect and within the Young and Old groups (Table 4).

DISCUSSION

The present investigation used a large animal model to assess the function and structure of the spatially distinct sub-populations of cardiac mitochondria under normal conditions in young and old females and with aldosterone infusion that induced transient hypertension, modest LV wall thickening, and impaired LV filling. There are three main findings from this study. First, we found that respiratory capacity and resistance to Ca$^{2+}$-induced MPT were enhanced in IFM compared with SSM in LV myocardium in both young and old dogs (15, 35). Second, we observed that aldosterone infusion in old dogs reduced the yield and increased the size of SSM, but not IFM. Third, aldosterone infusion decreased the capacity for Ca$^{2+}$ uptake in SSM but had no effect on IFM. Thus, in the healthy female heart, mitochondria located in the outer region of the cell are more susceptible to permeability transition and have a lower capacity for ATP generation. Furthermore, in response to aldosterone infusion, SSM became enlarged and less resistant to stress, whereas the mitochondria found among the myofibrils appear unchanged.

We found that the rate of oxidative phosphorylation with pyruvate + malate, palmitoylcarnitine and succinate + rotenone was ~30–50% greater in IFM than SSM in a large
animal. This finding was evident in both young and old dogs and with aldosterone infusion and in both RV and LV myocardium. In a previous study in young male mongrel dogs, we did not observe any differences in state-3 or state-4 respiration between IFM and SSM in healthy animals or in dogs with chronic heart failure due to irreversible injury caused by multiple microinfarctions (43). This suggests that enhanced respiration in IFM from the canine heart may be unique to females. Sex differences between IFM and SSM have not been reported; however, a previous study that examined only SSM found that both young (4 mo) or mature (32 mo) female rabbits had a significantly higher state-3 rate with either complex I or II substrates compared with males. On the other hand, SSM from adult mice showed no sex differences in state 3 or 4 with pyruvate + malate as substrate (47). Thus, at present, it is unclear whether the greater respiratory capacity in IFM than SSM that we observed in female dogs is present in males.

The greater resistance to stress-induced permeability transition in IFM compared with SSM is consistent with previous results from young and old healthy rats (15, 20, 35). Hoppel et al. (35) showed that isolated IFM from rat heart had a 50% greater Ca2+ uptake capacity than SSM. Furthermore, SSM released cytochrome c and matrix enzymes in response to added Ca2+, whereas IFM were relatively unaffected. On the other hand, we previously observed similar Ca2+ retention capacities in IFM and SSM in normal rats or rats with infarct-induced heart failure and in healthy hamsters (8, 30). Hofer et al. (15) found that IFM from senescent rats are more suscep-
tible to Ca\textsuperscript{2+}-induced MPT, whereas this parameter was unaffected by age in SSM. We did not observe any effect of age in indexes of Ca\textsuperscript{2+}-induced MPT in the present study; however, 8-yr-old beagles should be considered middle aged, as the typical life span of this strain is 13.5 ± 0.2 yr (24). A previous study found that healthy 11 ± 2-yr-old male beagles had a decrease in succinate dehydrogenase activity in myocardial tissue homogenates and a qualitative increased vacuolization of mitochondria by visual analysis of electron micrographs compared with 4 ± 1-yr-old animals; however, mitochondrial respiration and MPT were not assessed (3). Future studies should evaluate these parameters in senescent dogs, where greater mitochondrial dysfunction would be expected.

The effects of LVH and heart failure on MPT in humans or large animal models are not well understood. Young dogs with advanced heart failure due to microembolizations have a greater susceptibility to MPT in permeabilized cardiomyocytes (50), but the effects of early stage heart failure associated with LVH have not been reported in humans or large animals. Recent studies found that atrial mitochondria from middle-aged diabetic patients without heart failure are more sensitive to Ca\textsuperscript{2+}-induced MPT and have greater production of H\textsubscript{2}O\textsubscript{2} than mitochondria from nondiabetic patients (1). We found IFM from cardiomyopathic hamsters had greater susceptibility to Ca\textsuperscript{2+}-induced MPT than normal healthy hamsters, whereas SSM were similar (8). The implications of these findings are not clear, as the role of MPT in normal cardiac physiology and the progression of heart failure are unresolved and remain under extensive investigation.

