Mitochondrial Respiration in Mononuclear Cells and Heart Fibers in Spontaneously Hypertensive Rats

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Abstract
Mitochondrial function is altered in organs in hypertension and may be implicated in subclinical organ damage. Therefore measuring mitochondrial function may be clinically useful but, a biopsy of organ/tissue is required to measure mitochondrial function and that is invasive in the clinical setting. The aim of this study was to measure mitochondrial function from easily obtainable circulating mononuclear cells (MC) and the heart tissue of spontaneously hypertensive rats (SHR) at an early hypertensive and a compensated hypertrophic stage. Six and 14 months age SHR and Wistar control rats were divided into four groups and mitochondrial function was measured in MC and permeabilized heart fibers. MC showed decreased respiratory control ratios and decreased electron transport system capacity attributable to Complex I in SHR. These findings suggest that MC were less efficient at producing adenosine triphosphate (ATP) and were less dependent on CI substrates. In contrast, heart tissue from SHR and Wistars showed similar mitochondrial function at both ages. Altered mitochondrial function was present in MC at a stage in the disease when it was not detectable in the heart. This study provides a protocol to study mitochondrial function from MC which may be correlated with subclinical organ damage in patients with hypertension.

Keywords — hypertension, mitochondria, oxidative phosphorylation, electron transport system, subclinical organ damage, spontaneous hypertensive rat, mononuclear cells.

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INTRODUCTION
Hypertension affects 33% of US adults who are ≥ 20 years of age and over 900 million people worldwide, and increases the risk of coronary disease, congestive heart failure, stroke and renal insufficiency. Assessment of hypertensive subclinical organ damage can demonstrate whether or not there are underlying abnormalities in the structural and functional properties of vital organs which can increase the chance of a future adverse clinical event. Currently, electrocardiography, echocardiography, proteinuria or microalbuminuria are used to measure subclinical organ damage in specific organs. While multiple organ damage carries a worse prognosis than single organ involvement, there is no sole measure for multiple organ damage in hypertension.

Tissue mitochondrial function is altered in multiple organs such as heart, kidneys and vascular endothelium in hypertension. For example in the compensated spontaneous hypertensive rat (SHR) heart, adenine triphosphate (ATP) supply and handling was reported to be impaired and in failing heart muscle fibers a 40% depression in mitochondrial respiration with all substrates except for glutamate and malate has been demonstrated. Since mitochondria are affected in multiple organs in hypertension, measuring mitochondrial function may be a prognostic advantage in hypertension. However, measuring mitochondrial function in tissues such as heart, kidneys or blood vessels would currently require tissue biopsies to be obtained from these vital organs. This is clearly an impractical solution since obtaining multiple tissue biopsies from these vital organs is invasive and would pose significant morbidity from the risk of bleeding and infection for the otherwise well patient. Instead, we propose there may be merit in understanding what happens to mitochondrial function in blood mononuclear cells (MC) which can be easily obtained from peripheral blood in hypertensive patients.

Blood MC are known to play an important part in the pathophysiology of hypertension. Mice lacking both B and T cells have blunted hypertensive responses and do not develop abnormalities of vascular function to prolonged angiotensin II infusion or deoxycorticosterone acetate salt challenge. In addition, signs of oxidative stress that occur in hypertensive hearts also occur in circulating MC. These cells show elevated ratios of oxidized to reduced glutathione pools and depressed activities of anti-oxidant enzymes. This collectively points to intricate involvement of MC in the hypertensive state, however there has been no detailed study measuring mitochondrial function in blood MC in hypertension and comparing it to mitochondrial function in a known organ affected by hypertension such as the heart.

