Sympathetic cardiac hyperinnervation and atrial autonomic imbalance in diet-induced obesity promote cardiac arrhythmias


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METHODOLOGY

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were individually housed in a temperature controlled room (22 ± 2°C) set to a 12:12-h dark-light cycle. Rats were provided chow.
and water ad libitum. All experimental protocols were conducted in agreement with the National Institutes of Health’s Guide for the Health and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University.

**DIO Model**

Rats (57–59 days, 250–275 g) were placed on a HFD (33% kcal fat, Purina 571R, Richmond, IN) for 4–6 wk, as previously described (25, 41). After 2 wk, the bottom tertile of weight gain was classified as the OR group, while the top tertile was classified as the OP group. The middle third was not used. In addition, an aged-matched CON group was fed normal chow (Purina 5001, 13% kcal from fat) for the same period of time. The nutritional profile of the CON diet is 23% protein, 4.5% fat, and 6% fiber. The HFD is a derivative of the 5001 diet with 10% lard added to the mix, composed of 22% protein, 15% fat, and 5% fiber. Experiments commenced when the animals were on the HFD for 4–6 wk.

**Experimental Protocols**

**Protocol 1: Does DIO promote sympathetic hyperinnervation of the heart? IMMUNOHISTOCHEMISTRY.** Hearts were rapidly excised from rats, measured for weight and length, and divided into four main transverse sections. The top and bottom quarters were discarded. The second quarter of the heart was immersed in 4% paraformaldehyde for 1–2 h, rinsed with 1X PBS, cryoprotected in 30% sucrose (15%–30%) overnight, and frozen in Tissue Tek OCT compound (Fisher Scientific, Pittsburg, PA). The third quarter was divided into the left and right ventricle, flash frozen on dry ice, and stored at −80°C for subsequent quantification of norepinephrine (NE) by HPLC (see protocol 4). Immunohistochemistry was performed on 10-μm cryostat sections on a set of five slides per animal with three serial sections per slide. Heart sections were incubated overnight with a primary rabbit anti-rat tyrosine hydroxylase (TH)-specific antibody (1:300; AB152, Millipore, Billerica, MA), followed by incubation for 1.5-h with an anti-rat IgG-specific antibody (1:300; Life Technologies, Grand Island, NY). Slides were analyzed under a Zeiss Axioshot II fluorescence microscope (Thornwood, NY) with a ×40 objective. Photomicrographs of the heart were taken in two representative areas of the left ventricle, septum, and right ventricle (Fig. 1A). The fluorescent TH nerve fiber area was quantified with threshold discrimination by two blinded reviewers using ImageJ software and expressed relative to the total sectional cardiomyocyte area (%TH-immunoreactive area). Values for each region were averaged from five equidistant sections 50 μm apart. Data shown for each region are the average of two separate analyses.

**Protocol 2: Does DIO stimulate sympathetic nerve growth? SYMPATHETIC NEURITE OUTGROWTH FROM SCG EXPLANTS.** Rat SCGs were removed from the same group of animals used in protocol 1. Once removed, SCGs were halved and explanted into a 12-well tissue culture plate and covered with 5 μl Matrigel (BD Biosciences, San Jose, CA). Serum-free DMEM/F-12 media (Life Technologies, Grand Island, NY) + 1% penicillin/streptomycin was layered over the solidified Matrigel, and wells were treated with 2 ng/ml NGF. Culture plates were placed in a humidified incubator of 95% O2/5% CO2 at 37°C. Axon outgrowth was visualized with phase-contrast microscopy, and axon length was measured in the images using Nikon Elements AR 3.0 software (Melville, NY). Initial images for “time zero” were taken 24 h after plating. Images were taken at time zero and again 24-h later (time 24-h). Images were analyzed blindly, and the rate of neurite outgrowth (μm/h) was calculated from the difference in axon growth at time zero and time 24 h, and then dividing this value over the duration of the treatment. Since SCGs were halved, neurite outgrowth was measured in two wells for each animal. The values from each well were averaged to determine the mean rate of neurite outgrowth for each animal.

**Protocol 3: Does DIO increase the risk of evoked arrhythmic events? SURGERY.** In a separate group of rats, OR (n = 3) and OP (n = 5) rats were anesthetized (2.5% isoflurane in 100% O2) and aseptically instrumented with a femoral arterial catheter (PE-50) to measure pulse pressure and heart rate (HR) and two venous catheters (PE-10 attached to PE-50) for intravenous access. The catheters were tunneled subcutaneously and exteriorized between the scapulae. Catheter patency was maintained by flushing with sterile heparinized saline (100 U/ml) at least three times per week. At least 5 days were allowed for recovery before experiments were performed.

