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Metabolic syndrome (MetS) is emerging as a global health threat due to its strong association with increased risk for cardiovascular disease and diabetes. Currently, 20-25% of the world's population exhibits some traits of MetS, namely obesity, dyslipidemia, hyperinsulinemia, hypertension, and hyperglycemia. In addition, MetS also promotes the development of impaired short-term regulation of mean arterial pressure (AP) by baroreflexes, which normally act to stabilize AP. The resulting increased AP variability, which is an independent risk factor for poor outcomes, is overlooked as a trait of MetS and goes without evaluation or treatment. People who have controlled hypertension without minimizing elevated AP variability are still at significant risk for detrimental cardiovascular events such as stroke and cognitive decline. Therefore, understanding mechanisms impairing baroreflexes with MetS will help determine appropriate therapeutic management to restore baroreflexes and promote stability of AP. Furthermore, because sex differences in the development of impaired baroreflexes with obesity have been reported, an understanding of how females are protected would provide valuable insights for underlying causes for early onset of impaired baroreflexes in obese males and eventual development of impaired baroreflexes in obese females.

In this project, I utilized a rodent model of MetS, obese Zucker rats (OZR), to examine contributions of hypertension and hyperglycemia in the development of impaired baroreflexes in male OZR, and whether hypertensive female OZR have delayed onset of impaired baroreflexes because they have the ability to maintain glycemic control. Male and female OZR have excess weight gain from an early age because the mutation of a leptin receptor renders them insensitive

to leptin's actions to regulate appetite and metabolism, promoting excess intake of standard chow and storage of ingested calories. Like obese humans, OZR develop dyslipidemia, hypertension, and insulin resistance that eventually progresses to type 2 diabetes, making them a suitable model for the consequences of MetS. Young adult male OZR (12-15 weeks) develop sympathetically driven hypertension with pronounced attenuation of baroreflex control of heart rate (HR) and sympathetic nerve activity (SNA) compared to juvenile OZR and lean Zucker rats (LZR). In male OZR, the development of impaired baroreflexes coincides with blunted activation of the NTS, the brain stem region that receives baroreceptor afferent inputs to promote baroreflex-mediated changes in HR and SNA, and this deficit likely yields diminished baroreflexes observed in young adult male OZR.

In the first project I examined whether improvement of impaired glycemic control in young adult male OZR restores baroreflex-mediated bradycardia and activation of the NTS. Both type 1 and type 2 diabetic rats have impaired vagally-mediated activation of the NTS, in agreement with the reported loss of glucose's ability to enhance glutamatergic neurotransmission within the NTS of hyperglycemic, diabetic rodents. Male OZR develop insulin resistance at an early age, characterized by elevated insulin and triglycerides with impaired glucose tolerance but normal fasting hyperglycemia. We examined glucose homeostasis using chronic measures of blood glucose by telemetry in undisturbed rats because of previous reports of exaggerated stress responses. We observed that although young adult (12-14 weeks old) male OZR have normal fasting blood glucose, they are chronically hyperglycemic with access to food. Treatment of OZR with metformin or pioglitazone restored fed blood glucose levels with access to food and enhanced baroreflex-mediated bradycardia and activation of the NTS, as suggested by phenylehphrine-induced c-Fos expression. In contrast, treatment of LZR did not alter glucose or

affect baroreflex-mediated bradycardia and activation of the NTS. Neither treatment reduced elevated AP and insulin in OZR, suggesting the lowering of blood glucose was effective for restoring baroreflexes in young adult male OZR, even in the face of hypertension.

In the second project I examined whether the delayed onset of impaired baroreflexes in hypertensive female OZR could be due to their ability to maintain a normal blood glucose and baroreflex-mediated activation of the NTS. Premenopausal obese women protected from diabetes, suggesting they would be protected from deficits produced by hyperglycemia. I observed that intact baroreflex-mediated bradycardia in young adult female OZR extended to preserved sympathetic baroreflexes and baroreflex-mediated activation of the NTS in 12-15-week-old female OZR. Furthermore, although these OZR were hypertensive and hyperinsulinemic, fed glucose levels and glucose tolerance are comparable to LZR. In contrast, by 6 months of age, baroreflex-mediated bradycardia was blunted in female OZR. However, fed glucose was only mildly elevated and baroreflex-mediated activation of the NTS was comparable in OZR and LZR. These data suggest the ability to maintain glucose homeostasis in young adult female OZR coincides with a preservation of baroreflex-mediated bradycardia and activation of the NTS. However, the later development of impaired baroreflex-mediated bradycardia in female OZR occurs through mechanisms distinct from those observed in male OZR.

The third project examined whether preventing hypertension in male OZR protected against the development of impaired baroreflexes and activation of the NTS. Treatment with losartan or hydralazine normalized baseline AP in male OZR without affecting hyperinsulinemia, dyslipidemia, or hyperglycemia. Furthermore, these treatments enhanced baroreflex-mediated bradycardia and activation of the NTS in male OZR. However, even when AP was normalized in male OZR, baroreflex-mediated bradycardia was still smaller in treated OZR compared to

like-treated LZR, suggesting other mechanisms also contribute to the blunted baroreflexes.

Together these studies suggest that the development of hyperglycemia and hypertension in male OZR contribute to impaired baroreflex-mediated bradycardia and activation of the NTS in male OZR. However, the ability of female OZR to maintain glucose homeostasis preserves baroreflexes despite the presence of hypertension and hyperinsulinemia. Furthermore, when female OZR later develop impaired baroreflex-mediated bradycardia, this deficit occurs by mechanisms that differ from male OZR, highlighting the need to examine both sexes for the development of cardiovascular and metabolic disorders.

BRAINSTEM MECHANISMS THAT IMPAIR AUTONOMIC REGULATION

OF BLOOD PRESSURE WITH OBESITY

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ADN	Aortic depressor nerve
AHA	American Heart Association
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	Activated protein kinase
ANOVA	Analysis of variance
Ang II	Angiotensin II
AP	Arterial pressure
AP-1	Activator protein 1
ARB	Angiotensin II AT1 receptor antagonist
AT1	Angiotensin II receptor type 1
AUC	Area under the curve
BMI	Body Mass Index
CCK	Cholecystokinin
CVLM	Caudal ventrolateral medulla
EPSC	Excitatory postsynaptic potential
Fra	Fos related antigen
GABA	gamma-aminobutyric acid
GLUT	Glucose transport protein
GTT	Glucose tolerance test
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
HR	Heart rate
HSD	Honestly Significant Difference
HYD	Hydralazine
IML	Interomediolateral
i.p	Intraperitoneal

IPSC	Inhibitory postsynaptic potential
LOS	Losartan
LZR	Lean Zucker rat
MET	Metformin
MetS	Metabolic Syndrome
NA	Nucleus Ambiguus
NMDA	N- methyl-D- aspartate
N.S	Not Significant
NTS	Nucleus Tractus Solitarius
OZR	Obese Zucker rat
PBG	Phenylbiguanide
PE	Phenylephrine
PPAR	Peroxisome proliferator-activated receptor
PTP1B	Protein tyrosine phosphatase1B
RVL	Rostral ventrolateralis
RVLM	Rostral ventrolateral medulla
SE	Standard error
SNA	Sympathetic nerve activity
TBS	Tris-buffered saline
TG	Triglyceride

CHAPTER I

DISSERTATION OVERVIEW

The prevalence of obesity has risen at an alarming rate, with approximately 37% of the adult US population classified as obese when defined by a body mass index of greater than 30 (178). In 2015-2016, this percentage represented 93.3 million adults within the United States who were affected by obesity (93). With the rising incidence of obesity, the prevalence of metabolic syndrome (MetS) has also grown significantly. MetS is now considered to be a global epidemic, occurring in approximately one-quarter of the world's population (212). The American Heart Association defines MetS as the presence of at least three of the following attributes 1) abdominal obesity (waist circumference of >40 inches in men and >35 inches in women), 2) elevated plasma triglyceride levels (>150 mg/dl), 3) reduced HDL cholesterol (<40 mg/dl in men and <50 mg/dl in women), 4) hypertension (≥130/85 mmHg), or 5) elevated fasting blood glucose (≥100 mg/dl). Because the traits of MetS are significantly associated with increased risks for the development of cardiovascular disease and diabetes, MetS poses significant social, economic and clinical burdens by promoting premature mortality and morbidity.

Not only are the presence of these attributes of MetS associated with deleterious outcomes, individual traits such as hypertension and hyperglycemia appear to exacerbate each other. For example, the presence of MetS enhances the prevalence of hypertension-associated dysfunctions like left ventricular hypertrophy, diastolic dysfunction, coronary heart disease,

retinopathy and microalbuminuria (203). Furthermore, in a prospective study of hypertensive patients without cardiovascular disease, the rate of cardiovascular events almost doubled in patients that also had MetS, particularly with diabetes (215). The development and exacerbation of hypertension in MetS patients is also aggravated by the presence of insulin resistance before the presence of fasting hyperglycemia (37). Hypertension and insulin resistance appears to have a reciprocal relationship with approximately 50% of hypertensive patients having insulin resistance (199) and approximately 80% of diabetic patients presenting with hypertension (136, 271). These observations strongly suggest that negative interactions of attributes of MetS further advance premature morbidity and mortality, and suggest treatments of individual attributes should consider the presence of MetS

Although the absolute level of arterial pressure (AP) is the diagnostic measure for hypertension, elevated levels of absolute AP in MetS are also associated with increased variability of AP (AP) (60, 98). This increase in AP variability is associated with worse prognosis in subjects with hypertension (155), and in obese individuals increased variability of AP known to further aggravate the progression of diabetes (181, 228) thereby, contributing towards increased incidence of cardiovascular events (155). Furthermore, independent of hypertension, increased variability of AP is a significant independent risk factor for end-organ damage, stroke and cognitive decline (73, 182, 210, 258, 260). These data suggest that both absolute levels of AP and the variability of AP should be assessed for a more complete prognosis, particularly in the setting of MetS.

The variability of AP is tightly controlled by arterial baroreflexes, which act to reduce moment-to-moment fluctuations in AP and maintain AP homeostasis. Baroreflexes minimize the variability of AP by altering sympathetic and parasympathetic outflow to the heart, blood

vessels, and kidneys to produce changes in cardiac output, vasomotor tone, and blood volume that yield a consistent AP. The baroreflex pathway begins with the sensation of AP by mechanoreceptors in the aortic arch and carotid sinus. These baroreceptors give rise to afferent nerve fibers within the vagus and glossopharyngeal nerves, which communicate moment-to-moment changes in AP to the brain stem by projecting to the nucleus tractus solitarius (NTS). The information from the NTS is then relayed to other autonomic-related brainstem regions to regulate efferent autonomic outflow to vessels and heart to maintain a stable level of AP. In MetS patients, arterial baroreflexes are impaired (241), which is very likely to contribute to the observed increased variability of AP in this population (60, 98).

Multiple attributes of MetS are associated with impaired baroreflexes including elevated AP, insulin, glucose, glucocorticoids, inflammatory factors, oxidative stress, and dyslipidemia. In particular, hypertension is strongly linked with the impairment of the gain and range of baroreflexes (26, 81, 172). Hyperglycemia associated with type I diabetes, type II diabetes or stroke is associated with reduced baroreflex sensitivity independent of hypertension (74, 162, 229). Moreover, increased insulin resistance (57, 149), inflammation (196) , lipids (69) and high-fat diet (10, 166) are all independently and positively correlated with reduced baroreflex sensitivity. However, whether the correction of hypertension or hyperglycemia alone in the setting of MetS is sufficient to restore baroreflex function and reduce AP variability is not known.

Obesity in both men and women is associated with the MetS attributes of hypertension, hyperinsulinemia, hyperlipidemia, and increased susceptibility to diabetes. However, the degree of development of these MetS attributes differs between the sexes. Compared to obese women, men with a comparable degree of obesity exhibit a higher degree of hypertension,

hyperlipidemia, hyperinsulinemia, and impairment of glycemic control (130). In the setting of obesity, elevated muscle sympathetic nerve activity (SNA) and cardiac sympathetic tone are generally observed to be higher in obese males compared to obese premenopausal women (61). Moreover, in women, basal levels of muscle SNA do not directly correlate with obesity unlike men (133, 239). These observations highlight the importance of understanding how MetS affects both men and women in order to optimize treatments to combat this deleterious cluster of attributes.

Differences in consequences of MetS between men and women are likely impacted by sex differences in the regulation of cardiovascular and metabolic function in healthy individuals. For instance, young adult, healthy premenopausal women present with enhanced baroreflexes compared to age-matched healthy men (96). However, women do develop reduced baroreflex efficacy as they age, particularly after menopause (41). In conjunction with impaired baroreflexes function (41), postmenopausal women present with a significant decline in insulin sensitivity (249). In agreement, estradiol impacts glucose metabolism both centrally and peripherally, and estrogen replacement therapy in postmenopausal women protects against the development of diabetes (88). Although estradiol appears to preserve glycemic function in women, it is not known whether women with MetS have delayed development of impaired baroreflexes coincident with the timing of changes in glycemic control.

Similar to MetS patients, animal models of MetS also present with impaired baroreflexes (141, 218). Analogous to obese humans, Obese Zucker rats (OZR) develop metabolic, autonomic and cardiovascular deficits as they gain excess body weight. The OZR have a leptin receptor mutation rendering the receptor unresponsive to leptin (110). Shortly after weaning this absence of leptin signaling promotes excessive weight gain due to impaired resting metabolic rate and

hyperphagia of standard rat chow (56). In contrast, lean Zucker rats (LZR) with functional leptin receptors do not develop these traits and serve as normal weight control. By 6-7 weeks of age, OZR already have elevated insulin and triglycerides compared to age-matched LZR, and these persist into the adulthood (67). By 12-14 weeks of age, male OZR develop elevated SNA that drives elevated AP, and these changes coincide with the development of impaired baroreflexes and NTS function (218). Interestingly, although female OZR at this age develop excessive weight gain and hypertension they do not have impaired baroreflex-mediated control of HR. Instead, baroreflex impairment emerges later in life and is present in female OZR by six months of age (240). However, the mechanisms contributing to the delayed development of impaired baroreflexes observed in females in the setting of MetS is not known.

Therefore, the overall goals for this dissertation were to determine whether poor glycemic control or hypertension contribute to the impairment of baroreflexes and baroreflex-mediated activation of the NTS in the setting of MetS in male OZR, and to determine whether delayed onset of impaired baroreflexes in female OZR coincides with preserved glycemic control and baroreflex-mediated activation of the NTS.

OVERALL HYPOTHESIS AND SPECIFIC AIMS

We hypothesized that in young adult male OZR poor glycemic control and elevated AP contributes to impaired baroreflexes and activation of the NTS and that preserved baroreflexes in young adult female OZR coincide with delayed onset of impaired glycemic control and activation of the NTS

To address our hypothesis 3 specific aims were developed to determine glycemic control and baroreflex function in male and female obese and lean Zucker rats.

<u>Specific Aim 1</u>: To determine whether preventing the development of impaired glycemic control by treatment with a biguanide or a thiazolidinedione improves impaired baroreflexes and activation of the NTS in young adult male OZR

To selectively restore glycemic control independent of hyperinsulinemia and hypertension OZR were treated with 1 of 2 anti-diabetic treatments, 1) Metformin, a biguanide that primarily acts to reduce gluconeogenesis in liver and improve insulin sensitivity and 2) Pioglitazone, which acts on PPAR receptors to improve glycemic control. To control for nonspecific effects of the drugs, LZR were also treated. Because OZR have been shown to develop stress reactivity (86, 144), blood glucose was measured chronically by telemetry.

<u>Specific Aim 2</u>: To determine whether the preserved baroreflex-function in female OZR may be due to their ability to maintain glucose homeostasis and whether the presence of normal baroreflex-mediated bradycardia response reflects preserved sympathetic baroreflexes and NTS function.

Cardiac baroreflex efficacy was examined in female OZR and LZR at 2 age ranges to confirm a previous report of normal baroreflex-mediated bradycardia at 3 months of age becomes blunted by 6 months of age. To confirm that the change in baroreflex-mediated bradycardia was reflective of changes in baroreflex-mediated changes in SNA, splanchnic SNA was examined over a wide range of AP. To determine whether intact baroreflexes in 3-month-old

female OZR coincided with a preserved glycemic control and baroreflex-mediated activation of the NTS, these attributes were assessed in female OZR and LZR.

<u>Specific Aim 3</u>: To determine whether preventing hypertension by treatment with vasodilator or an angiotensin receptor antagonist enhances impaired baroreflexes and activation of the NTS in young adult OZR.

The impact of two anti-hypertensive drugs with differing mechanisms of action were examined to control for potential effects of the drugs unrelated to their ability to reduce AP, 1) Losartan, an angiotensin II AT1 receptor antagonist (ARB) that reduces MAP by blocking the AT1 receptor in the periphery and the brain to reduce SNA and peripheral resistance and 2) Hydralazine, a vasodilator lowers MAP by decreasing peripheral resistance and does not affect SNA. The impact of these treatments upon baroreflex-mediated bradycardia and activation of the NTS were examined in age-matched young adult male OZR and LZR.

This series of studies aims to provide novel insights into the relationship between glycemic control and baroreflex-mediated activation of the NTS and bradycardia in male and female Zucker rats to provide a model for future investigations into cellular mechanisms underlying the deleterious effects of impaired regulation of glucose and AP in the setting of obesity. The OZR provides a rodent model of obesity that mimics the traits of MetS observed in humans, suggesting results obtained in these rats will enhance our understanding of MetS in obese humans.

LITERATURE REVIEW 1

METBOLIC SYNDROME: ATTRIBRUTES, INTERACTIONS, AND CONSEQUENCES

Metabolic syndrome (MetS) describes a cluster of interrelated traits that foster cardiovascular disease and diabetes to promote premature morbidity and mortality (113). In 1988, the initial concept of MetS, then referred to as Syndrome X, was focused on a potential causative relationship between insulin resistance and increased risk of cardiovascular disease (201). Since then, the definition of MetS has been revised several times to provide criteria for diagnosis to initiate treatment based on risk of later disease, rather than as a means to understand the causative relationships among the traits. Currently, the American Heart Association (AHA) defines MetS as the presence of at least 3 of the following attributes with stated threshold values:

1) abdominal obesity defined by a waist circumference of > 40" in men and > 35" in women

2) elevated triglycerides (TG) ≥ 150 mg/dl

3) reduced HDL cholesterol < 40 mg/dl in males, and < 50mg/dl in females

4) hypertension with systolic AP ≥ 130 mmHg or diastolic AP ≥ 85 mmHg

5) elevated fasting glucose ≥ 100 mg/dl.

This diagnostic tool has value because the presence of these traits together increases risk of cardiovascular disease more than the presence of any one of them alone (84). The defining traits are generally accepted, but definitions differ slightly in terms of the threshold values, whether obesity is a required attribute, and whether treatment of a trait can also define its presence.

Although this definition recognizes the traits often occur together, it does not provide any insights into how these traits impact each other or how one trait should be treated in the presence of another.

Obesity

Obesity is the only visible trait of metabolic syndrome and is the most modifiable risk factor, because lifestyle changes can yield weight loss-related restoration of function (34, 127). For the International Diabetes Foundation obesity is required for diagnosis of MetS, along with 2 of the other traits outlined by the American Heart Association (4). Obesity itself is not a disease, but its presence increases the likelihood that homeostatic processes have become overwhelmed and developed into pathological compensatory mechanisms. The utility of its inclusion is that obesity is readily observable and can prompt examination for other MetS traits, because obesity increases the likelihood of other traits of MetS (189). However, the requirement of obesity as a trait for MetS may delay identification of individuals who are significantly insulin resistant without excess adiposity (83).

Initially, body mass index was used as the rubric for obesity because height and weight are routinely measured with accuracy and have predictive value (209). However, studies suggesting excess abdominal fat near the viscera was more causally related to the development of MetS traits (114) prompted the use of waist circumference as the measure of excess adiposity. In agreement, with equivalent degree of excess adiposity, premenopausal women tend to accumulate more subcutaneous fat and are at lower risk for developing MetS than men, who tend to accumulate visceral fat (186, 257). Unfortunately, measures of waist circumference are less

reliable, and the relationship between degree of abdominal adiposity and risk for disease varies significantly by ethnicity (49). Furthermore, data from a National Health and Nutrition Survey showed a very tight correlation between body mass index and waist circumference regardless of age, sex, or ethnicity (64). Likewise, the European Group for the Study of Insulin Resistance reported the use body mass index or waist circumference were equally effective for predicting the presence of insulin resistance (62).

Excessive weight gain occurs when the ingestion of calories significantly and consistently exceeds energy expenditure, regardless of underlying causes. The creation of a persistent positive energy balance that results in a cascade of pathological compensatory responses involving dysfunctional adipocytes, inflammation, dyslipidemia, hypertension and insulin resistance with compensatory hyperinsulinemia (46, 51, 75). As discussed below, insulin resistance appears to be at the core of many of these pathologies (199, 201, 213, 214). However, not all obese people are insulin resistant, and significant insulin resistance can be present in the absence of excess adiposity (1, 70, 167, 168, 185). The overlapping but incomplete coexistence of obesity and insulin resistance serves as a reminder that although the presence of either one increases the likelihood of the observing the other, the expression of these traits is also influenced by genetic, environmental, and lifestyle factors.

Obesity, Adipocyte Dysfunction, and Insulin Resistance

Obesity promotes a vicious cycle of deleterious interactions between adipocytes and insulin that foster dyslipidemia, impaired glucose homeostasis, and cardiovascular disease. With excess weight gain, adipose tissue expands in cell number and size (33, 140), and hypertrophied,

insulin-resistant adipocytes secrete less beneficial and more harmful factors into the circulation. For example, reduced release of adiponectin by hypertrophied adipocytes impairs the ability of adiponectin to maintain insulin sensitivity, combat atherosclerosis, and vasoconstriction (95, 148), because adiponectin normally stimulates AMPK to promote fatty acid oxidation and glucose uptake in muscle, endothelial nitric oxide production to promote vasodilation, and PPARα activity to decrease inflammation (46). In addition, hypertrophied, insulin-resistant adipocytes secrete less transforming growth factor β, interleukin10, and nitric oxide, which normally promote insulin sensitivity and combat atherosclerosis (33, 91). Instead these adipocytes secrete more proinflammatory cytokines such as tumor necrosis factor α, interleukin 6, leptin, and resistin to further promotes insulin resistance (33, 254, 261-263). Hypertrophied adipocytes also become less sensitive to insulin-induced suppression of lipolysis, causing increased release of fatty acids into the circulation that compete with glucose in muscle and the liver to lead to an imbalance in the glucose-fatty acid cycle (20, 91). Excess fatty acid taken up by muscle is either stored as triglycerides or used for oxidation, and excess fatty acid stored in the liver contributes to MetS-related hepatic steatosis or non-alcoholic fatty liver disease (82). The free fatty acids also promote insulin resistance by impairing the ability of insulin to suppress hepatic glucose output and stimulate glucose uptake by skeletal muscle. Additionally, free fatty acids inhibit insulin secretion from the pancreas (121). Thus, excess weight gain leads to reduced responses of adipocytes to insulin, and in turn these dysfunctional adipocytes impair insulin secretion and actions to further develop insulin resistance. Like obesity, insulin resistance is not a disease unto itself, but rather serves as a gateway for the development of attributes that promote cardiovascular disease and type 2 diabetes (200).

As a primary regulator of blood glucose, insulin is released by pancreatic beta cells after ingestion of a meal to increase oxidation and uptake of glucose by muscle and adipocytes and reduce gluconeogenesis by the liver (264). The binding of insulin to its dimeric receptor activates a tyrosine kinase domain to trigger a cascade of phosphorylation reactions that ultimately phosphorylate and inactivate glycogen synthase kinase 3 to promote glycogen synthesis. Additionally, insulin promotes glucose uptake by facilitating the transport of GLUT 4 (glucose transport protein) to the cell surface in skeletal muscle and adipose tissue (264). As insulin's actions at adipose tissue and skeletal muscle become diminished in MetS, the pancreas releases more insulin in order to maintain euglycemia. This hyperinsulinemia is compounded by the development of leptin resistance, as leptin continues to be secreted by adipocytes in proportion to degree of adiposity, but can longer inhibit the production of insulin by pancreatic beta cells (80, 269). Unabated overproduction of insulin by the pancreas can eventually lead to pancreatic failure, that transforms type 2 diabetes to the type 1 phenotype whereby reduced insulin exacerbates impaired glucose homeostasis (54). Conversely, although most insulin-resistant people can maintain a normal or close to normal glucose tolerance, the requisite hyperinsulinemia itself has adverse effects upon other targets that do not have the same degree of insulin resistance (200). For example, hyperinsulinemia in the presence of excess fatty acids fosters overproduction of triglycerides by the liver, which was the initial abnormality in lipid metabolism shown to be linked with hyperinsulinemia due to insulin resistance (66, 72). The presence of hypertriglyceridemia and other insulin-induced lipid abnormalities increases the risk of developing cardiovascular diseases (145, 233).

The elevated SNA associated with obesity also appears to promotes insulin resistance (17, 120). In this case, augmented vasoconstriction of the microcirculation impairs the

postprandial rise in skeletal muscle blood flow and uptake of insulin, thereby promoting additional insulin production (245). Furthermore, obesity is associated with diminished insulin-mediated vasodilation and capillary recruitment via endothelial nitric oxide synthesis (47, 118). Although this microvascular dysfunction appears not to play a role in mediating hypertension (104, 147), it directly affects insulin-mediated glucose uptake in skeletal muscle (48). In addition, chronic hyperglycemia is associated with arterial dysfunction that promotes vasoconstriction. The ability of obese women to better maintain glycemic control with less hyperinsulinemia than obese men may underlie their resistance to the development of MetS-associated cardiovascular diseases during premenopausal years (130).

Obesity and Hypertension

Excess weight gain positively correlates with the development of hypertension (59, 227, 250), and hypertension observed twice as often in the obese population compared to individuals of recommended body weight (107). In addition, with similar degrees of obesity, men exhibit more severe hypertension than women, suggesting sex hormones may alter the body's responses to obesity or protect from consequences of deleterious obesity-related attributes (130). Likewise, in animal models of obesity, hypertension develops much earlier in males than females, suggesting that examination of sex differences could provide novel insights into the mechanisms underlying obesity-related hypertension (191, 206, 238). As is the case with diabetes, hypertension arises from multiple, distinct but related disturbances within the regulatory mechanisms for the maintenance of a normal AP.

Although historically the kidney was proposed as the long-term regulator of AP (90), strong evidence suggests elevated sympathetic nerve activity to cardiovascular targets plays an important role in promoting elevated AP (58). The cardiovascular targets themselves are also likely contributors to hypertension, with changes in basal vascular tone brought about by altering responses of the microcirculation to circulating and neural inputs to increase vascular resistance (112). Furthermore, the kidneys contribute to hypertension with maladaptive changes in excretion of water and sodium along with enhanced release of renin to promote production of circulating angiotensin II (Ang II) (23, 220). Angiotensin II, in turn, acts in the periphery to constrict vessels, promote adipocyte dysfunction, stimulate release of adrenal aldosterone, and enhance neurotransmission at sympathetic nerve terminals (21, 32, 68, 118, 175, 204). In addition, circulating Ang II acts at circumventricular organs within the central nervous system to stimulate sympathetic nerve activity, release of vasopressin, and ingestion of fluids (68, 138). As discussed above, diseased adipose tissue also releases excess Ang II and aldosterone-releasing factors to stimulate the release of adrenal aldosterone, thereby contributing to excessive sodium retention by the kidneys, vascular stiffening, and vascular dysfunction by oxidative stress, inflammation, endothelin I, and reduced nitric oxide (28, 108, 115). The interactive nature of these circulating and neural factors complicates the management of hypertension, which is sometimes ineffective even with multiple simultaneous treatments (22, 247). Conversely, one treatment may combat hypertension by altering multiple AP-raising factors. For instance, antagonism of Ang II-AT1 receptors can lower AP by reducing vasoconstriction, aldosterone release, sodium retention, inflammation, and elevated SNA to cardiovascular targets (170).

With the ingestion of calories the sympathetic nervous system contributes to beneficial homeostatic processes such as thermogenesis (12, 135, 180), but excess food intake fosters

obesity and promotes harmful elevations in SNA, particularly to cardiovascular-related targets (11, 59). The sympathetic nervous system has the capacity to alter cardiac output via HR and stroke volume and to alter total peripheral resistance via vasoconstriction and blood volume. In obese people and animals, autonomic balance is shifted toward less parasympathetic control of HR and more sympathetic control of the heart, blood vessels, muscles, and kidneys to promote hypertension (119, 157). In men, basal levels of muscle SNA positively correlate with degree of obesity (133, 239), but in women muscle SNA is more closely related to AP (133), suggesting interactions for obesity, SNA, and AP that are also influenced by other factors.

Sympathetic nerve activity is stimulated by multiple mechanisms in the setting of obesity. Circulating factors such as insulin are elevated with MetS and can activate neurons in the arcuate nucleus of the hypothalamus to stimulate sympathetic nerve activity (8, 59, 68). However, insulin appears to predominantly activate lumbar SNA to aid in the disposal of glucose by muscle (142, 195), and insulin's sympathoexcitatory effects on AP are offset by insulin's ability to directly dilate blood vessels (47). The expanding adipose tissue that occurs with obesity provides a significant source of circulating factors to activate the sympathetic nervous system, and visceral adiposity is strongly linked with increased sympathetic activity (6, 7). Leptin is secreted by adipocytes, and plasma levels of leptin rise with increasing adiposity (33, 38, 134). Like insulin, circulating leptin acts at the arcuate nucleus to stimulate sympathetic nerve activity (94, 146), but obesity promotes resistance to the actions of both leptin and insulin (27, 38, 121, 177). A selective leptin resistance theory has been proposed as a mechanism for leptin to continue elevating sympathetic nerve activity and AP in the setting of obesity (38). However, leptin is not essential, because obesity still fosters hypertension in the absence of leptin's actions

(97, 102, 218), and leptin can increase sympathetic nerve activity without raising AP (97). Furthermore, in obese people, leptin is correlated with body mass index, but not AP (255).

Several key regions within the brain that contribute to elevated SNA and hypertension with obesity have been identified. The rostral ventrolateral medulla is the brain stem source of sympathetic drive to cardiovascular targets (44), and this drive is elevated in obesity-related hypertension (106, 234) as well as many other forms of hypertension (225). Local Ang II contributes to the overactivation of presympathetic neurons in the rostral ventrolateral medulla in obese rats (15, 106) in addition to its ability to stimulate SNA via actions at circumventricular organs (68, 184). Similarly, circulating insulin and leptin can act at the arcuate nucleus to promote activation of hypothalamic melanocortin 4 receptors (197, 226, 251), which also contributes to elevated SNA and AP in several forms of sympathetically-driven hypertension including obesity (42, 53, 237).

In addition to altering basal sympathetic and parasympathetic tone, MetS is associated with impaired autonomic reflexes that affect AP. In particular, baroreflex-mediated changes in HR and SNA are diminished with insulin resistance and hypertension (78, 81, 139, 246, 270). Impaired baroreflexes often appear before hypertension in animal models of obesity (166, 218, 244), and obese people are more likely to have impaired baroreflexes with or without hypertension (19, 77, 79, 230). In early stages of MetS, baroreflex-induced bradycardia appears to be particularly affected (87, 103), and this vagal defect is later followed by impaired sympathetic baroreflexes (103). Diminished baroreflexes likely contribute to hypertension (89, 150), and chronic stimulation of baroreceptor inputs can produce lasting decreases in AP (152, 259). In addition to hypertension, impaired baroreflexes promote increased variability of AP (29, 160, 248, 252), an independent risk factor for deleterious cardiovascular incidents such as

stroke and cerebrovascular-related cognitive decline (123, 159, 171, 187, 210). These topics are discussed in further detail in the second literature review that follows.

Summary

Stedman's Medical Dictionary defines a syndrome as "The aggregate of signs and symptoms associated with any morbid process, and constituting together the picture of the disease" (232). In agreement with this definition, MetS is a cluster of deleterious attributes with co-dependence that exacerbate each other. People diagnosed with MetS are at increased risk for developing cardiovascular diseases and type 2 diabetes in conjunction with other devastating conditions such as malignancy, degenerative arthritis and infertility. Early detection and clinical intervention have the capacity to prevent or lessen the risk of these complications and associated permanent damage. However, successful treatments for the deleterious consequences of MetS are hindered by the silent, significant progression of risk factors long before overt symptoms are present. Insulin resistance has multiple deleterious physiological consequences before fasting blood glucose is impaired. Furthermore, recent evidence shows that static threshold values for attributes such as hyperglycemia and hypertension do not adequately identify abnormal fluctuations in glucose and AP that are now recognized to independently promote deleterious cardiovascular incidents and cognitive decline (123). Because early stage insulin resistance fosters elevated triglycerides and reduced HDL cholesterol, screening obese individuals with dyslipidemia for glucose intolerance could significantly accelerate the diagnosis of MetS to permit restoration of insulin sensitivity before the occurrence of irreversible organ damage and deleterious cardiovascular events. Studies within this dissertation seek to elucidate connections between the development of MetS-associated traits such as insulin resistance-induced

hyperglycemia and hypertension and the impairment of autonomic regulation of AP by baroreflexes to determine whether current standards of care are adequate for ameliorating this overlooked, deleterious attribute of MetS. Furthermore, these studies will examine whether delayed onset of impaired baroreflexes in obese females coincides with preserved glycemic control that eventually succumbs to dysfunctions analogous to those observed in obese males.

LITERATURE REVIEW II

THE ARTERIAL BAROREFLEX: ANATOMY, PHYSIOLOGY, AND PURPOSE

Over the centuries, the causes and significance of fluctuations in arterial pressure (AP) and heart rate (HR) have intrigued clinicians and researchers. The identification of arterial baroreflexes as a negative feedback mechanism for stabilizing AP provided an explanation for how the body could regulate these variations in HR and AP. Moment-to-moment changes in AP are detected by baroreceptors, which are mechanoreceptors that sense the extent and timing of stretch of the vascular wall of the aorta and carotid arteries. This information is then relayed from baroreceptors to the central nervous system via afferent nerves to promote adjustments in sympathetic and parasympathetic autonomic outflow to vessels and heart and buffer against changes in AP. Because increased variability of AP leads to negative consequences, the importance of this baroreflex mechanism was recognized for its ability to minimize variations in AP by producing alterations in HR and peripheral vascular resistance.

Historical landmark observations in the discovery of the baroreflex pathway

The baroreflex phenomenon were first described in 1836 by surgeon Astley Paston Cooper, who noticed the consequences of producing a rise in AP by carotid occlusion (36, 100). However, the phenomenon was attributed to cerebral ischemia. Interestingly, because various sites involved in the baroreflex pathway were discovered in different experimental contexts, their

collective roles were not appreciated until much later. For example, in 1853 the sympathetic nerve effects on vasomotor tone were discovered independent of the baroreflex phenomenon. The discovery of aortic depressor nerves (ADN) in 1866 led to a trajectory of discoveries related to the baroreflex phenomenon. In 1885, Sewall and Steiner discovered that bilateral sectioning of ADN caused a rise in AP (243). Determination of the aortic arch to be origin of ADN in 1902 led to two discoveries in 1908 that significantly advanced the understanding of ADN and their properties. First, Williem Einthoven determined that aortic distension generated action potentials in the ADN that were pulse-associated with each heart beat (243). Second, Eyster and Hooker discovered that dilatation of the aortic arch caused bradycardia (243). Thus, the concept of baroreceptors and ADN with their possible function came into existence.