The hypothesis for this study is that MC mitochondrial respiration function is altered in the hypertensive state. This study aims to measure blood MC and heart fiber mitochondrial respiratory function in male SHR at an early hypertensive stage
as well as a compensated hypertrophic non-failing stage and compare to age matched normotensive male Wistar rats. The SHR model is an accepted mimic of idiopathic human hypertension demonstrating an early increase in blood pressure (BP), a long progression of compensated hypertrophy and eventual cardiac failure late in life.\textsuperscript{12}

METHODS

A. Animals

All experiments were approved by the Animal Ethics Committee of the University of Auckland and fulfilled requirements of the National Institutes of Health \textit{Guide for the Care and Use of Laboratory Animals}.\textsuperscript{13} Male Wistar rats and SHR were housed in pairs and kept under a constant 12 h light: dark cycle with 50-70\% humidity at a temperature of 19-21°C. All rats were fed standard Harlan Tekland 2018 rodent diet (Madison, WI, USA) and allowed tap water ad libitum. Twenty-eight animals were divided into four groups (i) Wistar 6 months (mo), n=6 (ii) SHR 6 mo, n=6 (iii) Wistar 14 mo, n=8 and (iv) SHR 14 mo, n=8.

B. Animal characterization and Doppler ultrasonography

The following signs of overt heart failure: 1) pericardial effusions, 2) pleural effusions, 3) ascites and 4) dyspnea at rest were monitored in all rats.\textsuperscript{14} To avoid a stress related immune response, blood pressure (BP) was measured in a different cohort of 6 mo and 14 mo SHR and Wistars that were co-bred under identical conditions in our laboratory to the animals used in this experiment. BP was measured indirectly by tail cuff (MC4000 Blood Pressure Analysis System, Hatteras Instruments) (n=3 in each group). Body weight was measured in isoflurane-anesthetized rats and for fundamental measures of heart function an 8-mHz ultrasonic Doppler probe (Sonotrax A, Contec Medical Systems) was used with lubricating gel to record heart rate and heartbeat duration under constant flow rates of 3 l/min of 3\% vol/vol isoflurane. Once a clear heart beat was detected, sonograms were generated for at least one minute.\textsuperscript{15} Heart rate, beats per minute (BPM) and beat duration (period of an individual beat) was determined directly from sonograms using Audacity 1.2.6.

C. Preparation of blood mononuclear cells

Two minutes after an injection of heparin (100 IU/kg) a midline thoracotomy was carried out and blood was retrieved through a euthanizing cardiac puncture. Peripheral blood MC were retrieved from whole blood using the density gradient separator Ficoll-Paque\textsuperscript{TM} Plus (GE Lifesciences) according to published protocols.\textsuperscript{16} After washing with phosphate buffered saline, cells were re-suspended in mitochondrial respiration medium (MiR05) containing (in mmol/l) 0.5 EGTA, 3 MgCl\textsubscript{2}, 60 K-lactobionate, 20 taurine, 10 K\textsubscript{2}HPO\textsubscript{4}, 110 sucrose, and 1 mg/ml BSA in 20 mmol/l HEPES. A cell count was performed using trypan blue and the Countess Automated Cell Counter (Invitrogen), and respiration assays normalized per million live cells.

D. Preparation of heart and skinned myofibers

The heart was stopped by exsanguination then excised and immediately immersed in ice-cold saline (0.9\% NaCl). Heart
weight was recorded after trimming the major vessels and blot drying. Approximately 25mg of myocardium from the apex of the left ventricle was trimmed and dissected into fiber bundles as described previously.15

To permeabilize myofibers for mitochondrial analysis, fiber bundles were transferred into 2 ml fresh high-energy relaxing solution with saponin (50 µg) and agitated for 30 min at 4°C, followed by three 10 minute washes in MiR05 as described previously.15 Fiber bundles were blot dried on lint-free lens paper, and ~2 mg weighed for respiration assays.

E. Respiration assays
A multiple substrate-inhibitor titration protocol was employed because it is known to better reflect respiration in vivo and to explore the relative capacity of the electron transport system (ETS) and phosphorylation system (Oxphos) components.6 Two OROBOROS Oxygraph-2K (Anton Paar, Graz, Austria) were employed for oxygraphy and respiratory measurements were performed at 37°C in 2 ml MiR05, and the oxygen concentration at air saturation of the medium was 215 nmol O2/ml at 95 kPa barometric pressure. Respiration was measured as oxygen flux per million live cells for blood MC (pmol O2·millon cells) and as weight-specific oxygen flux (pmol O2·mg wet wt−1) for heart fibers. Oxygen flux was calculated as the time derivative of oxygen concentration using the DatLab 4 Analysis Software, OROBOROS (Innsbruck, Austria).