EXPERIMENTAL PROTOCOL. Baseline mean arterial pressure and HR was measured for ~30 min in conscious animals. Rats were then challenged with increasing intravenous doses of epinephrine (0.625, 1.25, 2.5, and 5.0 μg/kg). After each bolus was administered, HR and pulse pressure were allowed to return to baseline before the next dose was applied.

Data were sampled at 2,000 Hz using the Biopac MP100 data acquisition system and analyzed online (AcqKnowledge 3.0, Biopac, Goleta, CA). Arrhythmias were defined as a continuous irregular HR for more than five beats. Arrhythmia duration was calculated as the number of seconds over which arrhythmias persisted without interruption.

**Protocol 4: Does DIO alter atrial neurotransmitter release? FIELD STIMULATION OF ATRIAL EXPLANTS.** Using a separate group of rats, the right atrium was removed (n = 4–7 per group) from rats under isoflurane anesthesia and pinned to a thin layer of Sylgard (Dow Corning, Midland, MI) in a preheated (37°C), continuously oxygenated, water-jacketed organ bath containing 2 ml of Ringer solution (120 mM NaCl, 1.2 mM KH2PO4, 4.7 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 25 mM NaHCO3, 11 mM glucose; pH 7.4). The atrial tissue was placed between platinum stimulating electrodes and stimulated as in our laboratory’s previous study (17). Briefly, a baseline sample was taken (5-min incubation) followed by atrial stimulation using an S88X Stimulator (Grass Technologies, West Warwick, RI) in constant-voltage mode (15 V, 5 Hz, 0.1-ms pulse duration) for 1 min to trigger neurotransmitter release. Two 5-min samples were collected poststimulation. Each 1-ml sample was split into 0.5-ml samples for analysis of ACh and NE. Baseline levels of transmitter were subtracted from the amounts recovered during the stimulation and recovery periods. At the end of the experiment, atria were incubated in 1 ml of 0.1 M perchloric acid (PCA) for 24 h to extract total remaining ACh and NE, which were quantified by HPLC coupled with mass spectrometry (HPLC-MS) or electrochemical detection (HPLC-ED), respectively. Stimulation-evoked release was the amount of ACh or NE in the bath after stimulation, less the ACh or NE present before stimulation.

HPLC-MS FOR ACH QUANTIFICATION. The tissue used for this set of experiments was the same as for the field stimulation experiments. As in our laboratory’s previous study (17), 0.5-ml aliquots of the collected samples and a 0.5-ml aliquot of the PCA extract of the atrial tissue were spiked with deuterated ACh (d4 ACh; 0.1 pg/μl, CDN Isotopes, Quebec, Canada). Deuterated ACh was also added to standards of known concentrations of ACh (10 fg/μl to 100 pg/μl, Sigma-Aldrich). Samples were chromatographically separated on a hydrophilic interaction chromatography mode column (HILIC; PolyLC, Columbia, MD; 30-μl injection volume, mobile phase A: ammonium formate; B: acetonitrile) and detected and quantified by a linear ion trap mass spectrometer (Applied Biosystems MDS SCIEX 4000 QTrap mass spectrometer, Carlsbad, CA). Multiple reaction monitoring was used to quantify d4 ACh (mass-to-charge ratio 150→91.1) and ACh (mass-to-charge ratio 146→87). Injections of 2.05 fmol (300 fg in 30 μl) ACh consistently gave signal-to-noise ratios above 3, indicating the detection limit for ACh in our system (lower limit mean for 21 HPLC-MS runs = 2.40 ± 0.04 fmol). Data were acquired using Analyst software (AB SCIEX, Framingham, MA). For both atrial content and stimulus-evoked release measurements, the amount of ACh was calculated by comparing the ratio of...
ACh to d4 ACh in samples to those ratios from known standards for ACh and d4 ACh run in parallel.