The contribution of carotid sinus nerve to the baroreflex phenomenon was not appreciated until decades later. In 1923, Heinrich Edwald Hering discovered that stimulation of the carotid sinus wall caused hypotension in addition to bradycardia, and he described this phenomenon as the carotid sinus reflex (188, 243). It took a turn of the century from the original discovery of the baroreflex phenomenon before the concept of cerebral ischemia was abolished and instead attributed to a reflex response. The development of this new concept of a reflex response was based on experiments by Heymans and Ladon, who used a donor dog to perfuse the head of a recipient dog and learned that sectioning of the vagus abolished the heart responses to induced changes in AP (101). In addition, in 1931, Eberhardt Koch recognized a more precise role of baroreceptors in the baroreflex pathway by describing quantitative properties of baroreceptors such as gain, operating point, and threshold AP (243). Additional studies confirmed baroreceptor properties for encoding AP by investigating impulses from afferent nerve preparations from carotid sinus in relation to changes in AP (55, 131, 231). Collectively,

these observations elucidated anatomical and physiological properties of the sensory limb of the baroreflex and some of the resultant motor effects on the heart and vasculature, providing a rudimentary understanding of baroreflex-mediated responses to changes in AP.

By 1948 the baroreflex-related afferent and efferent end organ responses were well established, and attention was drawn toward central integration sites giving rise to the baroreflex phenomenon. In the 1950s and 1960s several experiments confirmed that the brain stem region of the nucleus tractus solitarius (NTS) was essential for baroreceptor afferent nerves to evoke decreases in HR and AP, because lesions of the NTS abolished these baroreceptor-mediated reflex responses (224). Further study of the properties and site of baroreflex afferent integration at the NTS (224) established the NTS as the site of first synapse of baroreflex afferent nerves (9, 35, 169). The discovery of histochemical fluorescence in 1964 led to a significant breakthrough in identifying and mapping catecholaminergic neurons in NTS and vasopressor area of ventrolateral part of the medulla (30). In 1974, the critical role of rostral ventrolateralis, now known as the rostral ventrolateral medulla (RVLM) in the maintenance of baseline AP was established based on the observation that bilateral inhibition of this specific ventral medullary region produced a significant fall in AP (85). This observation was followed by a number of studies that corroborated the notion of the RVLM as a presympathetic region that generated sympathetic drive (44, 45, 205, 223, 256). Subsequently, direct neuronal projections from RVLM to sympathetic preganglionic neurons in the thoracic spinal cord were identified by retrograde immunocytochemical (207, 208) and electrophysiological methods (16, 164, 173). Thus, the discovery of critical roles of NTS and RVLM for the generation of sympathetic tone and its regulation by baroreceptor afferents provided an anatomical basis for baroreflexes.

Regulation of AP by the autonomic nervous system

The afferent limb of the baroreflex: Arterial baroreceptors are located in the walls of aortic arch and carotid bifurcations of all vertebrate animals. These baroreceptors consist of mechanosensitive afferent nerve endings that detect stretch of the arterial walls proportionate to AP. This information is transduced into electrical signals carried by afferent nerves and relayed to the central nervous system. The arterial baroreflex afferent pathway is comprised of carotid sinus nerves that join cranial nerve IX (glossopharyngeal nerve) and aortic depressor nerves that join cranial nerve X, (vagus nerve), with their associated cell bodies located in the petrosal ganglion and nodose ganglion respectively. These afferent fibers consist of myelinated A fibers and unmyelinated C fibers. The A fibers generally display an initiation pressure threshold of 40-120 mmHg and are capable of discharging with a high frequency of >100 Hz (9). The C fibers have a higher threshold discharge 60->200 mmHg, but discharge at a lower frequency than the A fibers at the same level of AP (9). Large-caliber A fibers, known as Type 1 fibers, are usually inactive at baseline AP owing to a high pressure threshold, but are recruited once threshold AP is reached with a discharge rate that rises with AP (221). Whereas Type II fibers, which include small caliber A fibers and unmyelinated C fibers, are spontaneously active at baseline AP and continuously fire with a discharge related to AP in a sigmoid manner (222).

Central component of the arterial baroreflex: Type 1 and II baroreceptor afferents integrate as excitatory monosynaptic inputs to neurons within the nucleus tractus solitarius (NTS), which serves as the brain stem site for the first synapse in the arterial baroreflex. The NTS is a bilateral structure that receives these monosynaptic inputs from afferents primarily using glutamate as a neurotransmitter (9, 31). Glutamate activates ionotropic (ion channel response) and metabotropic (second messenger system response) glutamatergic receptors within the NTS. Three types of

ionotropic glutamatergic receptors have been identified: N- methyl-D- aspartate (NMDA) and two non-NMDA receptors, a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (9). Barosensitive NTS neurons can be identified as receiving monosynaptic (second order neuron) or polysynaptic (higher-order neuron) inputs based on their response to afferent nerve stimulation. Non-NMDA (AMPA /Kainate) receptors appear to predominate on NTS neurons receiving monosynaptic inputs from baroreceptor afferent nerves (268), which are likely to transmit high-frequency dynamic information to the caudal ventrolateral medulla (CVLM) to regulate sympathetic discharge (13, 266). Whereas, NMDA receptors appear to predominate on NTS neurons receiving polysynaptic connections from baroreceptor afferents (265), these neurons are more likely to project to the forebrain (13).

Integrated baroreceptor afferent information at the NTS is then relayed to other brain stem sites via polysynaptic pathways to regulate sympathetic and parasympathetic efferent nerves. Barosensitive, glutamatergic NTS neurons modulate sympathetic efferent nerve activity by sending glutamatergic projections to the CVLM (3, 143), which in turn activates GABAergic CVLM neurons that project to pre-sympathetic neurons in the rostral ventrolateral medulla (RVLM) (2, 216), thereby, inhibiting RVLM and decreasing sympathetic drive to cardiovascular-related targets. In contrast, parasympathetic efferent nerves are regulated by NTS-mediated, glutamatergic activation of nucleus ambiguus, which contains parasympathetic preganglionic neurons of vagal efferent nerves that innervate cardiac parasympathetic ganglia. Beyond the scope of this discussion, multiple supramedullary areas such arcuate nucleus, subfornical organ, and hypothalamic nuclei such paraventricular nucleus, perifornical nucleus, and dorsomedial nucleus also modulate baroreflex brainstem network (50).

Efferent pathways for arterial baroreflex: For producing baroreflex-mediated changes in HR and AP the major autonomic efferent nerves and targets are parasympathetic inputs to the heart and sympathetic inputs to the heart and resistance blood vessels. Sympathetic efferents of the baroreflex pathway are driven by presympathetic RVLM neurons that innervate the interomediolateral cell column of the thoracic spinal cord. The sympathetic preganglionic fibers leave the central nervous system to innervate sympathetic ganglionic neurons, which are activated by release of acetylcholine from the preganglionic neurons that binds to nicotinic cholinergic receptors on the ganglionic neurons. Sympathetic postganglionic fibers from sympathetic ganglia release norepinephrine to bind to β adrenergic receptors in the heart and kidney and α adrenergic receptors in blood vessels. Sympathetic efferent nerves are activated by a decrease in AP to mitigate hypotension (89). Sympathetic activation raises AP by increasing HR and contractility to increase cardiac output, constricting blood vessels to increase total peripheral resistance, and stimulating renin release from the kidneys to produce angiotensin II (Ang II) (52). Circulating Ang II also constricts blood vessels and acts at the central nervous system to further stimulate sympathetic efferent nerve activity and intake of fluids to increase blood volume. Thus, a hypotensive stimulus can produce neural, hormonal and behavioral responses to act in concert to maintain homeostasis.

In the parasympathetic efferent pathway for the arterial baroreflex, preganglionic neurons in the nucleus ambiguus exit the brain stem as part of the vagus nerve to innervate parasympathetic ganglia near the heart. As seen with sympathetic preganglionic neurons, parasympathetic preganglionic neurons release acetylcholine to activate nicotinic receptors on cardiac parasympathetic ganglionic neurons. These neurons give rise to postganglionic fibers that release acetylcholine to activate muscarinic receptors at the heart and reduce HR. These

efferent nerves are activated by an increase in AP to produce a baroreflex-mediated reduction in cardiac output to decrease AP. A simplistic summary schematic of the arterial baroreflex pathways are depicted below in Figure. 1.

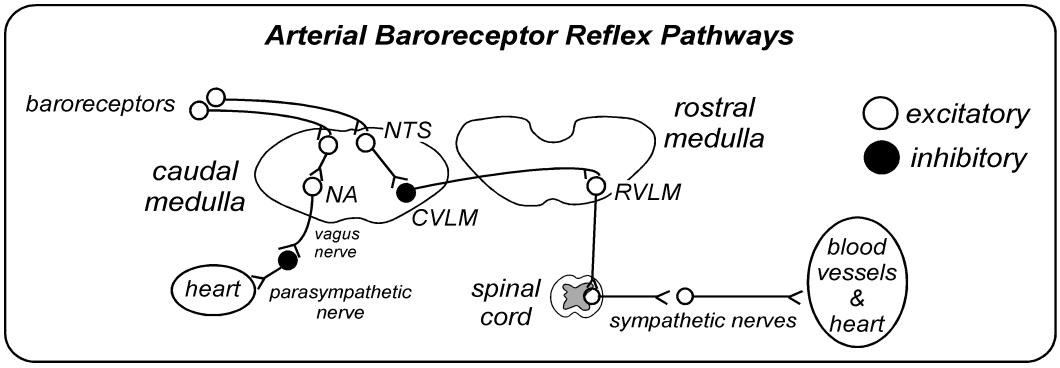

Figure 1. Schematic of pathways for arterial baroreflex-mediated regulation of the heart and vessels. The 2 pathways are bilateral, but shown once for clarity.

Relationships between AP, baroreceptors, and baroreflexes

Clearly, baroreceptor afferent nerve activity and AP are related and impact each other. Some studies suggest a higher AP causes changes in baroreceptor sensitivity as well as impairments in baroreflexes to permit a higher AP both in physiological conditions like exercise or pathophysiological conditions like hypertension (109, 125, 193, 194, 198). Hypertensive people and animals are more likely to have impaired baroreflexes (81, 172, 246), although impaired baroreflexes can occur in the absence of hypertension (65, 79, 137). In some cases, impaired baroreflexes appear to precede hypertension (166), leading support to the notion that this deficit contributes to later chronic rises in AP. In addition, Ang II-induced hypertension can be prevented by denervation of baroreceptor inputs, suggesting these afferents play a key role in this form of hypertension (18). Alternatively, hypertension can exist in the apparent absence of

diminished baroreflexes (172), suggesting other causes underlie the rise in AP. In this case, although baroreflexes can provide a tonic influence upon AP, they may be less effective if the higher AP is associated with reduced activity of baroreceptor afferents (165) or if changes in AP occur beyond the working range of the baroreflex (125).

Early studies suggested baroreflexes were short-term regulators for moment-to-moment changes in AP, with evidence that changes in AP produced resetting of baroreceptors in hours, days, or weeks (129, 174, 198). Baroreceptor resetting to a higher AP shifts the relationship between AP and baroreceptor afferent nerve activity rightward to allow baroreflexes to more effectively defend a higher AP (126). In addition, the brain's response to incoming inputs is also altered by elevated AP, whereby hypertension promotes resetting within the central nervous system. For example, despite the resetting of individual baroreceptor afferents, more baroreceptor afferent nerves are tonically active with hypertension coincident with enhanced GABAergic inhibition within the NTS that could protect these individual NTS neurons from overactivation (117, 267). These types of adaptations are also beneficial for conditions of acute rises in AP or altered cardiovascular needs such as exercise (193, 194). In the short-term, resetting is readily reversible and appears to involve raising the threshold AP for mostly A-fibers, namely myelinated afferents with a high range of firing and narrow threshold (151). In contrast, chronic resetting resulting from a sustained increase in AP is not readily reversible and may result in reduced baroreflex sensitivity (128). Chronic resetting is more associated with unmyelinated C-fibers that operate at over a broader range of AP (128). In 1956, McCubbin et al. first proposed the possibility of long-term baroreceptor resetting based on their observation of reduced rate of baroreceptor afferent firing in chronically hypertensive dogs (165). In support of the notion that baroreflexes are strictly short-term regulators of AP, denervation of baroreceptor

afferent nerves produces the expected acute rise in AP (63, 129, 235), but within a week AP returns to preoperative levels, albeit with increased variability of AP (179, 183, 236). Instead, brain stem nuclei that connect baroreceptor afferents to autonomic efferent nerves adapt to the loss of baroreceptor inputs to maintain pre-denervation levels of AP. Paradoxically, the chronic absence of baroreceptor eliminates the tonic influence of the intermediate NTS upon AP (219). Instead, in the chronic absence of a drive from baroreceptors or the NTS, the CVLM is driven by glutamatergic inputs from unknown sources to restore GABAerigc inhibition the RVLM and maintain sympathetic vasomotor tone and AP at pre-denervation levels (40, 43, 71). This baroreceptor-independent glutamatergic drive to the CVLM is also present in rats with intact baroreceptors and is enhanced by increasing respiratory drive (161). Together, these observations suggest that baroreceptor-independent mechanisms within the central nervous system produce a set-point for AP, and baroreflexes primarily act to buffer against moment-to-moment fluctuations in AP.

Although the lack of change in AP with chronic baroreceptor denervation and the ability baroreceptors and the brain to adapt to changes in AP argue against a significant role for baroreceptors in the long-term regulation of AP, a closer look at their behavior over a variety of conditions suggests otherwise. Clearly baroreceptors can adapt their activity with a rise in AP, but multiple studies suggest that this resetting is incomplete (132, 151, 153), yielding a hypertension-resistant baroreceptor tone that continues to tonically restrain AP. In rabbits with renovascular hypertension, the impact of baroreceptor resetting in individual afferents is offset by the recruitment of active baroreceptor afferent neurons at the higher baseline AP and more activation of the NTS (117). Furthermore, chronic electrical stimulation of carotid sinus nerve produces long-term reductions in AP in hypertensive humans and in dogs with no apparent

impact on baroreflexes or central adaptations (99, 152). Similarly, angiotensin II (Ang II) appears to preserve baroreflexes in the face of hypertension. Ang II-induced hypertension is associated with a chronic activation of the NTS and CVLM in dogs (154) , and no sign of shifting of the sympathetic baroreflex to higher AP in rabbits (18). In addition, the chronically suppressed renal SNA with Ang II-induced hypertension is prevented by sino-aortic denervation (18). On the other end of the spectrum, unlike the effects of baroreceptor denervation, chronic unloading of baroreceptors in the carotid sinus of dogs produces sustained hypertension that is characterized by elevated SNA and plasma renin, reminiscent of the acute responses to hypotension (242). As with most debates in physiology, the answer to the question of whether baroreflexes are important for short-term or long-term control of AP is that baroreflexes likely contribute to both types of control with relative impact changing depending on current cardiovascular needs and underlying disease states.

Baroreflexes and variability of AP

Physiological variations in AP occur in healthy subjects, and in some cases, such as dipping of AP during sleeping hours, are correlated with positive outcomes (92). However, when fluctuations in AP become excessive, target tissues are adversely affected (14, 155). Changes the variability of AP commonly coincide with presence of hypertension, suggesting a causative relationship (158). Compared to normotensive individuals, hypertensive patients more often present with impaired baroreflexes and excessive AP variability (156, 190, 252). However, controlling hypertension with different classes of medications has differing efficacy for reducing the AP variability, suggesting hypertension is only one of the contributors for this instability of AP. Although increased AP variability may have more than one cause, impaired baroreflexes are

highly correlated with reduced HR variability and elevated AP variability (29, 109, 116, 160, 248, 252). In agreement, destruction of baroreceptor inputs causes persistent decreased or absent baroreflexes with a marked increase in AP variability and reduced variability of HR with no change in baseline AP (5, 39, 217, 219). Conversely, carotid sinus stimulation therapy can produce a sustained decrease in AP without altering short-term regulation of AP by baroreflexes (122) . Reduced variability of HR in and of itself is not deleterious, but this state has prognostic value because it reflects impaired baroreflex-mediated changes in HR that stabilize AP. Because impaired baroreflexes can occur without hypertension (5, 39, 65, 79, 111, 137, 166, 217, 219), elevated AP variability may go undetected. In agreement, patients successfully treated for hypertension with residual elevations in their variability of AP are still at significant risk for deleterious cardiovascular incidents such as stroke (211). In addition, increased AP variability is an independent risk factor for development and progression of end organ damage, cardiovascular events, stroke, and cognitive decline (123, 159, 163, 171, 187).

Diagnostic measures of AP variability vary in the time frame they are measured and calculation of their magnitude, but multiple methods are predictive of poor outcomes. Recent studies suggest that long-term measures of AP variability, particularly systolic AP, are indicative of short-term changes with equal value in assessing cardiovascular-related risks (192, 211). Even visit-to-visit variations in AP measures in the clinic over the course of a year have prognostic value (176). Conversely, short-term measures of AP variability are of prognostic value because they indicate a chronic pathology (156, 190, 252). Quantification of AP variability can be measured as the coefficient of variance to control for differences in absolute AP (202, 253), because a higher mean yields a higher standard deviation with a similar percent change. Alternatively, an argument could be made that the number of excursions of AP above a

threshold or the standard deviation of the mean gauges the actual changes the target organ experiences. In a recent study by Kim et al, the prognostic value of both coefficient of variance and standard deviation were comparable (123), suggesting either could be utilized. In addition, when differences in baseline AP are small or absent, coefficient of variance does not provide more information than standard deviation of the mean.

Increased variability of AP is frequently observed in patients with metabolic syndrome (MetS) (60). The observation that impaired baroreflexes can occur independent of hypertension in MetS patients (76) has led to suggestions of other potential causative factors such as external stress, poor diet, diabetes, dyslipidemia, or cardiovascular disease (24, 25, 124). Multiple rodent models of obesity display attributes of MetS in combination with impaired baroreflexes and increased variability of AP. In Sprague-Dawley rats made obese by a high fat diet or rats genetically predisposed to overeat and gain excess weight, such as obese Zucker rats, deficits in baroreflex efficacy appear early and persist with the later development of hypertension (105, 166, 218). Thus, these obese rats provide an excellent model for understanding how MetS impairs baroreflexes independent of hypertension and for determining how the addition of hypertension impacts baroreflex efficacy in the setting of obesity.

Conclusions

Although the relative roles of baroreflexes in short-term and long-term control of AP have been debated, arterial baroreflexes are clearly an essential determinant of the variabilities of HR and AP (29, 109, 116, 160, 248, 252). Changes in AP can promote resetting at the afferent limb and central components of the baroreflex to enhance function at the new AP, but this

phenomenon is not consistent for all circumstances of hypertension. In addition, impaired baroreflexes can occur in the absence of hypertension due to other causes and remain a contributor to increased AP variability with its associated risks. A better understanding of how baroreflexes are altered independent of hypertension will lead to improved treatments to reduce risks of premature morbidity and mortality. Furthermore, inclusion of an assessment of AP variability in standard clinical practice would likely provide further insights into potential causes for this detrimental trait and promote improved treatment to reduce risk of cardiovascular disease, end-organ damage, and cognitive decline.

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CHAPTER II

IMPROVED GLUCOSE HOMEOSTASIS IN MALE OBESE ZUCKER RATS COINCIDES WITH ENHANCED BAROREFLEXES AND ACTIVATION OF THE NUCLEUS TRACTUS SOLITARIUS

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ABSTRACT

Young adult male obese Zucker rats (OZR) develop insulin resistance and hypertension with impaired baroreflex-mediated bradycardia and activation of nucleus tractus solitarius (NTS). Because type 1 diabetic rats also develop impaired baroreflex-mediated NTS activation, we hypothesized that improving glycemic control in OZR would enhance compromised baroreflexes and NTS activation. Fasting blood glucose measured by telemetry was comparable in OZR and LZR at 12-17 weeks. However, with access to food, OZR were chronically hyperglycemic throughout this age range. By 15-17 weeks of age, tail samples yielded higher glucose values than those measured by telemetry in OZR but not LZR, consistent with reports of exaggerated stress responses in OZR. Injection of glucose (1g/kg, i.p.) produced larger rises in glucose and areas under the curve in OZR than LZR. Treatment with metformin (300 mg/kg/day) or pioglitazone (5 mg/kg/day) in drinking water for 2-3 weeks normalized fed glucose levels in OZR with no effect in LZR. After metformin treatment, area under the curve for blood glucose after glucose injection was reduced in OZR and comparable to LZR. Hyperinsulinemia was slightly reduced by each treatment in OZR, but insulin was still greatly elevated compared to LZR. Neither treatment reduced hypertension in OZR, but both treatments significantly improved the blunted phenylephrine-induced bradycardia and NTS c-Fos expression in OZR with no effect in LZR. These data suggest that restoring glycemic control in OZR enhances baroreflex control of heart rate by improving the response of the NTS to raising AP even in the presence of hyperinsulinemia and hypertension.

INTRODUCTION

Excess weight gain is associated with the development of a cluster of attributes known as metabolic syndrome that foster premature cardiovascular morbidity and mortality (33, 52, 54). Obese people are prone to hypertension with an autonomic imbalance favoring reduced cardiac vagal tone and elevated sympathetic nerve activity (SNA) to multiple cardiovascular-related targets (75, 81). In addition, obesity is linked with the development of compromised short-term control of arterial pressure (AP) by baroreflexes (66, 78, 81), which promotes reduced variability of heart rate (HR) and increased variability of AP. Diminished baroreflexes can occur independently from hypertension (28), and increased AP variability confers a significant independent risk for coronary heart disease, renal disease, stroke and cerebrovascular-related cognitive decline (25, 70, 88). Mechanisms underlying the development of impaired baroreflexes in obese people are not well understood.

Obesity is also associated with the development of pre-diabetic attributes that impact autonomic regulation of cardiovascular function before progression into frank type 2 diabetes mellitus. Whether or not fasting hyperglycemia or hypertension are present, hyperinsulinemia and glucose intolerance coincide with impaired baroreflex-mediated control of HR (36, 45, 66, 76). Weight loss improves insulin sensitivity and baroreflexes in obese people (21, 27, 82), but the relationship between these deficits has not been elucidated. Insulin has the ability to raise SNA, alter baroreflexes, and increase AP variability (46, 83). However, diminished cardiac baroreflexes and HR variability observed in patients with type 1 diabetes with normal weight with elevated hemoglobin A1c (HbA1c) suggests that poor glucose homeostasis is a causative factor for impaired control of HR and AP (10, 18, 51).

These deleterious cardiovascular and metabolic attributes are also observed in obese rodents, allowing for more invasive study of underlying mechanisms of disease. Rats or mice made obese by high fat diet or by genetically-driven hyperphagia of standard rodent chow, such as obese Zucker rats (OZR) and *db/db* mice, develop elevated SNA that drives hypertension (23, 40, 59, 80). Before the onset of hypertension, both models of rodent obesity develop impaired baroreflexes (59, 63, 74), which likely contributes to their reduced HR variability (4, 55, 64) and increased AP variability (9, 35). In obese rats, impaired baroreflexes coincide with changes in the brain stem baroreflex pathway (31, 59). Normally, an evoked rise in AP reduces HR via excitation of arterial baroreceptor afferent nerves that activate nucleus tractus solitarius (NTS) neurons in the brain stem, which in turn excite cholinergic neurons in nucleus ambiguus to activate cardiac parasympathetic efferent nerves and GABAergic neurons in the caudal ventrolateral medulla (CVLM) to inhibit pre-sympathetic neurons in the rostral ventrolateral medulla (RVLM) (1). In adult male OZR and LZR, changes in baroreceptor afferent nerve activity over a wide range of evoked changes in AP are comparable, suggesting sensory mechanisms for detection of AP are intact (40). In contrast, direct electrical stimulation of baroreceptor afferent fibers evokes smaller decreases in SNA and mean AP in adult male OZR (40). Likewise, in rats made obese by a high fat diet, electrical stimulation of myelinated baroreceptor afferent nerves evokes smaller decreases in HR, which is due to reduced vagal activation and sympathetic withdrawal (59). As further evidence of centrally-mediated changes, acutely raising mean AP produces fewer c-Fos-positive neurons in the NTS of adult OZR (31) and less inhibition of pre-sympathetic RVLM neuronal activity in rats made obese by a high fat diet (37). In agreement with diminished baroreflex-mediated activation of the NTS, microinjection of glutamate into the intermediate NTS evokes smaller reductions in splanchnic

SNA and mean AP in adult male OZR (31). The NTS appears to be a critical central site of impairment, because glutamatergic activation of the CVLM and GABAergic inhibition of the RVLM produce equivalent decreases in SNA and AP in adult male OZR and LZR (31, 39). In contrast to adult OZR, juvenile OZR and LZR have equivalent baroreflex-mediated changes in SNA and HR that coincide with comparable physiological responses to activation of the NTS (31, 74). These data suggest that the progression of metabolic syndrome reduces baroreceptor afferent-mediated activation of the NTS to yield impaired inhibition of pre-sympathetic RVLM neurons, SNA, and HR. Because OZR become obese with excess intake of standard rat chow (79, 86), the development of obesity-related deficits in autonomic regulation of SNA, HR, and AP can be examined without altering diet composition.

In addition to hypertension and impaired baroreflexes, young adult male OZR have hyperinsulinemia and glucose intolerance in the presence of normal fasting glucose levels (24, 44). As reported in humans, poor glycemic control in rats is associated with diminished short-term control of AP even in the absence of other traits of metabolic syndrome. Streptozotocin-induced hyperglycemia in Sprague-Dawley rats produces diminished baroreceptor-mediated activation of the NTS without increasing body weight, insulin, or AP (26), and this treatment also produces impaired baroreflexes and excess AP variability that are improved by reducing blood glucose (91) . Therefore, the present study examined the hypothesis that improved glycemic control after treatment with metformin or pioglitazone enhances blunted arterial baroreflex-mediated control of HR and activation of the NTS in conscious adult male OZR. In addition, we measured blood glucose continuously by telemetry to determine whether fasting glucose reflects basal glucose levels with *ad libitum* access to food in conscious, undisturbed rats. These experiments provide the first determination of whether improved glucose

homeostasis in pre-diabetic male OZR enhances diminished baroreflex-mediated changes in HR and activation of the NTS.

MATERIALS AND METHODS

Animals

Male OZR [Lepr (*fa/fa*)] and LZR [Lepr (+/+) and (+/*fa*)] were purchased from Charles River (Houston, TX) and were individually housed in centralized animal care facilities with consistent humidity (60 ± 5%), temperature (24 ± 1°C), and light cycle (lights on 7:00 am – 7:00 pm). Rats were fed a standard chow (Prolab RMH 1800, LabDiet). Experiments were performed on young adult (12-17 weeks), age-matched Zucker rats. Animal experiments were conducted according to the National Institutes of Health's *Guide for Care and Use of Laboratory Animals* and the American Physiological Society's *Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training*. All protocols were approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee.

Implantation of glucose-sensing transmitters

After the rats were anaesthetized with Isoflurane, a laparotomy was performed using aspectic conditions. The tip of the transmitter catheter (HD-XG, Data Sciences International) was inserted rostrally into the abdominal aorta distal to the renal arteries. The aortic wall was sealed around the catheter with a piece of mesh and a small drop of cyanoacrylate adhesive. The transmitter was secured to the abdominal wall using 4.0-prolene suture. After closing the incision, rats were kept warm and monitored until fully conscious. Each cage was placed on a receiver (DSI) to continuously measure blood glucose by telemetry. Calibration of the

transmitters was verified using blood samples from the tail with rats in fasted and fed states and with glucose tolerance tests.

Glucose measures and treatment with metformin or pioglitazone

To determine blood glucose levels from rats in fasted (18 hours) and fed states, blood samples were taken from the tail between 8:00 a.m. – 9:00 a.m. with at least 3 days between the samples. To minimize disturbance to the rat during glucose measures, the tip of the tail was snipped with scissors while the rat remained in their home cage with nesting material. A drop of blood from the tail was applied to a glucose test strip that was inserted into a calibrated, hand-held glucometer (Accu-Chek® Aviva Plus). Duplicate blood samples were taken to generate a mean value for each measurement. In fasted and fed rats, analogous baseline values were generated using telemetry by taking the average of 20-second samples every 5 minutes for 1 hour. To determine blood glucose values over the course of one day when rats had access to food, hourly blood glucose values were generated by averaging 5-minute samples spanning 60 minutes from each rat over a 24-hour period that was at least 2 days apart from a fasting period.

The first glucose tolerance test (GTT) was performed 3-4 days after implantation of the glucose transmitter. Rats were fasted overnight for 18 hours with access to water. The next morning cages were removed from their rack and lined up on carts with their telemetry receivers, alternating lean and obese rats. A duplicate baseline blood sample was taken from the tail of each rat at approximately 8 a.m., and then 30-60 minutes later each rat was briefly lifted from their cage and injected with glucose (1g/kg from a 0.5g/ml solution, i.p.). While the rat rested in its home cage, additional tail blood samples were taken at 15, 30, 45, 90, and 120 minutes after administration of glucose. Periodic fed glucose samples were also taken every 1-2 weeks to

ensure calibration of the transmitters. All tail blood sample readings were entered into the telemetry data acquisition software in duplicate for each time point as they were collected (Dataquest® A.R.T. software platform, DSI).

Subsets of LZR and OZR (13 -14 weeks old) began treatment with metformin (300mg/kg/day) in their drinking water (14, 22, 50) during the week after their first glucose tolerance test. These rats were housed next to age-matched OZR and LZR with untreated drinking water. Fluid intake and weight were monitored every 48 hours, and the concentration of metformin was adjusted to provide the correct daily dose. The metformin-treated drinking water was sweetened with an artificial sweetener (2 packets of Splenda©/ 1L water) (42), and these rats had access to HydroGel cups (Clear H_2O) in their cages to ensure proper hydration. Metformin was chosen for its ability to selectively normalize glucose homeostasis in the continued presence of hyperinsulinemia and hypertension (14, 50). Additionally, metformin does not impact glucose levels in LZR (22), allowing for treatment in LZR to control for other potential effects of metformin. Treatment was limited to 2-3 weeks to minimize weight loss (22, 89). After 2-3 weeks of drug treatment, a second GTT was performed in treated and untreated rats to ensure calibration of the transmitter and examine the efficacy of drug treatment.

In order to control for potential effects of weight loss with metformin, another set of rats was treated with pioglitazone (5 mg/kg/day suspended in 0.5% methylcellulose), which tends to promote weight gain while improving glucose homeostasis. In addition, this dose produces minimal effects upon AP in OZR (19, 53). As described below, these rats were used for assessment of AP, baroreflex-mediated bradycardia, and activation of the NTS as indicated by c-Fos expression. Blood samples were taken in the fed state at approximately 9 a.m. in conscious rats to confirm effects of pioglitazone treatment upon glucose, insulin, and lipids.

Phenylephrine infusion in conscious rats to activate baroreflexes and the NTS

After 2 - 3 weeks of treatment, rats were anesthetized with isoflurane (5% in a ventilated box and then 2.0 - 2.5% through a nose cone). A catheter was implanted in the left femoral artery to record AP and HR and the left femoral vein to infuse fluids. As previously described, the free ends of the catheters were tunneled subcutaneously to exit between the scapulae (31). The rats were fitted with a button tether and dual channel swivel (Instech Laboratories, Inc.) attached to a counter-balanced lever arm to allow them to move freely in a covered plexiglass cylindrical cage (MTANK/W and MTOP, Instech). The rats were allowed to recover for 24 - 48 hours with access to food and water. On the morning of the experiment, food and water were removed, and the cages were surrounded with a cover to minimize disturbance to the rat. At approximately 9:00 a.m. a baseline blood sample (0.5 ml) was drawn through the arterial catheter, and the volume was replaced by sterile saline. Then, the arterial line was connected through the swivel to a transducer (NL108T2, Digitimer), and the venous line was connected through the swivel to an infusion pump (Model A-99, Razel). After 30 minutes of baseline recording of AP and HR, phenylephrine was infused to raise mean AP by 40 mmHg for 90 minutes (13 – 31 µl/minute of 0.5 mg/ml of phenylephrine in saline, i.v.). The infusion rate was continuously adjusted to maintain the 40 mmHg rise in mean AP over the 90-minute period. This protocol allowed for measurement of baroreflex-mediated bradycardia within the first 5 minutes and later activation of c-Fos expression in the brain stem. During the last 30 minutes of the infusion, the phenylephrine-filled syringe was replaced by a saline-filled syringe to slowly replace the phenylephrine in the line by the end of the 90-minute period. Mean AP remained elevated throughout the 90-minute protocol. Rats were deeply anesthetized with urethane (1.5 g/kg, i.v. bolus) after the 90-minute infusion and perfused transcardially with 250 ml of

phosphate-buffered saline (pH 7.4) followed by 500 ml of 4% phosphate-buffered paraformaldehyde (Electron Microscopy Sciences). The brains were removed and stored in the same fixative for 48 hours.

Measurement of plasma insulin, cholesterol, and triglycerides

Blood drawn from the arterial catheter of conscious rats was collected into a heparinized tube and immediately centrifuged to isolate plasma. Plasma samples were stored frozen as aliquots for later analysis by ELISA. Measurements were made using a Rat Ultrasensitive Insulin ELISA kit (80-INSRTU-E01, ALPCO) for plasma insulin, a Cholesterol E kit (439-17501, Wako Diagnostics) for total plasma cholesterol, and L-Type TG M reagents for triglycerides (Color A 461-08992, Color B 461-09092 and Multi-Lipid calibrator 464-01601, Wako Diagnostics). Samples were run in duplicate to obtain an average value for each rat.

Immunohistochemistry for c-Fos

Brainstems were sectioned with a Vibratome (30 μm, coronal plane), and sections were placed consecutively in a 24-well dish containing a cryoprotectant solution. The free-floating sections were stored at -20°C until further processing. Immunohistochemistry for c-Fos protein was performed on free-floating sections on an orbital shaker in solutions prepared in Tris-buffered saline (TBS, pH 7.4) at room temperature unless specified otherwise. Sections from rats of different groups were run in adjacent columns within the same staining dishes to ensure comparable staining conditions. The sections were incubated with 1% hydrogen peroxide (30 minutes) to block endogenous peroxidases, rinsed in TBS, and blocked in 10% horse serum (45 minutes). Then, sections were incubated with a goat-anti c-Fos primary antibody (1:2,000; 4°C; 48 hours; Santa Cruz Biotechnology; sc-52G), as previously described (31). After rinsing in TBS, sections were incubated with a biotinylated donkey anti-goat secondary antibody (1:400; 1

hour; #705–066-147, Jackson Laboratories) followed by an avidin-biotin solution (1 hour; PK-6100; Vector Laboratories). The c-Fos immunoreactivity was revealed by incubation with a nickel-intensified 3–3'diaminobenzadine solution. The reaction was closely monitored for 8-10 minutes and terminated with TBS rinses when staining became visible. Processed sections were mounted onto gelatin-coated slides and air dried overnight. Slides were submerged in a series of alcohols and xylenes and then coated with DPX mounting medium (Sigma-Aldrich) to affix coverslips. The c-Fos-immunoreactive (Fos+) neurons were mapped and counted bilaterally in the NTS at 4 rostro-caudal levels using Neurolucida (MFB Bioscience) using blinded conditions as previously described (31).

Data acquisition and analysis

Glucose levels were recorded by telemetry for 20 seconds every 5 minutes for 3 - 4 weeks. For 24-hour measures, hourly averages were produced from the 5-minute data samples. Variability of glucose was calculated as the standard deviation of the mean using 2 hours of recordings by telemetry. The AP was measured through a femoral artery catheter, and the mean AP and HR were derived from the AP pulse using a calibrated low-pass filter (N125) and a spike trigger (NL201), respectively (Neurolog System, Digitimer). Analog signals were converted to digital (Micro 1401, Cambridge Electronic Design) and viewed online using Spike2 software (Cambridge). All group data are expressed as mean ± SEM. Significant statistical difference was set at $P < 0.05$. Before treatments, baseline parameters in age-matched OZR and LZR were compared using unpaired t-tests. Values at multiple time points were compared using an ANVOA with repeated measures. After drug treatments, all measures were compared using an appropriate ANOVA, and pairwise comparisons were made using Tukey's Honestly Significant Difference (HSD) *post hoc* tests. (SigmaStat, version 3.5).