Approximately 10 million MC were added per 2ml chamber and endogenous respiration was measured. Digitonin (25µg/ml) was then added to disrupt the outer cholesterol laden plasma membrane so that mitochondrial substrates could be titrated in the organelle (Fig. 1A). In parallel, from the same animal, 2–3 mg of permeabilized heart fibers were added to chambers in another oxygraph and allowed to equilibrate (Fig. 1B). For heart fibers only, oxygen was then added to chambers and maintained above 280 nmol/ml to ensure saturation. In both MC and heart fiber assays, Complex I leak (CI leak, state 2 respiration) was measured by the addition of Complex I substrates alone (10 mmol/l glutamate, 2 mmol/l malate). Subsequent addition of excess ADP (1.25 mmol/l for MC and 2.5mmol/l for heart fibers) stimulated oxidative phosphorylation (CI Oxphos, state 3 respiration), and by then adding cytochrome c (10 µmol/l), the functional integrity of mitochondria was measured. To assess for any additional effect of CI Oxphos, pyruvate (5mmol/l) was added. In MC only, the reduced form of nicotinamide adenine dinucleotide (NADH) (5mmol/l) was added to measure integrity of the mitochondrial outer membrane.17 Phosphorylating respiration with Complex I and II substrates (CI,II Oxphos) was assessed by addition of succinate (10 mmol/l). The “leak rate” with Complex I and II substrates (CI,II leak or state 4 respiration) was measured by addition of oligomycin (5µmol/l) in MC and atractylside (1 mmol/l) in heart myofibers, followed by repeated titrations of carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone (FCCP, 0.5 µmol/l) to
uncouple mitochondria to measure ETS capacity. Complex I and Complex II activity were selectively inhibited by the addition of rotenone (1 μmol/l) and malonate (15mmol/l). In MC glycerol-3-phosphate (10mmol/l) (G3P) was then added.

Statistical analysis

All data are presented as means ± SE, and analyses were performed using the statistical package Systat SigmaPlot 11 and graphs produced using Graphpad Prism 5. Characteristics of the animal model as well as Doppler measurements were analyzed using two-way ANOVA with post hoc Tukey tests. Where appropriate, log transformations were applied to the data before model fitting. Differences were considered significant at $P < 0.05$.

RESULTS

F. Animals and cardiac function

SHR weighed significantly less than the Wistars at both ages (38% at 6 mo and 27% at 14 mo, Table 1). There was no difference in heart weight between Wistars and SHR at 6 mo of age but at 14 mo the SHR heart was markedly heavier than the controls (p<0.001). For SHR the systolic BP was 176 ± 6 mmHg and 189 ± 11 mmHg for 6 mo and 14 mo respectively. For the Wistars, the systolic BP was 132 ± 8 and 154 ± 9 mmHg (P<0.05; both ages). None of the signs of overt heart failure (pericardial effusion, pleural effusion, ascites or dyspnea at rest) were present in any of the Wistars or SHR. SHR hearts weighed significantly less at 6 mo compared to SHR hearts at 14 mo. As a percentage of body weight the heart weighed more in SHR at both ages compared to Wistars (p<0.001; both ages). We found no change in either heart beat duration (SHR 6 mo 128.6 ± 5.6μs, SHR 14 mo 133.1 ± 4.2μs, Wistar 6 mo 135.3 ± 5.0μs, Wistar 14 mo 136.9 ± 3.4μs), or beat rate (SHR 6 mo 338.4 ± 6.6 bpm, SHR 14 mo 345.9 ± 7.4 bpm, Wistar 6 mo 350.2 ± 13.3 bpm, Wistar 14 mo 346.4 ± 1.4 bpm). These data indicate that while there was an apparent cardiac hypertrophy, there was no apparent arrhythmia or perturbation of heart beat among groups consistent with compensated non failing cardiac hypertrophy.21

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Fig. 3. Permeabilized heart fiber respiration of SHR and Wistar rats at 6 mo and 14 mo of age (meansSE) The different respiratory states measured were: CLII Oxphos [Complex II oxidative phosphorylation rate] (A), ETS [Electron Transport System capacity] (B), CIV [Complex IV] (C), Ratios of respiratory states were calculated, including the RCR [Respiratory Control Ratio] (D) and % CI ETS [percentage Complex I relative to total electron transport system capacity] (E). Two-way ANOVA effects and interactions are shown underneath each respiratory state, and results of post-hoc Tukeys are shown in the figure, NS p>0.05.