HPLC-ED FOR NE QUANTIFICATION. The atrial tissue used for this set of experiments was the same as for the field stimulation experiments. The ventricular tissue used was from the same set of heart samples as in protocol 1. With a similar protocol to that previously described (17), a 0.5-ml aliquot of each sample, and a 0.5-ml aliquot of the PCA extract from atria, left ventricle, and right ventricle were spiked with the internal standard dihydroxybenzylamine (DHBA, 9 nM; Sigma-Aldrich). A similar amount of DHBA was also added to standards of known amounts of NE (4 – 40 nM), and catecholamines were purified from samples and standards by alumina extraction (15 mg, 30 min). Samples were chromatographically separated by reverse-phase HPLC (50 μl injection volume with C18 column; 15 × 0.46 cm, 5-μm particle size; Varian, Lake Forest, CA) using a mobile phase containing 75 mM NaH2PO4 (pH 3.0), 1.7 mM sodium octane sulfonate, and 4% (vol/vol) acetonitrile. A coulometric detector (ESA, Bedford, MA) was used to detect and quantify NE (electrode potential 180 mV, 50 nA) with area under curve normalized to DHBA area. Detection limits were ~50 fmol with recoveries >60%. Data were acquired with LCsolution software (Shimadzu, Columbia, MD). NE levels were determined by comparison of the ratios of NE to DHBA in the samples to those ratios from known standards for NE and DHBA run in parallel with the experimental samples.

Statistical analysis. All between-group differences were compared with one-way ANOVA. Specific comparisons were assessed using a Neuman-Keuls post hoc test, and *P < 0.05 indicated significance.

RESULTS

Baseline Measurements

As shown in Table 1, OP rats weighed more (*P < 0.05) than OR and CON rats. Furthermore, the heart weight (*P < 0.05) and heart length (*P < 0.05) were greater in OP compared with OR and CON rats. Body weight, heart weight, and heart length were similar between OR and CON rats, even though the OR rats were fed the HFD.
Table 1. Body weight, heart weight, and heart length were greater in OP rats compared with CON and OR rats. In agreement with our hypothesis, SCG neurite outgrowth rate was 25% higher in OP rats (n = 5) compared with CON rats (n = 3; Fig. 2A, P < 0.05). However, the rate of growth was also higher (42%) in OR rats (n = 4) compared with CON (Fig. 2A, P < 0.05) and similar to OP rats. Interestingly, the elevated outgrowth parallels the hyperinnervation observed in OR and OP rats.

DIO Increases the Prevalence of Cardiac Arrhythmic Events

To determine whether DIO is associated with a higher occurrence of arrhythmias, we assessed the duration of arrhythmic events after an intravenous epinephrine challenge in conscious OR (n = 3) and OP (n = 5) rats. Baseline mean arterial pressure (OR: 105 ± 5 mmHg, OP: 101 ± 3 mmHg) and HR (OR: 354 ± 9 beats/min, OP: 333 ± 7 beats/min) did not differ between groups. The duration of arrhythmias was directly dose-dependent in both groups (P < 0.05), with the minimal occurrence of arrhythmias at the lowest doses, and the longest events at the highest dose. The lowest dose of epinephrine (0.625 μg/kg) evoked arrhythmias in one OP rat. The higher doses of epinephrine (1.25, 2.5, and 5 μg/kg) evoked arrhyth-
miotachic autonomic parasympathetic and sympathetic cardiac nerves. However, normal atrial ACh content and release were not significantly different between groups, and these weights were used for normalizing both the stimulus-evoked release of neurotransmitter and the total pool of neurotransmitter remaining in atria after stimulation. The total pool of ACh in atria from OP rats ($n = 7$) was lower than that in CON ($57\%, P < 0.05$) and OR ($n = 4; 54\%, P < 0.05$) atria (Fig. 4, left). The fraction of ACh released after field stimulation constituted a small fraction (0.2–0.5%) of the total pool of ACh in all groups. Following field stimulation, all groups demonstrated a statistically significant increase in ACh release from baseline ($P < 0.05$, data not shown). However, ACh release levels from OP atria were significantly less (48%, $P < 0.05$) than those from CON rat atria (Fig. 4, right).

**DIO Elevates Atrial NE Content, But Lowers Release**

In contrast to ACh, the total pool of NE in atria from OP rats ($n = 7$) was significantly greater than that in CON ($n = 4; 87\%, P < 0.01$) and OR ($n = 4; 84\%, P < 0.01$) atria (Fig. 5, top left). The fraction of NE released after atrial field stimulation was limited, being only 0.1–0.2% of the total pool of atrial NE across all groups. Following stimulus-evoked release, NE release from OP rats was significantly lower (76%, $P < 0.05$) than that from CON atria (Fig. 5, top right). Even when normalized to atrial content, or to atrial weight as a fraction of body weight, NE release from OP atria was still significantly lower than CON tissue (data not shown). In contrast to the atria, NE content of the left (Fig. 5, bottom left) and right (Fig. 5, bottom right) ventricles was similar between groups.