RESULTS

Blood glucose values in OZR and LZR before metformin treatment

Young adult male OZR weighed 59% more than age-matched LZR (Table 1). Morning fasted blood glucose measured by telemetry was comparable in OZR and LZR (Fig. 1*A*). In contrast to fasting conditions, access to food produced a rise morning blood glucose levels in OZR but not LZR, resulting in a significantly elevated morning fed glucose level only in OZR (Fig. 1*A*). When rats were undisturbed in their cages with access to food, hourly averages of blood glucose over a 24-hour period revealed that OZR had higher blood glucose levels than LZR at all hours of the day and night (Fig. 1*B*). In addition to chronically elevated blood glucose in OZR, the 24-hour variability of blood glucose was higher in OZR compared to LZR (8.0 ± 0.8 vs. 4.7 ± 0.4, $P<0.05$, unpaired t-test), with the blood glucose variability being highest in OZR during the night (Fig. 1*C*).

The evening before the first glucose tolerance test (GTT), food was removed from the cages at approximately 5:00 p.m. Within 8 hours of fasting, blood glucose levels in OZR and LZR were equivalent to baseline measures taken the next morning, providing comparable and consistent blood glucose values for 7 hours before the GTT (*data not shown*). Injection of glucose (1g/kg, i.p.) produced a higher blood glucose in OZR compared to LZR (Fig. 2) with a peak that occurred later in the OZR (20 minutes for 8 of 12 rats) than in LZR (15 minutes for 8 of 11 rats; Table 1, Fig. 2*A*). In addition, the return of blood glucose toward baseline levels took longer in OZR than in LZR (180 vs. 90 minutes, Fig. 2*A*), contributing to a larger area under the curve in OZR (Table 1). By 95 minutes blood glucose levels were not different between OZR and LZR (Fig. 2*A*). Return of food to the cages 3 hours after injection of glucose produced a rise in blood glucose that peaked by 5 hours in both groups (Fig. 2*B*). The peak blood glucose was

significantly greater in OZR compared to LZR (Fig. 2*B*), and blood glucose levels remained higher in OZR thereafter, as seen in baseline measures with access to food (Fig. 1*B*).

Blood glucose values in OZR and LZR after metformin treatment

At 15 - 17 weeks of age, body weight was 57% higher in untreated OZR and 83% higher in metformin-treated OZR compared to like-treated, age-matched LZR (Table 2). Treatment with metformin tended to retard weight gain in both LZR and OZR, consistent with reported reductions in food intake with this dose of metformin in Zucker rats (71). In this particular set, metformin-treated LZR weighed less than untreated LZR, but no differences were observed between the OZR groups (Table 2). As seen at 12 - 14 weeks of age, at 15 -17 weeks of age the fasted blood glucose measured by telemetry was comparable in untreated OZR and LZR, but higher in OZR than LZR with access to food (Fig. 3*A*). Treatment with metformin had no effect on fasted blood glucose levels in OZR or LZR (Fig. 3*A*). However, metformin treatment effectively ameliorated the elevated morning blood glucose in OZR with access to food (Fig. 3*A*), and dramatically reduced blood glucose levels in OZR at all hours of a 24-hour period (Fig. 3*B*). Analysis of this 24-hour period showed metformin treatment did not alter fed blood glucose levels in LZR, and metformin-treated OZR had fed blood glucose levels equivalent to LZR (Fig. 3*C*). With the progression of metabolic syndrome from 12-14 weeks to 15-17 weeks of age, variability of blood glucose with access to food over a 24-hour period was greatly increased in untreated OZR (from 8.0 ± 0.8 to 22.7 ± 2.7, $P<0.05$, paired t-test), and treatment with metformin normalized blood glucose variability in OZR with no effect in LZR (Fig. 3*D*). Blood glucose levels were normalized in these young adult male OZR within approximately 1 week with this dose and route of administration of metformin (*data not shown*), providing 1-2 weeks of normalized blood glucose with *ad libitum* access to food before experiments began.

The 15-17-week-old rats were fasted 18 hours before the glucose tolerance test, and at this age 10 hours were required for blood glucose levels to be equivalent in untreated OZR and LZR (*data not shown*). As seen at 12 - 14 weeks of age, injection of glucose (1 g/kg, i.p.) into fasted, untreated rats evoked a larger rise in blood glucose in OZR than LZR at 15 - 17 weeks of age (Fig. 4*A*). Treatment with metformin had no effect on the response to injected glucose in LZR (Figs. 4*B* and *E*), but metformin significantly reduced the evoked rise in blood glucose, time to peak, recovery time, and area under the curve in OZR compared to untreated OZR (Figs. 4*C* and *F*; Table 2). Comparison of metformin-treated OZR and LZR revealed a slightly higher peak in blood glucose in the treated OZR (Fig. 4*D*), but time to peak and area under the curve for these metformin-treated OZR and LZR were not different (Table 2). With the return of food 3 hours after glucose injection, the untreated OZR displayed a significant rise in blood glucose that was virtually absent in metformin-treated OZR and unaffected by metformin treatment in LZR (Figs. 4*E and F*, 5*D*).

Comparison of blood glucose values from tail samples and telemetry

Because treatment with metformin did not completely normalize the peak in blood glucose with the GTT in OZR (Fig. 4*D*) but normalized the elevated basal blood glucose with access to food (Figs. 3C and 4*F*), we examined whether moving and handling of the rats altered blood glucose levels more in OZR than LZR. Direct comparison of baseline blood glucose levels by tail sample, which involved moving and opening cages and handling the rats' tails, with measures of blood glucose by telemetry one hour prior to any disturbance, revealed striking differences that were impacted by age, rat genotype, and feeding status. At 12-14 weeks of age fasting blood glucose values were comparable in LZR and OZR whether measured by tail sample or telemetry (Fig. 5*A, left panel*). In contrast, at 15-17 weeks of age fasted blood glucose levels

measured by tail sample were significantly higher than values measured by telemetry in OZR (Fig. 5*A, middle panel*), and this difference persisted after metformin treatment (Fig. 5*A, right panel*). In contrast, comparisons of blood glucose levels by tail sample and telemetry in LZR did not differ with or without metformin treatment at 15-17 weeks of age (Fig. 5*A middle and right panels*). Thus, fasted blood glucose levels were higher in 15-17-week old OZR compared to age-matched LZR when measured by tail samples, but fasted blood glucose levels were comparable in OZR and LZR when measured by telemetry (Fig. 5*A*).

When rats had access to food, the comparison of glucose values from tails samples and telemetry also revealed some differences that varied by age. As seen in these rats when they were fasted, at 12-14 weeks of age glucose readings by both measures were comparable in LZR and OZR (Fig. 5*B, left panel*), and with access to food OZR had higher glucose levels than LZR whether measured by tail samples or telemetry, in agreement with Fig. 1*A* (Fig. 5*B, left panel*). At 15-17 weeks of age, glucose levels measured by tail sample or telemetry were comparable in LZR with or without metformin treatment (Fig. *B, middle and right panels*). However, as seen with fasting, when OZR had access to food their blood glucose levels were higher when measured by tail sample than with telemetry whether or not the OZR were treated with metformin (Fig. 5*B, middle and right panels*). In contrast to the fasted state, in the fed state the sampling method did not alter conclusions regarding differences between OZR and LZR. With access to food, untreated 15-17-week old OZR had higher blood glucose levels than LZR, but metformin-treated OZR and LZR had comparable blood glucose levels whether measured by tail sample or telemetry (Fig. 5*B, middle and right panels*).

Similar observations regarding the impact of handling the rats upon blood glucose levels can be made by examining the 15-17-week-old rats immediately before and after a GTT (using

segments of data from Fig. 4). Fasting blood glucose levels were comparable in treated and untreated OZR and LZR when measured by telemetry (time span -90 to -70 minutes in Fig. 5*C,* taken from Figs. 4*A* and *D*). However, with moving the cages and taking a blood sample from the tail, blood glucose levels measured by telemetry rose in OZR but not LZR during the hour before the injection of glucose that occurred at time 0 (Fig. 5*C*). Although analysis of the entire time period in Fig. 4*A* (4 hours of 5-minute samples) did not detect differences observed before the injection of glucose, analysis of a shorter time span before the injection of glucose (80 minutes of 5-mintues samples) revealed clear differences between OZR and LZR (Fig. 5*C*). Blood glucose was higher in untreated OZR than LZR for 40 minutes (-50 to -10 minutes, Fig. 5*C*, from Fig. 4*A*), and metformin treatment in OZR and LZR reduced the duration of the difference in fasted blood glucose after tail sampling to 10 minutes (at -25 and -20 minutes; Fig. 5*C*, from Fig. 4*D*).

Differences between OZR and LZR can also be seen following the GTT when food was returned to the cages, and the cages were moved back to their rack 3 hours after the injection of glucose (5-minute samples starting at 180 minutes, Fig. 5*D*, after time frame shown in Figs. 4*A* and *D*). The rise in blood glucose with the return of food is larger in untreated OZR than LZR (Fig. *5D*), and this difference is also apparent in the hourly averages (Figs. 4 *E* and *F*). In metformin-treated rats, analysis of 5-minute samples for 90 minutes after the return of food also revealed a significant rise in blood glucose in OZR compared to LZR during the time when cages were being moved, although the difference was smaller compared to untreated rats and occurred only for 10 minutes (Fig. 5*D*). Within 30 minutes after return of food to their cages and moving them, blood glucose levels in metformin-treated OZR were comparable to LZR (Fig. 5*D*),

whereas untreated OZR remained significantly elevated above metformin-treated OZR (Fig. 5*D*), as observed in the hourly averages (Fig. 4*F*).

Baseline plasma insulin and lipids in OZR and LZR after treatment with metformin

Before the infusion of phenylephrine, a blood sample was taken from the arterial catheter in conscious, undisturbed rats after 1 day of recovery from surgery to implant indwelling femoral vascular catheters. As observed with the rats used for telemetry, metformin tended to retard weight gain. In this particular set, unlike those in Table 2, the metformin-treated LZR were comparable to untreated LZR, whereas metformin-treated OZR weighed less than untreated OZR (Table 3). As seen in Table 2, comparison of like-treated rats showed OZR weighed significantly more than LZR, (by 67% in untreated rats and 46% in metformin-treated rats; Table 3). Insulin levels were slightly reduced by metformin treatment in OZR, with no effect in LZR (Table 3). However, compared to like-treated LZR, untreated and metformin-treated OZR had insulin levels that were 5 times and 7 times higher, respectively (Table 3). Plasma cholesterol and triglycerides were higher in metformin-treated OZR compared to LZR and neither measure was affected by metformin treatment or differences in body weights of treated and untreated OZR (Table 3).

Impact of metformin on phenylephrine-induced bradycardia and NTS c-Fos expression

Mean AP was elevated in untreated OZR compared to LZR, and metformin treatment did not reduce mean AP in either LZR or OZR (Fig. 6*A*). Instead, mean AP was slightly but significantly higher in metformin-treated OZR compared to untreated OZR (Fig. 6*A*). There were no differences in baseline HR among the groups (*data not shown*), as previously reported in untreated conscious male OZR and LZR of a similar age (31). Raising mean AP by 40 mmHg with infusion of phenylephrine evoked a baroreflex-mediated bradycardia that was markedly

reduced in untreated OZR compared to LZR (Figs. 6*B* and *C*), as previously reported (31). Treatment with metformin enhanced phenylephrine-induced bradycardia in OZR with no effect in LZR (Fig. 6*C*). However, the bradycardia in metformin-treated OZR was still smaller compared to LZR (Fig. 6*C*), suggesting a partial restoration of the lower plateau of the cardiac baroreflex in the metformin-treated OZR.

As expected, the number of phenylephrine-induced c-Fos expressing neurons in the caudal and intermediate NTS of OZR was reduced compared to LZR at all 4 rostro-caudal levels examined (Figs. 7*A, B,* and *C* and Figs. 8*A* and *B*) (31). Treatment with metformin did not alter phenylephrine-induced c-Fos expression in the NTS of LZR, but enhanced c-Fos expression in OZR comparable to counts observed in LZR at all 4 rostro-caudal levels of the NTS examined (Figs. 7 and 8). Regions of the NTS known to receive baroreceptor inputs (13) expressed fewer c-Fos+ neurons in untreated OZR compared to LZR (Fig. 7*C*, *left side* and Figs. 8*A* and *B*), and expression in metformin-treated OZR was restored within these regions (Fig. 7*C*, *right side* and Fig. 8*D*).

Baseline plasma insulin, and lipids in OZR and LZR after treatment with pioglitazone

Because metformin reduced weight gain in OZR and LZR, another set of rats were treated with pioglitazone and compared to untreated OZR and LZR. As expected (19), pioglitazone accelerated weight gain in OZR (Table 4). Compared to like-treated LZR, untreated and pioglitazone-treated OZR weighed 47% and 56% more, respectively (Table 4). Like metformin, treatment with pioglitazone caused a small reduction in plasma insulin levels in OZR but had no significant effect in LZR (Table 4). However, compared to like-treated LZR, insulin levels were 4.8 times higher in untreated and pioglitazone-treated OZR. Plasma cholesterol was elevated in OZR compared to LZR and was not affected by treatment with pioglitazone (Table

4). Plasma triglycerides were elevated in untreated OZR compared to LZR, and pioglitazone treatment had no significant effect in OZR or LZR (Table 4).

Impact of pioglitazone on glucose and phenylephrine-induced changes in HR and NTS c-Fos expression

Blood glucose was measured at approximately 9 a.m. in rats with access to food because these rats were not instrumented to record blood glucose by telemetry, and differences in blood glucose in untreated OZR and LZR were present only when rats had access to food (see Figs. 3*A* and 5*B*). Like metformin, treatment with pioglitazone did not alter morning fed blood glucose levels in LZR (Fig. 9*A*). In contrast, treatment with pioglitazone greatly reduced morning fed blood glucose levels in OZR comparable to those observed in LZR (Fig. 9*A*). Although glucose tolerance tests were not performed, previous reports show normalized fed glucose in OZR coincides with restoration of glucose response to glucose challenge (19). Pioglitazone did not alter mean AP in conscious LZR or OZR, leaving the pioglitazone-treated OZR with higher mean AP than LZR (Fig. 9*B*). As seen with metformin, pioglitazone did not alter phenylephrine-induced bradycardia in LZR (Fig. 9*C*). In contrast, the phenylephrine-induced bradycardia that was blunted in untreated OZR was fully restored by treatment with pioglitazone (Fig. 9*C*). Treatment with pioglitazone did not alter c-Fos expression in the NTS of LZR, but the diminished c-Fos expression observed untreated OZR and was significantly enhanced by treatment with pioglitazone (Fig. 9*D*). However, after treatment with pioglitazone, phenylephrine-induced c-Fos expression was still slightly reduced in OZR compared to LZR (Fig. 9*D*).

CHAPTER II – Figure II-1. Glucose by Telemetry in Male Zucker Rats at 12-14 weeks

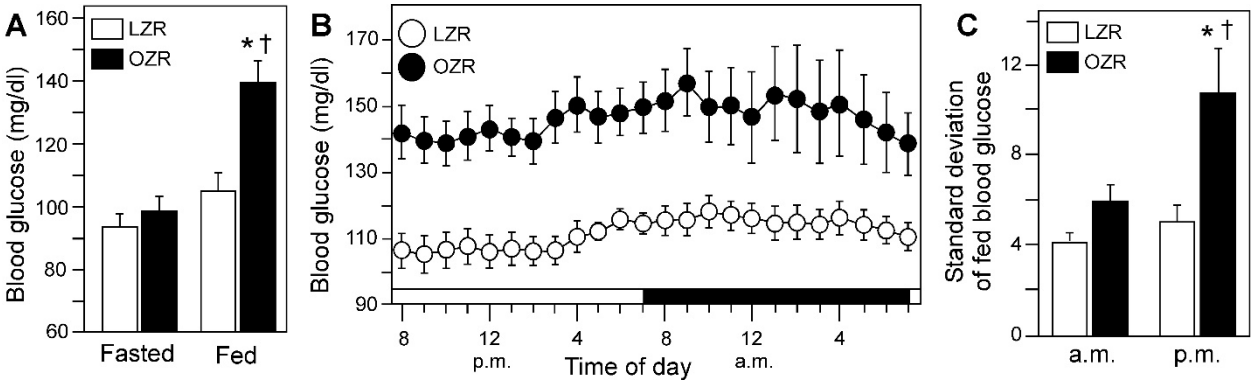

Figure 1. Baseline blood glucose levels measured by telemetry in young adult (12-14 weeks old), male lean Zucker rats (LZR, $n = 11$) and obese Zucker rats (OZR, $n = 12$). (*A)* Morning blood glucose levels in fasted and fed states, * $P < 0.05$ vs. fasted OZR, † $P < 0.05$ vs. fed LZR. (*B)* Hourly averages over a 24-hour period in these OZR and LZR with access to food, $P < 0.05$, OZR vs. LZR for all hours. *(C)* Standard deviation of glucose in these LZR and OZR during peak hours of the night (9:30 p.m. – 11:30 p.m.) and lowest hours of the morning (5:30 a.m. – 7:30 a.m.), *$P < 0.05$ vs. LZR during the same time period, † $P < 0.05$ vs. OZR during the morning. Data were analyzed by ANOVA with repeated measures followed by Tukey's HSD tests.

CHAPTER II – Figure II-2. Glucose Tolerance Tests in Male Zucker Rats at 12-14 Weeks

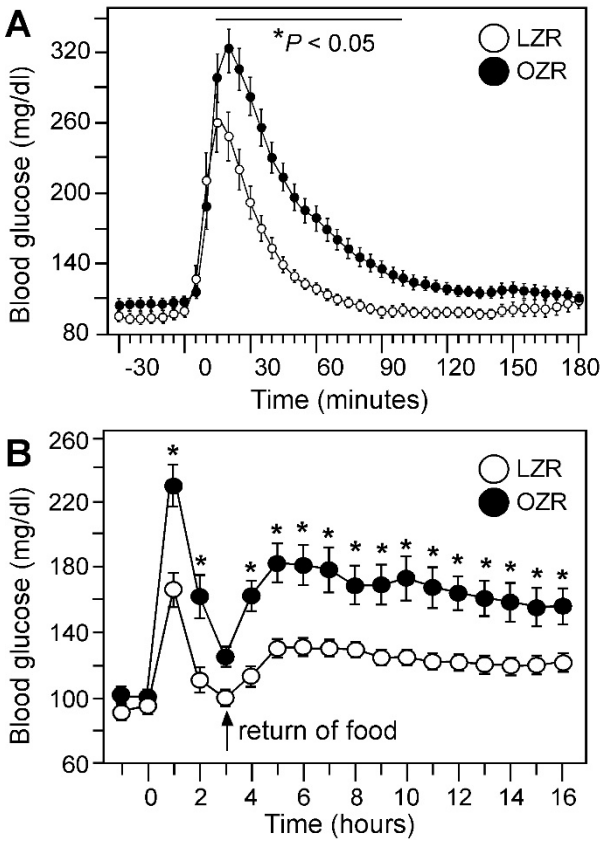

Figure 2. Glucose tolerance test (GTT) in OZR and LZR at 12 - 14 weeks of age. See Fig. 1 for baseline glucose values for these rats. *(A)* Blood glucose values before and after injection of glucose (at time 0) measured by telemetry every 5 minutes over 210 minutes, * $P < 0.05$ vs. LZR at that time point. *(B)* Same glucose tolerance test with glucose measured in hourly averages over 18 hours with return of food at hour 3, *$P < 0.05$ vs. LZR at that time point. Pairs of groups were analyzed by ANOVA with repeated measures followed by Tukey's HSD tests. See Table 1 for single value comparisons during the GTT.

CHAPTER II – Table II-1. Glucose Tolerance Test in Male Zucker Rats at 12-14-weeks

Group	*n*	age (days)	body weight (g)	time to peak (minutes)	AUC (mg/dl x 3hrs)
LZR	11	90.5 ± 0.7	296.1 ± 6.4	15.4 ± 0.8	333.3 ± 40.2
OZR	12	91.2 ± 0.8	471.7 ± 10.7*	19.2 ± 0.8*	523.2 ± 59.5*

Data are expressed as mean ± SE. Age and weight were determined the morning of the glucose tolerance test (Fig. 2). AUC, area under the curve measured as difference from baseline in 5-minute increments from -15 to 165 minutes in relation to the injection of glucose, * $P < 0.05$, unpaired t-tests.

CHAPTER II – Figure II-3. Metformin: Baseline Glucose in Male Zucker rats at 15-17 weeks

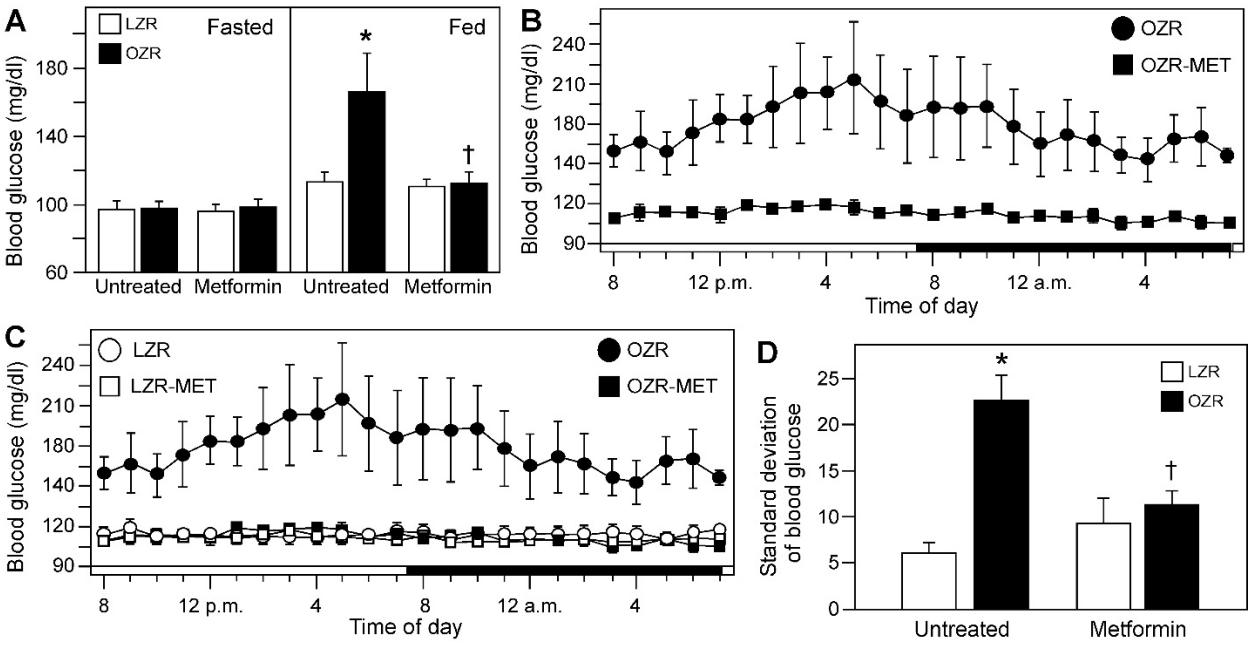

Figure 3. Baseline blood glucose values measured by telemetry in untreated and metformin-treated LZR and OZR at 15 - 17 weeks of age. Data are from the same rats from Figs. 1 and 2 with each group divided into untreated rats (5 LZR and 6 OZR) and metformin-treated rats (6 LZR and 6 OZR). (*A)* Morning blood glucose levels in untreated and metformin-treated rats during fasted (*left*) and fed (*right*) states. Data were analyzed separately within the same feeding status by ANOVA with Tukey's HSD tests, * P <0.05 vs. untreated fed LZR, † P < 0.05 vs. untreated fed OZR. *(B)* Hourly glucose values from untreated and metformin-treated OZR over a 24-hour period analyzed by ANOVA with repeated measures and Tukey's HSD tests, P < 0.05 for untreated and metformin-treated OZR for all hours. *(C)* Hourly glucose values over a 24-hour period from untreated and metformin-treated LZR and OZR (same OZR as in *B*). ANOVA with repeated measures for metformin-treated LZR and OZR and ANOVA for untreated and metformin-treated LZR did not yield significant differences. *(D)* Standard deviation of blood glucose values over a 24-hour period with access to food. Data were analyzed by ANOVA with Tukey's HSD tests, * P < 0.05 vs. untreated LZR, † P < 0.05 vs. metformin-treated OZR.

CHAPTER II – Table II-2. Glucose Tolerance Tests in Male Zucker Rats at 15-17 weeks

Group	*n*	age (days)	body weight (g)	time to peak (minutes)	AUC (mg/dl x 3hrs)
Untreated					
LZR	5	108.2 ± 0.2	351.4 ± 10.5	21.0 ± 1.0	310.8 ± 80.6
OZR	5	108.2 ± 0.2	552.6 ± 20.1*	27.0 ± 2.0*	940.6 ± 138.5*
Metformin-treated					
LZR	6	108.0 ± 0.0	273.3 ± 22.0†	19.2 ± 0.8	341.6 ± 66.4
OZR	6	109.5 ± 1.5	500.0 ± 22.6*	22.5 ± 1.1†	547.7 ± 91.6†
P values					
Rat type		0.383	<0.001	0.002	<0.001
Treatment		0.520	0.005	0.023	0.074
Interaction		0.383	0.536	0.308	0.040

Data are expressed as mean ± SEM. Age and weight were determined the morning of the glucose tolerance test (Table 2). The AUC was measured as the difference from baseline at 5-minute increments from -15 to 165 minutes in relation to the injection of glucose, *$P < 0.05$ vs. LZR of like treatment. † $P < 0.05$ vs. untreated rat of same type. Each measure was analyzed by 2-way ANOVA for rat type x treatment followed by Tukey's HSD tests. See Figs. 3 and 4 for blood glucose values in these rats.

CHAPTER II – Figure II-4. Metformin: Glucose Tolerance Tests, Male Zuckers at 15-17 weeks

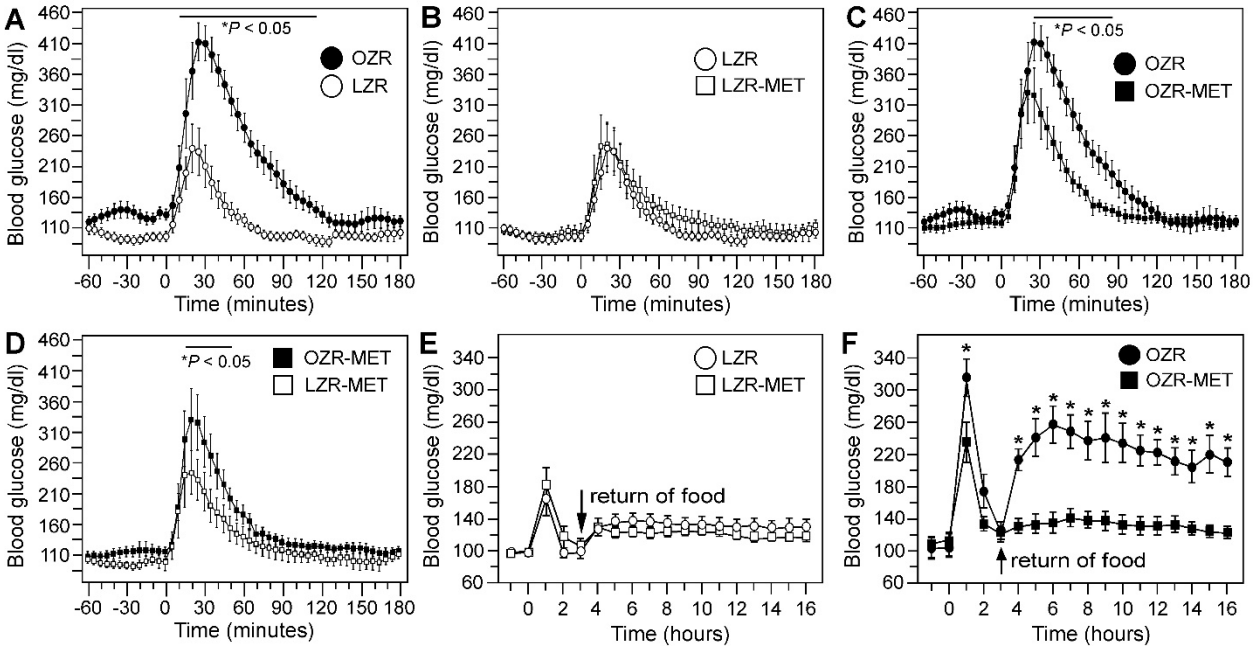

Figure 4. Glucose tolerance tests in untreated and metformin-treated LZR and OZR at 15 -17 weeks of age. See Table 2 and Fig. 3 for baseline values of these rats. *(A – D)* Blood glucose levels measured by telemetry before and after injection of glucose (at time 0) shown in pairs of groups for clarity. Each pair was analyzed by ANOVA with repeated measures with Tukey's HSD tests. * $P < 0.05$, differs at that time point. *(E)* Same glucose tolerance test in LZR from (*B*) with blood glucose measured in hourly averages and return of food at hour 3. *(F)* Same glucose tolerance test in OZR from (*C*) with blood glucose measured in hourly averages and return of food at hour 3. For *E* and *F*, the data were analyzed by ANOVA with repeated measures with Tukey's HSD tests, *$P < 0.05$ vs. metformin-treated OZR at that time point.

CHAPTER II – Figure II-5. Comparison of Blood Glucose by Tail Sample and Telemetry in Male Zucker rats at 12 -17 weeks with effects of Metformin

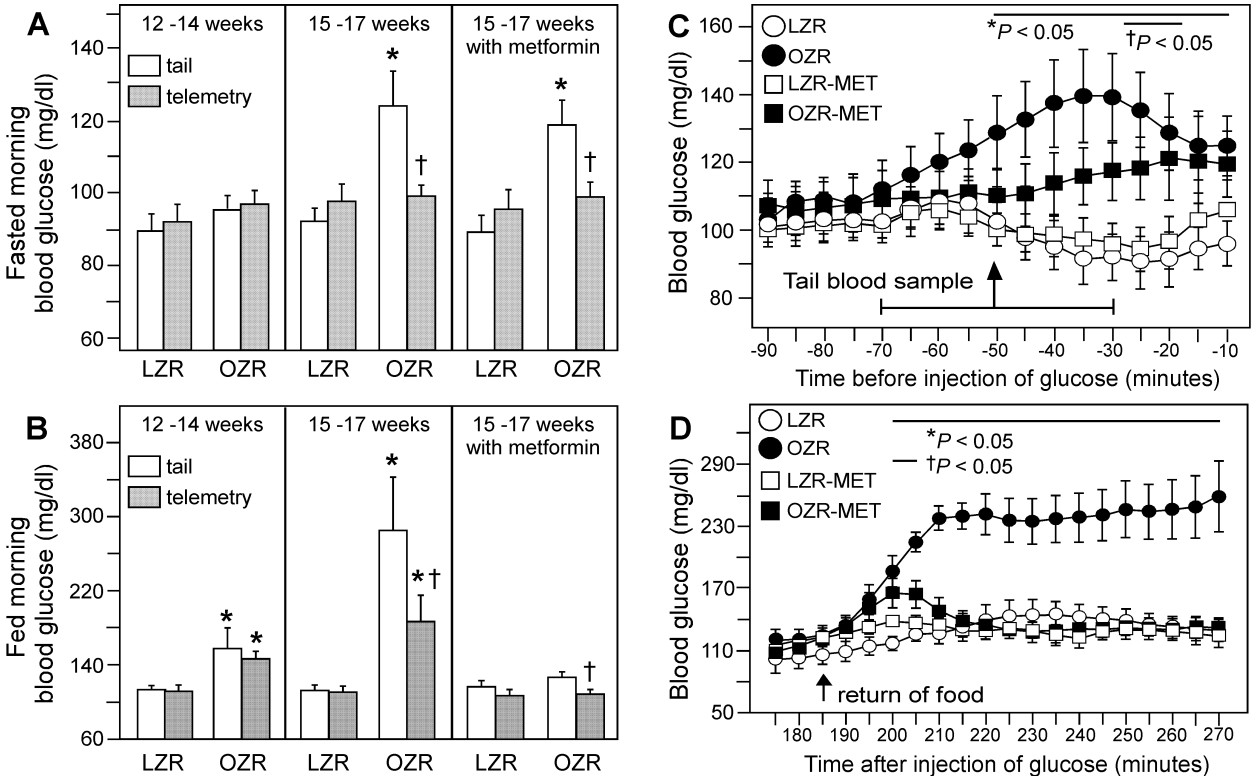

Figure 5. Development of enhanced reactivity to tail sampling of blood glucose in OZR at 15 – 17 weeks of age. Comparisons of fasted (*A*) and fed (*B*) blood glucose values from morning tail samples and telemetry values 1 hour before the tail samples in OZR (12) and LZR (11) at 12 – 14 weeks of age (*left*), in OZR (6) and LZR (6) at 15 – 17 weeks of age (*middle*) and in OZR (6) and LZR (6) at 15 – 17 weeks of age after metformin treatment (*right*). Each of the 3 sets were analyzed by ANOVA with repeated measures for sample type with Tukey's HSD tests. * $P <$ 0.05 vs. tail sample in LZR, † $P < 0.05$ vs. tail sample in OZR. *(C)* Enlargement of data shown in Fig. 4*A* and *D* with a tail sample taken ~1 hour before injection of glucose at time 0. The arrow denotes the mean time of all tail samples (51 minutes) with a horizontal line to denote the range of times a baseline tail sample was taken (-30 to -70 minutes). Like-treated rats were

compared by ANOVA with repeated measures with Tukey's HSD tests. * $P < 0.05$ vs. untreated LZR, † $P < 0.05$ vs. metformin-treated LZR. *(D)* Expansion of data shown in Figs. 4*E* and *F* to show 5-minute averages over the time period of 95 minutes to 270 minutes (3 hours) after injection of glucose. Like-treated rats were compared by ANOVA with repeated measures with Tukey's HSD tests. * $P < 0.05$ vs. untreated LZR (circles) during that time period, † $P < 0.05$ vs. metformin-treated LZR (squares) during that time period.

CHAPTER II – Figure II-6. Metformin: Baseline Arterial Pressure and Baroreflex Bradycardia in Conscious Male Zucker rats at 14-17 weeks

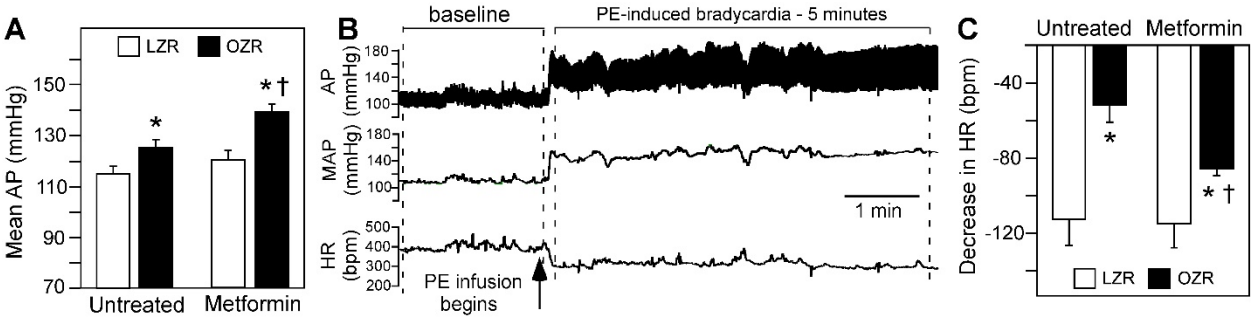

Figure 6. Baseline mean arterial pressure (AP) and phenylephrine-induced bradycardia in untreated and metformin-treated LZR and OZR. (*A*) Mean AP in untreated and metformin-treated, conscious LZR and OZR. See Table 3 for more baseline values. Data were analyzed by ANOVA with Tukey's HSD tests. * $P < 0.05$ vs. like-treated LZR, † $P < 0.05$ vs. untreated OZR. (*B*) Representative tracing from an untreated LZR illustrating baseline period for mean AP (MAP) and period of analysis for phenylephrine-induced decrease in heart rate (HR). (*C*) Phenylephrine-induced decrease in HR in untreated and metformin-treated LZR and OZR. Data were analyzed by ANOVA with Tukey's HSD tests. * $P < 0.05$ vs. like-treated LZR, † $P < 0.05$ vs. untreated OZR.

