Thermoneutrality induces vascular dysfunction and impaired metabolic function in male Wistar rats: a new model of vascular disease

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Objective: Cardiovascular disease is of paramount importance, yet there are few relevant rat models to investigate its pathology and explore potential therapeutics. Housing at thermoneutral temperature (30 °C) is being employed to humanize metabolic derangements in rodents. We hypothesized that housing rats in thermoneutral conditions would potentiate a high-fat diet, resulting in diabetes and dysmetabolism, and deleteriously impact vascular function, in comparison to traditional room temperature housing (22 °C).

Methods: Male Wistar rats were housed at either room temperature or thermoneutral temperatures for 16 weeks on either a low or high-fat diet. Glucose and insulin tolerance tests were conducted at the beginning and end of the study. At the study’s conclusion, vasoreactivity and endothelial nitric oxide synthase activity and nitrotyrosine, and elevated glutathione activity were observed in aorta from rats housed under thermoneutral conditions, indicating a climate of lower nitric oxide and excess reactive oxygen species in aorta. Thermoneutral rat aorta also demonstrated less mitochondrial respiration with lipid substrates compared with the controls (P < 0.05).

Conclusion: Our data support that thermoneutrality causes dysfunctional vasoreactivity, decreased lipid mitochondrial metabolism, and modified cellular signaling. These are critical observations as thermoneutrality is becoming prevalent for translational research models. This new model of vascular dysfunction may be useful for dissection of targetable aspects of cardiovascular disease and is a novel and necessary model of disease.

Keywords: cardiovascular disease, endothelial nitric oxide synthase, mitochondria, thermoneutrality, vasodilation

INTRODUCTION

Cardiovascular disease (CVD), the leading cause of death in the United States [1], includes all conditions impacting the heart and vasculature, such as hypertension, heart failure, and atherosclerotic cardiovascular disease (ASCVD). CVD risk is significantly elevated in those with diabetes compared with the general population [2], and its progression includes impaired vasoreactivity (dilation and constriction), compromised vascular endothelial function, structural stiffness, increased tone, and vascular mitochondrial dysfunction [3–6]. Vasodilation is regulated by endothelial nitric oxide synthase (eNOS), and this enzyme’s activity is compromised in CVD and diabetes [7–11]. We and others have demonstrated that mitochondria are critical to vascular function [12–14] and are

Journal of Hypertension 2022, 40:2133–2146

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Received 26 October 2021 Revised 3 March 2022 Accepted 4 March 2022

DOI:10.1097/HJH.000000000003153
regulated by eNOS activity in the vasculature [14–16]. Endothelial-mediated vasoreactivity, calcium signaling associated with vascular relaxation, smooth muscle cell proliferation, and apoptosis are processes regulated, in part, by eNOS. Each of these processes require healthy mitochondrial function [17–19]. Vascular mitochondrial dysfunction has been reported in association with smooth muscle cell apoptosis, vascular inflammation, and vascular stiffness [20–22]. In summary, mitochondria are a dominant factor regulating vascular function in myriad ways. Thus, elucidating the connection between mitochondria, eNOS activity, and vascular dysfunction in CVD is critical to a more complete understanding of the pathogenesis of ASCVD.

Animal models demonstrating dysfunction in vascular reactivity and mitochondrial activity within the context of diabetes, metabolic syndrome, or obesity have limitations. Behavioral rat models of diabetes and obesity, such as consuming a high-fat diet, are difficult to calibrate, demonstrate inconsistent vascular structural and functional changes and align poorly with human physiology (such as development of chronic hypertension or development of ASCVD). Rodents can be resistant to the metabolic and cardiovascular impact of cage dwelling (sedentary behavior) and the development of diet-induced obesity and carbohydrate intolerance. As such, most rodent models incompletely and inconsistently recapitulate metabolic disease. Further, many rodent models of ASCVD require genetic changes, such as manipulation of apolipoprotein E or the low-density lipoprotein (LDL) receptor, only available in mice.

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METHODS AND MATERIALS

Reagents

Western blotting gels were from BioRad (Hercules, California, USA) and PVDF membranes were from Millipore (Burlington, Massachusetts, USA). Collagenase, EDTA, ethylene glycol tetaecetic acid (EGTA), sodium pyrophosphate, sodium orthovanadate, sodium fluoride, okadaic acid, 1% protease inhibitor cocktail, dithiothreitol, magnesium chloride, K-lactobionate, taurine, potassium phosphate, HEPES, digoxin, pyruvate, malic acid, glutamic acid, adenosine diphosphate, succinic acid, oligomycin, carbonyl cyanide 4 (trifluoromethoxy)phenylhydrazone (FCCP), antibody to β-actin (mouse), phenylephrine and acetylcholine, trypsin inhibitor and cytochrome c were procured from Sigma-Aldrich (St Louis, Missouri, USA). Dimethyl sulfoxide (DMSO), sodium chloride, sucrose, and bovine serum albumin were purchased from Fisher Scientific (Pittsburg, Pennsylvania, USA).