**DISCUSSION**

The purpose of this study was to test the hypothesis that obesity-induced sympathetic outgrowth and cardiac hyperinnervation increases the risk of arrhythmic events. In agreement with our hypothesis, we showed that OP rats exhibit accelerated SCG neurite outgrowth, express hyperinnervation of the ventricles compared with CON rats, and are more susceptible to arrhythmic events during an epinephrine challenge. In contrast to our hypothesis, OR rats also exhibit sympathetic outgrowth and hyperinnervation of the ventricles, but are not as susceptible to arrhythmic events, despite hyperinnervation of the heart. Interestingly, OP rats have attenuated atrial ACh content and release compared with OR and CON atria. OP rats also exhibit elevated atrial NE content, but NE release is dramatically suppressed. Together, these findings suggest that the consumption of the HFD enhances sympathetic growth and cardiac hyperinnervation expressed in both OR and OP rat populations. However, normal atrial ACh content and release may protect OR rats from the development of potentially lethal arrhythmias in both groups; however, the OP rats exhibited a sensitization to the epinephrine challenge, as reflected by a prolonged duration of arrhythmias compared with OR rats (Fig. 3A; $P < 0.05$ at each dose).

**Fig. 3.** Assessment of arrhythmic events in OR ($n = 3$) and OP ($n = 5$) rats. A: the duration of arrhythmic events evoked by intravenous epinephrine was greater in OP compared with OR rats. B: representative arterial pulse pressure trace in an OR and OP rat during an epinephrine challenge. Values are means ± SE. *$P < 0.05$ vs. OR.

**Fig. 4.** HPLC-mass spectrometry quantification of atrial acetylcholine (ACh) content and release. Total atrial ACh content (left) and release (right) were lower in OP rats ($n = 7$) compared with CON ($n = 4$) and OR ($n = 4$) rats. Values are means ± SE. *$P < 0.05$ vs. CON. †$P < 0.05$ vs. OR.
arrhythmias that would normally be triggered by hyperinnervation of the heart.

Obesity is associated with a higher occurrence of arrhythmic events (30, 32, 37, 40), but few mechanistic details are understood. Since human obesity is rarely attributed to a specific genetic mutation (8), we chose to investigate these mechanisms in rats with DIO. The DIO model utilizes outbred genetically diverse rats, which represents the polygenetic profile of clinical obesity (24). Excessive weight gain in OP rats is attributed to a high food intake and a dysregulation of energy balance, most likely due to central overexpression of neuropeptide Y (23). Furthermore, OP rats have insulin resistance (6, 9, 22, 41) and lower rates of fat oxidation (6), modeling the characteristics of human obesity.

In the present study, OP rats were fed a HFD for 4–6 wk, unlike other DIO models that utilize a longer high-fat feeding regimen (10–12 wk) (10). Our present and previous findings show that OP rats fed a short-term HFD do not develop arterial hypertension, but exhibited severely impaired cardiac baroreflex function and autonomic imbalance (25, 41), similar to human obesity (1, 15, 36). This suggests that changes in autonomic tone may precede, and perhaps be causal, in the development of hypertension in obesity (14, 20, 21). Indeed, OP rats have atrial norepinephrine (NE) content (top left) but low NE release (top right) compared with CON (n = 4) and OR rats (n = 4). NE content in the LV (bottom left) and RV (bottom right) did not differ between groups. Values are means ± SE. *P < 0.05 vs. CON. †P < 0.05 vs. OR.

Further analysis showed that the hyperinnervation was specific to the right ventricle, which is in agreement with clinical findings that the development of right ventricular dysfunction is directly related to body mass index (39). Despite the absence of overt changes in systemic arterial pressure, elevated pulmonary artery pressure in our model cannot be ruled out, especially since obesity is a risk factor for pulmonary hypertension (5, 13). If pulmonary hypertension is present, increased load on the right ventricular cardiomyocytes may be causal to the right-sided hyperinnervation. However, this mechanism will require further investigation. Although our assessment of arrhythmias cannot distinguish between atrial and ventricular events, the pronounced hyperinnervation in the right ventricle may trigger preventricular contractions in OP rats, especially since preventricular contractions mainly originate from the right ventricle in humans (19).