CHAPTER II – Figure II-7. Metformin: Phenylephrine-induced c-Fos Expression in NTS

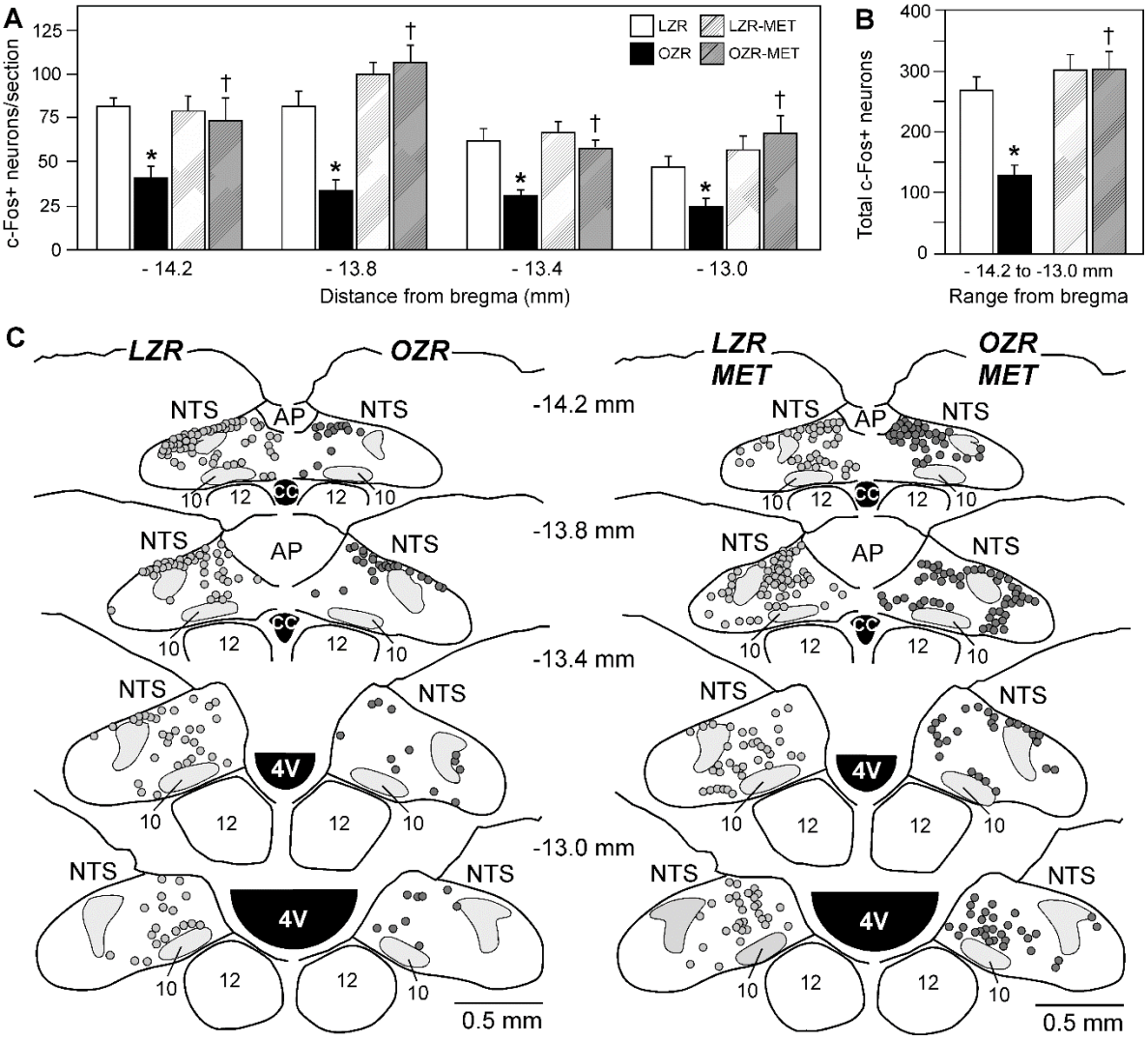

Figure 7. Phenylephrine-induced c-Fos expression in the nucleus tractus solitarius (NTS) of untreated and metformin-treated LZR and OZR. (*A*) Number of c-Fos-positive neurons from bilateral counts of NTS at 4 rostro-caudal levels. Each bregma level was analyzed separately by ANOVA with Tukey's HSD tests. * $P < 0.05$ vs. untreated LZR, † $P < 0.05$ vs. untreated OZR. (*B*) Total counts of c-Fos-positive neurons from all 4 rostro-caudal levels of the NTS. Data was analyzed by ANOVA with Tukey's HSD tests. * $P < 0.05$ vs. untreated LZR, † $P < 0.05$ vs. untreated OZR. (*C*) Representative maps of c-Fos-positive neurons at 4 rostro-caudal levels of the NTS in untreated (*left*) and metformin-treated (*right*) LZR and OZR.

CHAPTER II – Figure II-8: Metformin: Photomicrographs of Phenylephrine-induced c-Fos Expression in the NTS of Male Zucker Rats

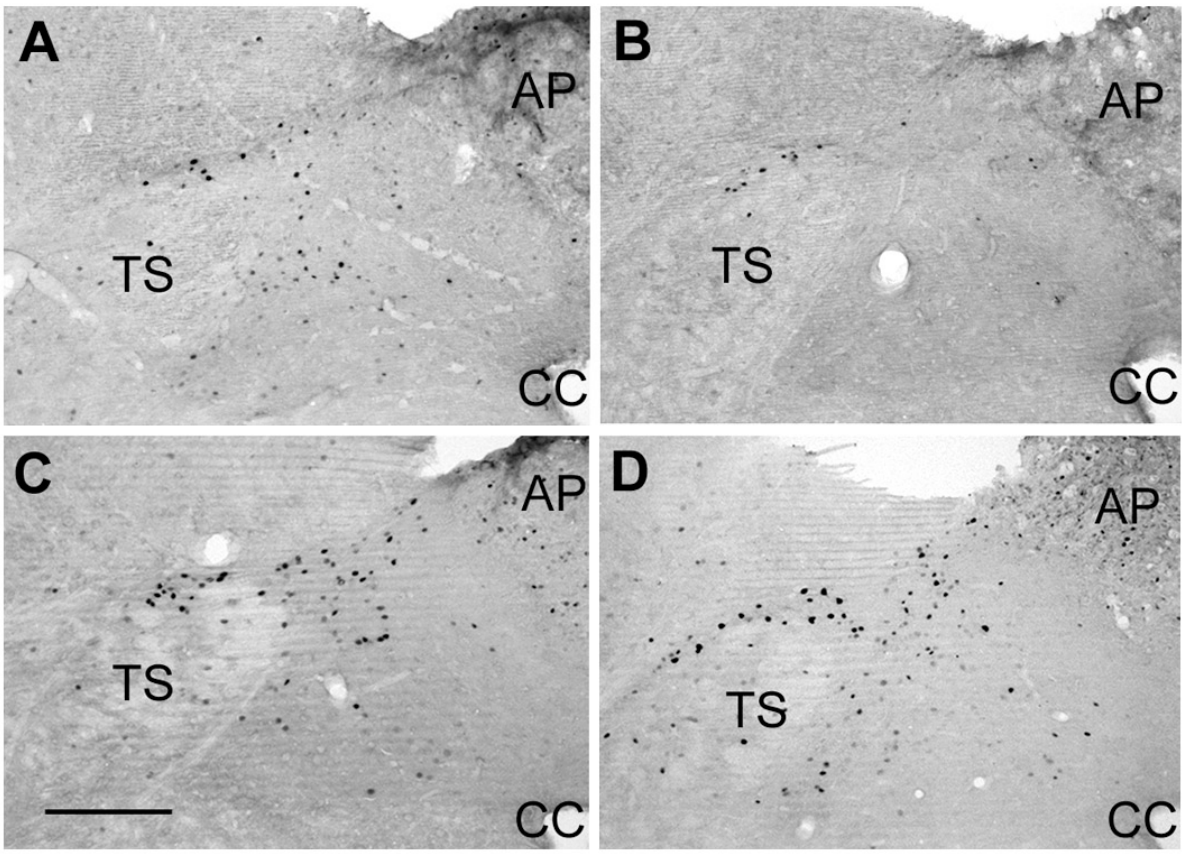

Figure 8. Representative brightfield photomicrographs of the left NTS at -13.8 mm caudal to bregma from an untreated LZR (*A*) and OZR (*B*) and a metformin-treated LZR (*C*) and OZR (*D*). TS, tractus solitarius; AP, area postrema; CC, central canal; Scale bar, 250 μm.

CHAPTER II – Table II-3. Metformin and Plasma Values in Male Zucker Rats at 15-17 weeks

Group	*n*	age (days)	body weight (g)	insulin (ng/dl)	cholesterol (mg/dl)	triglycerides (mg/dl)
Untreated						
LZR	7	115.3 ± 1.7	351.7 ± 10.5	3.8 ± 1.3	107.5 ± 7.8	16.3 ± 2.9
OZR	6	114.0 ± 1.3	583.5 ± 28.7*	22.9 ± 0.4*	227.1 ± 16.2*	52.6 ± 16.0
Metformin						
LZR	8	116.5 ± 0.3	338.1 ± 11.8	2.3 ± 0.4	124.4 ± 7.5	18.2 ± 3.0
OZR	8	113.0 ± 1.4	493.6 ± 14.2*†	18.5 ± 2.1*†	206.2 ± 17.8*	68.6 ± 18.5*
P values						
Rat type		0.064	<0.001	<0.001	<0.001	0.002
Treatment		0.972	0.004	0.046	0.880	0.478
Interaction		0.364	0.029	0.312	0.166	0.574

Values are mean ± SEM. Blood samples were collected from arterial line at approximately 9:00 a.m. in conscious rats with access to food and water before infusion of phenylephrine. *P <0.05 vs. LZR of like treatment. † P <0.05 vs. untreated rat of same type. Each measure was analyzed by 2-way ANOVA for rat type x treatment followed by Tukey's HSD tests.

CHAPTER II – Figure II-9. Pioglitazone: Glucose, Mean Arterial Pressure, and Baroreflexes in Male Zucker rats at 15 -17 weeks

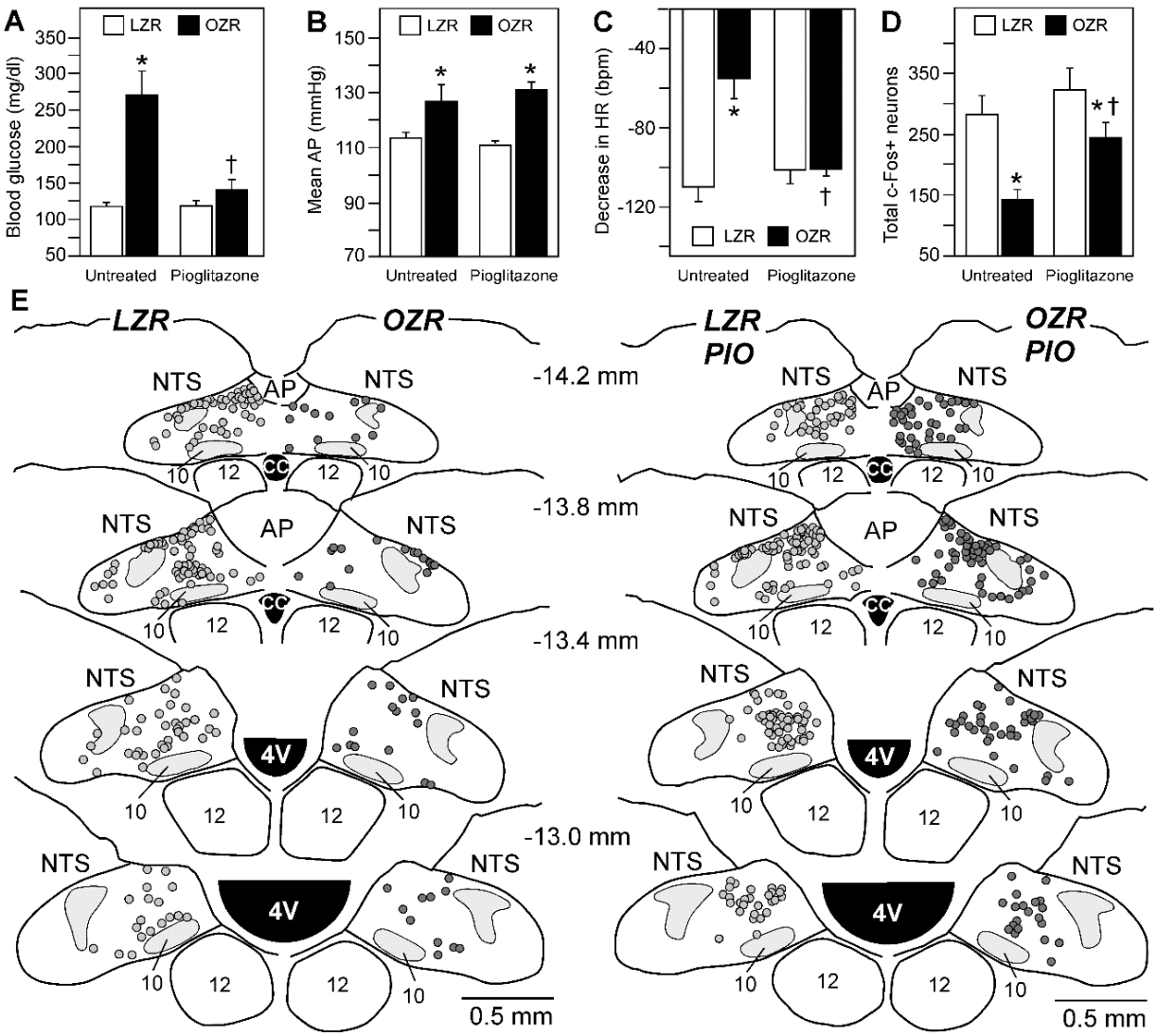

Figure 9. Baseline blood glucose, mean AP with phenylephrine-induced bradycardia and total counts of c-Fos-positive neurons in the NTS of untreated and pioglitazone-treated OZR and LZR. (*A*) Morning fed glucose taken from tail samples. * $P < 0.05$ vs. untreated LZR, † $P < 0.05$ vs. untreated OZR. (*B*) Baseline mean AP (MAP) in conscious rats before infusion of phenylephrine. * $P < 0.05$ vs. untreated rat of same type. (*C*) Phenylephrine-induced decrease in HR using same protocol shown in Fig. 6*B*. * $P < 0.05$ vs. untreated LZR, † $P < 0.05$ vs. untreated

OZR. (*D*) Total counts of c-Fos-positive neurons from 4 rostro-caudal levels of the NTS (-14.2, -13.8, -13.4, and -13.0 mm caudal to bregma) using same protocol as in Fig. 7*B*. * $P < 0.05$ vs. untreated LZR, † $P < 0.05$ vs. untreated OZR. Each measure was analyzed by ANOVA with Tukey's HSD tests. (*E*) Representative maps of c-Fos-positive neurons at 4 rostro-caudal levels of the NTS in untreated (*left*) and pioglitazone-treated (*right*) LZR and OZR.

CHAPTER II – Table II-4. Pioglitazone and Plasma Values in Male Zucker Rats at 15-17 weeks

Group	*n*	age (days)	body weight (g)	insulin (ng/dl)	cholesterol (mg/dl)	triglycerides (mg/dl)
Untreated						
LZR	8	115.5 ± 1.6	378.4 ± 8.2	4.0 ± 1.0	83.4 ± 7.9	8.6 ± 1.8
OZR	6	113.3 ± 2.1	557.0 ± 12.2*	23.2 ± 0.1*	148.7 ± 16.2*	30.0 ± 8.8*
Pioglitazone						
LZR	7	112.4 ± 0.4	400.1 ± 11.6	3.3 ± 0.4	58.9 ± 9.0	15.9 ± 2.1
OZR	8	111.9 ± 0.4	624.1 ± 24.8*†	19.1 ± 2.0*†	123.5 ± 11.3*	31.1 ± 9.5
P values						
Rat type		0.314	<0.001	<0.001	<0.001	0.010
Treatment		0.093	0.012	0.050	0.034	0.525
Interaction		0.498	0.180	0.212	0.973	0.639

Values are mean ± SEM. Blood samples were collected from arterial line at approximately 9:00 a.m. in conscious rats with access to food and water before infusion of phenylephrine. For triglycerides *n*=5 for untreated OZR due to the removal of one outlier (385.2 mg/dl). **P* <0.05 vs. LZR of like treatment. † *P* <0.05 vs. untreated rat of same type. Each measure was analyzed by 2-way ANOVA for rat type x treatment followed by Tukey's HSD tests.

DISCUSSION

Obesity is associated with the development of a constellation of attributes, known as metabolic syndrome, that promote premature morbidity and mortality (33, 52, 55). Current clinical guidelines use threshold values of these attributes that may not provide adequate triggers for intervention to reduce significant health risks. In the present study, fasting blood glucose levels measured by telemetry were comparable in adult male LZR and OZR up to 17 weeks of age. However, with access to food OZR were chronically hyperglycemic with impaired glucose tolerance by 12-14 weeks of age. By this age, OZR also develop hypertension and impaired baroreflexes coincident with reduced baroreflex-mediated activation of the NTS (31, 74). Treatment of OZR with metformin or pioglitazone dramatically improved glucose homeostasis and simultaneously enhanced the blunted baroreflex-mediated bradycardia and c-Fos expression in NTS with no effect in treated LZR. These attributes were restored in the persistence of hyperinsulinemia and hypertension, suggesting changes in insulin and AP did not contribute to the effects of the treatments. These data strongly suggest that impaired glucose homeostasis in prediabetic, insulin-resistant OZR contributes to reduced baroreceptor-mediated activation of the NTS and bradycardia before the onset of frank type 2 diabetes mellitus.

Metformin and pioglitazone are both highly effective for restoring glucose homeostasis without producing hypoglycemia, but their underlying primary mechanisms differ. Pioglitazone stimulates peroxisome proliferator-activated receptor gamma (PPAR-γ) to increase insulin sensitivity particularly in liver and adipose tissue (3), whereas metformin stimulates AMP-activated protein kinase to reduce hepatic gluconeogenesis and enhance glucose uptake by muscles (93). Although unidentified effects of metformin and pioglitazone may have also contributed to improvement of baroreflexes, several controls in the present study strengthen the

argument that improved glucose homeostasis enhanced the brainstem's response to baroreceptor inputs in insulin-resistant OZR. Neither treatment altered baroreflex-mediated bradycardia or activation of the NTS in the LZR, suggesting these treatments were effective by ameliorating obesity-related attributes. Metformin tended to retard weight gain in all treated rats, and weight loss can improve baroreflexes in obese subjects (21, 27, 82). However, pioglitazone accelerated weight gain while restoring glycemic control and baroreflex efficacy, providing confidence that improvement with metformin was not just a consequence of weight loss. Although plasma insulin was slightly reduced by both treatments in OZR, plasma insulin was still 5-7 times higher in OZR than in LZR. Furthermore, insulin appears to act in the forebrain to alter baroreflex-mediated tachycardia without a significant effect on baroreflex-mediated bradycardia (62, 69). Hypertension is also associated with compromised baroreflexes (29), but both treatments enhanced baroreflex-mediated responses without reducing mean AP. Elevated cholesterol and triglycerides in OZR were not reduced by these treatments. Thus, the data suggest the enhanced glycemic control observed in treated OZR contributed to the improvement of baroreceptor-mediated activation of the NTS and bradycardia.

Metformin and pioglitazone both improved phenylephrine-induced bradycardia and activation of the NTS in OZR, but the degree of recovery for each measure varied by treatment. Pioglitazone was more effective than metformin for restoring baroreflex-mediated bradycardia in OZR. The partial restoration observed in metformin-treated OZR may be related to the increase in baseline mean AP or the use of a suboptimal dose, as metformin-treated OZR retained some differences in glucose compared to treated LZR. In addition, although pioglitazone-treated OZR and LZR had comparable phenylephrine-induced bradycardia, the enhanced phenylephrine-induced c-Fos expression in the NTS was still significantly less in OZR than in LZR. These data

suggest that pioglitazone may also enhance baroreflexes independent of improved activation of NTS. Furthermore, a related compound, rosiglitazone, increases baroreflex gain in correlation with or independent of improved insulin sensitivity in obese rats, depending upon the dose (92). Alternatively, the apparent mismatches in degree of improvement may also be related to the rudimentary assessment of baroreflex efficacy and excitation of baroreflex-related NTS neurons. Quantification of the baroreflex was limited to the maximal bradycardia evoked by a rapid rise in AP using a protocol to optimize c-Fos expression in the NTS. Bradycardia was chosen as the dependent measure because it could be readily quantified in conscious rats, and the maximal response to an evoked rise in AP is a prominent deficit observed in obese rodents (12, 59, 74). Likewise, overweight, insulin-resistant human subjects with normal or elevated fasting blood glucose have can have diminished baroreflex-mediated bradycardia in the absence of diminished baroreflex-mediated tachycardia or changes in SNA (36). In obese rats both sympathetic and parasympathetic contributions to baroreflex-mediated bradycardia are impaired (6, 59), but further study is needed to determine how metformin and pioglizatone improve the autonomic regulation of HR in OZR. In addition, although phenylephrine-induced c-Fos expression in NTS depends upon inputs from arterial baroreceptors (13), many of activated NTS neurons are not likely to be directly involved in producing the baroreflex-mediated bradycardia. Most NTS neurons that express c-Fos after phenylephrine infusion are glutamatergic, but only a small portion of these neurons project to the region of the CVLM and nucleus ambiguus (87). Therefore, although the number of phenylephrine-induced c-Fos expressing neurons roughly indicates a magnitude of regional activation within the NTS, future studies will be necessary to determine which neurons are altered in OZR and affected by treatments that improve glycemic control. Nevertheless, in the present study treatment of OZR with metformin or pioglitazone

increased c-Fos expression in regions of the NTS containing neurons that project to the ventrolateral medulla (87). Despite these methodological limitations, these data show that restoration of glucose homeostasis in OZR coincides with improved baroreflex-mediated activation of the NTS and bradycardia in conscious rats.

Although the present study cannot delineate the cellular mechanisms underlying diminished baroreceptor-mediated activation of the NTS in OZR or its reversal by treatments that normalize glucose homeostasis, these changes are consistent with the impact of hyperglycemia upon the afferent limb of the baroreflex. In absence of obesity, hypertension or hyperinsulinemia, the presence of elevated blood glucose is accompanied by diminished phenylephrine-induced c-Fos expression in the NTS (26) and impaired baroreflexes without altering the relationship between aortic depressor nerve activity and AP (17, 20, 38). Likewise, adult male OZR develop impaired activation of the NTS with no overt changes aortic depressor nerve activity at an age when they are chronically hyperglycemic with access to food (31, 40). The threshold AP for onset of aortic depressor nerve activity is comparable in these OZR and LZR, and both the percent change and raw voltage of the aortic depressor nerve are equivalent over a wide range of AP. Furthermore, the modest hypertension in these OZR is accompanied by a slightly baseline higher aortic depressor nerve activity, suggesting no resetting of baroreceptor afferent nerve activity (40). In contrast, electrical stimulation of the baroreceptor afferent fibers or microinjection of glutamate into the NTS evokes smaller physiological responses in adult male obese rats (40, 92). These observations are consistent with a hyperglycemia-induced reduction in the ability of baroreceptor afferent nerves to activate the NTS.

Neurons within barosensitive regions of the NTS can be excited by raising circulating glucose within a physiological range and by local changes in glucose concentration (90). Within the NTS, glucose increases glutamate release from vagal afferent nerve terminals to enhance vagal activation of NTS neurons, and glucose produces excitatory postsynaptic effects in some NTS neurons (85, 90). In the setting of metabolic syndrome many of these homeostatic mechanisms are disrupted or lost altogether. Just as hyperinsulinemia promotes insulin resistance, hyperglycemia fosters glucose insensitivity within the NTS. In type I diabetic mice, glucose fails to modulate the frequency of sEPSCs in NTS neurons, suggesting chronic hyperglycemia abrogates the glucose-enhanced glutamate release from vagal afferent nerve terminals (11). Furthermore, with streptozotocin-induced hyperglycemia, glucose-mediated augmentation of NTS neuronal excitability is lost coincident with reduced expression and function of glucokinase within the NTS (32). Thus, acute local changes in glucose concentration play an important role in facilitating the activation of NTS neurons by afferent inputs, and the loss of glucose-mediated enhancement of neurotransmission with chronic hyperglycemia likely contributes to diminished activation of the NTS in the setting of diabetes.

Although the loss of glucose-enhanced activation of NTS neurons has been proposed to underlie impaired gastrointestinal function with diabetes (11), these changes in NTS neurotransmission with chronic hyperglycemia would also impair autonomic regulation of cardiovascular function. Whether or not the barosensitive NTS neurons are also glucose-sensitive, vagal activation of NTS neurons that regulate ingestive behaviors and digestion clearly converge to modulate the activity of barosensitive neurons in the ventrolateral medulla. Ingestion of calories stimulates duodenal release of cholecystokinin (CCK), which activates subdiaphragmatic vagal afferents that project to the NTS (65) and selectively inhibits splanchnic

SNA to increase mesenteric blood flow (61, 72). In agreement, some baro-activated GABAergic CVLM neurons are also excited by CCK (61), and some baro-inhibited bulbospinal RVLM neurons are inhibited by CCK with timing that corresponds to inhibition of splanchnic SNA (72). Thus, vagal activation of NTS neurons that regulate gastrointestinal function also impact autonomic control of cardiovascular targets to coordinate digestion and blood flow.

In agreement with the notion of a more widespread depression of vagal activation of NTS neurons with chronic hyperglycemia, OZR have impaired sympatho-inhibitory responses to direct stimulation of vagal afferent fibers and activation of the von Bezold-Jarisch reflex by phenylbiguanide (40). Although only a small proportion of NTS neurons activated by phenylbiguanide are also excited by increased AP (68), these reflexes converge at the ventrolateral medulla as phenylbiguanide excites baro-activated GABAergic neurons in the CVLM (73) and inhibits pre-sympathetic RVLM neurons (84). Deficits in sympatho-inhibitory reflexes initiated by vagal afferents are not specific to OZR, because obese Sprague-Dawley rats on a high fat diet have diminished CCK-induced inhibition of splanchnic SNA that coincides with preserved subdiaphragmatic vagal afferent nerve responses and reduced CCK-induced c-Fos expression in the NTS and CVLM (37) . Furthermore, in these rats, bulbospinal RVLM neurons display significantly reduced barosensitivity and inhibition by CCK (37). Similarly, Wistar rats on a high fat diet become hypertensive with impaired baroreflexes and blunted inhibition of renal SNA to volume expansion (43). Renal denervation restores these sympatho-inhibitory reflexes in the obese rats (43), and this denervation improves glycemic control in insulin-resistant rats by reducing hepatic glucose production and increasing insulin sensitivity (15). Thus, poor glycemic control appears to foster a widespread impairment of autonomic reflexes that are mediated by activation of the NTS.

The long-term measurement of blood glucose by telemetry in conscious, undisturbed rats provided more in-depth and accurate assessment of baseline values and changes in blood glucose over time. Analysis spanning 12 - 17 weeks of age demonstrated a comparable fasting blood glucose in OZR and LZR that was chronically elevated in OZR with access to food by 12 weeks of age. In addition, telemetry provided real-time changes in blood glucose that could not be readily achieved with periodic blood samples. For example, the time required for fasting to produce equivalent blood glucose levels in OZR and LZR grew longer as metabolic syndrome progressed over time, and neither group showed significant periods of hypoglycemia with fasting or drug treatments. Furthermore, within this age range one week of metformin treatment was sufficient to reduce fed glucose levels in OZR to match LZR. During glucose tolerance tests, measurement of blood glucose by telemetry was essential for determining the time to reach peak glucose, which was usually longer in OZR than LZR, and an accurate determination of the peak, which would not be possible with the use of timed tail samples.

Measures of blood glucose by telemetry also revealed the development of enhanced glucose responses to mild stressors in OZR. Great care was taken to minimize disturbance to the rats during blood sampling from their tails, and LZR and OZR showed similar alerting and exploration responses with no obvious behavioral differences. At 12-14 weeks of age, comparison of morning blood glucose values measured by tail and telemetry showed no differences in LZR or OZR whether they were fasted or fed, even though OZR displayed glucose intolerance when challenged. However, by 15-17 weeks of age, tail sample values were much higher than telemetry values in fasted OZR but not in LZR. This discrepancy yielded striking contradictory results for fasted blood glucose levels between LZR and OZR, as OZR would be considered hyperglycemic with tail samples but not with telemetry measures. Telemetry also

revealed differences in blood glucose between OZR and LZR after baseline tail samples before glucose tolerance tests and return of food to cages afterward. Metformin treatment greatly reduced but did not eliminate discrepancies between tail sample and telemetry values in 15-17-week-old OZR or differences in peak responses to glucose challenge between OZR and LZR. Thus, this dose of metformin was sufficient to maintain normal blood glucose levels in undisturbed OZR with access to food, but not completely effective for counteracting exaggerated rises in glucose to mild stressors. These data highlight the importance of considering that conscious rats may respond differently to the same environment and handling in healthy and disease states, and that these differences may not be detected by observing their behavior.

Adult male OZR have exaggerated sympatho-excitatory reflexes (41), but brief immobilization stress in adult male OZR evokes enhanced rises in glucose that are accompanied by normal increases in plasma norepinephrine and epinephrine in comparison to LZR (49). Instead, these hyperinsulinemic OZR have larger reductions in plasma insulin after immobilization stress (49), consistent with augmented corticosterone-mediated inhibition of insulin secretion (7). Without functional leptin receptors, OZR are not protected by leptin-mediated suppression of corticosterone release from the adrenal cortex during stressors like immobilization (34). In addition to amplifying stress responses, corticosterone can raise AP and impair glutamatergic activation of the NTS (8, 77). However, corticosterone-induced impairment of baroreflex-mediated changes in HR are related only to the gain, with no changes in the maximal phenylephrine-induced bradycardia (8). Furthermore, the dose and duration metformin used in the present study appear to have no effect on comparable morning plasma levels of corticosterone in OZR or LZR of this age (70). These data suggest the beneficial effects of metformin and pioglitazone were not caused by reductions in corticosterone.

However, enhanced corticosterone actions may contribute to the hypertension and exaggerated stress responses observed in OZR (16, 30, 34).

PERSPECTIVES

Metabolic syndrome significantly increases the risk for development of cardiovascular disease and type 2 diabetes, and the diagnosis is defined by the presence of 3 of 5 attributes with stated threshold values for central adiposity, AP, and fasting blood levels of HDL cholesterol, triglycerides, and glucose. However, mounting evidence supports the importance of dynamic measures of AP and blood glucose. Increased variability of AP is a significant, independent risk factor for coronary heart disease, renal disease, stroke and cognitive decline, and this deficit can be detected with short-term measures such as white coat hypertension or long-term measures such as visit-to-visit variability in AP (25, 48, 57, 67, 70, 88). Furthermore, the variability of AP has been shown to be a better predictor than mean AP for end organ damage and cognitive decline (2, 47, 60). White coat hypertension is significantly associated with a higher prevalence of glucose dysregulation that can be detected by glucose challenge, but not a threshold value for fasting blood glucose (56, 57). Similarly, in the absence of diabetes, a prediabetic level of fasting glucose(>100 mg/dl) is a better predictor of impaired HR variability and HR turbulence than any other attribute of metabolic syndrome (5). Furthermore, in type 2 diabetics the variability of blood glucose has a highly significant inverse relationship with baroreflex sensitivity (58).

The present study postulates that chronic hyperglycemia with access to food is a critical link between insulin resistance and increased variability of AP, because this state impairs the brain stem's response to acute changes in AP. Furthermore, the resulting diminished baroreflex-mediated control of HR occurs independent of hypertension and hyperinsulinemia. Because

insulin resistance and poor glycemic control are not typically managed until a significant increase in fasting blood glucose is observed, a pre-diabetic population may not be treated while hyperglycemia impairs stability of AP. The present study underscores the need for early detection of poor glycemic control in metabolic syndrome to reduce the risks of stroke, cognitive decline, and irreversible organ damage that could occur before the onset of frank type 2 diabetes.

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DISCLOSURES

The authors have no financial disclosures related to this study.

AUTHOR CONTRIBUTIONS

Both authors designed the experiments, collected and analyzed data, prepared figures, and drafted the text of the manuscript.

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CHAPTER III

PRESERVED BAROREFLEXES AND GLYCEMIC CONTROL

IN YOUNG ADULT HYPERTENSIVE FEMALE OBESE ZUCKER RATS

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ABSTRACT

Obese Zucker rats (OZR) have excess weight gain, hyperinsulinemia, and hypertension compared to lean Zucker rats (LZR). By 12 weeks of age, male OZR have impaired baroreflex-mediated activation of nucleus tractus solitarius (NTS) and bradycardia, and both are improved by restoration of glycemic control. In contrast, hypertensive female OZR at this age do not have impaired baroreflex-mediated bradycardia. This study examined whether 12-15-week-old female OZR maintain sympathetic baroreflexes, activation of the NTS, and glucose homeostasis. We also determined whether later development of impaired baroreflex-mediated bradycardia in female OZR (6-month-old) coincides with hyperglycemia and diminished NTS activation. Female OZR (12-15-week-old) had elevated arterial pressure and sympathetic nerve activity (SNA), but sympathetic baroreflexes were equivalent to LZR. Likewise, activation of the NTS by phenylephrine infusion or microinjection of glutamate yielded comparable responses in female OZR and LZR. Coincident with preserved baroreflexes, these female OZR maintained 24-hour glucose homeostasis. In contrast, 24-27-week-old female OZR exhibited markedly attenuated phenylephrine-induced bradycardia. However, phenylephrine-induced c-Fos expression in NTS was comparable in these OZR and LZR, and fed glucose levels were only modestly elevated in female OZR. These data suggest that maintenance of glycemic control may contribute to preserved activation of NTS and baroreflexes in 12-15-week-old female OZR, in support of the notion that impaired glucose homeostasis in male OZR contributes to diminished activation of NTS and baroreflexes. In contrast, older female OZR develop impaired baroreflex-mediated bradycardia independent of changes in activation of NTS, suggesting baroreflex deficits occur by different mechanisms in male and female OZR.