Antibodies

Antibodies to total adenosine monophosphate kinase (AMPK, Cell Signaling #2532S, 1: 500, mouse), phosphorylated AMPK (pAMPK, Cell Signaling #2532S, 1: 500, rabbit), Sirtuin 1 (SIRT1, Cell Signaling #9475S, 1: 250, rabbit), Sirtuin 3 (SIRT3, Cell Signaling #2627S, 1: 500, rabbit), total endothelial nitric oxide synthase (eNOS, Cell Signaling #9572S, 1: 500, mouse), Ser177 phosphorylated eNOS (Cell Signaling #9571S, 1: 500 Rabbit), were obtained from Cell Signaling (Danvers, Massachusetts, USA). Nitrotyrosine was purchased from Cayman Chemical (#10189540, 1: 500, rabbit, Ann Arbor, Michigan, USA). For the ratio of phosphorylated to total protein, alternate host animal antibodies and alternate secondary antibodies were used with different wavelengths, to eliminate the possibility of signal bleed-through. Antibody cocktail to representative subunits of mitochondrial oxidative phosphorylation (Total OXPHOS Rodent WB Antibody Cocktail Abcam #ab110413, 1: 1500, mouse) complexes I (subunit NDUF88), II (subunit SDHB), III (subunit UQCRC2), IV (MTCO1), and V (subunit ATP5A), PPARγ coactivator 1 alpha (PGC-1α, Abcam #ab54481, 1: 500, rabbit) and MnSOD antibody (anti-SOD2/MnSOD antibody [2a1], Abcam, #ab16956, 1: 500) were obtained from Abcam (Cambridge, Massachusetts, USA). Secondary antibodies were IRDye 800RD goat antimouse #926-68070 at 1: 10,000, IRDye 800RD goat antirabbit #926-68071 at 1: 10,000 were purchased from LI-COR (Lincoln, Nebraska, USA) and Starbright Blue 700 goat antimouse at 1: 5000 #12004159 from Bio-Rad Laboratories.

In-vivo experiments

Animals (male Wistar rats, 5 weeks old), kept at two animals per cage, were housed at either room temperature (22°C) or thermoneutrality (29–30°C). Body temperature was taken superficially and elevated temperature was achieved in those housed at thermoneutral conditions as compared...
with those house at room temperature (30.4 ± 0.1 vs. 27.4 ± 0.1°C, P < 0.001, data not shown). Animals were fed either a customized diet containing 13% kcal fat (LFD) or 42% kcal fat (HFD) (Envigo [Teklad]) for 16 weeks. Blood (approximately 50 μl) was collected in 0.5 ml/1 EDTA and spun at 12 000g for 10 min at 4°C. Plasma was extracted and stored at −80°C. Fasting blood (6h) was taken at the beginning and end of the study, fed blood was taken biweekly for glucose and insulin concentrations, and body weight and food consumption were measured weekly. Endpoint parameters were taken at sacrifice, and all animals were euthanized in the morning following ad libitum food consumption.

**Vasoreactivity**

Sacrifice of animals occurred at 16 weeks, and aorta and carotid vessels were taken from rats at sacrifice, cleaned of fat and tissue, and measured for vasoreactivity using force tension as previously described [30–33]. Denuding was completed mechanically; interior tissue was either rubbed gently with tweezers (aorta) or bubbled with air using a syringe (carotid), and we have previously determined that these techniques result in significantly less response to ACh as compared with intact. We also conducted a Student’s t test to ensure denuding caused no mechanical damage; we observed no significant differences in paired aorta or carotid. To quantify vasoreactivity, tissue (2 mm rings) was subjected to the carbohydrate experiment. In the lipid experiment (palmitoylcarnitine-driven respiration), rates were measured with 5 μmol/l palmitoylcarnitine + 1 mmol/l malate, 2 mmol/l ADP, 2 mmol/l glutamate + succinate, 4 mg/ml oligomycin, and 0.5 mmol/l stepwise titration of 1 mmol/l carbonyl cyanide 4-trifluoromethoxy)phenylhydrazone (FCCP) until maximal uncoupling (uncoupled state). Only aorta was subjected to the carbohydrate experiment. In the lipid experiment (palmitoylcarnitine-driven respiration), rates were measured with 5 μmol/l palmitoylcarnitine + 1 mmol/l malate, 2 mmol/l ADP, 2 mmol/l glutamate + succinate, 4 mg/ml oligomycin, and 0.5 mmol/l stepwise titration of FCCP. Cytochrome c (10 mmol/l) was used to determine mitochondrial membrane integrity. Both vessels were subjected to the lipid experiment. Vessels were dried overnight at 60°C and weighed for dry weight normalization. Using the Oroboros DatLab software, traces were analyzed by averaging a stable trace segment of 3–5 min for each state.