The underlying mechanisms responsible for the differences in respiration and resistance to MPT between IFM and SSM are not known. High-resolution scanning electron microscopy analysis revealed clear structural differences between the two subpopulations in rat cardiomyocytes, with SSM having more lamelli form and less tubular cristae than IFM (40), which could affect function. Mitochondrial phospholipid composition can clearly impact respiration and MPT (54); however, there are no differences between IFM and SSM in phospholipid composition, cardiolipin molecular species, or phospholipid fatty acid composition in normal or heart failure dogs or rats (20, 30, 41). On the other hand, mitochondrial sphingolipid composition tremendously varies between IFM and SSM in rat heart, particularly for long-chain ceramides (26). While this could impact mitochondrial respiration or susceptibility to permeability transition, the role of these compounds in mitochondrial physiology are not yet clear; thus it is premature to postulate a mechanistic link to the present findings (26). It is also possible that differences in key proteins involved in MPT pore structure or regulation differ between subpopulations; however, there was no difference in protein levels for the voltage-dependent anion channel, cyclophilin D or the adenine nucleotide translocator between SSM and IFM in young or old rat hearts (15). Clearly, additional work is needed to elucidate...
the underlying reasons for the distinctions between the two subpopulations.

A novel finding of the present investigation is that IFM from the RV exhibited enhanced respiratory capacity relative to SSM, consistent with current and previous findings from LV mitochondria (30, 33, 34, 38). While the functional and metabolic differences between the two chambers have been described (23, 48, 49), little is known about differences in mitochondrial structure and function. Direct comparisons of the LV and RV in healthy young male rats found a 20% greater mitochondrial volume density in the RV as assessed by electron microscopy, but no difference in mitochondrial respiratory capacity in permeabilized cardiomyocyte bundles or in the activity of complexes I–IV or citrate synthase in myocardial homogenates (29). In contrast, mitochondrial volume density was not different between LV and RV myocardium in healthy young pigs (52). Here we show that mitochondrial yield and functional differences between SSM and IFM were qualitatively similar in the LV and RV with responses to aging and aldosterone infusion (Figs. 3–8). The present investigation was not designed to statistically compare the RV with the LV, as this would require a three-way ANOVA (chamber/mitochondrial population/age/treatment group) and thus a much larger sampled size to achieve acceptable statistical power. We did not assess Ca2+–induced MPT in RV mitochondria, which could have been differentially effected by aldosterone compared with LV mitochondria. It has recently been proposed that mitochondrial dysfunction in the RV plays a key role in the pathological adaptations to pulmonary hypertension and that

![Graph](https://via.placeholder.com/150)

**Fig. 11.** Comparison between IFM and SSM from the LV for each group of dogs for extramitochondrial Ca2+ concentration plotted as a function of the cumulative Ca2+ load. Mito Prot, mitochondrial protein. *P < 0.05, IFM compared with SSM.

![Graph](https://via.placeholder.com/150)

**Fig. 12.** Extramitochondrial Ca2+ concentration plotted as a function of cumulative Ca2+ load for SSM (top) and IFM (bottom) from the LV. *P < 0.05 compared with Young; †P < 0.05 compared with Old.

The underlying reasons for the distinctions between the two subpopulations.

A novel finding of the present investigation is that IFM from the RV exhibited enhanced respiratory capacity relative to SSM, consistent with current and previous findings from LV mitochondria (30, 33, 34, 38). While the functional and metabolic differences between the two chambers have been described (23, 48, 49), little is known about differences in mitochondrial structure and function. Direct comparisons of the LV and RV in healthy young male rats found a 20% greater mitochondrial volume density in the RV as assessed by electron microscopy, but no difference in mitochondrial respiratory capacity in permeabilized cardiomyocyte bundles or in the activity of complexes I–IV or citrate synthase in myocardial homogenates (29). In contrast, mitochondrial volume density was not different between LV and RV myocardium in healthy young pigs (52). Here we show that mitochondrial yield and functional differences between SSM and IFM were qualitatively similar in the LV and RV with responses to aging and aldosterone infusion (Figs. 3–8). The present investigation was not designed to statistically compare the RV with the LV, as this would require a three-way ANOVA (chamber/mitochondrial population/age/treatment group) and thus a much larger sampled size to achieve acceptable statistical power. We did not assess Ca2+-induced MPT in RV mitochondria, which could have been differentially effected by aldosterone compared with LV mitochondria. It has recently been proposed that mitochondrial dysfunction in the RV plays a key role in the pathological adaptations to pulmonary hypertension and that

| Table 4. LV mitochondrial H2O2 production with succinate + rotenone or glutamate + malate |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | Young           | Old             | Old + Aldo      | Main Effect     |
| Succinate + Rotenone, SSM      | 128 ± 8         | 120 ± 8         | 132 ± 8         | Versus SSM      |
| Succinate + Rotenone, IFM      | 127 ± 10        | 130 ± 8         | 135 ± 15        |                 |
| Glutamate + Malate, SSM       | 62.8 ± 10.5     | 69.6 ± 8.1      | 54.5 ± 9.4      |                 |
| Glutamate + Malate, IFM       | 46.5 ± 13.3     | 37.7 ± 4.7      | 45.9 ± 7.8      | 0.001           |