INTRODUCTION

With the rising global epidemic of obesity (47), the prevalence of metabolic syndrome (MetS) has also grown significantly. Currently, almost one-quarter of the world's population exhibits signs of MetS (53). MetS is associated with a cluster of traits that greatly increases risk of poor cardiovascular outcomes (22, 26). In addition to hypertension, impaired baroreflex-mediated control of heart rate (HR) and sympathetic nerve activity (SNA) are commonly observed in both humans and animals with MetS (56, 57, 60, 65). Because these baroreflexes are essential for maintaining stability of arterial pressure (AP), compromised baroreflexes are closely linked to increased arterial pressure variability, a deleterious trait that is present but often overlooked in patients with MetS (14, 30). In the absence of hypertension, AP variability is an independent risk factor for end-organ damage, stroke, and cognitive decline (18, 36, 48, 52, 64). Consequently, understanding mechanisms underlying the impairment of baroreflexes in the setting of MetS is essential for reducing these risks.

Multiple attributes of MetS are strongly and individually linked to baroreflex efficacy and AP variability (14), namely hypertension (8, 21, 44), dyslipidemia (16), insulin resistance (45), and hyperglycemia (19, 42, 57). These attributes of MetS also exacerbate each other, contributing to their comorbid presentation. For example, insulin resistance furthers the development and severity of hypertension in patients with MetS (50). In addition, the presence of hypertension in obese patients worsens baroreflex impairment, providing an indirect link between insulin resistance and baroreflex deficits (20). Even in the absence of obesity and hypertension, insulin resistance is also associated with impaired baroreflexes, particularly parasympathetic baroreflex-mediated bradycardia (45). These interactions complicate the treatment of attributes in MetS, necessitating consideration of comorbidities.

Sex differences have been observed in the development of MetS attributes, with many appearing earlier or in worse severity in males. Compared to women, a similar degree of obesity in men is associated with higher levels of hypertension, triglycerides, hyperinsulinemia, and impaired glycemic control (38). Sex differences appear to be related to where fat depositions occur, with men more likely to accumulate abdominal visceral fat than women (38). Furthermore, obese women who have excess abdominal fat exhibit a male obesity-related metabolic risk profile (38). The elevation in SNA to cardiovascular targets is also generally higher in obese men than premenopausal women (15), and SNA is reduced by weight loss in obese men but not women. Although body mass index (BMI) is highly correlated with SNA in men, neither BMI nor waist circumference are related to SNA in women (39, 58). However, SNA is positively correlated with AP in both obese men and women (39). Thus, the regulation of SNA in obese women appears to be influenced by additional factors that are not present in obese men, but in both sexes elevated SNA drives the obesity-related hypertension.

Sex differences for baroreflexes have also been reported, even in healthy adults. Young adult premenopausal women exhibit greater baroreflex efficacy compared to age-matched men (27) and postmenopausal women (10). Estrogen replacement therapy in postmenopausal women improves baroreflex function (34) and also appears to protect against the development of insulin resistance and diabetes (25, 51). Therefore, the loss of estrogen-related preservation of glycemic control may contribute to the negative impact of menopause upon baroreflexes. However, connections between sex-based differences for impairment of glycemic control and baroreflexes in the setting of MetS have not been elucidated.

Obese Zucker rats (OZR) develop MetS with sex differences in the onset of impaired baroreflex-mediated changes in HR (23, 56, 59). Male and female OZR have nonfunctional

leptin receptors owing to a genetic mutation, which promotes hyperphagia and excess weight gain before adulthood (35). By 12 weeks of age, young adult male OZR develop hypertension and blunted baroreflexes (23, 56), coincident with impaired baroreflex-mediated activation of the NTS (23, 33). At this age, male OZR have insulin resistance, characterized by hyperinsulinemia and glucose intolerance but normal fasting blood glucose (9). However, when these OZR have access to food, blood glucose measured by telemetry is chronically elevated. Furthermore, restoration of normal fed glucose levels enhances baroreflex-mediated activation of the NTS and bradycardia, even in the persistence of hyperinsulinemia and hypertension (9).

In contrast to male OZR, at 3 months of age female OZR have hypertension, but baroreflex-mediated bradycardia is comparable to age-matched female LZR (59). However, by 6 months of age female OZR have impaired baroreflex-mediated bradycardia, suggesting female OZR develop the same dysfunction with a delayed onset, as observed in obese humans (10, 27). This study tested the hypothesis that preserved baroreflexes in young adult female OZR coincides with their ability to maintain glucose homeostasis and baroreflex-mediated activation of the NTS. Because fasting blood glucose does not reflect fed glucose levels in the presence of insulin resistance (9), and OZR have exaggerated rises in glucose with brief stressors (40), blood glucose was measured by telemetry in undisturbed rats. Furthermore, we determined whether older female OZR develop impaired baroreflex-mediated bradycardia coincident with a decline in baroreflex-mediated activation of the NTS and elevated fed glucose levels.

MATERIALS AND METHODS

Animals

Male and female OZR [Lepr (fa/fa)] and LZR [Lepr (+/+) and (+/fa)] from Charles River (Houston, TX) were individually housed in centralized animal care facilities kept at a consistent humidity (60±5%), temperature (24±1°C), and light cycle (lights on 7:00 am – 7:00 pm). All the rats were fed on standard rat chow (Prolab RMH 1800, LabDiet). Experiments were performed on young adult (13-17 weeks) and older adult (25-27 weeks), age-matched OZR and LZR. The University of North Texas Health Science Center Institutional Animal Care and Use Committee approved all animal experiments, which were performed in accordance with guidelines from the National Institutes of Health's *Guide for Care and Use of Laboratory Animals* and the American Physiological Society's *Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training*.

Surgical preparation for cardiovascular measures in conscious rats

Young (13-14-week-old) and older (24-27-week-old) adult female Zucker rats were anesthetized with isoflurane (5% in a ventilated box and then 2.4% through a nose cone). Catheters were implanted into the left femoral artery to record AP and HR and the left femoral vein to infuse fluids. In 24-27-week-old rats a 0.5 ml blood sample was taken from the arterial line, and the volume was replaced by flushing the line with isotonic saline. As previously described, the free ends of the catheters were tunneled subcutaneously to exit between the scapulae (23). The rats were fitted with a tether and dual channel swivel (Instech Laboratories) that was attached to a counter-balanced lever arm to allow them to move freely in a covered

plexiglass cylindrical cage (MTANK/W and MTOP, Instech). The rats were allowed to recover after surgery for 24 - 48 hours with access to food and water.

Assessment of baroreflex-mediated bradycardia in conscious rats

On the day of the experiment, the cages were surrounded with a cover to minimize disturbance to the rat. The arterial line was connected through the swivel to a transducer (NL108T2, Digitimer), and the venous line was connected through the swivel to an infusion pump (Model A-99, Razel). Following 30 minutes of baseline recording of AP and HR, PE was infused to raise mean AP by 40 mmHg for 90 minutes (13-31 µl/minute of 0.5 mg/ml of PE in saline, i.v.). This protocol allowed for measurement of baroreflex-mediated bradycardia within the first 5 minutes and later activation of c-Fos expression in the brain stem. During the last 30 minutes of the PE infusion, the PE-filled syringe was replaced with a saline-filled syringe to flush the PE from the line. After 90 minutes of infusion, rats were deeply anesthetized with urethane (1.5 g/kg, i.v. bolus) and perfused transcardially with 250 ml of phosphate-buffered saline (pH 7.4) followed by 500 ml of 4% phosphate-buffered paraformaldehyde (Electron Microscopy Sciences). The brains were extracted and stored in the same fixative for 48 hours.

Immunohistochemistry for c-Fos

Brain stems were sectioned with a Vibratome (30 µm, coronal plane) and stored at -20°C in a cryoprotectant solution. Immunohistochemistry for c-Fos protein was performed on free-floating sections (every 1 in 6 sections) on an orbital shaker in solutions prepared in Tris-buffered saline (TBS, pH 7.4) at room temperature unless specified otherwise. Subsets of OZR and LZR were run together to ensure consistent conditions between groups. The sections were

incubated with 1% hydrogen peroxide (30 minutes) to block endogenous peroxidases, rinsed in TBS, and blocked in 10% horse serum (45 minutes). Then, sections were incubated with a goat-anti c-Fos primary antibody (1:2,000; 4°C; 48 hours; sc-52G, Santa Cruz Biotechnology), as previously described (23). After rinsing in TBS, sections were incubated with a biotinylated donkey anti-goat secondary antibody (1:400; 1 hour; 705–066-147, Jackson Laboratories) followed by an avidin-biotin solution (1 hour; PK-6100; Vector Laboratories). Incubation with a nickel-intensified 3–3'diaminobenzadine solution was used to reveal the c-Fos immunoreactivity. The reaction was carefully monitored for 8 - 10 minutes and terminated with a TBS rinse when staining became visible under a dissecting microscope. Prepared sections were mounted onto gelatin-coated slides and air-dried overnight. Slides were submerged through a series of alcohols and xylenes and then coated with DPX mounting medium (Sigma-Aldrich) to affix coverslips. The c-Fos-immunoreactive (Fos+) neurons were mapped and counted bilaterally in the NTS at four rostro-caudal levels using a Ludl-motor-driven stage and Neurolucida software (MicroBrightfield) as previously described (23).

Surgical preparation for measures in anesthetized rats

Young adult (15 week) female Zucker rats were anesthetized with isoflurane (5% in a ventilated box and then 2.4% through a nose cone) with 100% oxygen. After confirming the adequacy of anesthesia by toe pinch, femoral arterial and venous catheters were implanted. A 0.5 ml blood sample was taken from the arterial line, and the volume was replaced by flushing the line with sterile saline. After insertion of a tube into the trachea, rats were artificially ventilated. For LZR the ventilation rate was ~55–65 strokes/min of 1 ml/100 g LZR body weight (Model 683; Harvard Apparatus). For OZR the initial ventilation was based on the tidal

volume of the age-matched LZR, with further adjustment slightly upward to achieve an end-tidal CO_2 comparable to the LZR (3.5- 4.0%; CapStar-100; CWE) at a similar rate of ventilation, as previously described (33). The rat was placed in a stereotaxic instrument (David Kopf Instruments) with the bite bar set at -11 mm to facilitate exposure of the dorsal brainstem. The left greater splanchnic nerve was exposed retroperitoneally, isolated immediately distal to the adrenal branch, and placed on the bared tips of two Teflon-coated silver wires (A-M Systems). After carefully removing surrounding fluid, the nerve and exposed wires were encased in silicone elastomer (Kwik-Sil; World Precision Instruments), as previously described (33). To expose the dorsal surface of the brainstem, the occipital bone was exposed and removed, and the underlying meninges were clipped and retracted. Rectal temperature was maintained continuously at 37°C. After completion of the surgical preparations, the isoflurane anesthesia was replaced by urethane (1.5 g/kg LZR body weight administered intravenously using 1.5 g/5 ml solution at 50 ml/min). After 30 - 45 minutes of recovery under urethane anesthesia, the rat was paralyzed with pancuronium (0.1 ml/100g from a 1mg/ml solution with 1/3 dose supplements hourly; Hospira).

Assessment of sympathetic baroreflexes in anesthetized rats

To produce a steady rise in mean AP, phenylephrine (PE) was infused (2 mg/100g from a 1 mg/ml solution, at 34 - 42 ml/min, iv) until the splanchnic SNA reached an obvious lower plateau. Then after the mean AP and SNA had returned to within 90% of baseline, sodium nitroprusside was infused (2mg/100g from a 1mg/ml solution, infused at 34-42 ml/min, iv) to steadily reduce mean AP until SNA reached an obvious upper plateau. The rat was allowed to recover to baseline mean AP and SNA before receiving microinjections into the NTS.

Microinjections into the NTS in anesthetized rats

Microinjections into the brainstem were performed using single-barrel glass pipettes pulled and cut to a 40 - 50 mm diameter tip that were fixed on a stereotaxic arm and connected to a pressure microinjection apparatus (Pressure System IIe; Toohey). Glutamate (1 nmol in 50 nl) was prepared in artificial cerebrospinal fluid and injected over a period of 8 - 10 seconds (Pressure System IIe; Toohey). The stereotaxic coordinates for the NTS were 0.5 mm lateral to the midline, 0.5 mm rostral to calamus scriptorius, and 0.5 mm ventral to the dorsal surface of the brain stem (23). After bilateral injections of glutamate, the pipette was cleaned and filled with the $GABA_A$ receptor agonist muscimol (100 pmol in 100 nl) for microinjection into the NTS bilaterally. Following the microinjections, the rat was treated with a ganglionic antagonist (mecamylamine; 5 mg/kg iv) to estimate the minimum SNA. After the completion of the experiment, the rat was euthanized with urethane and decapitated.

Measurement of plasma insulin, cholesterol, and triglycerides

Arterial blood samples were collected in heparinized tubes and centrifuged immediately to isolate plasma. Plasma samples were aliquoted and stored at -20°C for analysis by ELISA. Rat Ultrasensitive Insulin ELISA kit (80-INSRTU-E01, ALPCO) was used to quantify plasma insulin concentration. Measurements of plasma cholesterol and triglyceride concentrations were performed using a Cholesterol E kit (439-17501, Wako Diagnostics) and L-Type TG M reagents (Color A 461-08992, Color B 461-09092 and Multi-Lipid calibrator 464-01601, Wako Diagnostics) respectively.

Implantation of glucose sensing transmitters

Using aseptic conditions, a laparotomy was performed while the rat was under isoflurane anesthesia. The tip of the transmitter catheter (HD-XG, Data Sciences International) was inserted rostrally into the abdominal aorta distal to the kidneys of 13-14-week-old Zucker rats. The aortic wall was sealed around the catheter with a piece of mesh and a small drop of cyanoacrylate adhesive. The catheter and its connected transmitter were secured to the abdominal wall using 4.0-prolene sutures. After closing the incision, rats were kept warm and monitored until fully conscious. Each cage was placed on a receiver (DSI) to continuously measure blood glucose by telemetry. The transmitters were calibrated using blood samples from the tail with rats in fasted and fed states and with glucose tolerance tests. Glucose levels were recorded continuously for approximately 4 weeks.

Glucose measures by telemetry

A glucose tolerance test (GTT) was performed 3 - 4 days after implantation of the glucose transmitter. Rats were fasted for 18 hours with access to water, and then a baseline blood sample was taken at approximately 8:00 a.m. While rats remained in their home cage, the tip of the tail was snipped with scissors to obtain a drop of blood, and the blood was applied to a glucose test strip that was inserted into a calibrated hand-held glucometer (Accu-Chek® Aviva Plus). One hour later each rat was briefly lifted from their cage and injected with glucose (1g/kg from a 0.5g/ml solution; 0.5-1.0 ml, i.p.). While the rats rested in their home cage, additional tail blood samples were taken at 15, 30, 45, 90, and 120 minutes after administration of glucose. Periodic fed glucose samples were taken every 1-2 weeks. All blood sample readings were

performed in duplicate and entered into the telemetry data acquisition software (Ponemah software platform, DSI). A second GTT was performed at 15 weeks of age.

Data acquisition and analysis

The AP was measured through a femoral artery catheter, and the mean AP and HR were derived from the AP pulse using a low-pass filter (NL110) and a spike trigger (NL201), respectively (Neurolog System, Digitimer). Raw splanchnic SNA was amplified and filtered (10 Hz to 3 kHz with 60 Hz notch filter, differential AC amplifier 1700, A-M Systems), and baseline voltage was obtained after subtraction of voltage due to noise (33). Raw SNA was full-wave rectified and integrated into 1-second bins, and changes in integrated SNA were measured as percent change from baseline (33). The analog signals were converted to digital form (Micro 1401, Cambridge Electronic Design) to view them online using Spike2 software (Cambridge Electronics). All group data are shown as mean ± SE. The significant statistical difference was set at $P < 0.05$. Baseline parameters, quantified measures for SNA baroreflex curves, and NTS microinjections in age-matched OZR and LZR were compared using unpaired t-tests. Hourly and daily glucose values, standard deviation of glucose, glucose tolerance test, and counts of Fos+ neurons were compared using the appropriate ANOVA followed by Tukey post hoc tests (SigmaStat software version 3.5). Baroreflex-mediated changes in SNA were fit and analyzed with a sigmoid curve (Origin lab Software 2017).

RESULTS

Phenylephrine (PE)-induced bradycardia and NTS c-Fos expression: 13-14-week-old females

In age-matched young adult females (96.4 ± 1.7 days in 7 LZR vs. 96.1 ± 1.3 days in 9 OZR) body weight was higher in OZR (481.3 ± 15.0 g) compared LZR (222.3 ± 6.3 g, $P<0.05$, unpaired t-test). Baseline mean AP was higher in conscious female OZR compared to LZR (Figure III-1A), but HR was not different (392.6 ± 4.9 bpm in LZR vs. 388.3 ± 10.2 bpm in OZR). Infusion with PE to raise MAP by 40 mmHg evoked a comparable baroreflex-mediated bradycardia response in female OZR and LZR (Figure III-1B). In contrast, as shown in Chapter II, baroreflex-mediated bradycardia was significantly reduced in male OZR compared to LZR at this age (Figure III-1C, rescaled from Figure II-6).

In the same female rats (Figure III-1A and B), the PE infusion was continued to maintain a 40-mmHg rise in mean AP for 90 minutes and induce c-Fos expression in the NTS. Compared to the LZR, the PE-induced c-Fos expression in OZR was slightly but significantly reduced at -14.2 mm from bregma, but at 3 more rostral levels of NTS (-13.8, -13.4, -13.0 mm from bregma) the number of c-Fos+ neurons were comparable between OZR and LZR examined (Figure III-2A). The total number of c-Fos-expressing neurons at the 4 rostro-caudal levels of caudal and intermediate NTS of OZR was equivalent to LZR (Figure III-2B). The distributions of c-Fos+ neurons within the 4 levels of the NTS were similar in LZR and OZR (representative LZR and OZR in Figure III-2C).

Baroreflex-induced changes in SNA in female rats at 15 weeks of age

In age-matched young adult females (108.6 ± 0.9 days in 5 LZR vs. 107.8 ± 0.6 days in 5 OZR) body weight was higher in OZR (466.0 ± 5.1 g) compared LZR (230.4 ± 4.2 g, $P<0.05$, unpaired t-test). The OZR had a significantly elevated baseline splanchnic SNA and mean AP

compared to LZR under isoflurane at the end of surgical preparation and under urethane anesthesia immediately before baroreflex assessment (Table III-1). Baseline HR was not different between groups under either anesthesia, although HR tended to be higher under urethane anesthesia (Table III-1). The PE was infused to raise mean AP until SNA reached an obvious lower plateau. Once the mean AP returned to baseline, sodium nitroprusside was infused to reduce mean AP until SNA had reached an upper plateau. The changes in SNA over the full range of mean AP were fit to a sigmoid curve for analysis (see Figure III-3 for representative examples in an LZR and OZR). In agreement with the higher baseline mean AP in OZR (Table III-1), the MAP_{50} of the curve was also higher in OZR than LZR (Table III-2). However, the slope, upper and lower plateaus, and range of SNA over the full range of mean AP was not different between LZR and OZR (Table III-2), in agreement with the PE-induced bradycardia in conscious OZR and LZR (Figure III-1B).

Microinjections into the NTS in female Zucker rats at 15 weeks of age

The NTS was activated on each side by microinjection of glutamate (1 nmol in 50 nl), and the decreases in SNA and mean AP from both sides were averaged for each rat (representative tracing of physiological responses in an LZR in Figure III-4A). Microinjection of glutamate into NTS of 15-week-old females evoked comparable changes in SNA and mean AP in LZR and OZR (Fig III-4B), unlike the smaller physiological responses observed in 14-week-old male OZR (Fig III-4C). Likewise, microinjections of muscimol into the NTS produced comparable rises in SNA, HR, and mean AP in female LZR and OZR (Fig III-4D-F), in contrast to previously reported smaller rises in SNA and HR with muscimol-induced inhibition of the NTS in young adult, male OZR compared to LZR (32).

Table III-3 shows metabolic attributes in 15-week-old rats. The OZR weighed significantly more than age-matched females LZR. As seen with males (Table II-3), the OZR had elevated plasma insulin, cholesterol, and triglycerides compared to age-matched LZR. However, fed glucose levels were comparable in female OZR and LZR, unlike the hyperglycemia observed in male OZR with access to food at this age (Figure II-5B).

Blood glucose measured by telemetry in female Zucker rats at 13-14 weeks and 15 weeks

At 13-14 weeks of age female OZR and LZR had comparable morning fasting and fed glucose when measured by telemetry (Figure III-5A), although access to food yielded higher blood glucose levels in both OZR and LZR (Figure III-5A). Likewise, with access to food their 24-hour blood glucose levels recorded by telemetry were comparable (Fig III-5B). In addition, these OZR and LZR also had equivalent 24-hour standard deviation of blood glucose (4.6 ± 0.3 in LZR and 5.6 ± 0.4 in OZR) that was reflected in their daytime and nighttime blood glucose variability (Figure III-5C). For contrast, see males in this age range in Figure II-1 with markedly elevated fed glucose levels and glucose variability in OZR compared to LZR.

As seen 13-14-week-old female Zucker rats, at 15 weeks of age female OZR and LZR had comparable fasting and fed glucose, although fed glucose levels were higher than fasted glucose levels in both groups (Fig. III-5D). However, at 15 weeks of age female OZR began to show elevations in blood glucose late in the day and into the early evening compared to LZR (Figure III-E). In addition, in these 15-week-old OZR standard deviation of blood glucose was approximately doubled in the day and night hours compared to LZR (Figure III-F).

Glucose tolerance test (GTT) in female Zucker rats at 13-14 weeks and 15 weeks

After 18 hours of overnight fasting with access to water, rats were injected with glucose (1g/kg, i.p.) to determine their glucose tolerance. The 13-week-old female OZR and LZR had comparable rises in blood glucose with equivalent peak glucose values (Figure III-6A and B) and return of glucose to baseline with comparable areas under the curve (Figure III-6C). In contrast, by 15 weeks of age, impairment in glycemic control emerged as female OZR exhibited a higher peak blood glucose in response to glucose challenge compared to LZR (Figure III-6D and E) and a delayed return to baseline with a larger area under the curve (Figure III-6F).

Time course of development of changes in fed blood glucose in female and male Zucker rats

With continuous measures of glucose by telemetry from 12 - 17 weeks of age, the time courses of changes in daily blood glucose and standard deviation were determined in female and male Zucker rats. Female OZR exhibited comparable daily blood glucose levels from 12 - 15 weeks of age, with modest but significant increases at 16 – 17 weeks of age that remained below 140 mg/dl (Figure III7-A). In contrast, in male OZR daily blood glucose levels were elevated above male LZR at 12 weeks of age with daily blood glucose levels rising and averaging above 140 mg/dl at 13-16 weeks of age (Figure III7-B). In both female and male OZR the standard deviation of blood glucose began to rise above LZR at 13 weeks of age with consistent differences by 14 weeks of age (Figure III-7C, D).

Metabolic parameters in female Zucker rats at 24-27 weeks

As observed with 15-17-week-old female OZR (Figure III-7A), at 24-27 weeks of age female OZR had markedly elevated plasma insulin, cholesterol and triglycerides compared to

LZR (Table III-4). Fed glucose levels were modestly elevated female OZR compared to age-matched female LZR (Table III-4). The fed glucose levels in these females OZR are similar to those observed in pioglitazone-treated male OZR at 15- 17 weeks of age (Fig. II-9A).

PE-induced bradycardia and NTS c-Fos expression in female Zucker rats at 26-27 weeks

As seen at 13-14 weeks of age (Figure III-1A), female OZR were hypertensive (Figure III-8A). In contrast to 13-14-week-old female OZR (Figure III-1B), 26-27-week-old female OZR had markedly reduced PE-induced bradycardia compared to age-matched female LZR (Figure III-8B and C). However, in these 26-27-week-old rats, PE-induced c-Fos expression in the NTS was comparable in OZR and LZR at all 4 rostro-caudal levels examined (Figure III-8D and E).

Figure III-1. Mean Arterial Pressure and Phenylephrine-induced Bradycardia in Conscious Adult Female Zucker Rats at 13-14 weeks with Males for Comparison

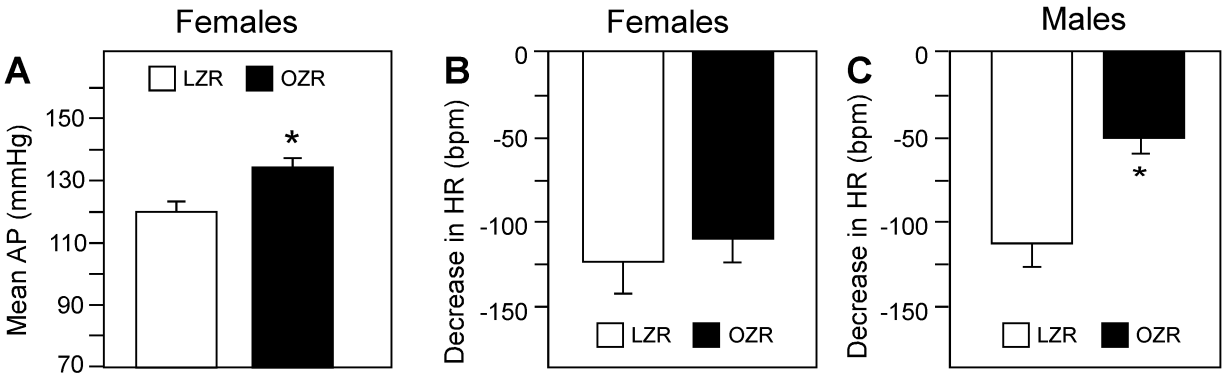


Figure 1. Baseline mean arterial pressure (AP) and phenylephrine (PE)-induced bradycardia in conscious 13-14-week-old in rats. (*A*) Baseline mean AP in young adult (13-14-weeks old) female LZR (7) and OZR (9). (*B*) Decrease in heart rate (HR) evoked by a 40-mmHg rise in MAP with infusion of PE in young adult (13-14-weeks old) female rats. *N.S.*, unpaired t-tests. The PE-induced c-Fos expression in these female rats in shown in Figure III-2. (*C*) The PE-induced bradycardia in 14-week old male rats with scale adjusted for comparison with females (from Figure II-6). $*P < 0.05$, vs. male LZR, unpaired t-tests.

Figure III-2. Phenylephrine-mediated c-Fos Expression in the Nucleus Tractus Solitarius (NTS) in Conscious Female Zucker Rats at 13-14 Weeks

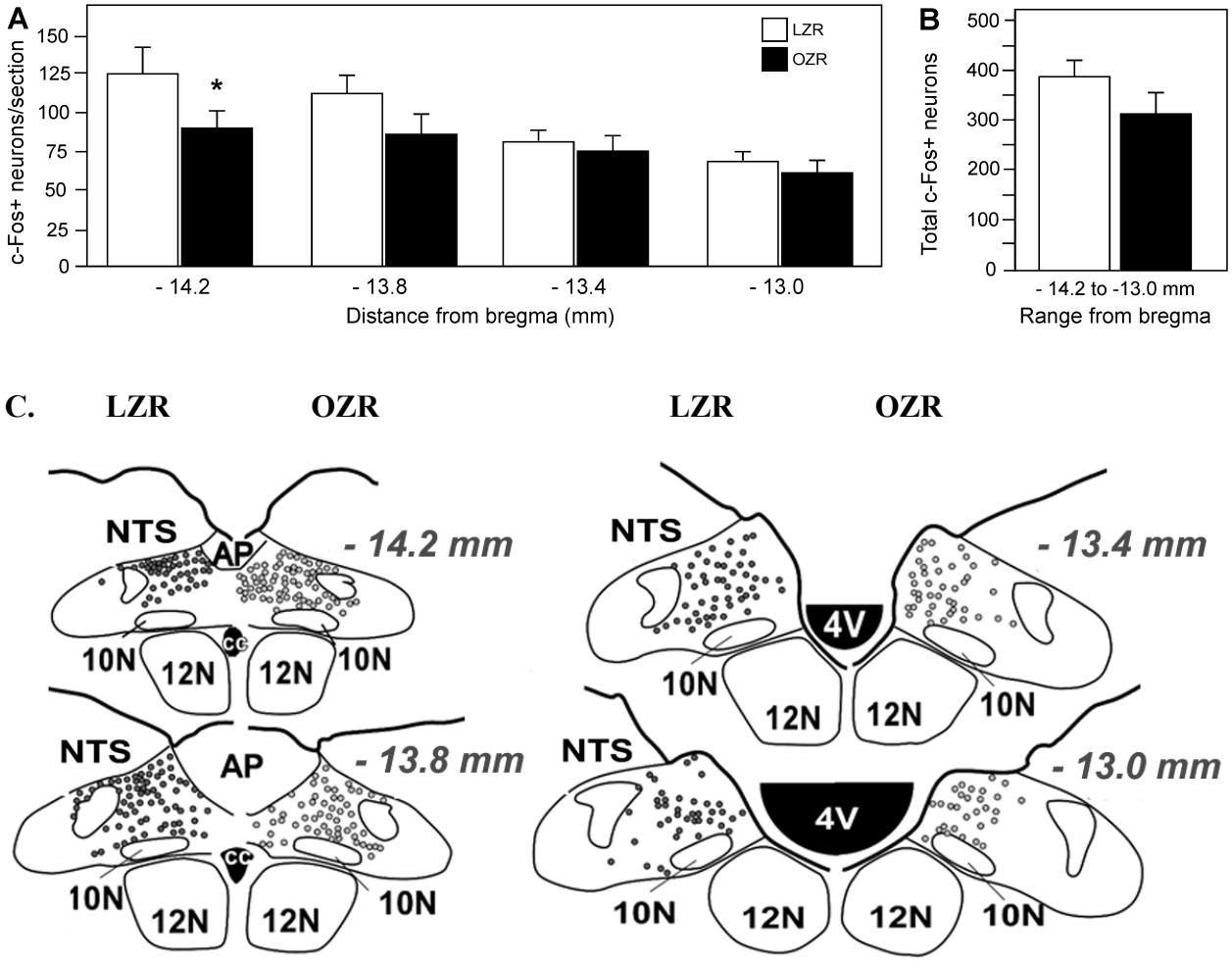


Figure 2. Phenylephrine-induced c-Fos expression in the nucleus tractus solitarius (NTS) of conscious, 13-14-week-old female LZR and OZR. (*A*) Number of c-Fos-positive neurons from bilateral counts of NTS at 4 rostro-caudal levels. * $P < 0.05$ vs. LZR at that bregma level, 2-way ANOVA with Tukey post hoc tests for rat type x bregma level. (*B*) Total counts of c-Fos-positive neurons from all 4 rostro-caudal levels of the NTS. *N.S.*, 2-way ANOVA with Tukey post hoc tests (*C*) Representative maps of c-Fos-positive neurons at 4 rostro-caudal levels of the NTS with LZR on the left side and OZR on the right side of each section.

TABLE III-1. Baseline Mean Arterial Pressure, Sympathetic Nerve Activity, and Heart Rate in Anesthetized Female Zucker Rats at 15 Weeks

		isoflurane anesthesia			urethane anesthesia		
Group	*n*	SNA (mV)	mean AP (mmHg)	HR (bpm)	SNA (mV)	mean AP (mmHg)	HR (bpm)
LZR	5	1.7 ± 0.3	98.5 ± 1.9	387.4 ± 12.4	0.98 ± 0.1	94.1 ± 4.7	436.6 ± 7.3
OZR	5	3.3 ± 0.6*	119.2 ± 1.4*	342.4 ± 12.7	2.25 ± 0.4*	113.4 ± 2.9*	412.5 ± 14.4

Data are expressed as mean ± SE. Surgical preparation was performed with rats under isoflurane anesthesia. Afterwards, the rats were switched to urethane anesthesia before examining sympathetic baroreflexes (Figure III-3, Table III-2). * $P < 0.05$, unpaired t-tests.

Figure III-3. Representative Sympathetic Baroreflex Curves in Female Zucker rats at 15 weeks

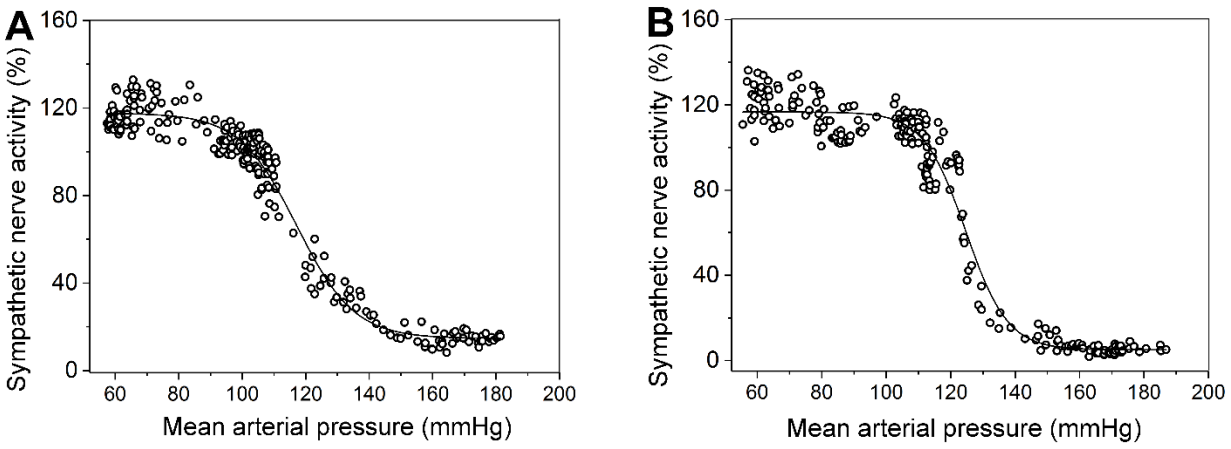


Figure 3. Representative sigmoidal analysis of the sympathetic baroreflex in a 15-week old female LZR (*A*) and OZR (*B*). Rats were anesthetized with urethane (1.5 g/kg in a 1.5g/5ml solution infused at 51 ml/min, iv), artificially ventilated, and paralyzed (pancuronium, 0.1 ml/100g from a 1mg/ml solution). Mean arterial pressure (MAP) was raised by infusion of PE (2mg/100g from a 1mg/ml solution, infused at 34-42 ml/min, iv) until spanchnic SNA reached a lower plateau. After MAP returned to baseline levels, MAP was lowered by infusion of sodium nitroprusside (2μg/100g from a 1mg/ml solution, infused at 34-42 μl/min, iv) until SNA reached an upper plateau. See Table III-1 for group data of baseline values and Table III-2 for group values for the sympathetic baroreflex.

Table III-2. Sympathetic baroreflexes in Anesthetized Female Zucker Rats at 15 Weeks

Group	*n*	MAP_{50} (mmHg)	slope (%/mmHg)	lower plateau (%)	upper plateau (%)	range (%)
LZR	5	117.3 ± 5.2	- 2.8 ± 0.4	19.6 ± 2.7	113.1 ± 1.7	93.5 ± 3.3
OZR	5	132.5 ± 1.9*	- 2.2 ± 0.6	23.6 ± 7.5	113.0 ± 10.2	89.4 ±16.1

Data are expressed as mean ± SE. In urethane-anesthetized, artificially ventilated, paralyzed rats changes in MAP and SNA were measured during infusions of PE and nitroprusside. Baseline values are in Table III-1. Figure III-3 shows representative examples of the relationship between MAP and SNA in an LZR and OZR. * $P < 0.05$, unpaired t-tests.