Glycerol-3-phosphate is a substrate used by glycerol-3-phosphate dehydrogenase (GpdH) in MC mitochondria and feeds electrons directly into the Q junction. Antimycin A (1 μmol/l) was then added to block Complex III in both MC and heart myofibers. This gave a measure of non-mitochondrial oxygen consumption or ROX. Finally, the activity of cytochrome c-oxidase (CIV) was measured by the addition of the electron donor couple N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD, 0.5 mmol/l) and ascorbate (2 mmol/l). Activities were determined per million live cells for MC and relative to wet mass (mg) of tissue for heart fibers. Chemical backgrounds were measured and subtracted from all experiments. Respiratory control ratio (RCR) and the percentage of Complex I as a function of ETS (%CI ETS) were calculated as CLII Oxphos/CLII leak and ETS-rot/ETS * 100, respectively.20
respiration was apparent with age (Fig 2e). In Wistars a 57% depression (p=0.014) with G3P was seen with increasing age and in SHR a non significant drop of 41% was apparent with increasing age (p = 0.104).

**H. Effect of hypertension and age on heart tissue mitochondrial function**

Permeabilized fibers from SHR and Wistar hearts showed similar CI,II Oxphos and there were no significant differences in ETS or CIV flux between any of the groups (Fig. 3A,B,C). Similarly, the RCR and %CI ETS did not differ between groups (Fig. 3D,E). There were also no significant changes in heart mitochondrial function with progressive age within Wistar or SHR.

**DISCUSSION**

This study examined mitochondrial function in two tissues, circulating MC and cardiac myofibers, taken from young (6 mo) and older (14 mo) SHR and control Wistar rats. Heart weights as a percentage of body weight increased in SHR rats with age compared to Wistars and the lack of systemic heart failure signs was consistent with compensated non failing cardiac hypertrophy. The mitochondrial respiration of MC differed for several parameters. There was an increase in respiration globally in the SHR MC compared to Wistars. MC endogenous respiration was increased two fold relative to age-matched Wistars at both ages examined. After cell permeabilization, phosphorylating flux was higher with all substrates (Oxphos) in SHR of both ages relative to controls, with the only exception being Complex I linked substrates (glutamate, malate, and pyruvate) where there was no difference at 6 mo or 14 mo.

While there was a higher ETS capacity, the %CI ETS was lower in SHR at 14 mo. Even though, the altered mitochondrial respiration in MC anticipated differences in the heart fibers, there was no difference in mitochondrial respiration in permeabilized heart fibers between either rat strains or ages.

SHR are a well-established model of genetic hypertension that produces an increase in systolic wall stress resulting in concentric hypertrophy. Although the Wistar-Kyoto (WKY) rat is often the control for the SHR model, up to 28% of commercially available WKY rats have severe congenital cardiac abnormalities associated with biventricular hypertrophy with ventricular septal defect and pulmonary regurgitation being the commonest cardiac defect. Papillary muscles isolated from the left ventricles of WKY rats have been found to be stiffer than those from Wistarrats, with evidence of a greater level of myocardial fibrosis and WKY rats from different sources have been reported to have significantly varied blood pressures. Of further concern for this present study which explores MC, WKY are reportedly more prone to infection relative to SHR and Wistarrats which could confound our MC findings. Due to these reasons we elected to use age matched male Wistars as the most appropriate controls in this study.
Mitochondrial respiration in MC was significantly different in SHR compared to Wistars. The endogenous respiration of MC was approximately double in the SHR to that of the Wistars. The endogenous cellular respiration is the sum of Oxphos, respiration attributed to proton leak across mitochondrial inner membranes (which do not contribute to ATP synthesis), and non-mitochondrial oxygen consumption by numerous oxidases. Since the respiration attributable to proton leak and non mitochondrial oxygen fluxes were similar in SHR and Wistar MC (data not shown) the increase in endogenous respiration is most likely from an increase in respiration by Oxphos and this is in fact what we have found (see below). Consistent with our study, when MC were activated with Concanavalin A, endogenous respiration increased by 53% and this increase was associated with increased ATP demands. Taken together it appears that the hypertensive state in SHR chronically activates MC probably through stimuli such as angiotensin II and the result is increased endogenous mitochondrial respiration and Oxphos to cope with the increased ATP demand.