**Respiration**

Mitochondrial respiration was measured using Oroboros Oxygraph-2k (O2k; Oroboros Instruments Corp., West Warwick, Rhode Island, USA) while incubated in a bath at 1.5 g basal tension for aortae and 1.0 g for carotids; baths contained Krebs buffer (119 mmol/l NaCl, 4.7 mmol/1 KCl, 2.5 mmol/l CaCl2, 1 mmol/l MgCl2, 25 mmol/l NaHCO3, 1.2 mmol/l KH2PO4, and 11 mmol/l D-glucose) and continuously bubbled with 95% O2 and 5% CO2. Constriction was conducted by exposure to 80 mmol/l KCl. A phenylephrine dose–response curve was also done with doses ranging from 0.002 to 0.7 μmol/l. To investigate vasodilation, a dose–response curve with ACh was performed with a range of 0.05–20 μmol/l secondary to phenylephrine constriction. Data was collected using AcqKnowledge software.

**Insulin and glucose intraperitoneal tolerance tests**

Insulin tolerance testing (ITT) was done at 1 and 16 weeks of the study, following a 6h fast, by injection of 1 U/kg body weight of insulin. Blood glucose concentrations were sampled at 0, 15, 30, 45, 60, and 120 min postinjection. Glucose tolerance testing (GTT) followed the same protocol using 1.5 g/kg body weight of glucose, injected intraperitoneally, and was separated from ITT testing by 4 days. Baseline concentrations were subtracted for area under the curve (AUC) analyses. Fasting glucose and insulin concentrations were taken as the 0-min blood collection during GTT.

**Western blotting**

Aortae were flash-frozen in nitrogen and later processed in mammalian lysis buffer (MPER with 150 mmol/l sodium chloride, 1 mmol/l of EDTA, 1 mmol/l EGTA, 5 mmol/l sodium pyrophosphate, 1 mmol/l sodium orthovanadate, 20 mmol/l sodium fluoride, 500 mmol/l okadaic acid, 1% protease inhibitor cocktail). Aortae were ground under nitrogen with a mortar and pestle, and homogenized at 4°C and centrifuged first at 1000g for 2 min, and supernatants subsequently centrifuged 16 400g at 4°C for 10 min. The Bradford protein assay was used to measure the protein concentration of the lysate. Protein samples (15–40 μg) in Laemmli sample buffer [LSB, boiled with 100 mmol/l dithiothreitol (DTT)] were run on precast SDS–4–15% polyacrylamide gels. Proteins were transferred to PVDF membranes. Quantity One, Bio-Rad, was used to evaluate protein loading. Blots were probed with antibodies described above and left overnight at 4°C. Fluorescent secondary antibodies were applied following the primary antibody incubation.
Glutathione and thiobarbituric acid reactive substances

GSH and thiobarbituric acid reactive substances (TBARS) concentrations were assessed in plasma using kits and instructions from Abcam (Cambridge, UK) and Cayman Chemical (Ann Arbor, Michigan, USA), respectively. For GSH measurements, samples were deproteinized using trichloroacetic acid (TCA) precipitation, according to the kit protocol.

Statistical analysis

To analyze data with time/dose along with diet and temperature, we employed a repeated measures ANOVA, along with a mixed-effects model. For data without a time or dose component, we employed a two-way ANOVA for the variable temperature and diet. Tukey's post hoc analyses were conducted within ANOVA tests. A P value of less than 0.05 was used as the cutoff for statistical significance in all tests. A P value of equal or less than 0.08 was considered indicative of data trends approaching significance.

RESULTS

Metabolic parameters

Rat metabolic parameters were impacted by diet, temperature, or the interaction of these variables across the study (Table 1). Body weights and insulin significantly increased over time (P < 0.05 for all), with a high-fat diet resulting in higher weight gain (P < 0.05, Table 1); Thermoneutral housing resulted in less weight gain than other groups (P < 0.05, Table 1). Fasting insulin concentrations increased to a greater degree in animals housed at thermoneutrality, as well as those on a high-fat diet (P < 0.05 for all, Table 1). There was a significant interaction effect of all variables on fasting glucose concentrations (P < 0.05, Table 1). AUC of GTT and ITT were higher in those housed at thermoneutrality as compared with diet-matched controls, approaching significance (P < 0.05 for all, Table 1). Animals on a high-fat diet had significantly greater gonadal epididymal fat depots than those on a low-fat diet (P < 0.05, Table 2). Cardiac fat was unaffected (Table 2).

Housing temperature significantly alters vascular reactivity

To gauge differences in vasoreactivity, aorta and carotid tissue rings were hung on a force transducer and exposed to dose–response curves of vasoconstrictor phenylephrine and vasodilator acetylcholine (ACh). When exposed ex situ to vasodilator ACh doses, aorta from rats housed at thermoneutrality demonstrated significantly less response as compared with aorta from RT-housed animals (*P < 0.05 for dose x temperature interaction, Fig. 1Aa). The compromised vasodilation activity was also seen in carotids from rats housed at thermoneutrality (*P < 0.05, Fig. 1Ad). When further analyzed, no effect of diet was noted on aorta or carotid rings.