Figure III-4. Physiological effects of activation and inhibition of the NTS in Zucker rats at 14-15 Weeks

Activation of the NTS by microinjections of glutamate in female and male Zucker rats

Inhibition of the NTS by microinjections of muscimol in female Zucker rats

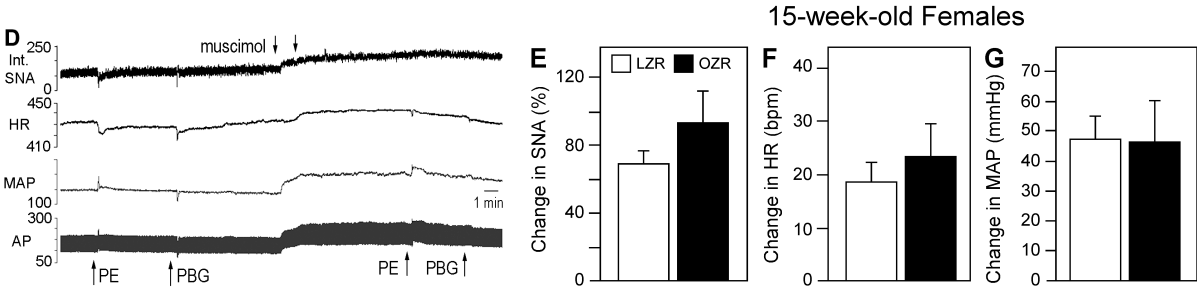


Figure 4. Microinjections of glutamate (1 nmol/50 nl) and muscimol (100pmol/100nl) into the NTS of urethane-anesthetized, artificially ventilated, paralyzed rats. (*A*) Representative traces of changes in splanchnic SNA and MAP after microinjection of glutamate into the NTS. (*B*) Glutamate evoked comparable decreases in SNA and MAP in female OZR (4) and LZR (5). (*C*) Glutamate evoked smaller decreases in SNA and MAP in male OZR (12) and LZR (10), * $P<0.05$, unpaired t-test. (*D*) Representative traces of changes in SNA, heart rate (HR), and MAP with bilateral microinjections of $GABA_A$ agonist, muscimol (arrows, left and right). Confirmation of inhibition of the NTS by absence of PE-induced bradycardia and phenyl

biguanide (PBG)-induced decreases in SNA, HR, and SNA. (*E*-*G*) Muscimol-induced rises in SNA, HR, and MAP are comparable in female OZR (4) and LZR (5).

Table III-3. Baseline Plasma Values in Fed Female Zucker Rats at 15 Weeks

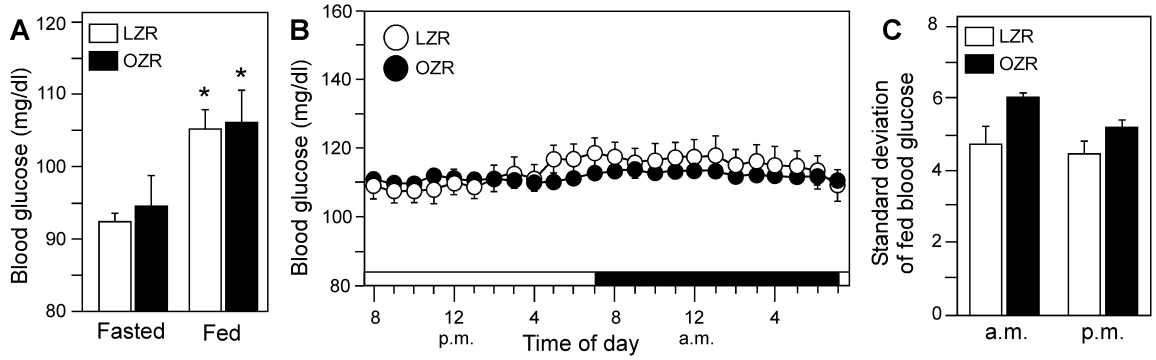
Group	*n*	age (days)	body weight (g)	insulin (ng/ml)	cholesterol (mg/dl)	triglycerides (mg/dl)	fed glucose (mg/dl)
LZR	8	107.3 ± 0.9	222.4 ± 3.0	1.0 ± 0.1	69.8 ± 4.6	102.3 ± 18.9	108.2 ± 1.6
OZR	7	107.6 ± 1.0	488.7 ± 14.7*	7.4 ± 2.0*	119.8 ± 6.8*	567.4 ± 101.0*	109.3 ± 3.4

Data are expressed as mean ± SE. Samples were obtained between 8:00 – 10:00 am rats that were not fasted. After insertion of a femoral arterial catheter under isoflurane, a blood sample was drawn to obtain plasma for measurement of insulin, cholesterol, and triglycerides. Blood glucose was obtained from a tail snip in the rat's home cage on the morning before experiments.

* $P < 0.05$ vs. LZR, unpaired t-tests.

Figure III-5. Baseline Blood Glucose Values and Their Variability Measured by Telemetry in Female Zucker rats at 13-15 Weeks

13-14 weeks old



15 weeks old

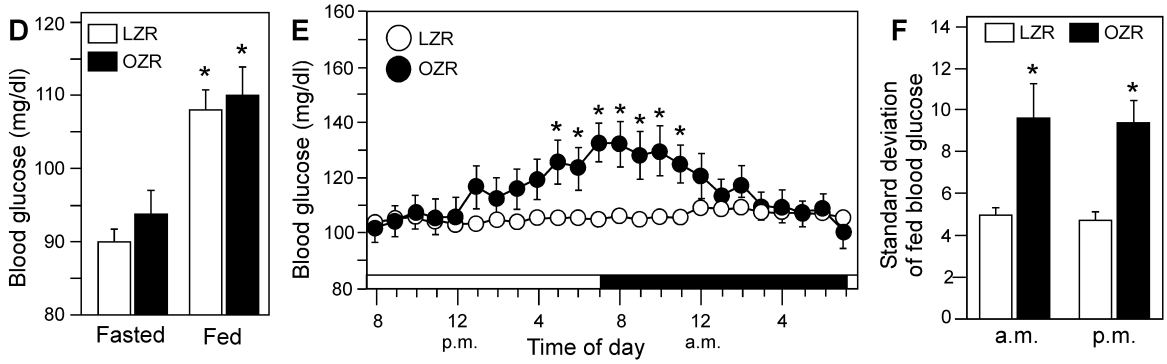
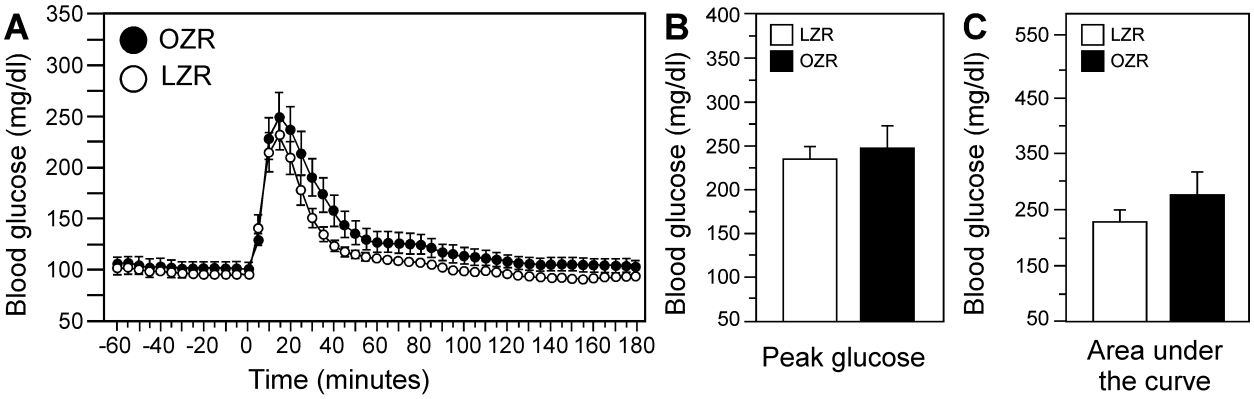


Figure 5. Blood glucose levels measured by telemetry in young adult, female OZR (12) and LZR (11) at 12-13 weeks and 15 at weeks (13/group). (*A, D)* Morning blood glucose levels in fasted and fed states, * $P < 0.05$ vs. fasted state of same rat type. (*B, E)* Hourly averages over a 24-hour period in these OZR and LZR with access to food, *$P<0.05$ vs. OZR at that hour, *(C, F)* Standard deviation of glucose in these LZR and OZR in the morning (5:30 a.m. – 7:30 a.m.) and in the evening (9:30 p.m. – 11:30 p.m.), *$P<0.05$ vs. LZR at that time period. Data were analyzed by ANOVA with repeated measures followed by Tukey post hoc tests.

Figure III-6. Glucose Tolerance Tests in Female Zucker rats at 13-15 Weeks

13 weeks old



15 weeks old

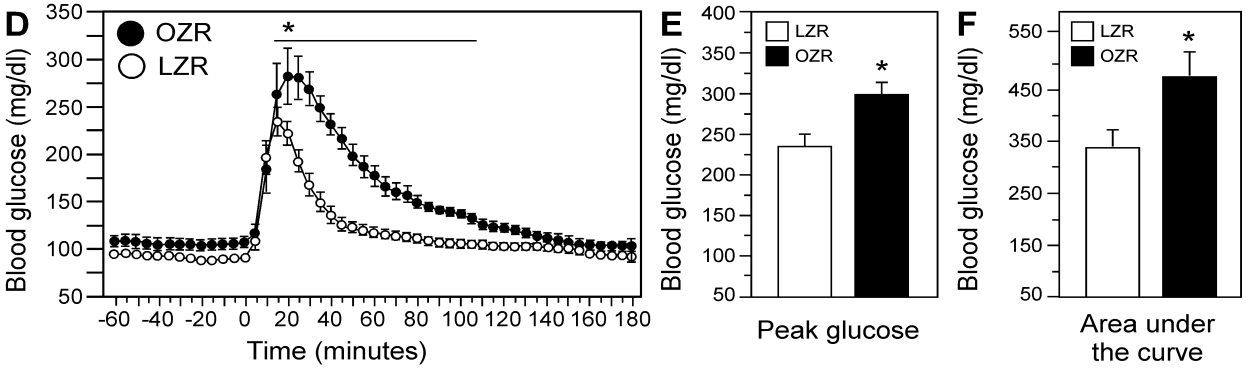


Figure 6. Glucose tolerance test (GTT) in OZR and LZR at 13 and 15 weeks of age. See Figure III-5 for baseline glucose values for these rats. *(A, D)* Blood glucose values after injection of glucose (at time 0) measured by telemetry every 5 minutes over 180 minutes, **P<0.05*, vs. LZR at that time point, 2-way ANOVA with repeated measures and Tukey post hoc tests. *(B-C, D-F)* Peak glucose values and areas under the curve for GTTs. (*B-C, E-F*) **P<0.05* vs. LZR, unpaired t-tests.

Figure III-7. Time Courses for Development of Increased Blood Glucose and Variability of Blood Glucose in Conscious Female and Male Zucker rats over 12-17 Weeks

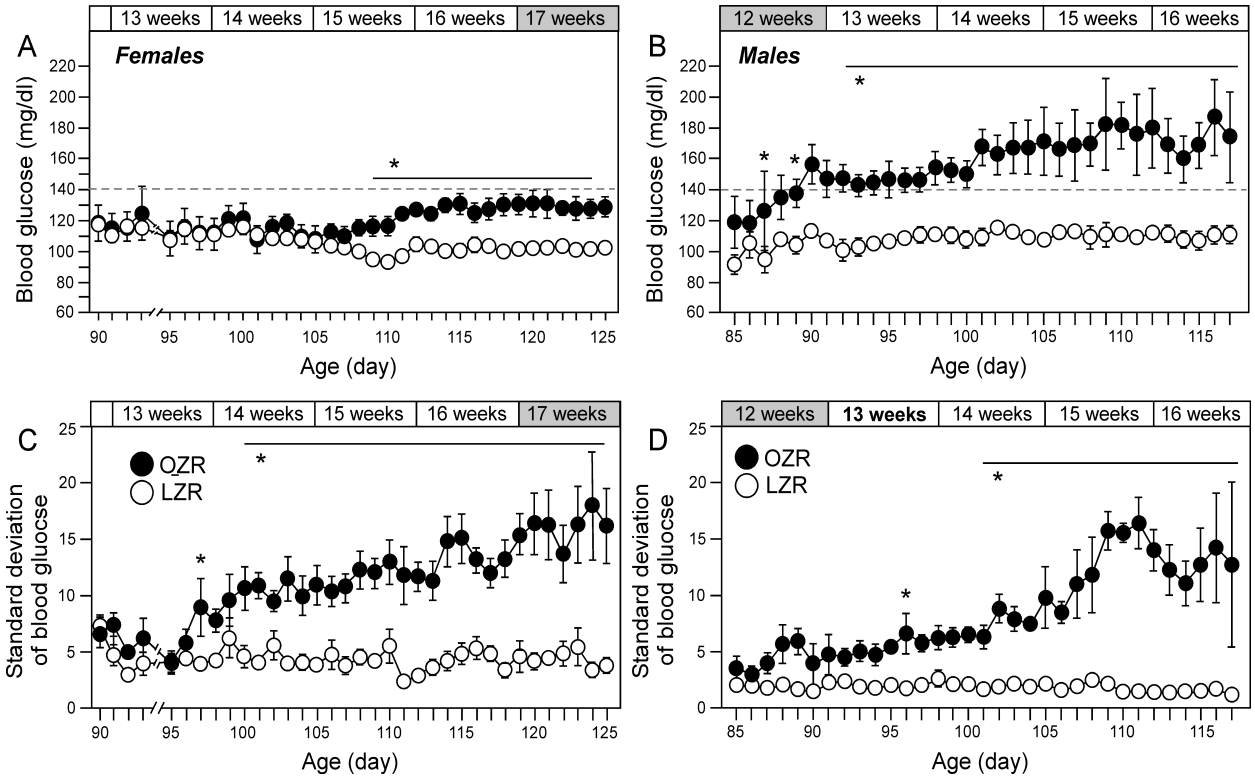


Figure 7. Time courses of blood glucose levels measured by telemetry in female LZR and OZR at 13-17 weeks and male LZR and OZR at 12-16 weeks with access to food. (*A)* Average mean daily blood glucose levels of female LZR (n = 13, 5-13 values/day) and OZR (n = 13, 5-13 values/day), (*B)* Average mean daily blood glucose levels of male LZR (n = 5, 3-5 values/day), and OZR (n = 5, 3-5 values/day), *(C)* Average mean standard deviation of daily blood glucose in female rats in *(A)*, *(D)* Average mean standard deviation of daily blood glucose levels in male rats in *(B)*. Day 94 was removed from the females (*A* and *C*) due to a large number of rats undergoing fasting and glucose tolerance tests on that day. A day was defined as the time period between 12 a.m. – 11 p.m. Each set of data were analyzed by 2-way ANOVA with repeated measures followed by Tukey post hoc tests. *P<0.05 vs. LZR on that day.

Table III-4. Baseline Plasma Values in Female Zucker Rats at 24-27 Weeks

Group	*n*	age (days)	body weight (g)	insulin (ng/ml)	cholesterol (mg/dl)	triglycerides (mg/dl)	fed glucose (mg/dl)
LZR	8	186.4 ± 0.2	278.0 ± 7.5	0.8 ± 0.2	88.0 ± 5.4	138.5 ± 22.0	108.9 ± 1.8
OZR	7	185.2 ± 2.1	669.4 ± 23.5*	7.3 ± 1.7*	480.5 ± 60*	845.8 ± 45.0*	126.1 ± 4.8*

Data are expressed as mean ± SE. Samples were obtained between 8:00 – 10:00 am from rats that were not fasted. After insertion of a femoral arterial catheter under isoflurane, a blood sample was drawn to obtain plasma for measurement of insulin, cholesterol, and triglycerides. Blood glucose was obtained from a tail snip on the morning before experiments. * $P < 0.05$ vs. LZR, unpaired t-tests.

Figure III-8. Baseline MAP and Phenylephrine-mediated Bradycardia and c-Fos expression in Conscious Female Zucker rats at 24-27 weeks

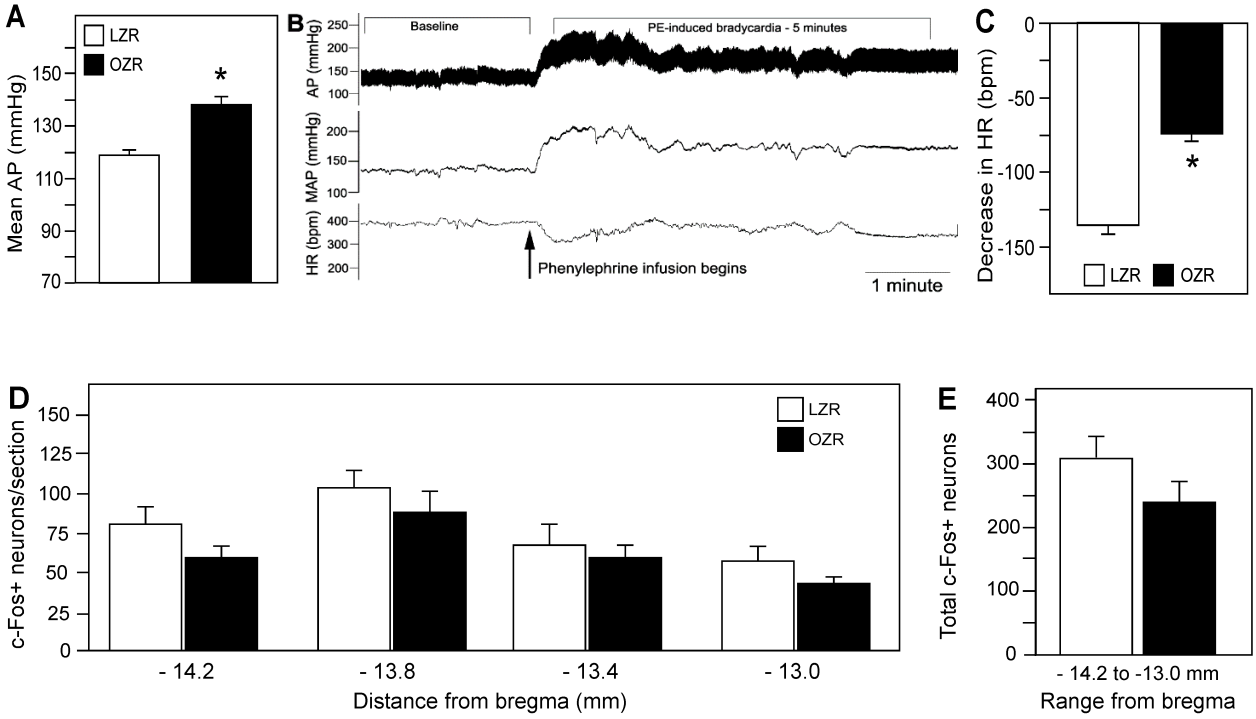


Figure 8. Baseline mean arterial pressure (AP) and PE-induced bradycardia in female LZR and OZR at 24-27 weeks of age. (*A*) Mean AP in conscious LZR and OZR before infusion of PE in conscious rats. *$P<0.05$ vs. LZR, unpaired t-test. See Table III-4 for more baseline values. (*B*) Representative tracing from an OZR illustrating baseline period for mean AP and period of analysis for PE-induced decrease in heart rate (HR). (*C*) The PE-induced decrease in HR in 24-27-week-old female LZR and OZR. * $P < 0.05$ vs. LZR, unpaired t-test. (*D*) The PE-induced c-Fos expression in the NTS at 4 rostro-caudal levels in relation to bregma of rats in (*A*-*C*). *N.S.,* 2-way ANOVA. (*E*) Total c-Fos expression from the 4 rostro-caudal levels shown in (*D*). *N.S.*, unpaired t-test.

DISCUSSION

Metabolic syndrome describes a cluster of interconnected, deleterious attributes that exacerbate each other to foster premature morbidity and mortality. Although hypertension is an identifying trait for MetS, the stability of AP is also negatively affected with MetS. Short-term control of AP by baroreflex-mediated modulation of HR and SNA is compromised in MetS, contributing to a rise in AP variability and excursions of AP to dangerously high levels in obese people and animals (56, 57, 60, 65). Premenopausal women are protected from the development of some of these cardiovascular and metabolic disorders, compared to men and postmenopausal women, suggesting estrogen ameliorates traits of MetS (4, 15, 38). We recently showed that young adult male OZR have hypertension and diminished baroreflexes along with impaired glycemic control in the presence of normal fasting glucose levels (9). Treatment of this prediabetic state to normalize glycemic control restores impaired baroreflex-mediated bradycardia and activation of the NTS despite persistent hypertension (9). In contrast, hypertensive female OZR at this age have intact baroreflex-mediated bradycardia that develops later at 6 months of age (59). The major findings of the present study are that preserved baroreflex-mediated bradycardia in hypertensive, young adult female OZR extended to sympathetic baroreflexes and baroreflex-mediated activation of the NTS. Likewise, glutamatergic activation of the NTS produced equivalent decreases in SNA and AP in female OZR and LZR. Furthermore, unlike young adult male OZR, female OZR had normal glycemic control with glucose tolerance comparable to female LZR. In contrast, by 6 months of age, female OZR had diminished baroreflex-bradycardia with no differences in PE-induced c-Fos expression in the NTS and a modest elevation in fed glucose. These data suggest that preserved glycemic control in female OZR protects baroreflexes and baroreceptor-mediated activation of

the NTS even in the presence of hypertension. Furthermore, when female OZR develop diminished baroreflex-mediated bradycardia later in adulthood, the underlying mechanisms differ from male OZR.

Despite differences in the onset of impaired baroreflexes in male and female OZR, both develop elevated SNA and hypertension by young adulthood (Table III-1; (23) (32). The inter-relationships of excess weight gain, elevated SNA, and hypertension are complicated by independent and co-dependent causes along with significant effects upon one another. In humans, hypertension is associated with elevated SNA with or without obesity, but obesity exacerbates each of these attributes (20). In the absence of hypertension, muscle SNA is higher in obese subjects than leans, and the presence of hypertension is associated with a further rise in muscle SNA in obese subjects (20). The incomplete overlap of obesity with these two attributes may be partially related to the use of BMI as a defining measure for excess weight gain, because the location of accumulated fat influences the presence of other attributes of MetS that significantly affect the impact of obesity upon SNA and AP. Men tend to accumulate abdominal fat, and rising BMI is closely associated with elevated SNA that is reduced by weight loss (15, 38). In contrast, women are more likely to gain gluteal and femoral fat instead of abdominal fat, and elevated SNA in obese women is not directly related with BMI or waist circumference and is not reduced by weight loss (15, 38). Interestingly, women who do gain excess abdominal fat tend to exhibit a male metabolic risk profile (38), suggesting the danger of abdominal fat accumulation is related to its propensity to also produce other MetS-related traits. Despite these differences, obese males and females both develop hypertension that is positively correlated with elevated SNA (39).

Hypertension is also strongly correlated with the development of impaired baroreflexes, which are worsened by obesity (21, 44, 61). However, diminished baroreflex-mediated control of HR and SNA has been observed in obese individuals in the absence of hypertension (20). This incomplete overlap between hypertension and baroreflex efficacy in obese subjects suggests distinct underlying causative mechanisms contribute to each deficit (20). As observed in obese humans, male and female OZR develop impaired baroreflexes in addition to elevated SNA and hypertension, although the relative onsets differ (Table III-1; 37). In male OZR and rats made obese by a high fat diet, impaired baroreflexes emerge earlier than hypertension (43, 56). In contrast, in female OZR impaired baroreflex-mediated bradycardia develops long after the onset of hypertension (59). In both cases, the onset of impaired baroreflexes occurs independent of the onset of hypertension, suggesting other mechanisms are responsible for impairment of baroreflexes with obesity.

Several observations strongly suggest that the development of hyperglycemia in the setting of obesity is a critical catalyst for impaired baroreflexes, particularly in males. First, type 1 diabetes mellitus-associated hyperglycemia impairs baroreflex control of HR in humans and rodents in the absence of obesity, hyperinsulinemia, and hypertension (41, 46). Likewise, insulin resistance-associated hyperglycemia in the absence of obesity reduces baroreflexes in fructose-fed rats, who are not hypertensive when AP is measured by telemetry (11, 23). Although baroreflex-mediated bradycardia in insulin-resistant, fructose-fed rats can occur before the onset of fasting hyperglycemia (45), the fasted state is not adequate to detect chronic hyperglycemia in prediabetic subjects. For example, fasting blood glucose is normal in insulin-resistant male adult OZR with impaired baroreflex-mediated bradycardia, but these rats have chronically elevated glucose with access to food (9). Second, restoration of glycemic control in fructose-fed rats or

male OZR improves baroreflex-mediated bradycardia (9, 24). Likewise, impaired baroreflex-mediated activation of the NTS is the blunted in type 1 diabetic rats and with insulin-resistant male OZR (9, 19), and restoration of glycemic control in male OZR also improves baroreflex-mediated activation of the NTS (9). Third, obese females and fructose-fed female rats are protected from the development impaired glycemic control and hyperglycemia (49) coincident with delayed development of impaired baroreflexes (4, 59). In the present study young adult female OZR had excess weight gain, hypertension, hyperinsulinemia, and dyslipidemia compared to female LZR, but baroreflex-mediated bradycardia and activation of the NTS were comparable to LZR coincident with a maintenance of normal glycemic control. Together, these observations strongly suggest that females are protected from developing hyperglycemia associated with excess ingestion fat, fructose, or total calories, and that maintenance of glycemic control preserves baroreflex-mediated bradycardia and activation of the NTS.

The mainstay diagnostic measure for diabetes is elevated fasting blood glucose, and clearly at this stage the inability of insulin to regulate blood glucose fosters a host of deleterious consequences and outcomes. However, more recent guidelines have focused on periodic hyperglycemia with ingestion of calories by use of HbA1c levels and glucose tolerance tests. Indeed, a higher percent HbA1c suggests elevations in blood glucose over the previous several months, because circulating glucose binds to the hemoglobin of red blood cells in proportion to glucose concentration in the blood. Although an elevated HbA1c does indicate periods of hyperglycemia, this test is not as sensitive as measuring the ability to reduce blood glucose after intake of calories (1). The latest guidelines for diagnosis of prediabetes includes a blood glucose of 140 – 199 mg/dl with an oral glucose tolerance test, which can detect impaired glycemic control before measures of fasting blood glucose or elevated HbA1c (1). Although this guideline

is used to assess future risk of developing diabetes, this prediabetic state obviously has physiological consequences before the onset of frank diabetes (17, 31). For example, Holwerda et al. (31) observed that baroreflex efficacy is significantly reduced in "obese healthy control" subjects compared to lean controls, but is comparable to obese diabetic subjects (31). Furthermore, plasma insulin was elevated in these nondiabetic obese subjects compared to lean subjects, while fasting glucose, HbA1c, and AP were comparable (31). Likewise, examination of differences in male and female OZR with regard to fed glucose levels and baroreflex impairment corroborates the negative impact of prediabetes on autonomic regulation of AP in male OZR. Young adult male OZR develop a daily blood glucose that exceeds 140 mg/dl (Figure III-7) coincident with the development of impaired baroreflexes, which are improved by restoring glycemic control (9). In contrast, female OZR maintain a daily blood glucose below 140 mg/dl (Figure III-7), and these rats do not have impaired baroreflex-mediated bradycardia or activation of the NTS. Together, these data suggest that in the absence of fasting hyperglycemia, elevated HbA1c, or hypertension, insulin resistance promotes hyperglycemia that can be detected with oral glucose tolerance tests or chronic measures of glucose by telemetry, and this insulin resistant state negatively impacts autonomic control of AP.

Estrogen has important roles in protecting females from developing metabolic and cardiovascular disorders. Premenopausal obese women have resistance to obesity-related elevations in SNA and AP, and this resistance declines after menopause (4). Consistent with estrogen's ability to reduce SNA, muscle SNA is lower by ~30% after transdermal estrogen treatment in postmenopausal women (4). In ovariectomized rats, local injections of estrogen into autonomic regulatory regions such as NTS or rostral ventrolateral medulla can evoke decreases in SNA, suggesting multiple regions of the brain could contribute to the sympatholytic actions of

estrogen (54, 55). However, given the opposing effects of the NTS and rostral ventrolateral medulla upon SNA to cardiovascular targets, the acute sympatholytic effect of estrogen in both of these regions suggests nonuniform or complex effects of estrogen upon neuronal activity. In agreement, local application of estrogen by iontophoresis onto unidentified NTS neurons produces a rapid inhibition of spontaneous activity and activation by excitatory amino acids in most recorded neurons, with a minority of NTS neurons being activated by estrogen (63). Whether these acute, non-genomic effects of estrogen reflect the impact of longer lasting genomic effects of estrogen related to the state of the animal require further study.

In addition to its effects upon basal SNA and AP, estrogen also enhances baroreflex efficacy (28, 55). Baroreflex gain fluctuates during the menstrual cycle with changes in estrogen (3), and baroreflex gain is reduced after ovariectomy (28, 29). In contrast, peripheral administration of estrogen in ovariectomized rats enhances baroreflex function (13, 28, 29). Likewise, microinjection of estrogen into the NTS enhances phenylephrine-induced bradycardia (55). In the present study, estrogen's apparent ability to activate the NTS may explain why phenylephrine-induced c-Fos expression in the NTS was not reduced in female OZR of either age examined despite the presence of other attributes of MetS. Further study is required to determine the identity and projections of estrogen-responsive neurons in the NTS and to distinguish between rapid, non-genomic effects of estrogen from its longer lasting genomic effects in order to better understand how estrogen affects NTS neurons that regulate basal autonomic tone to cardiovascular targets and its modulation by baroreceptor inputs.

Estrogen's ability to enhance glucose homeostasis may also aid in the maintenance of baroreflex efficacy in the setting of MetS. Premenopausal obese females have superior glycemic control compared to obese males that is lost after menopause or ovariectomy (66, 67). Even

male rats treated with estrogen are protected against the negative impact of diet-induced obesity upon glucose homeostasis, suggesting this sex difference can be explained by the presence of estrogen itself (12). Numerous actions of estrogen in the periphery have the capacity to enhance glucose homeostasis, including direct effects upon insulin signaling in insulin-sensitive tissues, release of insulin from the pancreas, adipose tissue metabolism and pattern of distribution, and hepatic glucose production (6, 12, 25, 51). Thus, in the present study, estrogen is very likely to aid in the maintenance of glucose homeostasis in female OZR despite the development of other aspects of MetS. In Zucker diabetic fatty rats, a strain variant of OZR with early onset severe diabetes, the obese females have a delayed onset of hyperglycemia compared to obese males, and this delay is abolished by ovariectomy and restored by replacement of estrogen (62). The maintenance of glucose homeostasis also likely contributes to preserved baroreflex efficacy in females, because fructose-feeding in ovary-intact female rats produces impaired vagal function that is associated with insulin resistance (2). Likewise, HR is elevated and baroreflex-mediated bradycardia is impaired in obese, hyperinsulinemic humans regardless of sex (31). Thus, in the present study preservation of baroreflex-mediated bradycardia and activation of the NTS in female OZR is likely to be the result of the maintenance of glycemic control in addition to direct excitatory actions of estrogen in brain regions such as the NTS.

The development of impaired baroreflex-mediated bradycardia in 26-27-week-old female OZR in the present study was unexpected, given that estrogen should still be present at this age, and glycemic control is only modestly impaired. Indeed, fed glucose levels in 24-27-week old female OZR were similar to those observed in metformin- and pioglitazone-treated male OZR, and these treatments restored baroreflex-mediated bradycardia in male OZR (9). In addition, the comparable PE-induced c-Fos expression in the NTS of 26-27-week-old OZR and LZR suggests

baroreflexes are not impaired by the same mechanisms observed in male OZR (23). Because sympathetic baroreflexes were not evaluated in the present study, it is not clear whether reduced baroreflex-mediated bradycardia is the result of altered autonomic efferent nerve activity or parasympathetic actions at the heart. Furthermore, reduced bradycardia to direct electrical stimulation of the vagal efferent nerve has been reported in obese rats (7). A role for ovarian hormones cannot be ruled out since estrogen and progesterone levels were not measured in the present study, and both of these hormones can affect cardiac muscarinic receptor density (37). Additionally, the further progression of existing traits could also begin to impact baroreflex efficacy. For instance, plasma lipids continue to rise with age in female OZR, and fat content in liver is strongly and inversely associated with reduced cardiac vagal tone and efficacy of baroreflex-mediated changes in HR (68). Future studies will be necessary to determine whether any of these attributes contribute to impaired baroreflex-mediated bradycardia in female rats at 26-27 weeks of age and whether these changes are specific to obese females.

Summary

Both male and female OZR develop elevated SNA and hypertension by young adulthood, but only the males also develop impaired baroreflexes at this age (56). In agreement with a causative role of the NTS for this sex difference in baroreflex efficacy, activation of the NTS by glutamate or an acute rise in AP produces diminished responses in male but not female OZR at this age (23). Although both males and females have exaggerated weight gain along with elevated triglycerides, cholesterol, and insulin, only the males have impaired glycemic control at this age, suggesting a causative connection between chronic hyperglycemia and the ability of the brain to respond to changes in AP. In agreement, the ability of glucose to enhance release of

glutamate from afferent terminals in the NTS appears to be lost in the presence of chronic hyperglycemia (5), and restoration of glycemic control in male OZR improves baroreflex-mediated bradycardia and activation of the NTS (9). Although female OZR begin to develop elevated glucose with access to food by 15 weeks of age, the rise in female OZR is modest and below threshold for prediabetic diagnosis even at 24-27 weeks of age (1). In agreement, the development of impaired baroreflex-mediated bradycardia by 26-27 weeks of age does not coincide with impaired baroreceptor-mediated activation of the NTS, suggesting a different cause for diminished baroreflexes in female OZR that will require further study. These data highlight limitations of using males to understand obesity-related disease progression in females, both in terms of onset for the development of MetS-related traits in relation to excess weight gain and for understanding the underlying mechanisms for impaired autonomic regulation of AP.

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CHAPTER IV

REDUCTION OF ARTERIAL PRESSURE IN MALE OBESE ZUCKER RATS ENHANCES BAROREFLEXES AND ACTIVATION OF THE NUCLEUS TRACTUS SOLITARIUS

Parul Chaudhary and Ann M. Schreihofer

ABSTRACT

Male obese Zucker rats (OZR) develop attributes of metabolic syndrome (MetS) analogous to obese humans. Young adult male OZR develop modest hypertension along with impaired baroreflexes and baroreceptor-mediated activation of the nucleus tractus solitarius (NTS). This study tested the hypothesis that preventing hypertension would enhance impaired baroreflexes and activation of the NTS in young adult male OZR. Treatment with a vasodilator, hydralazine, or an angiotensin receptor antagonist, losartan, in drinking water for 5 weeks prevented the development of hypertension in male OZR. Although losartan had no effect on AP in LZR, mean arterial pressure (AP) was slightly reduced by hydralazine in LZR. In OZR, both treatments improved baroreflex-mediated decreases in the heart rate to a phenylephrine (PE)-evoked rise in AP with no effect in treated LZR. However, neither treatment fully restored the impaired baroreflex-mediated bradycardia in OZR compared to like-treated LZR, suggesting a partial improvement of baroreflexes. Losartan restored PE-induced c-Fos expression in the NTS in treated OZR without a significant effect in LZR. In contrast, hydralazine treatment greatly enhanced PE-induced c-Fos expression in treated OZR and LZR. Across all groups of treated and untreated rats, PE-induced c-Fos expression was most strongly correlated with baseline mean AP and less so with magnitude of baroreflex-induced bradycardia. These data suggest that reducing AP in male OZR partially restores baroreflexes and augments baroreceptor-mediated activation of the NTS. The persistent impairment of baroreflexes in normotensive adult male OZR suggests other MetS attributes contribute to the development of diminished baroreflexes in these rats.