Values are means ± SE (expressed as nmol-mg mitochondrial protein$^{-1}$-min$^{-1}$). *P < 0.05, interfibrillar cardiac mitochondria (IFM) compared with subsarcolemmal cardiac mitochondria (SSM).
mitochondrial targeted interventions may be therapeutic (37). Additional work focused on interventricular differences in the mitochondrial response to left- and right-side failure is warranted.

In the present investigation, we aimed to develop a new model of pathological LVH in dogs using a continuous infusion of aldosterone with an implanted motorized infusion pump. This general approach has been used in young unilaterally nephrectomized rats to induce hypertrophic heart failure with mitochondrial dysfunction (19) but to our knowledge has not been attempted in dogs. Our goal was to mimic key aspects of heart failure with preserved ejection fraction; thus we used old animals as they are more susceptible to myocardial pathology in response to stress (18, 28). Females were used rather than males because they make up ~55–65% of heart failure patients with preserved ejection fraction (4, 31). Furthermore, dogs were ovarioctomized to eliminate the confounding effects of ovulation and to better reflect conditions in postmenopausal women. We wished to avoid the confounding effects of thoracic or renal surgery required for aortic constriction, nephrectomy, or the Page renal wrap procedure (13, 14, 32); thus we used a controlled intravenous infusion of aldosterone via a pump implanted in the neck. Previous studies showed that aldosterone at 12 to 15 μg·kg⁻¹·day⁻¹ increased mean arterial pressure by ~15 mmHg for up to 10 days in young mongrel dogs (5, 10, 27). We aimed to increase mean pressure by 15 to 20 mmHg, and in dose escalating pilot studies in our model system, we found that this required ~30 μg·kg⁻¹·day⁻¹. While we clearly accomplished the desired increase in serum aldosterone and blood pressure out to 8 wk (Fig. 1), we were surprised to see a fall in both parameters by 15 wk of infusion. In hindsight, a progressive increase in the aldosterone infusion rate would likely have maintained hypertension and resulted in clear pathological LVH. Furthermore, simultaneous infusion of other potent vasoconstrictor compounds with toxic myocardial effects, such as angiotensin II or endothelin, should be considered to further accelerate the development of hypertension, LVH, and heart failure. Further investigation is needed with a more severe and prolonged hormonal stress to elicit advanced heart failure with preserved ejection fraction.

A limitation of our experimental design is the lack of a control group implanted with an infusion pump and treated with vehicle for 16 wk. This group was not included for ethical reasons. Blood pressure transiently increased following initiation of aldosterone infusion (Fig. 1), suggesting that this was due to aldosterone infusion. However, in the absence of a parallel vehicle-treated group, it is not possible to definitively attribute the observed differences in blood pressure and other parameters to aldosterone infusion.

A hallmark of heart failure with preserved ejection fraction is impaired diastolic filling as reflected by delayed mitral inflow (e.g., decrease in the E/A ratio) and elevated LV end-diastolic pressure. In the present investigation, we observed a modest but significant decrease in the E/A ratio but no increase in LV end-diastolic pressure. In contrast, Mathieu et al. (25) observed a similar modest but significant decrease in the E/A ratio (from 1.51 to 1.22), but also an increase in LV end diastolic pressure from 14 to 23 mmHg in beagles 2 mo after myocardial infarction induced by coronary ligation. This suggests that these modest reductions in the E/A ratio should be interpreted with caution, as they clearly do not necessarily correspond with changes LV filling pressure.

In conclusion, we demonstrate in a large animal model that resistance to MPT is greater in IFM than in SSM in young and old female dogs. When old dogs were stressed with aldosterone infusion, there was selective enlargement of SSM and greater susceptibility to MPT, with no change to IFM. Furthermore, we observed a greater capacity for oxidative phosphorylation in IFM than SSM in all three groups. Together, these findings suggest that in a large animal model, there are clear differences between cardiac mitochondrial subpopulations under normal conditions and response to 15 wk of aldosterone infusion. The implications of this finding to more advanced LVH and heart failure require further study.

REFERENCES


mitochondrial effects of aging and hypertrophy