**TABLE 1. Animal weight, fasting glucose and insulin concentrations, and glucose and insulin tolerance test area under the curve at 1 and 16 weeks of treatment**

| Housing | 1 week | | 16 weeks | | |
|---------|--------|--------|----------|--------|--------|
| | LFD | HFD | LFD | HFD | LFD | HFD |
| Weight (g) | 199.8 ± 2.6 | 218.8 ± 3.5 | 187.5 ± 4.2 | 182.4 ± 4.3 | 565.3 ± 12.2 | 623.3 ± 33.7 | 516.0 ± 34.1 | 578.1 ± 24.9 |
| Glucose (mg/dl) | 79.8 ± 1.8 | 86.8 ± 2.2 | 84.9 ± 3.0 | 89.1 ± 3.5 | 70.4 ± 2.6 | 67.0 ± 2.9 | 67.2 ± 2.3 | 72.0 ± 2.4 |
| Insulin (µg/mL) | 0.5 ± 0.1 | 0.9 ± 0.2 | 0.5 ± 0.2 | 0.7 ± 0.1 | 1.1 ± 0.2 | 1.7 ± 0.3 | 0.9 ± 0.2 | 1.7 ± 0.4 |
| GTT (AUC) | 4031 ± 464 | 5574 ± 482 | 4158 ± 523 | 4693 ± 388 | 7860 ± 2361 | 9599 ± 1271 | 10916 ± 1183 | 12651 ± 1577 |
| ITT (AUC) | 5366 ± 711 | 4958 ± 454 | 5214 ± 469 | 4155 ± 383 | 4290 ± 509 | 3754 ± 467 | 4483 ± 285 | 4163 ± 330 |

HFD, high fat diet; LFD, low fat diet; RT, room temperature; TN, thermoneutrality. All parameters had significant effect of time (P < 0.05 for all).

*P < 0.05 diet.

*Time x diet.

*Time x temperature.

*Time x diet x temperature effects.

*P < 0.08 temperature.

*P < 0.05 diet.

*Time x temperature, three-way ANOVA, mean ± SEM. GTT AUC reflects integrated glucose concentrations (mg/dl) at time 0, 15, 30, 45, 60, and 120 minutes following an IP glucose injection and ITT AUC is integrated glucose concentrations at the same timepoints following an IP insulin injection. Data were analyzed with mixed-effects and/or repeated measures three-way ANOVA. Data is mean ± SEM.
Thermoneutrality and vasculature in rats

TABLE 2. Animal cardiac and gonadal–epididymal adipose mass after 16 weeks of treatment

| Housing | 16 weeks | 16 weeks |
|---------|----------|----------|
|         | LFD      | HFD      | LFD      | HFD      |
|         | RT       |          | TN       |          |
| Cardiac fat (g) | 0.19 ± 0.02 | 0.22 ± 0.03 | 0.28 ± 0.04 | 0.26 ± 0.02 |
| GE fat (g) | 14.78 ± 1.47 | 22.02 ± 2.55 | 12.46 ± 1.98 | 19.49 ± 1.40 |

HFD, high fat diet; LFD, low fat diet; RT, room temperature; TN, thermoneutrality.

Thermoneutral housing impacts cellular signaling associated with vasoreactivity and nutrient sensing

To address effects of housing temperature on cellular signaling upstream of vasoreactivity and mitochondrial function, we measured protein expression of nutrient sensor and regulator of eNOS, AMPK, as well as vaso dilator eNOS itself. There was a significant interaction effect of diet and temperature on phosphorylated AMPK protein expression in aorta (P < 0.05, Fig. 1Da), but no significance was observed in AMPK specific activity (Fig. 1Da). Phosphorylated eNOS was significantly lower in those housed at thermoneutrality (P < 0.05, Fig. 1Da). There was a significant interaction effect of temperature × diet, resulting in a greater difference in expression between the room temperature low-fat diet and room temperature high-fat diet groups than between the thermoneutral groups (P < 0.05, Fig. 1D). Total eNOS protein expression was not different between groups (Fig. 1D). eNOS specific activity was lower in rats housed at thermoneutrality (Fig. 1D). A non-denatured gel was used to measure dimer and monomer eNOS concentrations in rat aorta (Fig. 1D) to ascertain the presence of the active dimer form or inactive monomer form of eNOS. Monomer protein expression was significantly reduced in those housed at room temperature and on a low-fat diet, indicative of less NO generation, as well as in both groups housed at thermoneutral (P < 0.05, Fig. 1D). There were no differences between groups in dimer expression (Fig. 1D). To ascertain whether nitric oxide contributed to a climate of excess reactive nitrogen species, we measured total nitrotyrosine via protein expression. Significantly less nitrotyrosine was observed in aorta from rats housed at thermoneutral (P < 0.05, Fig. 1Di). Lower lipid substrate mitochondrial respiration and altered protein expression of mitochondrial complexes with thermoneutrality