In parallel with high levels of endogenous respiration, CI,II Oxphos was also two fold higher in SHR at both ages relative to Wistars. This is in contrast with respiration with Complex I substrates alone (glutamate, malate and pyruvate) which were similar between SHR and Wistars at both ages. Therefore the increased CI,II Oxphos appears to be primarily driven by increased Complex II flux. It is known that Complex II requires more oxygen to produce the equivalent ATP from Complex I substrates and is therefore less efficient at ATP production than Complex I. This is reflected by the decreased RCR which was used to quantify coupled Oxphos capacity. Although the RCR has not been studied before in MC in hypertension, the RCR in 4 mo old SHR brain mitochondria measured as CI Oxphos/CI leak was also found to be decreased compared to those from WKY, which is consistent with our findings in MC.

Additionally, the %CI ETS flux trended 37% lower (P=0.106) in 6 mo SHR than 6 mo Wistars and was 52% lower in 14 mo SHR relative to age-matched Wistars (p=0.001). This suggests a defect in Complex I linked respiration or a defect in organisation of Complex I within supercomplexes, when evaluated under conditions when any limitation by the phosphorylation system is removed by chemical uncoupling. Consistent with this finding, a progressive decrease in %CI ETS was recently reported in heart mitochondria from patients with heart disease and congestive heart failure compared to controls. Complex I linked respiration is more coupled to the generation of a proton gradient, with Complex I based respiratory chains producing approximately 40% more ATP than Complex II based chains. Therefore, a drop in the relative contribution of Complex I in SHR should depress the overall efficiency of ATP production and this is observed in the lower %CI ETS in the SHR.

In keeping with the overall increase in respiration, G3P fuelled respiration, CIV respiration and ETS were significantly higher in both 6 mo and 14 mo SHR compared to age-matched Wistars. GpDH resides on the outer surface of the inner membrane of the mitochondria and reduces a flavin prosthetic group that rapidly donates its reducing equivalents to an electron transport chain through Coenzyme Q in a similar manner to Complex II (i.e. GpDH does not itself directly contribute to the membrane potential). In SHR, the high levels of GpDH may reflect greater demands on rapid ATP production. However, as with Complex II, GpDH is less efficient at producing ATP than Complex I, indicating a general compromise of efficiency for increased ATP production rate. The increase in flux through CIV may be a result of elevated Complex IV concentration/abundance which can increase the mitochondrial oxygen affinity. Elevated Complex IV may be a compensation response to increased proportion of inhibited Complex IV molecules in vivo. Increased flux through CIV was also found in a Ren-2 rat model of hypertension when they were compared to Ren-2 rats treated with anti-hypertensives. The maximal uncoupled respiration reflects an overall increase in ETS capacity.

In MC, the increase in NADH-fuelled respiration at 14 mo is most likely mitochondrial in origin since background non-mitochondrial respiration was not significantly different in any of the groups after adding inhibitors specific for the various mitochondria ETS complexes (rotenone, malonate and antimycin-A, data not shown). In addition, the respiration changes observed with NADH were dependent on both underlying disease and age (disease-age interaction, p=0.018) with the older SHR rats having the greatest response. This suggests decreased mitochondrial outer membrane integrity in SHR that is progressive with disease and age.

Age had an independent effect in the MC with a drop in glycerol-3-phosphate fuelled respiration in both SHR and Wistars. Interestingly, a 57% depression (p=0.014) was seen at 14 mo compared to 6 mo old Wistar rats, while in SHR no significant drop was apparent with age (p=0.104). This is reflective of a more rapid decrease in G3P fuelled respiration with age in normotensive rats compared to hypertensive rats and this is consistent with an increase in rapid glucose oxidation in SHR.