INTRODUCTION

Hypertension is a prominent attribute of metabolic syndrome (MetS), which is a cluster of cardiovascular and metabolic abnormalities associated with obesity that increase risk of cardiovascular disease and diabetes. The presence of 3 of 5 traits including abdominal obesity, hypertension, dyslipidemia (low HDL or high triglycerides), or fasting hyperglycemia, establishes a diagnosis of MetS. Their comorbid presentation suggests that these traits have common underlying causes and interact with each other. Recent studies suggest that insulin resistance and prediabetes can contribute to the development of other attributes of MetS such as dyslipidemia and hypertension (33). In agreement, hypertension is twice as common in the obese population compared to healthy individuals (19), and weight gain is strongly correlated with elevated AP (8, 39, 40). Likewise, approximately 80% of people diagnosed with MetS also have hypertension (25) and conversely, hypertensive patients exhibit a higher prevalence of MetS (29, 30, 34).

Hypertension is also strongly correlated with impaired baroreflexes (14, 24, 27), irrespective of the etiology of hypertension (10). Given the strong association between MetS and hypertension, it is not surprising that impaired baroreflexes are commonly observed with obesity (7, 13), although the cause and effect relationship between hypertension and impaired baroreflexes is complex (5, 11, 33). Arterial baroreflexes are critical for short-term regulation of arterial pressure (AP) and for maintaining stability of AP over the long-term. The ability of baroreflexes to modulate cardiac output and total peripheral resistance in response to acute changes in AP via autonomic regulation of the heart and blood vessels provides moment-to-moment dampening of fluctuations in AP that would otherwise promote deleterious cardiac events and cognitive decline (9, 23, 31, 35, 41). Although hypertension and impaired

baroreflexes are associated with each other, baroreflexes can be impaired in the absence of hypertension (12, 26). The incomplete overlap of hypertension and impaired baroreflexes suggests they can have distinct causes but also interact to exacerbate each other. Although hypertension is routinely measured and treated in the clinic, baroreflex efficacy and its impact on variability of AP are rarely considered, leaving affected patients at risk for poor outcomes independent of hypertension (11, 35).

Obese Zucker rats (OZR) develop attributes of MetS analogous to deficits observed in obese humans. Unlike lean Zucker rats (LZR), OZR have a leptin receptor mutation rendering it nonfunctional (20). As a result, OZR exhibit hyperphagia-induced weight gain and develop elevated AP, dyslipidemia, and impaired glycemic control (15, 37). Moreover, young adult male OZR develop hypertension and impaired baroreflexes that coincide with the development of impaired activation of the nucleus tractus solitarius (NTS), the brain stem region that receives inputs from baroreceptors (37). In young adult male OZR, hypertension develops at approximately the same age as the emergence of impaired baroreflexes (15, 37), although juvenile OZR begin to have reduced baroreflex gain before changes in AP are observed (37). However, it is not known whether the presence of hypertension in adult male OZR exacerbates impaired baroreflexes.

The main objective of the study is to determine whether preventing hypertension enhances diminished baroreflexes and baroreceptor-mediated activation of the NTS in young adult male OZR. Two antihypertensive drugs with differing mechanisms were used to control for drug-specific effects unrelated to their anti-hypertensive properties. Male LZR were also treated with the same medications to control for effects of treatments unrelated to decreasing AP. The first medication, losartan, reduces AP by blocking angiotensin AT1 receptors in the periphery

and brain to reduce SNA and peripheral resistance. The second medication, hydralazine, acts as a vasodilator and reduces peripheral resistance to lower mean AP without inhibiting SNA. The impacts of these anti-hypertensive treatments upon baroreflex-mediated bradycardia and c-Fos expression in the NTS were examined in conscious male OZR and LZR.

MATERIAL AND METHODS

Animals

Male OZR [Lepr (fa/fa)] and LZR [Lepr (+/+) and (+/fa)] were purchased from Charles River (Houston, TX) and fed on standard rat chow (Prolab RMH 1800, LabDiet). To facilitate measurement of water intake for treatment dosing, rats were individually housed. The centralized animal care facilities regulated humidity (60 ± 5%), temperature (24 ± 1°C), and light cycle (lights on 7:00 am – 7:00 pm). At 9 weeks of age a subset of OZR and LZR began treatment with 10 mg/kg/day of losartan (17, 22) or 50 mg/kg/day of hydralazine (3, 21) added to their drinking water. The rats were treated for approximately 5 weeks and then studied at 14-15 weeks of age. Untreated rats were individually housed in cages adjacent to rats receiving treatments. All experiments were conducted in accordance with National Institutes of Health's *Guide for Care and Use of Laboratory Animals* and the American Physiological Society's *Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training*. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center.

Surgical preparation and measures in conscious rats.

Rats were anesthetized with isoflurane (5% in a ventilated box and then 2.5% through a nose cone). The left femoral artery and vein were catheterized for recording AP and infusing

drugs, respectively. A blood sample (0.5 ml) was taken from the arterial catheter for later analysis of plasma, and the volume was replaced by flushing the line with sterile saline. The free ends of the catheters were tunneled subcutaneously to exit between the scapulae (15). The catheters were run through a button tether that was sewn into the skin at one end and attached to dual channel swivel at the other end (Instech Laboratories). The swivel was supported by a counter-balanced lever arm to allow rats to move freely in a covered plexiglass cylindrical cage (MTANK/W and MTOP, Instech). The rats were allowed to recover for 24-48 hours with access to food and water.

Before the start of the experiment, food and water were removed from the cages, and the walls of the cages were covered to minimize disturbance to the rat. The arterial line was connected through the swivel to a transducer (NL108T2, Digitimer), and the venous line was connected through the swivel to an infusion pump (Model A-99, Razel). Baseline AP and HR were recorded for 30 minutes, and then phenylephrine (PE) was infused to raise mean AP by 40 mmHg for 90 minutes (13-31 μl/minute of 0.5 mg/ml of PE in saline, i.v.). During the first 5 minutes the PE-induced bradycardia was quantified, and the PE infusion was continued for 90 minutes to induce c-Fos expression in the brain stem. During the last 30 minutes of the infusion, the PE-filled syringe was replaced by a saline-filled syringe to clear the venous line of PE. Following the 90 minute infusion protocol, rats were deeply anesthetized with urethane (1.5 g/kg, i.v. bolus) and perfused transcardially with 250 ml of phosphate-buffered saline (pH 7.4) followed by 500 ml of 4% phosphate-buffered paraformaldehyde (Electron Microscopy Sciences). The brains were removed and stored in the paraformaldehyde for 48 hours at 4°C.

Immunohistochemistry for c-Fos expression

The brain stem was sectioned (30 mm, coronal plane) using a Vibratome and stored at 20°C in a cryoprotectant solution. Expression of c-Fos protein was performed using immunohistochemistry on free-floating sections (every 1 in 6) on an orbital shaker in solutions prepared in Tris-buffered saline (TBS, pH 7.4) at room temperature unless noted otherwise. The sections were incubated with 1% hydrogen peroxide to block endogenous peroxidases (1%, 30 minutes), rinsed in TBS, and then blocked by incubation in horse serum (10%, 45 minutes). Then, sections were incubated with a goat-anti c-Fos primary antibody (1:2,000, 48 hours at 4°C; Santa Cruz Biotechnology, sc-52G), as previously described (Guimaraes et al., 2014). After rinsing in TBS, sections were incubated with a biotinylated donkey anti-goat secondary antibody (1:400, 1 hour; Jackson Laboratories, #705–066-147) followed by incubation with an avidin-biotin solution (1 hour; Vector Laboratories, PK-6100). The c-Fos immunoreactivity was revealed by incubation with a nickel-intensified 3–3'diaminobenzadine (DAB) solution. The reaction was monitored carefully until the staining became visible (8-10 minutes), and then the reaction was terminated by rinsing with a TBS. The stained sections were mounted onto gelatin-coated slides and air-dried overnight. The slides were submerged in a series of alcohols and xylenes, and then DPX mounting medium was applied (Sigma-Aldrich) to affix coverslips. The neurons with c-Fos-immunoreactivity (Fos+ neurons) were mapped and counted bilaterally at 4 rostro-caudal NTS levels using Neurolucida software (MicroBrightfield) as previously described (15).

Plasma measurements

The blood drawn from the arterial catheter of isoflurane-anesthetized rats was collected into a heparinized tube and immediately centrifuged to isolate plasma. Plasma samples were stored as aliquots at -20°C for later ELISA analysis in duplicate. Plasma insulin was measured using a Rat Ultrasensitive Insulin ELISA kit (ALPCO, 80-INSRTU-E01). Total cholesterol was measured using a Cholesterol E kit (Wako Diagnostics, 439-17501). Triglycerides were measured with an ELISA using L-Type TG M reagents (Wako Diagnostics, Color A 461-08992, Color B 461-09092 and Multi-Lipid calibrator 464-01601).

Data acquisition and analysis

The AP pulse was measured through the femoral artery catheter connected to a transducer (NL108T2, Digitimer). This signal was used to derive mean AP and HR through a low-pass filter (NL110) and a spike trigger (NL201), respectively (Neurolog System, Digitimer). These analog signals were converted to digital (Micro 1401, Cambridge Electronic Design) and were viewed using Spike2 software (Cambridge). All group data were shown as mean ± SE. Significant statistical difference was set at $P < 0.05$. The baroreflex-mediated bradycardia and counts of Fos+ neurons from treated and untreated OZR and LZR were compared using a two-way ANOVA with repeated measures followed by Tukey post hoc tests (SigmaStat software version 3.5).

RESULTS

Losartan (LOS) or hydralazine (HYD) treatment and PE-induced bradycardia

Comparing treatments by rat type, mean AP in LOS-treated LZR was comparable to untreated LZR, but mean AP in HYD-treated LZR was slightly lower than untreated LZR. (Figure IV-1A, left). However, neither treatment altered PE-induced bradycardia in LZR (Figure IV-1B, left). In OZR, treatment with losartan (LOS) or hydralazine (HYD) reduced mean AP compared to untreated OZR (Figure IV-1A, right), and both treatments enhanced PE-induced bradycardia compared to untreated OZR (Figure IV-1B, right). Comparing OZR and LZR pairwise by treatment, mean AP was higher in untreated OZR compared to untreated LZR (Figure IV-1C, left), and PE-induced bradycardia was smaller in untreated OZR compared to untreated LZR (Figure IV-1D, left). Treatment with either LOS or HYD reduced mean AP in OZR to be comparable to like-treated OZR and LZR (Figure IV-1C, right). However, after treatment with LOS or HYD, PE-induced bradycardia was still smaller in OZR compared to like-treated LZR (Figure IV-1D, right).

Losartan (LOS) or hydralazine (HYD) treatment and PE-induced c-Fos expression

As previously reported (15), after infusion of PE to raise mean AP by 40 mmHg, untreated OZR had fewer c-Fos+ neurons in caudal and intermediate NTS compared to untreated LZR at all 4 rostro-caudal levels examined (Figure IV-2). Treatment with LOS enhanced c-Fos expression in OZR to be comparable to LOS-treated LZR at all 4 rostro-caudal levels of the NTS examined (Figure IV-2). In contrast, treatment with HYD significantly increased the number of c-Fos+ neurons in OZR and LZR compared to untreated rats of the same phenotype at all 4 bregma levels examined (Figure IV-3). However, HYD-treated OZR still had fewer c-Fos+

neurons than HYD-treated LZR at all 4 bregma levels examined (Figure IV-3). These data are summarized as total c-Fos+ neurons in Figure IV-4A.

Correlations of baseline mean AP, PE-induced bradycardia and c-Fos+ expression in the NTS

Losartan and hydralazine both reduced AP in OZR, but the impacts of these treatments upon PE-induced c-Fos expression differed. Therefore, we examined the relationships among the 3 variables, namely baseline mean AP, PE-induced bradycardia, and total number c-Fos+ neurons in the NTS. Scatter plots were made using the group mean ± SE value of each variable for each of the 6 groups (untreated, LOS-treated, and HYD-treated OZR and LZR). This analysis revealed a strong correlation between baseline mean AP and total number of PE-induced c-Fos+ neurons in the NTS (Figure IV-4B), suggesting baseline mean AP was a major factor affecting treatment-induced changes in c-Fos expression in the NTS regardless of rat phenotype or treatment type. The correlation between baseline AP and PE-induced bradycardia was not as strong (Figure IV-4C1 and D1). Furthermore, the PE-induced bradycardia was smaller in all 3 groups of OZR compared to all 3 groups of LZR, regardless of treatment (Figure IV-4C2). Examination of the relationship between baseline AP and PE-induced bradycardia suggested a reduction in baseline mean AP toward 105 mmHg enhanced PE-induced bradycardia in both LZR and OZR (Figure IV-4D2). However, further reduction of baseline mean AP below 105 mmHg did not yield an additional enhancement in the magnitude of PE-induced bradycardia in either OZR or LZR. Together these data suggest that baseline mean AP greatly influences how much a 40-mmHg rise in AP acutely activates the population of barosensitive NTS neurons as a whole, regardless of type of anti-hypertensive treatment or rat phenotype. In contrast, a comparable baseline AP yields a smaller PE-induced bradycardia in OZR than in LZR,

suggesting other factors besides baseline AP diminish PE-induced bradycardia in OZR (Figure IV-4D2).

Relationship between aortic depressor nerve activity and mean arterial pressure

To better understand the impact of differing baseline AP levels upon PE-induced c-Fos expression in the NTS, the span of mean AP encompassed by the PE-induced 40-mmHg rise in AP from each group was overlaid onto the previously reported aortic depressor nerve activity (ADNA) in OZR and LZR over a wide range of mean AP (18). The voltage of ADNA over a wide range in mean AP is not different between adult male OZR and LZR (Figure IV-5A). The threshold AP (>80 mmHg) for onset of ADNA is comparable in OZR and LZR. Likewise, the slope and upper plateau of ADNA does not differ between OZR and LZR. Furthermore, the full-wave rectified voltage and % change from baseline of ADNA are comparable in OZR and LZR (18). The modest elevation in baseline AP in untreated OZR compared to untreated LZR is accompanied by a higher baseline voltage of ADNA (16), suggesting no obvious resetting of ADNA.

In the untreated, control (CON) OZR, the 40-mmHg rise mean AP produced by PE infusion overlapped with the upper portion of the ADNA curves relatively close to the upper plateau of ADNA compared to the CON LZR, whose 40-mmHg rise coincided with the middle range of the ADNA curves (Figure IV-5B). The LOS-treated OZR and LZR spanned similar regions of the ADNA curves and likewise, had comparable PE-induced c-Fos expression, which was also similar to untreated LZR (Figure IV-2, Figure IV-5B). Compared to untreated (CON) OZR and LZR, the span of AP in the HYD-treated rats overlapped with the most linear portion of the ADNA curves (Figure IV-5B) and expressed the most PE-induced c-Fos+ neurons in the

NTS (Figure IV-3). Thus, a lower baseline mean AP may evoke a larger response in the NTS because the 40-mmHg change in AP occurs in a more sensitive part of the curve.

Impact of losartan treatment upon metabolic traits

Losartan treatment in OZR did not affect the body weight or fed blood glucose levels, therefore, OZR were higher than LZR for both measures. Likewise, plasma levels of insulin, cholesterol, and triglycerides were not affected by losartan treatment in either LZR or OZR (Table1). Thus, fed glucose, insulin, cholesterol, and triglycerides were higher in like-treated LZR and OZR (Table 1).

Figure IV-1. Prevention of Hypertension: Impact on Baroreflex-mediated Bradycardia in Conscious Male Zucker Rats

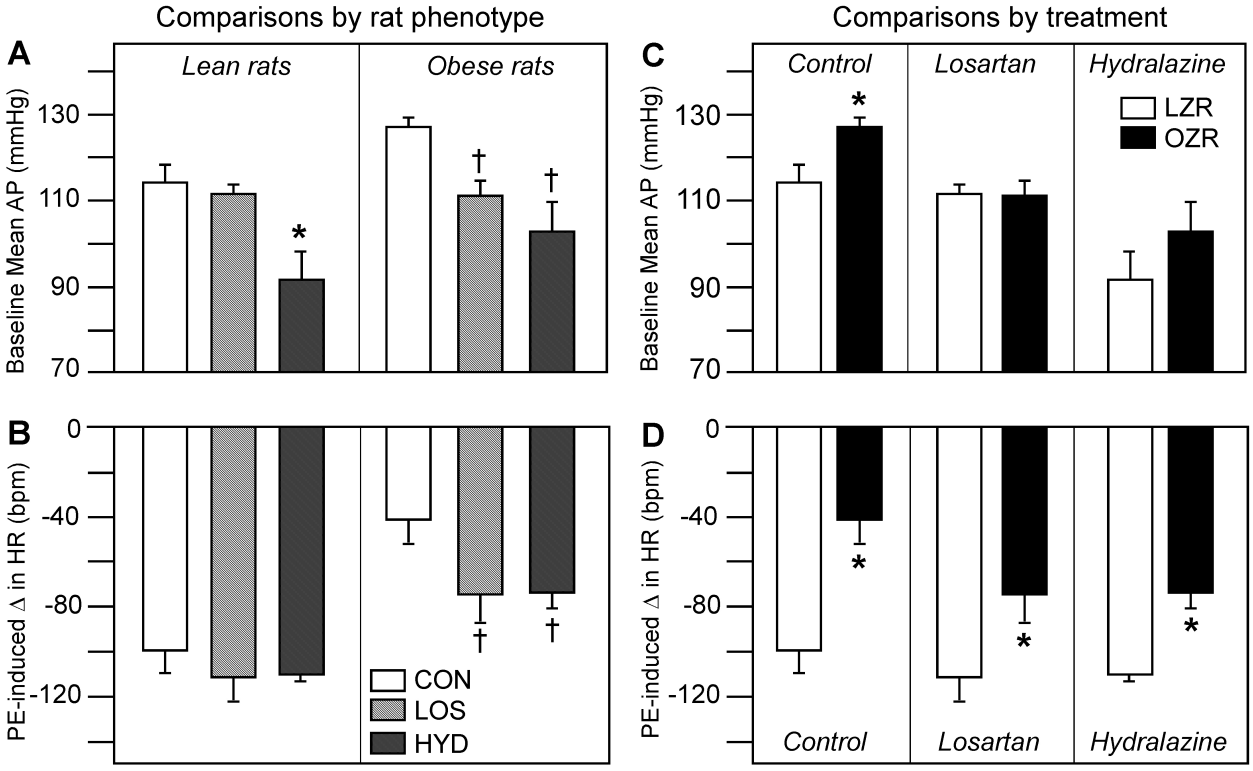


Figure 1. Treatment with losartan or hydralazine to prevent hypertension in obese Zucker rats (OZR) enhanced phenylephrine (PE)-induced bradycardia with no effect in lean Zucker rats (LZR). A. Losartan had no effect on mean AP in LZR, but hydralazine reduced mean AP compared to untreated LZR. Both treatments reduced mean AP in OZR. *$P<0.05$, compared to untreated LZR; † $P<0.05$, compared to untreated OZR. **B.** Neither treatment altered baroreflex-mediated bradycardia in LZR, but both treatments improved this response in OZR compared to untreated OZR. † $P<0.05$, compared to untreated OZR. **C.** Mean AP was higher in untreated OZR than LZR. Treatment with losartan or hydralazine reduced mean AP in OZR to be comparable to like-treated LZR. *$P<0.05$, compared to like-treated LZR. **D.** PE-induced bradycardia was impaired in untreated OZR compared to LZR. Treatment with losartan or hydralazine did not fully restore the diminished baroreflex-mediated bradycardia in OZR. *$P<0.05$, compared to like-treated LZR. Although data are displayed in 2 arrangements for

clarity, all data were analyzed by a 2-way ANOVA for rat phenotype and treatment. Mean AP and change in heart rate (HR) data were analyzed independently.

Figure IV-2. Losartan: Impact on Phenylephrine-induced c-Fos Expression in the NTS in Conscious Male Zucker Rats

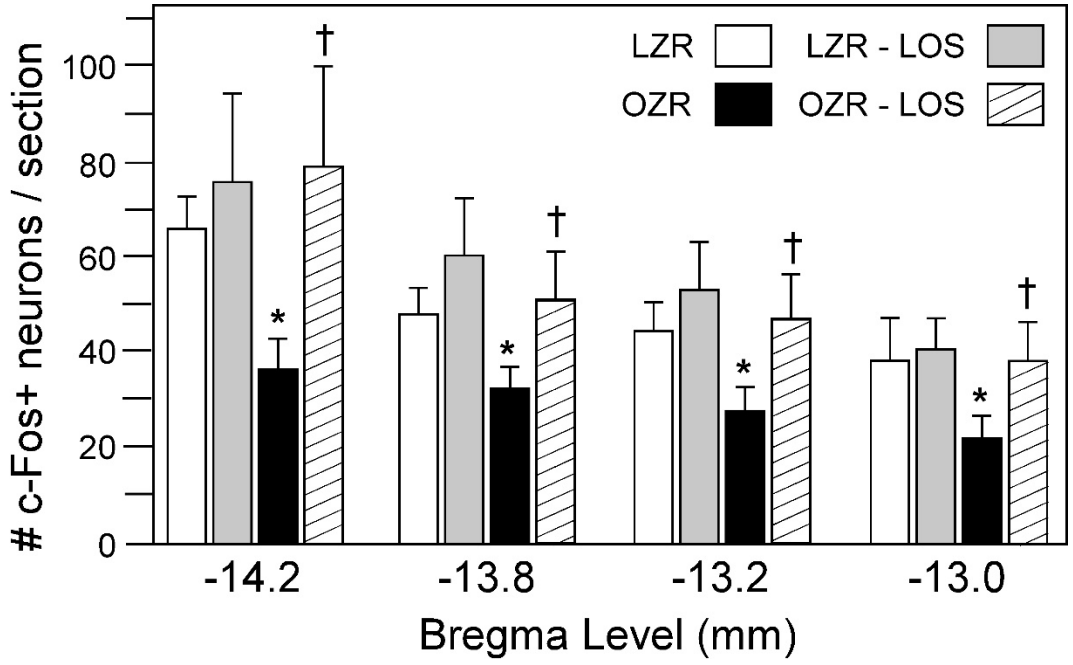


Figure 2. Effects of treatment with LOS on PE-induced c-Fos expression in the NTS. Infusion of PE to raise mean AP by 40 mmHg produced fewer c-Fos+ neurons in the untreated OZR compared to untreated LZR. Treatment with LOS did not alter PE-induced c-Fos expression in the NTS of LZR. However, in OZR treatment with LOS increased PE-induced c-Fos expression in the NTS of OZR at all bregma levels examined, comparable to counts of c-Fos+ neurons in the NTS of like-treated LZR. *$P<0.05$, compared to untreated LZR at that bregma level. † $P<0.05$, compared to untreated OZR at that bregma level. Each bregma level was analyzed separately by a 2-way ANOVA for rat phenotype and treatment condition.

Figure IV-3. Hydralazine: Impact on Phenylephrine-induced c-Fos Expression in the NTS in Conscious Male Zucker Rats

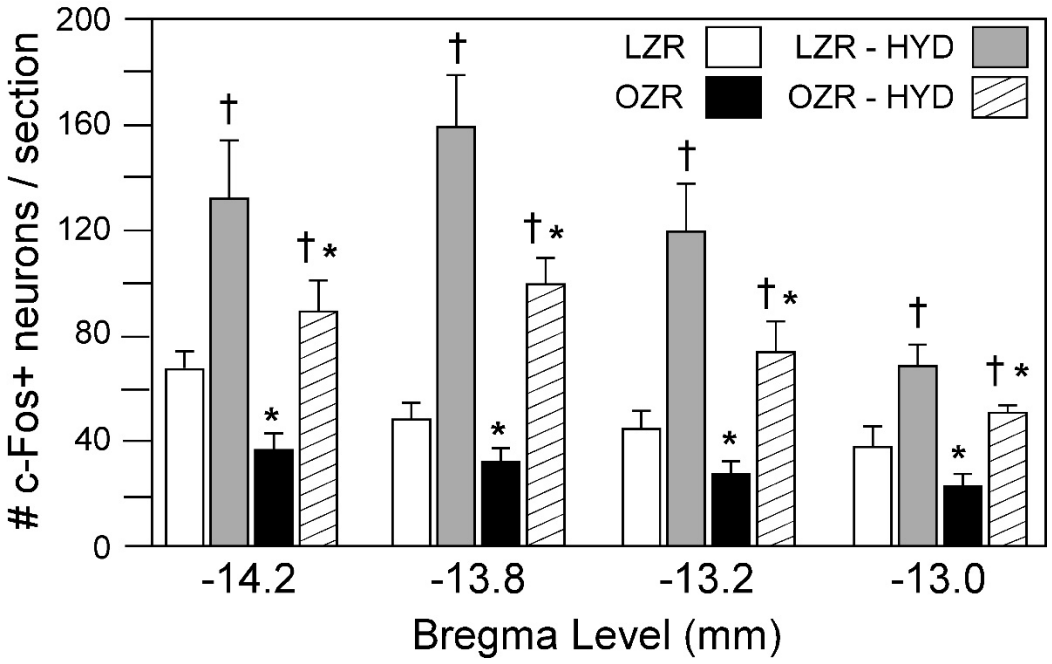


Figure 3. Effects of treatment with HYD on PE-induced c-Fos expression in the NTS. Infusion of PE to raise mean AP by 40 mmHg produced fewer c-Fos+ neurons in the untreated OZR compared to untreated LZR. In contrast, treatment with HYD increased PE-induced c-Fos expression in the NTS of LZR and OZR at all bregma levels examined. However, HYD treated OZR still had fewer c-Fos+ neurons than HYD-treated LZR at all 4 bregma levels examined. *$P<0.05$, compared to like-treated LZR at that bregma level. † $P<0.05$, compared to untreated rat of same type at that bregma level. Each bregma level was analyzed separately by a 2-way ANOVA for rat phenotype and treatment condition.

Figure IV-4. Relating Efficacy of Antihypertensive Treatments to Enhancement of

Phenylephrine-induced Bradycardia and c-Fos Expression in NTS in Male Zucker Rats

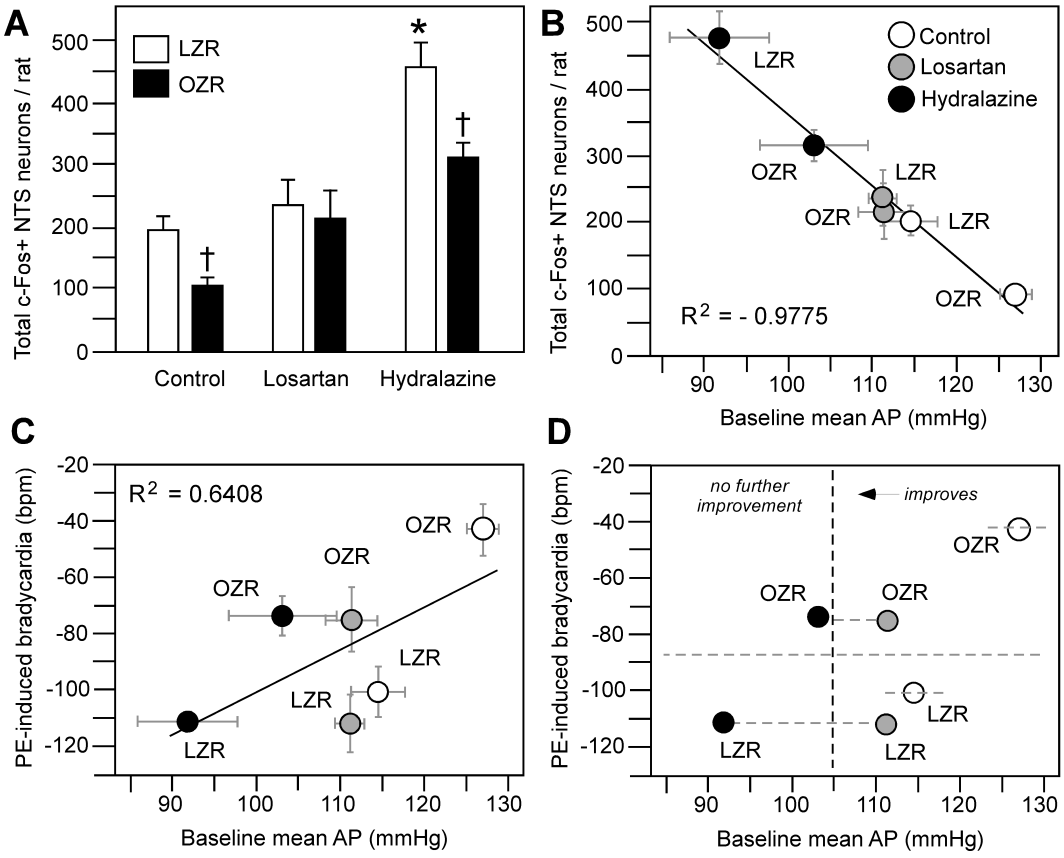
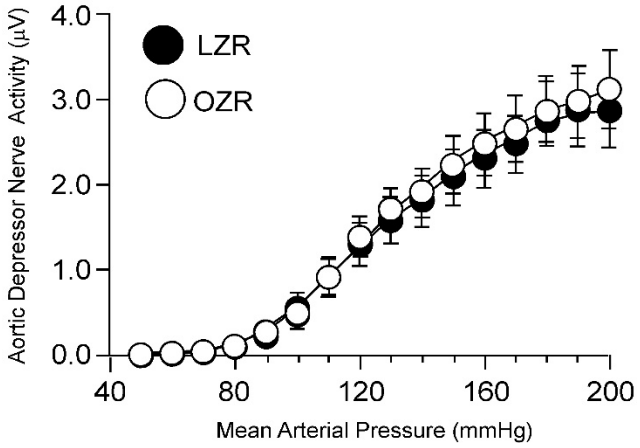


Figure 4. Relationships between baseline mean AP and number of PE-induced-c-Fos+ neurons in the NTS or bradycardia in OZR and LZR. Rats are from Figs. 1-3. **A.** Total counts of c-Fos+ neurons in NTS after infusion of PE to raise AP 40 mmHg. Data were analyzed by 2-way ANOVA with Tukey post hoc tests, *$P<0.05$ vs. LZR. † $P<0.05$ compared to like-treated LZR. **B.** Scatter plot of mean ± SE for each group relating baseline mean AP and the total number of PE-induced c-Fos+ neurons. Trendline shows correlation. **C.** Scatter plot of mean ± SE for each group relating baseline mean AP and magnitude of PE-induced bradycardia. Trendline shows correlation. **D.** Same relationship shown in **C,** Vertical dotted line approximates

threshold mean AP for improvement of PE-induced bradycardia. Horizontal line highlights separation between OZR and LZR regardless of treatment group.

Figure IV-5. Relationship Between Mean AP and Aortic Depressor Nerve Activity compared to PE-induced Changes in Mean AP in Untreated and Treated OZR and LZR

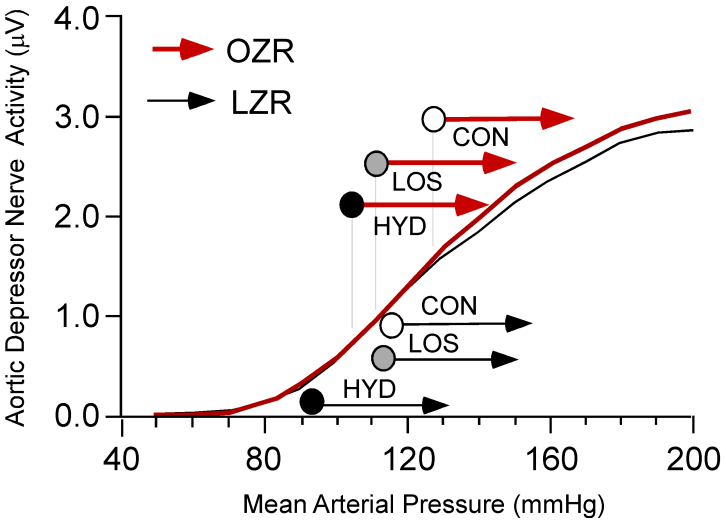


Adapted from:

Huber and Schreihofer, 2010,

J Physiol. 588: 1515-1525.

A. Aortic depressor nerve activity (ADNA) after ganglionic blockade with chlorisondamine in urethane-anesthetized, ventilated, paralyzed rats. Infusion of phenylephrine (iv) to raise mean AP evoked comparable rises in full-wave rectified ADNA in OZR (n=7) and LZR (n=7) at 14-18 weeks of age. Data were fitted by a four-parameter logistic sigmoidal regression.



B. Plot of ADNA and mean AP from (A) with the range of change in mean AP evoked by infusion of PE in control, losartan (LOS)-treated, and hydralazine (HYD)-treated OZR and LZR from Figures IV-1-4. Placement of the PE-induced 40 mmHg change in mean AP for each group in relation to the curve for ADNA activity to illustrate a potential explanation for PE-induced c-Fos expression in the NTS amongst the 6 groups (Figures IV-2 and 3).

Table IV-1. Baseline Plasma Values in Losartan-treated and Untreated Male Zucker Rats

Group	*n*	age (days)	body weight (g)	fed glucose (mg/dl)	insulin (ng/dl)	cholesterol (mg/dl)	triglycerides (mg/dl)
Untreated							
LZR	8	113 ± 3	407 ± 13	117 ± 2	1.3 ± 0.3	93.5 ± 3.3	61 ± 10
OZR	8	113 ± 3	593 ± 31*	343 ± 47*	6.6 ± 1.4*	89.4 ±16.1	492 ± 102*
Losartan-treated							
LZR	8	115 ± 3	405 ± 14	118 ± 3	0.9 ± 0.1	93.5 ± 3.3	114 ± 20
OZR	9	114 ± 3	598 ± 28*	387 ± 49*	8.9 ± 1.9*	89.4 ±16.1	349 ± 50*

Data are mean ± SE. Blood glucose was obtained from a tail snip on the morning before experiments with rats in their home cages. Plasma was obtained from blood samples drawn from a femoral arterial catheter while the rat was under isoflurane between 8:00 – 10:00 a.m.

* $P < 0.05$ vs. like-treated LZR, 2-way repeated measures ANOVA with Tukey post hoc tests.

DISCUSSION

Hypertension is strongly associated with impaired baroreflexes (4, 6, 10, 12, 14) and worsened by obesity, particularly in men (7, 12), because other MetS-related traits also contribute to the rise in AP (33). Similarly, impaired baroreflexes are also affected by multiple attributes of MetS (see Chapter II, (33)), and the onset of impaired baroreflexes can precede the development of MetS-related hypertension (26, 37). In agreement with the notion that impaired baroreflexes arise from hypertension-independent causes, anti-hypertensive medications vary in their capacity to simultaneously reduce elevated variability of AP (35). Furthermore, a residual elevation in AP variability in patients with controlled hypertension still leaves patients at significant risk for deleterious cardiovascular incidents such as stroke (35). Hypertension is one of the diagnostic traits of MetS, but dynamic measures such as baroreflexes or variability of AP are not considered or treated even though people with MetS are likely to have impaired baroreflexes (12). Therefore, understanding the efficacy of anti-hypertensive treatments for ameliorating impaired baroreflexes in the setting of MetS is essential for reducing risk of poor outcomes (35).

Using a rodent model of MetS, male OZR, the present study sought to examine whether prevention of hypertension by 2 different classes of anti-hypertensive medication effectively restored maximal baroreflex-mediated bradycardia. This parasympathetic reflexive decrease in HR, which can be readily measured in conscious subjects, appears to be impaired earlier and more prominently than deficits in sympathetic baroreflexes in humans and rodents with MetS-related traits (5, 16). In addition, because the development of impaired baroreflexes in male OZR occurs with a reduced baroreceptor-mediated activation of the NTS (15), we sought to determine the impact of anti-hypertensive treatments upon PE-induced c-Fos expression in the NTS. We

recently reported that improvement of glycemic control with metformin enhances baroreflexes in OZR, but the restoration is incomplete coincident with a rise in baseline AP (Chapter II, Figure II-6). Therefore, the present study also examined whether preventing hypertension improved baroreflex-mediated bradycardia and activation of the NTS in young adult male OZR in the continued presence of hyperglycemia.