To determine the impact of thermoneutrality on mitochondrial function, we measured mitochondria oxygen consumption using an Oroboros Oxygraph 2k closed chamber. Vessels were exposed to substrates and inhibitors mimicking carbohydrate and lipid metabolism in various states of oxidative phosphorylation. In both aorta and carotid vessels, mitochondrial states 3S (ATP production), 4 (membrane potential maintenance), and uncoupled (maximal uncoupled from ATP production) respiration were significantly diminished in thermoneutrally housed animals (P < 0.05 for all, Fig. 2A and B), regardless of diet. In aorta, thermoneutral resulted in significantly less mitochondrial respiration in state 2 (P < 0.05, Table 3) and carotid state 3 was diminished with diet, approaching significance (P < 0.08, Table 3). No impact on RCR was observed in either vessel (Table 3). To ascertain impacts of thermoneutral housing on mitochondrial complex protein expression, western blotting was used. Thermoneutral housing along with a high-fat diet elevated complex III expression, approaching significance (P = 0.08, Fig. 2Cc) Complex IV was also elevated in aorta from animals housed at thermoneutrality, near significance (P = 0.08, Fig. 2Cd).

Thermoneutrality is associated with decreased insulin secretion

As a gauge for glucose metabolism, an IP-GTT was performed on all animals. After 16 weeks, insulin secretion concomitant (AUC) with IP-GTT was less in those housed at thermoneutrality on a high-fat diet as compared with room temperature-housed animals (NS, Fig. 3).

Thermoneutrality modifies oxidative stress

To gauge oxidative stress and cellular response, plasma-reduced GSH and TBARS were measured, as well as aorta protein expression of SIRT1 and SIRT3 and cellular and mitochondrial SOD (Fig. 4a). There was a significant diet effect leading to elevated GSH in animals consuming a
FIGURE 1 (A–D) Vasoreactivity of aorta and carotid intact (A) and denuded (B) in response to acetylcholine or phenylephrine (C), and aorta protein expression of adenosine monophosphate protein kinase and endothelial nitric oxide synthase, western blot experiments (D), dimer and monomer eNOS protein expression, and total nitrotyrosine. Cleared, intact vessels were attached to a force transducer and exposed to an increased dose of either ACh or PE (A–C). ACh dose–response is expressed as a percentage of fully PE-constricted vessels (A–C). PE dose–response is expressed as mN/mg normalized to vessel wet weight (C). Effect of dose was significant for both ACh and PE ($P < 0.05$). Effects of temperature ($^*P < 0.05$, ACh/PE $^**P < 0.05$, diet $^***P < 0.05$, diet $^****P < 0.05$) mixed-effects and/or repeated measures three-way ANOVA (A–C). All vessels are combined according to housing temperature (A, B, and Ca and d) or analyzed together for temperature and diet effect (A and Bb, c, e, f). For D, aorta tissue was processed for protein analysis via western blot analysis, including specific activity (Da–i), and nondenatured samples were used for eNOS monomer and dimer detection. Interaction effect (long bar) $^*P < 0.05$, effect of temperature (elongated bracket), $^**P < 0.05$, $^*P < 0.07$ diet (symbol above data point), two-way ANOVA (C and D) two-way analysis of variance (ANOVA). Data are mean ± SEM. ACh, acetylcholine; AMPK, adenosine monophosphate protein kinase; eNOS, endothelial nitric oxide synthase; PE, phenylephrine; SEM, standard error of the mean.
FIGURE 1 Continued
high-fat diet ($P < 0.05$, Fig. 4), with elevated GSH in animals housed at thermoneutrality, approaching significance ($P = 0.06$, Fig. 4). There was a diet effect approaching significance in TBARS concentrations, resulting in elevated concentrations in those on high-fat diets ($P < 0.08$, Fig. 4d), and temperature effect diminishing SIRT1 protein expression ($P < 0.08$, Fig. 4b). There was also a significant interaction effect of temperature and diet on SOD expression ($P < 0.08$, Fig. 4e).

**DISCUSSION**

Our study tested the impact of thermoneutrality on vasoreactivity and metabolism in the context of low-fat and high-fat diet. We report here that thermoneutrality, in the context of a low-fat diet, led to the impairment of vasodilation and higher constriction in both aorta and carotid. At RT, diet had little-to-no impact on vasoreactivity or mitochondrial function and the impact of thermoneutrality and high-fat diet was not additive. Furthermore, thermoneutral housing lessens insulin secretion with an GTT and did not augment weight gain after 16 weeks. The finding that thermoneutral housing-induced disruption in vascular conduit and resistance artery function, independent of diet, has potential implications for studying human vascular disease. Thermoneutral manipulation generates a novel model of vascular impairment that has the classic clinically relevant functional profiles of endothelial dysfunction, enhanced vasoconstriction, and alterations in vascular mitochondrial function. By utilizing thermoneutral housing, we have characterized a novel driver of vascular disorder and mitochondrial dysfunction, illustrating a new paradigm in which to investigate CVD in both tissue-level functioning and upstream cell signaling.