The mitochondrial dysfunction in MC anticipates dysfunction in the heart fibers, but in contrast to MC, the mitochondrial respiration of SHR permeabilized heart fibers was comparable to age matched Wistars at both early hypertensive (6 mo) and compensated hypertrophic non failing stage (14 mo). Similar to the current study, no difference in CI,II Oxphos or CIV flux was found in 12 mo SHR permeabilized heart fibers by an earlier study in our lab compared to WKY rats of same age. Rimbaud et al also found no difference in basal and maximal mitochondrial respiration in skinned heart fibers between 15 and 25 week SHR and WKY controls. Unlike the present study however, a marginal elevation in CI Oxphos and ETS (both p<0.05) was reported previously in SHR. This discrepancy may relate to the differing methodology between the two studies. In our current study the control littermates were Wistars, whereas Hickey et al. used Wistar-Kyotos. In that study, mitochondrial function differences between WKY and...
SHR was minimal at rest but following maximal workloads, SHR hearts revealed considerable depression in ETS and Oxphos capacities relative to WKY hearts. This suggested that while SHR heart mitochondria appear normal at rest, under a stressful workload they may be more susceptible to oxidative damage. In this study we were specifically interested in how the resting heart mitochondrial function compared to mitochondrial function measured in circulating MC.

A consideration of this study is that the global differences in mitochondrial respiration in MC were due to the differences between the SHR and Wistars strains. However the relative trajectory of these changes were not the same between these two species at different ages, for example % CI ETS (Fig.2H) showed no difference at 6 mo between the SHR and the Wistars but there was a significant increase in respiration at 14 mo. This suggests there was no specific underlying species difference that were accounting for the results.

The differences in MC mitochondrial respiration in this study were found to be present from as early as 6 mo in SHR whereas no significant differences were seen in the heart tissue at either ages. However, a recent human heart study has shown similar mitochondrial findings of decreased %CI ETS in heart disease and congestive heart failure as seen in MC in this study. The early MC mitochondrial dysfunction changes compared to those in the compensated heart may be due to increased sensitivity of the MC to the hypertensive state. Although the aim of this study was not to establish the mechanism, one hypothesis for the observed altered mitochondrial respiration in MC is that they are exposed to increased oxidative stress as a result of endothelial dysfunction (Fig.4). Endothelial dysfunction is one of the hallmarks of hypertension and one of the main characteristics of endothelial dysfunction is an impaired vasomotor response. The normal vascular endothelium plays a pivotal role in regulating vascular tone by releasing various relaxing and contracting factors such as catecholamines, vasoactive peptides, reactive oxygen species (ROS) and nitric oxide (NO). NO is particularly important and when released from endothelial cells works in concert with prostacyclin to inhibit platelet aggregation, attachment of neutrophils to endothelial cells and expression of adhesion molecules. ROS such as superoxide rapidly react with NO limiting its bioavailability and its protective effects. Excessive scavenging of NO by ROS is one of the important mechanisms that result in endothelial dysfunction in hypertension. NO plays a crucial role in signaling to mitochondria, and recently, NO was shown to play a role in basal and adaptive mitochondrial biogenesis in the vasculature and regulation of mitochondrial turnover in hypertension. In hypertension, ROS is produced excessively by vascular endothelial cells which rapidly react with NO limiting its bioavailability and its protective effects. Oxidative stress has been reported to inhibit mitochondrial CI as witnessed by a decreased %CI ETS in MC mitochondria in this study. In addition, ROS is also produced in inflammatory leukocytes such as MC mitochondria and nicotinamide adenine dinucleotide phosphate oxidase which may contribute to the endothelial dysfunction.

CONCLUSIONS

In summary, MC are less efficient at producing ATP with a decreased Complex I as a function of ETS and a lower RCR in SHR compared to Wistars. From an early stage in the disease, the global increase in Oxphos and increased ETS capacity is suggestive of increased ATP demands in hypertensive MC. The differences in MC mitochondrial respiration were present at a stage in the disease when there were no detectable heart tissue mitochondrial respiration differences. Mononuclear mitochondrial respiration may now be measured using this protocol in patients with hypertension. MC mitochondrial respiration will now need to be correlated with indices of subclinical organ damage to assess its potential to be a marker of subclinical multiple organ damage in hypertensive patients.

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