In agreement with our hypothesis, the hyperinnervation of the heart in OP rats was paralleled by accelerated SCG neurite outgrowth in explant culture. In adult neurons, NGF stimulates nerve growth via TrkA signaling (12). Obesity is a chronic state of low-grade inflammation (38), but it is not clear if inflammatory cell-NGF release is prominent in the obese heart. However, in the present study, OR rats also exhibit greater sympathetic outgrowth and hyperinnervation of the ventricles, despite the absence of overt obesity. Since the common factor between OR and OP rats is the HFD, these data suggest that the neurite outgrowth and hyperinnervation are also stimulated by the consumption of the HFD. Our data are consistent with...
Aubin et al. (2), who found that female Sprague-Dawley rats fed a HFD that were not obese also exhibited sympathetic hyperinnervation of the heart. Higher circulating levels of free fatty acids from the HFD may directly stimulate growth and hyperinnervation, especially since fatty acids, by binding to residues on regulatory proteins, support growth cone function and neurite outgrowth (28). Alternatively, the consumption of the HFD may sensitize TrkA receptor signaling, but this mechanism would require further investigation.

If consumption of a HFD stimulates sympathetic outgrowth and hyperinnervation of the heart, then why were OR rats not sensitized to arrhythmias? Normal organization of ventricular gap junctions could lower the occurrence of arrhythmias, despite the hyperinnervation; however, this mechanism is unlikely since high-fat feeding in nonobese female rats stimulates abnormal connexin-43 expression and phosphorylation (2). Alternatively, the lack of arrhythmia generation in OR rats may be attributed to the difference in cardiac autonomic balance between OR and OP rats. Previously, our laboratory showed that the bradycardic response to aortic depressor nerve stimulation in OP rats is predominantly mediated by withdrawal of sympathetic nerve activity, rather than activation of central parasympathetic pathways (25). In contrast, OR rats exhibit robust decreases in HR for the same degree of stimulation, which are highly dependent on the parasympathetic system. In addition, our present study examined how DIO affects neurotransmitter release and content in the right atrium. In agreement with previous findings (25), we found that the OR rats have higher atrial ACh content and release compared with OP rats. Interestingly, parasympathetic stimulation of the heart suppresses the development of ventricular fibrillation (4, 35). Therefore, the parasympathetic nervous system may protect OR rats against arrhythmic events that would otherwise be triggered by the sympathetic hyperinnervation of the ventricles.

We also observed that OP rats had higher atrial NE content compared with OR and CON rats, while stimulus-evoked NE release from atrial terminals was diminished. Therefore, even without central input in our ex vivo field stimulation system, there appear to be basal differences in atrial sympathetic nerves from OP rats that promote attenuated release of NE from atrial terminals. Our field-stimulation data for NE parallel findings in obese humans (36) and our laboratory’s previous observation that the withdrawal of cardiac sympathetic activity is suppressed in OP rats after aortic depressor nerve stimulation (25).

Although stimulus-evoked NE release levels were only significantly decreased in OP rats, NE release in OR rats also trended in the same direction. Therefore, we suspect that both OR and OP groups may have deficiencies in atrial NE release. However, obesity also attenuates parasympathetic control of the heart (3, 25, 31) and may be the most physiologically relevant factor in protecting OR rats from developing potentially fatal arrhythmias.

In contrast to the atria, obesity did not change the NE content in the right ventricle, despite evidence of hyperinnervation. The lack of change in NE content of the OP rat right ventricle may reflect a disconnect between synthetic enzymes and NE processing. In particular, we have preliminary data suggesting that phosphorylation of TH, which occurs as TH is activated, may be decreased in OP rat sympathetic ganglia (data not shown); however, this suggestion warrants further study. It is evident that neurotransmitter synthesis, release, and cardiac distribution are all altered in obesity, resulting in autonomic imbalance at multiple sites within the heart.

Our novel findings, while largely ex vivo, suggest that sympathetic hyperinnervation of the heart and atrial autonomic imbalance may lower the threshold for evoked arrhythmic events in rats with DIO. However, the consumption of the diet itself enhances neurite outgrowth and sympathetic hyperinnervation of the heart. OR rats are less susceptible to arrhythmic events, despite hyperinnervation of the heart, perhaps due to a protective role of relatively elevated atrial ACh content and release in the OR heart. These complex findings open new avenues of research to investigate how the consumption of a HFD, with and without obesity, affects autonomic control and electrical remodeling of the heart, and how these factors contribute to the development of cardiac arrhythmias.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES

OBESITY STIMULATES CARDIAC HYPERINNERVATION AND ARRYTHMIAS