Rats housed at thermoneutral conditions had a significant impairment in both aorta and carotid ACh-mediated vasodilatation consistent with endothelial dysfunction, and thus a prominent role of eNOS. The loss of ACh-mediated vasodilatation in the denuded aorta aligns with endothelial dysfunction. We showed differential response to mechanical denuding in aorta and carotid. This may be because conduit vessels, such as aorta rely more heavily on NO and endothelium-dependent mechanisms for vasodilation than resistance vessels, such as carotid [35]. Our observation that the room temperature carotid responded to ACh could represent incomplete denudation or a difference between the arterial beds. The methods to denude the two vessels are different based on size. Given the similar changes in vasodilatation and vasoconstriction in the intact aorta and carotid, we believe this is a technical problem. Both conduit and resistance vessels from rats housed at thermoneutrality showed a significantly larger response to PE as compared with those from room temperature. Taken together, these functional data are consistent with endothelial dysfunction and eNOS failure. Further, we observed a significant decrease of eNOS activity as evidenced by phosphorylated eNOS protein expression and a lower monomer form of the enzyme in aorta of rats housed at thermoneutrality. This aligns with our observation of total nitrotyrosine, lower in thermoneutral aorta. Diminished eNOS activity and nitrotyrosine in thermoneutrally housed animals suggests a paradigm of ineffective nitric oxide signaling in the vasculature, likely because of NO scavenging in a climate of elevated ROS, agreeing with other studies in humans and rodents [13,14,36–39]. Additionally, our data point to the same mechanism of dysfunction in both conduit (aorta) and resistance (carotid) vessels. This strongly suggests that failed eNOS may be systemic in vasculature of rats housed at thermoneutrality, and thus an informative model of NOS dysregulation.

We demonstrate impaired ACh receptor vasodilation suggesting that NO is not effectively signaling to the smooth muscle. It is possible that nitric oxide is being immediately scavenged or not being produced in favor of eNOS.
production of superoxide instead, as known to happen in elevated ROS climates or disrupted eNOS function [40]. This interpretation is supported by the lower levels of nitrotyrosine in thermoneutrally housed rats, despite diminished monomer eNOS activity in these same animals. We observed significantly higher GSH concentrations in plasma from rats housed at thermoneutrality consistent with a climate of elevated ROS; however, TBARS concentrations were not different across groups, indicating that downstream damage from oxidative stress is not significant in this model. Localized excess ROS in the vascular is well known to diminish vasodilation [41,42], likely by scavenging NO, and is also observed in models of chronic disease [43,44], including CVD [7,8,42]. Diminished peNOS expression as well as diminished vasodilation were also observed in aorta of animals housed at RT on a high-fat diet, suggesting that the diminished endothelial and eNOS function we report and also observed in overnutrition, aging, and diabetes [9–11,45–47] may parallel our results from a thermoneutral environment. Taken together, our results support a paradigm of dampened eNOS activity, in turn resulting in diminished local NO production and bioavailability in both conduit and resistance vessels. We cannot rule out that signaling response may be dysfunctional at the

FIGURE 2 (A–C) Mitochondrial respiration in aorta (Aa–c) and carotid (Ba–c). lipid metabolism, and aorta protein expression of mitochondrial complexes (Ca–e). Permeabilized vessels were exposed to substrates and inhibitors mimicking lipid metabolism and background oxygen consumption or leak state (state 2), oxidative phosphorylation (+ADP, state 3), maximum oxidative phosphorylation [saccharate, state 35 (A and Ba)], state 4 [+ oligomycin (A and Bb), and uncoupled respiration [+ FCCP (A and Bc)] were determined. Respiration rates were normalized to tissue dry weight (n = 7–8). Effect of temperature *P less than 0.05, **P less than 0.08, two-way ANOVA. For (Ca–e), aorta tissue was processed for protein analysis via western blot analysis (n = 8). Blots were probed for mitochondrial complexes I–IV using a single antibody containing subunits of all complexes. *P = 0.08 interaction (long bar) and temperature (elongated bracket) effect, two-way ANOVA; (Ca–e) data are mean ± SEM. ANOVA, analysis of variance.
level of smooth muscle, such as damaged cGMP or muscle cell performance. Future work will also address the possibility of smooth muscle tissue remodeling and vascular stiffening.

The impact of the thermoneutral environment on vascular mitochondrial function may contribute to abnormal vasomotion. Our results show significantly dampened respiration in aorta and carotid vessels from rats housed at thermoneutrality when exposed to lipid substrates, and elevated mitochondrial complex III and complex V expression. In particular, state 4, a leak state or amount of oxygen consumed with the maintenance of proton gradient and membrane potential, was significantly dampened in aorta from rats housed at thermoneutrality. As damaged membrane potential alters the efficiency of ATP production [48], state 4 may be a driver of the other significantly diminished states in the vessels. Our observations of elevated complex III and IV expression may indicate compensatory activity at the cellular level; our functional data also align directionally with dampened SIRT3 protein expression, a known modulator of mitochondrial function [49,50]. As mitochondrial oxidative phosphorylation processes are coupled to substrates from the TCA cycle, it is intriguing that the diminished respiration observed in thermoneutrally housed rat vessels was only seen when using lipid substrates. This defect in lipid oxidation is suggestive of metabolic flexibility, likely resulting in mitochondrial contributions to a climate of elevated ROS, as previously reported [51–54].

Changes in mitochondrial regulation were observed both at room temperature in response to high-fat diet

| Respiration state/ratio | Aorta RT LFD | Aorta RT HFD | Aorta TN LFD | Aorta TN HFD |
|------------------------|--------------|--------------|--------------|--------------|
| 2*                     | 4.034 ± 0.79 | 3.924 ± 0.55 | 2.612 ± 0.54 | 2.527 ± 0.45 |
| 3                      | 9.211 ± 3.35 | 6.277 ± 1.09 | 6.042 ± 1.13 | 7.392 ± 0.75 |
| RCR                    | 1.351 ± 0.05 | 1.343 ± 0.05 | 1.332 ± 0.05 | 1.336 ± 0.04 |

| Respiration state/ratio | Carotid RT LFD | Carotid RT HFD | Carotid TN LFD | Carotid TN HFD |
|------------------------|----------------|----------------|----------------|----------------|
| 2                      | 4.955 ± 0.80   | 5.292 ± 0.66   | 6.065 ± 0.58   | 4.289 ± 0.92   |
| 3                      | 11.470 ± 1.31  | 8.395 ± 1.03   | 9.412 ± 1.26   | 7.528 ± 1.23   |
| RCR                    | 1.951 ± 0.15   | 1.816 ± 0.11   | 1.952 ± 0.21   | 2.079 ± 0.18   |

Vessels were exposed to lipid substrates and inhibitors to assess respiration rates at several states, including background oxygen consumption or leak state (state 2), oxidative phosphorylation (+ADP, state 3), maximum oxidative phosphorylation (succinate, state 3S), state 4 (+oligomycin), and uncoupled respiration (+FCCP). RCR was calculated as state 3S normalized to state 4. HFD, high-fat diet; LFD, low-fat diet; RCR, Respiratory control ratio; RT, room temperature; TN, thermoneutrality.

*P less than 0.05, temperature effect.

1P less than 0.05 diet effect, two-way ANOVA. Data are presented in O2 flux per mass pmol (s/mg).

FIGURE 3 (A) Insulin concentrations concurrent with intraperitoneal-glucose tolerance testing. Figures show glucose concentrations (mg/dl) at time 0, 15, 30, 45, 60, and 120 min following an i.p. glucose injection at 1 and 16 weeks of RT LFD (1), thermoneutral LFD (2), RT HFD (3), and thermoneutral HFD (4). Area under the curve (AUC) is represented for each group (5). Effect of time was significant (*P = 0.001). No other significance was observed, mixed-effects and/or repeated measures three-way ANOVA. Data is mean ± SEM. HFD, high-fat diet; i.p., intraperitoneal; LFD, low-fat diet; RT, room temperature; TN, thermoneutrality.
and at thermoneutrality on both high-fat and low-fat diet. Complex III and V expression are consistent with elevated pAMPK expression observed in rats housed at thermoneutrality. This finding suggests compensatory signaling from AMPK to mitochondrial activation and agrees with previous studies identifying AMPK as an upstream regulator of mitochondria [55,56] in its capacity as a nutrient sensor. These data may reflect that mitochondrial function is suboptimal because of overnutrition or thermoneutral housing; these conditions could decrease or damage bioenergetic demand [57], consistent with our functional respiration data. Conversely, in rats housed at thermoneutrality and fed a high-fat diet, adaptive increases in lipid oxidation would be expected; however, we did not observe this, indicating lower metabolic flexibility, consistent with previous reports [52–55]. Our cellular signaling data in aorta from rats housed at thermoneutrality and fed a high-fat diet underlie a model whereby AMPK is signaling mitochondria to process substrates and generate ATP but this operation is impaired, resulting in increased AMP/ATP ratio and even more AMPK activation further potentiated by overnutrition and/or thermoneutral housing. We and others have shown that NOS regulates mitochondrial function [15,16,58]. In the paradigm we outline here, NOS function upstream of mitochondria may be disrupted by elevated ROS or other factors. This may in turn cause diminished mitochondrial function and ATP production, resulting in altered vascular function because of lack of ATP availability or additional damaging factors cascading from NOS and mitochondrial dysfunction.

After 16 weeks of thermoneutral housing, rats showed a significant dampening of insulin secretion during a glucose tolerance test as compared with room temperature-housed rats. room temperature-housed rats on a low-fat diet

![Graph showing redox profile](image)
showed a higher insulin secretion resulting in the same glucose concentration area under the curve as those housed at thermoneutrality. We did not observe any differences in glucose handling because of either diet or housing temperature in IP-ITT (data not shown), suggesting that insulin secretion may be impacted, without significant impact on insulin sensitivity.

Our present study contributes to the understanding of thermoneutrality as an emerging method for the generation of animal models aligned with human physiology. Previous studies in mice have shown that thermoneutral housing aligns rodent physiology with that of humans, including similar energy expenditure to basal metabolic rate ratio [26], a cardiovascular profile more similar to that of humans [29], potentiation of high-fat diet-induced diabetes [59,60], and displays general differences in cardiovascular parameters of both rats and mice as compared with room temperature housing [61,62], agreeing with our observed data. Although many studies strongly suggest that thermoneutrality is likely a robust environment for rodent biomedical research and cardiometabolic profiles, the impact of thermoneutrality on the vasculature has not been explored in detail. Very little is known about how thermoneutral housing impacts cellular signaling regulating the vasculature, such as NOS and mitochondria. Here, we contribute to the emerging data on cardiometabolic effects of thermoneutrality by reporting altered vasoreactivity, vascular mitochondrial function, and cellular signaling in aorta and carotid of rats housed at thermoneutrality for 16 weeks. These functional changes make for a novel rat model of vascular dysfunction that recapitulates early endothelial dysfunction observed in human disease (including loss of Ach-mediated vasodilation and increased response to phenylephrine). These functional phenotypes plus the related changes in NOS and mitochondrial function present a highly clinically relevant mode of vascular disease.

Our study has limitations. We took external body temperature to ascertain whether animals were responding to housing climate; however, this was superficial and not an internal body temperature, and we observed significant physiological differences as a result of different housing temperatures. Housing room temperature was kept at 29–30 °C, which should take into account impacts of co-housed animals on body temperature. It may be possible that at exactly 30 °C, we would observe even more differences. Weight gain was greater in room temperature-housed animals as compared with thermoneutrally housed ones, which differs from reports in mice [59,60]. This could be attributed to the common issue of inconsistent weight gain with overfeeding in rodents in general. Also, we report differential responses in denuded aorta and carotid. Typically, mechanical denuding is different between these vessels and may explain our results. Secondly, aorta is more reliant on endothelial signaling for vasorelaxation that carotid [35], potentially explaining our observations. Also, we use cleaned vessels for our vasoreactivity experiments; they do not contain perivascular adipose tissue (PVAT), adventitia or connective tissue. PVAT acts as a paracrine organ and has considerable regulatory impact on vasodilation and constriction. Although we do not consider PVAT in this study, its impact on the vasculature is a current avenue of investigation in our laboratory. We acknowledge that more studies of rodent physiology comparisons between room temperature and thermoneutral housing are necessary, particularly those that address potential cold stress of housing at room temperature. Thermoneutral housing is an emerging metabolic technique, and additional data will bolster the use of this perturbation in chronic disease studies.

In conclusion, we report a novel rat model of vascular and endothelial dysfunction in response to housing animals at their thermoneutral conditions and independent of diet. The dysfunction of both vasoreactivity in the resistance and conduit vessels and local mitochondrial function aligns with initiating events in human ASCVD pathogenesis. This model also closely aligns with vascular disease profiles observed in human vascular disease, making it a new tool in translational cardiovascular disease in addition to studies of climate on vascular physiology. Our future directions will explore mechanisms connecting these functional observations with an eye on applications to atherosclerosis.

ACKNOWLEDGEMENTS

The authors wish to thank Ms Teri Armstrong and Ms Melissa Blatzer for their kind assistance with the in-vivo measurements, and Mr Jeremy Rahkola and Dr Lori Nield for technical prowess.

Funding sources: the authors wish to acknowledge the following funding sources: NIH/NCCR CCTS UL1 RR025780, VA Merit (J.E.B.R. BX002046), R01 DK124344-01A1 (J.E.B.R.), VA CDA2 (R.L.S. BX004533 and A.C.K. BX003185), Denver Research Institute, P30DK048520 Colorado Nutrition Obesity Research Center Pilot Award (R.L. S.), and the Ludeman Family Center for Women's Health Research at the University of Colorado Anschutz Medical Campus Junior Faculty Research Development Grants (R.L. S. and A.C.K.), Diabetes Research Center DK116073 (JEBR).

Previous presentations of the data herein:

Chun, J.H., Knaub, L.A., Walker, L.A., Reusch, J.E.-B., Keller, A.C. Delineating sex-specific mechanisms of impaired vasoreactivity in thermoneutrality. April 2021. Department of Medicine Research Day. Aurora, Colorado.

Chun, J.H., Knaub, L.A., Walker, L.A., Reusch, J.E.-B., Keller, A.C. Delineating sex-specific mechanisms of impaired vasoreactivity in thermoneutrality (update). September 2021. American Heart Association Hypertension Scientific Sessions. Virtual.

Author contributions: A.C.K. and J.E.B.R. generated the ideas, wrote the manuscript, housed the project and provided oversight. A.C.K., J.H.C., L.A.K., S.E.H. conducted experiments, generated data, and performed data analysis. M.M.H. and G.B.P. generated data and performed data analysis, and R.L.S. analyzed data. L.A.W. provided experimental oversight and data analysis input.

Data availability statement: the data within this manuscript and that support our reported findings are available from the corresponding author upon reasonable request.

Conflicts of interest

There are no conflicts of interest.
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