The major findings of the present study are: 1) Treatment with losartan or hydralazine reduced mean AP without affecting other diagnostic attributes of MetS in male OZR. 2) Compared to untreated OZR, both treatments improved baroreflex-mediated bradycardia and activation of the NTS. 3) The number of c-Fos+ neurons in the NTS after infusion of PE were strongly correlated to baseline mean AP regardless of treatment or rat type. 4) Although the magnitude of baroreflex-mediated bradycardia was correlated with baseline mean AP, this bradycardia was less in all OZR groups compared to LZR groups regardless of treatment. These data suggest that modestly elevated AP in male OZR contributes to impaired baroreceptor-mediated control of HR and activation of the NTS. However, independent of baseline AP, other attributes of MetS also significantly contribute to impaired baroreflex-related responses in adult male OZR.

Several observations suggest that treatments with losartan and hydralazine enhanced baroreflex-mediated bradycardia and activation of the NTS through their anti-hypertensive actions. Previous studies in other hypertensive models have reported improvement of baroreflexes with the classes of anti-hypertensive treatments used in the present study. For example, losartan is effective in reducing AP and improving baroreflexes in spontaneous hypertensive rats, which suggests the improvements seen in losartan-treated OZR were not specific for MetS (2). In agreement with our observed treatment effects of hydralazine, other

studies have reported that drugs exerting vasodilator effects also improve baroreflexes (28, 38). The absence of treatment effects upon baroreflex-mediated bradycardia in LZR suggests the drugs are enhancing baroreflexes in OZR by preventing hypertension. Furthermore, losartan and hydralazine were equally effective for improving baroreflexes in OZR, in agreement with the conclusion that reducing AP and not antagonism of angiotensin AT1 receptors or inhibition of SNA were required for improving baroreflexes.

Hypertension has been shown to alter baroreflexes by multiple mechanisms within different parts of the baroreflex pathway. For example, in SHR the threshold AP for the onset of baroreceptor afferent nerve activity, measured as whole nerve or single fibers of aortic depressor nerve, is shifted rightward to a much higher AP compared to normotensive Wistar Kyoto rats (36). This prominent resetting allows for the baroreflex to continue buffering against changes in AP. In addition, the development of hypertension coincides with increases in number of dendritic spines within the NTS and expression of the GluR1 subunit of the AMPA receptor for glutamate, the primary neurotransmitter released from baroreceptor afferent terminals (1). In contrast, with the more modest hypertension in adult male OZR, the threshold AP for onset of aortic depressor nerve activity (ADNA), the gain, and ADNA upper plateau AP are not different between LZR and OZR, suggesting there is no obvious rightward resetting of baroreceptors (18). In agreement with the notion that the relationship between ADNA and AP is not different in adult male OZR and LZR, baseline raw ADNA is higher in modestly hypertensive OZR compared to LZR (18). Whether this difference occurs by increased activity in active fibers or the recruitment of more fibers to be active is not known, but certainly both could be true (27, 36). Thus, assuming the anti-hypertensive treatments do not alter the relationship between ADNA and AP, when baseline

AP is reduced by losartan or hydralazine in OZR, some afferent fibers may have lower baseline firing rates or become silent.

Several observations suggest that baroreflex-related adaptations in the male OZR are occurring centrally within the NTS itself. First, bypassing the sensory end of the baroreceptors, direct electrical stimulation of baroreceptor afferent fibers yields smaller decreases in SNA and AP in male OZR compared to LZR (18). Likewise, direct activation of the NTS by microinjection of glutamate evokes smaller decreases in SNA and AP, but activation of the caudal ventrolateral medulla produces comparable physiological responses in adult male OZR and LZR. Furthermore, these changes in responses to glutamate in the NTS are absent in juvenile male OZR when the range of the baroreflex is comparable to LZR (15, 18, 37). In agreement with a reduced tonic activation of the NTS by baroreceptor inputs, inhibition of the NTS by microinjections of muscimol produces smaller rises in SNA and AP, but glutamate into the CVLM or GABA into the RVLM yield comparable responses in adult male OZR and LZR. In contrast, in female OZR a normal baroreflex corresponds with comparable OZR and LZR responses to PE-infusion, glutamatergic activation of the NTS, and GABAergic inhibition of the NTS (Chapter III). Further study will be required to determine the cellular changes within the NTS that are altered in the only in male OZR and how they are improved by reducing baseline AP in male OZR.

In the present study the number of NTS neurons expressing detectable levels of c-Fos protein after a 40-mmHg rise in AP was very closely related to baseline AP, regardless of treatment condition or rat phenotype. As speculated in Figure IV-5, if there was no significant change in the relationship between ADNA and AP before or after treatment, the lower baseline AP values would place the 40-mmHg rise in AP within the most sensitive part of the curve. In

contrast, although there was some relationship between baseline AP and baroreflex-mediated bradycardia, the responses in OZR were smaller at a given baseline AP than the LZR regardless of treatment condition. The apparent mismatch between baroreflex-mediated bradycardia and c-Fos expression is likely to be related to the nature of the measure. Whereas the number of neurons producing a detectable amount of c-Fos protein reflects a depolarization above threshold for detecting the c-Fos, with the number of neurons affected being related to the gain or relative recruitment and/or activation of afferent nerves with the rise in AP without being able to determine how much the neurons are activated. In contrast, the PE-induced bradycardia reflects the maximum response to a rise in AP, which is more reflective of the lower plateau of the baroreflex as well as the heart's response to changes in autonomic inputs. Further study will be necessary to determine how the treatments affected sympathetic baroreflexes, with a closer examination of the gain and range of the baroreflex. In addition, further study will be necessary to verify ADNA in relation to AP under these conditions in order to better understand how anti-hypertensive treatments may or may or may not change how they respond to acute rises in AP. Regardless, the reduction in baseline AP did enhance the ability of an acute rise in AP to activate NTS neurons and produce a baroreflex-mediated bradycardia, suggesting hypertension does contribute to these reduced responses to acute rises in AP in the male OZR.

Comparisons in like-treated LZR and OZR showed that restoration of baroreflex-mediated bradycardia is not fully restored in the hyperglycemic OZR. Unfortunately, this comparison to treated normotensive subjects is often not made in studies examining the ability of anti-hypertensive treatments to also restore baroreflexes, making it difficult to compare the results of the present study to previous reports in other models of hypertension. For example, the impact of losartan upon baroreflexes in hypertensive patients (32) and SHR (2) was only

examined in hypertensive subjects, leaving the question of whether normalization of AP was sufficient to fully restore baroreflex efficacy in comparison to normotensive subjects. In the present study, although the reduction in baseline mean AP toward ~110 mmHg appeared to enhance PE-induced bradycardia, further reduction in baseline AP did not yield a larger HR response in either OZR or LZR (Figure IV-4D). These data suggest an optimal baseline AP for baroreflex function that is likely to be related to the operating range of the baroreceptor afferent nerves and their impact on NTS neuronal activity for OZR and LZR.

In conclusion, this study demonstrates that losartan and hydralazine were equally effective for improving baroreflexes along with reducing AP, suggesting the treatments worked by lowering AP and not through drug-specific actions such as antagonism of angiotensin AT1 receptors or inhibition of SNA. However, even with prevention of hypertension, baroreflex-mediated bradycardia was still blunted in normotensive OZR compared to LZR, suggesting other attributes of MetS also contribute to impaired baroreflexes. Our previous study suggests that chronically elevated blood glucose is another significant contributor to impaired baroreflexes in OZR (Chapter II). This second causative attribute could provide an explanation for why some patients with controlled hypertension display residual elevated AP variability, which leaves them at risk for delirious cardiovascular outcomes (35). These observations highlight the complexity of the development of individual attributes of MetS that occurs both independent of and in concert with other detrimental attributes. Furthermore, these data emphasize the importance of understanding the interactions between cardiovascular and metabolic disorders to optimize treatments in the setting of MetS.

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CHAPTER V

DISSERTATION PERSPECTIVE AND SIGNIFICANCE

Metabolic syndrome (MetS) is a cluster of attributes namely excess central adiposity, low HDL cholesterol, and elevated AP, triglycerides, and fasting blood glucose that together greatly increase risk of cardiovascular disease and diabetes. In addition to these static measures with threshold values, recent data suggests pathological excess fluctuations in some of these attributes, such as glucose and AP, pose their own threat for poor outcomes independent of the more traditional diagnostic traits. Specifically, increased variability of AP is a significant independent risk factor for coronary artery disease, renal disease, stroke, and cognitive decline. Impaired baroreflexes is a major cause for increased variability of AP, in accord with its established role in the short-term control of AP. Multiple attributes of MetS are strongly associated with impaired baroreflexes, making the treatment of this deficit complex. The work presented here used Zucker rats to determine independent contributions of hyperglycemia and hypertension upon baroreflex-mediated control of heart rate and activation of the brain stem. In addition, we identified the sex differences in the development of impaired baroreflexes in MetS.

Glycemic control contributes to baroreflex function

By 12-14 weeks of age young adult male OZR have developed hyperinsulinemia, dyslipidemia, and sympathetically driven hypertension. In addition, these male OZR have developed impaired baroreflex efficacy concomitant with diminished baroreceptor-mediated activation of the NTS. In male OZR, analysis of glycemic control using long-term recordings by telemetry revealed a fasting glucose that comparable to age-matched LZR, but with access to

food blood glucose was chronically elevated in the male OZR. Furthermore, the hyperglycemia was accompanied by elevated variability of glucose and impaired glucose tolerance compared to LZR. Selective treatment of impaired glucose homeostasis without affecting hypertension and hyperinsulinemia in male OZR improved baroreflex function and enhanced baroreceptor-mediated activation of the NTS. These data suggest that poor glycemic control contributes significantly toward baroreflex impairment by diminishing baroreceptor-mediated activation of the NTS.

As observed in young adult male OZR, young adult female OZR develop hyperinsulinemia, dyslipidemia, and sympathetically driven hypertension. However, unlike male OZR, at 15 weeks of age female OZR still exhibit preserved baroreflex-mediated bradycardia and activation of the NTS. Analysis of glycemic control using long-term measures by telemetry in female OZR revealed fasting glucose, fed glucose, glucose variability and glucose tolerance levels compared to female LZR. These data suggest that maintenance of glucose homeostasis in these hypertensive female OZR preserves baroreflex-mediated bradycardia and activation of the NTS.

In male OZR fed glucose levels had steadily risen to exceed 24-hour values above 140mg/dl by 14 weeks of age. In contrast, in young adult female OZR fed glucose levels displayed a slow, gradual progression over 13-17 weeks of age that were slightly but significantly higher than age-matched female LZR, but 24-hour values were below 140 mg/dl. Together, these data suggest that the chronic hyperglycemia in male OZR contributes to impaired baroreflexes, but modestly elevated blood glucose in hypertensive female OZR was not sufficient to impair baroreflexes. These data highlight significant differences in glucose homeostasis between male and female OZR with the identification of a critical threshold for

hyperglycemia that is consistent with the 140 mg/dl threshold for diagnosis of prediabetes with oral glucose tolerance tests in humans. These data also highlight the importance of multiple assessments of dynamic values rather than a single static value. In agreement, a recent study reports the importance of considering variability of glucose, which is a strong, independent predictor of cardiovascular events and mortality (12). Widely accepted clinical diagnostic criteria for prediabetes from American Diabetes Association is based on elevated HbA1c and fasting blood glucose along with glucose intolerance (1). However, a specific diagnosis for impaired glucose tolerance is not routinely evaluated (9, 18). This is significant concern because using fasting glucose levels as an index of glycemic control in insulin resistant subjects, who have elevated insulin but normal fasting glucose levels, vastly underestimates glucose levels and their variability when food is available. Our study highlights the importance of detecting chronic hyperglycemia in the fed state, which alters autonomic function before elevated fasted glucose levels are detected. Thus, in obese patients an evaluation for impaired glucose tolerance and hyperinsulinemia should be implemented to routinely to trigger early interventions that could prevent the development of other deleterious attributes of MetS.

Glycemic control may affect NTS function

Young adult male OZR develop poor glycemic control coincident with impaired baroreceptor-mediated activation of the NTS and baroreflexes. The selective correction of impaired glucose homeostasis alone in presence of hypertension and hyperinsulinemia enhanced PE induced c-Fos expression in the NTS of young adult male OZR. In contrast, young adult female maintain glycemic control and preserved baroreceptor-mediated activation of the NTS in the presence of hypertension and hyperinsulinemia.

Male OZR exhibit a surge of ΔFosB expression in the NTS at 7-8 weeks of age, suggesting significant neuroplasticity occurring at that age within the NTS (See Appendix II). This neuroplasticity likely reflects changes that yield impaired baroreflex-mediated activation of the NTS within the weeks ahead. Although ΔFosB expression decreases in the NTS of male OZR after 11 weeks of age, the expression is still significantly higher than the ΔFosB expression seen in age-matched male LZR. Unlike male OZR, female OZR exhibit this early surge in ΔFosB expression seen in male OZR. Instead, ΔFosB expression in the NTS remains comparable in female OZR and LZR compared to age-matched female LZR even at 11-12 weeks of age. The absence of significant neuroplasticity and the preservation of NTS function in the setting of preserved glycemic control in female OZR suggest that the maintenance of fed blood glucose levels prevents the development of impaired activation by baroreceptor inputs.

Although it is not known whether the barosensitive NTS neurons are glucose-sensitive, changes in circulating blood glucose do influence the properties of NTS neurons. For example, impaired gastrointestinal function in diabetes is associated with loss of glucose-enhanced activation of NTS neurons (3). Although these NTS neurons may serve to regulate ingestive behaviors and digestion, the NTS neurons that respond to vagal afferents from the gut appear to converge to modulate the activity of barosensitive neurons in caudal ventrolateral medulla (17), which would serve to coordinate digestion and blood flow to the gut. Furthermore, a glucose-mediated increase of NTS neuronal excitability is the lost in streptozotocin-induced hyperglycemic model (8), suggesting a glucose resistance within the NTS. In agreement, in rats made obese by a high fat diet, autonomic responses to cholecystokinin are greatly diminished coincident with a reduced activation of the NTS and caudal ventrolateral medulla that impairs CCK-mediated changes in presympathetic neurons in the rostral ventrolateral medulla (10).

Thus, together these data indicate that in male OZR, chronic hyperglycemia contributes to diminished activation of NTS neurons by vagal afferent inputs that affect multiple regulatory systems within the body.

Impaired baroreflexes in female OZR occur by different mechanisms

In contrast to male OZR, the delayed development of baroreflex impairment in female OZR at 24-27 weeks does not coincide with impaired baroreceptor-mediated activation of the NTS. The 24-27-week-old female OZR and LZR display comparable PE-induced c-Fos expression within the NTS. Furthermore, at 24-27 weeks of age female OZR exhibit mildly elevated fed glucose levels compared to age-matched LZR that are similar to those observed in pioglitazone-treated male OZR (Chapter II, Figure II-9). This treatment also restored baroreceptor-mediated activation of the NTS and bradycardia (Chapter II, Figure II-9). Thus, it appears that although fed glucose levels are elevated in female OZR, they are still below the threshold levels to sufficiently alter baroreceptor-mediated activation of the NTS. Furthermore, when female OZR eventually develop impaired baroreflex-mediated bradycardia, the underlying mechanisms appear to be different than those observed in the male OZR. Future studies will be necessary to determine the underlying mechanisms for the development of impaired baroreflex bradycardia in female OZR. These findings highlight the importance of identifying the mechanisms that underlie the development of MetS attributes in women, and advocate redirecting efforts to identify sex difference to confer appropriate therapeutic management.

Mild stressors affect the glucose levels in OZR

In our study measurement of blood glucose by telemetry in male OZR revealed exaggerated rises in glucose in response to mild stressors like tail snips and movement of cages. Brief immobilization stress in male OZR is known to evoke exaggerated rises in blood glucose that are not accompanied by rises in epinephrine and norepinephrine (13), suggesting the deficit is not mediated by aberrant autonomic responses. Instead, in the absence of leptin-induced suppression of corticosterone, mild stressors evoke augmented rises in corticosterone that inhibit insulin secretion to allow glucose to rise (2). This was evident in our studies, particularly at 15-17 weeks of age, when male OZR displayed higher fasting glucose values when measured by tail samples compared to values generated by telemetry in undisturbed rats. For some comparisons between LZR and OZR the two methods of glucose measure yielded contradictory results as 15-17-week-old OZR would be considered hyperglycemic using tail samples but not with telemetry measures. In addition, although metformin-treated OZR and LZR had identical hourly glucose levels at all hours of the day and night when undisturbed, during glucose tolerance tests the male OZR still had a slightly but significantly higher peak in blood glucose compared to the male LZR (Chapter II). Future studies will be needed to determine whether this residual exaggerated response is due to the stress of glucose injection. These observations demonstrate the necessity of assessing blood glucose levels in stress-sensitive rats by measures that avoid even mild stressors.

Independence of hypertension and baroreflex impairment

Treatment with either losartan or hydralazine to prevent the development of hypertension in young adult male OZR improved PE-induced bradycardia and enhanced c-Fos expression in the NTS compared to untreated OZR. However, the baroreflexes were still impaired compared to the like treated LZR, suggesting partial restoration of baroreflexes. Both the drugs produced an equivalent improvements in baroreflexes in male OZR suggesting that this enhancement of baroreflexes can be attributed to the reduction in baseline AP independent of antagonism of angiotensin receptors or reduction of SNA. However, the selective correction of glycemic control in young adult male OZR also restored baroreflex and NTS function independent of hypertension, and hypertensive female rats do not develop impaired baroreflex-mediated activation of the NTS. These observations suggest that poor glycemic control may be necessary for the expression of impaired baroreflexes in the face of modest hypertension in OZR.

Although the hypertension is closely associated with impaired baroreflexes (6, 16, 20) our data suggest that other attributes of MetS cause the development of baroreflex-mediated activation of the NTS and bradycardia. This disconnect between hypertension and baroreflex efficacy is also observed in rats made obese by a high-Fat diet who develop diminished baroreflexes by central mechanisms before the development of hypertension (11, 15, 19). Likewise, obese people can also exhibit baroreflex impairment of heart rate and SNA in the absence of hypertension (5). Collectively these data suggest that in the setting of MetS, poor glycemic control is a critical catalyst for the development of impaired baroreflexes independent of the development of hypertension, but the later development of hypertension may exacerbate impaired baroreflexes.

Importance of combined therapy

Selective treatment of elevated mean AP or poor glycemic control in young adult male OZR improved baroreflexes compared to untreated OZR. However, baroreflexes in treated OZR were still diminished compared to like-treated LZR, suggesting only partial restoration of baroreflexes. Thus, these studies showed that treatment of individual attributes of MetS was ineffective in fully restoring baroreflex function. Standard of care for MetS patients is often directed toward treating individual attributes of MetS without consideration of comorbid traits (7). However, these MetS attributes clearly interact to initiate and exacerbate each other. Instead, a multidisciplinary approach to managing cardiovascular risks has been found to be more effective (4, 14). Thus, consideration of multiple attributes of MetS when determining a course of treatment will be needed to successfully combat traits like increased variability of AP in order to reduce risk of premature morbidity and mortality.

Summary

The studies contained within this dissertation postulate a critical link between impaired glucose homeostasis and diminished baroreflex-mediated control of heart rate. Moreover, the presence of preserved fasting glucose but exaggerated glucose responses to glucose challenge and stress highlight the challenge of detecting impaired glycemic control with routine clinical protocols. Clinically, hyperinsulinemia and fed glucose levels are not assessed as a routine procedure in MetS patients. Nevertheless, a recent study showed that the variability of AP and glucose as evaluated by their standard deviation contributes significantly to the development of cardiovascular disease (12). MetS embodies multiple attributes, in which treatment of one

attribute might not be sufficient for protection from later deleterious outcomes. Likewise, our studies showed that treatments of individual attributes hypertension or hyperglycemia were ineffective in fully restoring baroreflex efficacy. Thus, in the setting of obesity, the simultaneous consideration of multiple attributes is critical, and obese patients with elevated AP and triglycerides should be screened for prediabetes to diagnose insulin resistance to ensure a comprehensive treatment plan to minimize risks for development of deleterious conditions such as coronary artery disease, renal disease, stroke, and cognitive decline.

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CHAPTER VI

FUTURE DIRECTIONS

Assessment of anti-diabetic and anti-hypertensive treatment upon sympatho-inhibitory reflexes

The studies performed in males focused on baroreflex-mediated changes in heart rate (HR), because this measure could be readily performed in conscious animals to allow for examination of baroreflex-mediated activation of the NTS as indicated by expression of c-Fos. Furthermore, baroreflex-mediated bradycardia appears to be an early indicator of baroreflex impairment in human subjects with attributes of metabolic syndrome (5). A next step would be to examine whether treatments to restore glycemic control or AP in male OZR also improved sympathetic baroreflexes. Because changes in HR are a product of changes of autonomic responses to the heart and the response of the heart to autonomic inputs, the direct measure of SNA would provide a clear indication of how the efferent nerve activity leaving the CNS is affected by the treatments. Furthermore, the sympathetic baroreflexes would allow for a sigmoidal analysis of the relationship between SNA and a full range of AP, unlike HR responses which differ for baroreflex-mediated bradycardia and baroreflex-mediated tachycardia (10, 19). If differences in treatment effects are observed for HR compared to SNA, future studies could also examine whether the heart's responses to acetylcholine and norepinephrine have changed with treatments (3, 23).

In addition to baroreflexes, obese rodents also appear to develop other impaired sympatho-inhibitory reflexes that are initiated by vagal afferent stimulation of the NTS. For instance, injection of phenyl biguanide to elicit the Bezold-Jarisch reflex evokes smaller

decreases in splanchnic SNA in urethane-anesthetized, ventilated male OZR compared to LZR (7) . Likewise, Sprague-Dawley rats on a high fat diet have impaired cholecystokinin-induced inhibition of splanchnic SNA that is also coincident with impaired activation of the NTS and CVLM to produce less inhibition of presympathetic RVLM neurons (6). In agreement, volume expansion produced an impaired inhibition of renal SNA in rats fed a high fat diet. (8). The impact of restoring glycemic control or AP in the obese rats upon these other sympatho-inhibitory reflexes would provide a broader assessment of potential common mechanisms for reflexes initiated by vagal afferent nerves to the NTS.

Assessment of changes in NTS neurons in young adult male OZR

These studies provide a foundation for further examination of how the development of MetS in young adult male OZR affects neuronal function in the NTS. Phenylephrine-induced c-Fos expression in the NTS provides a time-dependent population response to raising AP. The identity of the activated neurons and their projections would provide additional information on which neurons are impacted by the development of MetS. In Chapter II, comparisons of treatment efficacies for metformin and pioglitazone upon enhancement of phenylephrine-induced bradycardia and c-Fos expression in the NTS yielded some mismatches in outcome that could not be clarified with the dependent variables measured. Determination of the identities and projections of c-Fos expressing neurons in the NTS would provide more conclusive information regarding whether neurons directly involved in the generation of baroreflexes were impacted by the treatments.

The activity of individually recorded NTS neurons in anesthetized rats could provide real time responses for second order neurons to their vagal inputs. Extracellular recordings of

individual NTS neurons while precisely and selectively activating baroreceptor afferent neurons would provide more in depth analysis of how this connection is altered in OZR compared to LZR, and the impact of treatments to restore glycemic control and hypertension. Extracellular electrophysiological recordings in NTS of LZR and OZR would help to determine the patterns of spontaneous discharge of second order NTS neurons upon modulating the duration, frequency or intensity of stimulus from baroreceptor afferent fibers. It has been observed that hypertensive rats exhibit reduced inhibition of action potential discharge with iontophoretic application of a $GABA_B$ receptor agonist (17). Thus, there is a need to evaluate GABA receptor-mediated alterations in hypertensive OZR and evaluate whether GABA receptors are altered by antihypertensive treatments. Moreover, these changes can be evaluated at different age groups to establish a time frame for the development of alterations in the NTS neurons of OZR.

Some barosensitive NTS neurons show altered excitatory postsynaptic effects with changes in glucose concentration within a physiological range (22). The effects of treatments to restore glycemic control upon spontaneous discharge patterns in NTS neurons in the presence of varying glucose concentrations could be analyzed and compared in treated and untreated groups. Using an in vitro preparation of the brainstem slice, patch clamp recordings from LZR and OZR could be used to study changes in synaptic neurotransmission. The stimulation the baroreceptor afferent fibers release glutamate to evoke post-synaptic currents (EPSCs and IPSCs) in second-order NTS neurons. The hypertensive rats exhibit a larger GABA receptor-mediated current in second order NTS neurons compared to normotensive rats (17). Therefore, brainstem slice preparations can be used to study the GABA-mediated neurotransmission in the NTS neurons of Zucker rats at different age groups to evaluate the time line for the development of these deficits.

Further, the effects of early and late antihypertensive treatments on the inhibitory current flow can be evaluated to differentiate between effects on onset versus maintenance of altered function.

Glucose modulates glutamatergic transmission at vagal afferent terminals. The frequency of EPSCs in NTS neurons is linearly dependent upon extracellular glucose concentration (21). Interestingly, in diabetic mice, the ability of glucose to modulate EPSCs in second order NTS neurons is lost in the presence of chronic hyperglycemia (1). Glucose-mediated EPSC responses can be analyzed in Zucker rats, and the impact of glucose treatment on EPSCs can be studied.

Assessment of glycemic control and baroreflexes in ovariectomized young adult female OZR

These studies suggest that young adult female OZR are protected from the development of impaired baroreflexes coincident with preserved glycemic control at this age compared to age-matched LZR. These observations are in agreement with superior maintenance of glycemic control in premenopausal women compared to men and postmenopausal women (4, 11-13, 20). Future studies could examine whether estrogen provides this protection by ovariectomizing female OZR and LZR to determine whether the absence of gonadal hormones worsens glycemic control in OZR coincident with the development of impaired baroreflexes. Using telemetry to record blood glucose chronically in undisturbed rats would provide a time course for changes in glycemic control after ovariectomy and whether female OZR developed exaggerated stress responses as observed in male OZR. At the same time these rats could be examined for the development of impaired baroreflexes and baroreflex-mediated activation of the NTS. This This concurrent evaluation could provide insights into potential glucose thresholds for having a significant effect upon sympatho-inhibitory reflexes mediated through the NTS.

Assessment of baroreflexes in 6-month-old female OZR and LZR

These studies showed that older adult female OZR have preserved baroreflex-mediated bradycardia, in agreement with a previous study in 6-month-old rats. In contrast to males, glycemic control in female OZR was much better maintained, even at this later age. Interestingly, baroreceptor-mediated activation of the NTS also appeared to be preserved in these females. These data suggest that the delayed development of baroreflex-mediated bradycardia may occur by different mechanisms. For instance, the ability of acetylcholine and norepinephrine to change HR could be altered in these older OZR compared to LZR. Dose response curves to these neurotransmitters could be examined in rats after ganglionic blockade to eliminate autonomic tone and baroreflex feedback to directly examine the responses of the heart to autonomic-related neurotransmitters. In addition, decreases in HR in response to vagal efferent nerve stimulation would provide more information regarding the ability of vagal inputs to the heart to change HR is affected in OZR compared to LZR. In addition, sympathetic baroreflexes measured by changes in splanchnic SNA to a wide range in AP would also determine whether apparent differences in autonomic efferent control of cardiovascular function are present in sympathetic efferent nerves in female OZR compared to LZR. These studies would provide more mechanistic insights into how baroreflexes are altered in older female OZR.

Determination of onset of poor glycemic control in male OZR and impact of early treatment

These studies examined adult male OZR and LZR after the onset of hypertension, impaired glycemic control, baroreflexes, and activation of the NTS in order to determine whether treatments could restore function. Data in Appendix II showed a time course of neuroplasticity in the NTS of male OZR and LZR by examination of DFosB/FosB expression at multiple age ranges (Figure AI-1) At 7-8 weeks of age, male OZR show a surge in DFosB/FosB expression

in the intermediate NTS that is not present in OZR at 4-5 weeks of age or in age-matched LZR. If the development of poor glycemic control is responsible for changes in how the NTS responds to vagal inputs, then early treatment to prevent the changes in blood glucose would reduce or eliminate the neuroplasticity observed in the NTS. Rats could be treated with metformin beginning at 5 weeks of age and followed for 2-3 weeks to examine blood glucose, AP, baroreflexes, and activation of the NTS (DFosB/FosB and c-Fos expression) to determine whether early intervention in these vulnerable rats prevents the development of impaired sympatho-inhibitory reflexes and activation of the NTS.

Effects of anti-hyperglycemic and anti-hypertensive treatments upon AP variability

A major rationale for examining the short-term control of HR and SNA by baroreflexes was that impaired baroreflexes promote increased variability of AP, which is an independent risk factor for deleterious cardiovascular events and cognitive decline (9, 14-16, 18). Adult male OZR and *db/db* mice develop MetS and have increased variability of AP (2) (Figure AI-1) that is likely to be brought about at least in part by the impairment of baroreflexes. Furthermore, deletion of PTP1B (molecular restraint on insulin signaling pathway), improves glycemic control in *db/db* mice by improving peripheral insulin sensitivity, and this genetic alteration reduces AP variability in these mice. These data support the notion that the development of poor glycemic control impairs baroreflexes to promote increased variability of AP. Examination of the impacts of treatments to restore glycemic control by metformin or hypertension by losartan upon the development of increased variability of AP in young adult OZR compared to LZR would provide further insights into the interrelationships of glycemic control, baroreflexes, and AP variability. In addition, these studies could refine the degree of deficit in glycemic control and duration needed to alter autonomic regulation of AP. Because these studies suggest that changes occur

before fasting hyperglycemia is observed, we would examine fed glucose levels by telemetry. We predict that measurements of AP by telemetry in undisturbed rats will show a reduction in AP variability, as measured by day and night standard deviations of AP, after metformin treatment in OZR that are still hypertensive. These studies will solidify our understanding of how attributes unrelated to AP directly have a significant impact upon the autonomic regulation of cardiovascular function. Together these studies strengthen the notion that attributes of MetS need to be treated in relation to the presence of other attributes of MetS that would otherwise seem to be unrelated. Earlier interventions could significantly improve outcomes and prevent irreversible damage to peripheral organs and the central nervous system.

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APPENDIX I

ALTERED VARIABILITY OF HEART RATE AND ARTERIAL PRESSURE IN ADULT MALE OBESE ZUCKER RATS

Arterial pressure (AP) is routinely measured in the clinic and if it exceeds recommended guidelines, hypertension is diagnosed and treated. In addition to the absolute level of AP, the excessive variability of AP is also diagnostic for poor outcomes. However, this dynamic measure is not routinely measured, and the guidelines for acceptable levels and necessary time frame of evaluation are not well defined. Furthermore, whether the important measure is one of variation or a matter of excursions above a particular value is not known. In addition, blood flow could be argued as the relevant variable as organs require a consistent blood flow to maintain the delivery of nutrients and disposal of waste. Alternatively, AP could be the relevant variable if organs are sensitive to pressure, as a higher pressure could weaken vessel walls and increase the force needed for the heart to pump blood. Whether one or all of these attributes are relevant for clinical outcomes, numerous studies have shown that the extent of variability of AP has predictive value for deleterious outcomes such as stroke, organ damage, and cognitive decline (8, 16, 36). Furthermore, the body is equipped with sensors to monitor AP in our major arteries and convey this information to the brain.

Although the evaluation and treatment of excessive AP variability are not part of standard clinical practice, evidence for the detrimental impact of this trait has been known for decades and is well documented. The presence of increased AP variability either over short-term (32, 43) or

long-term period (36) appears to be a strong predictor of poor cardiovascular outcome. An increasing number of studies demonstrates the association of higher diurnal AP varaibility with an increased risk of left ventricular hypertrophy (8, 30). Additionally, morning surges in AP and night dipping of AP are independently related to organ damage and cardiovascular events (15, 21). Increased AP fluctuations are also associated with cognitive impairment and brain atrophy (23, 42). Thus, it is now well recognized that an increase in AP variability is an independent risk factor for development and exacerbation of end-organ damage, cardiovascular event, stroke, and cognitive decline (20, 26, 29).

Physiologically, changes in AP is observed with an emotional response, physical activity and during sleep cycle (7).These AP variations are regulated by baroreflexes to maintain pressure homeostasis (22). At times the body requires an elevated AP to perform particular tasks, and baroreflexes are suppressed to allow AP to rise. Normally, a rise in AP leads to bradycardia to reduce cardiac output and relaxation of vessels to decrease peripheral resistance. However, during exercise a parallel rise in AP and heart rate (HR) occurs, which is due in part to a temporary resetting of the baroreflex (27). This shift in operating point allows for the rise in AP while also providing a continued mechanism to buffer against excessive changes in AP. Interestingly, regular exercise appears to permanently alter baroreflex efficacy in a beneficial manner. Repeated bouts of exercise sensitize baroreceptor afferents and their target neurons in the NTS, and this exercise-induced resetting of the baroreflex can be also beneficial for ameliorating diminished baroreflexes with hypertension (1).

As with many beneficial homeostatic processes, in a disease state a once beneficial mechanism can become pathological or a compensatory change in one part of the body sacrifices

other parts of the body that are not in need of this modification. For example, it could be argued that hypertension results from the body's need to maintain sufficient blood flow. The "selfish brain hypothesis" postulates that hypertension develops in response to reduced cerebral perfusion to maintain blood flow to the brain at the expense of deleterious effects of excessive AP to other parts of the body (11). In type 2 diabetes an analogous compensatory but debilitating rise in insulin occurs in an attempt to maintain glucose homeostasis. Although the insulin resistant muscle and adipose tissue require more insulin to store glucose, other organs such as the liver, which is still insulin sensitive, secrete excess triglycerides as insulin promotes conversion of free fatty acids in the liver to tryglycerides. This elevated level of circulating triglycerides increases risk of cardiovascular diseases such as atherosclerosis, endothelial dysfunction, and a procoagulant state (35). As reported in Chapter II, in obese subjects insulin resistance and its compensatory hyperinsulinemia promote elevated circulating triglycerides and hypertension even in the absence of fasting hyperglycemia (35).

Increased AP variability is strongly linked with the presence of hypertension (18, 19, 31, 44). However, correcting hypertension with different classes of medications yields inconsistent effects on the reducing variability of AP, suggesting other factors also contribute to this phenomenon. Hypertensive people have a higher likelihood of associated metabolic risk factors including obesity, insulin resistance, and glycemic impairment, suggesting some or all of these factors could contribute to elevated AP (12, 34). Consequently, increased AP variability is also observed in patients with metabolic syndrome (MetS) (5) and its associated traits of obesity (6) and diabetes (14).

Analogous to obese humans, OZR develop hyperphagia-induced obesity due to a leptin receptor mutation (13), and excess weight gain foster traits of MetS such as hypertension, insulin

resistance, and elevated lipid levels (4, 9). However, the variability of AP in OZR compared to LZR has not bee reported. Because a major rationale for examining baroreflexes is related to their impact on the variability of AP, assessment of AP variability is essential for understanding how this reflex alters the regulation of AP in the setting of MetS and whether treatments that improve baroreflexes also restore the stability of AP.

MATERIALS AND METHODS

Animals

Adult male (13- 14 weeks old) OZR [Lepr (fa/fa)] and LZR [Lepr (+/+) and (+/fa)] were purchased from Charles River (Houston, TX) and were individually housed in centralized animal care facilities. Optimal conditions were maintained with consistent humidity (60±5%), temperature (24±1°C), and light cycle (lights on 7:00 am – 7:00 pm) and were fed on standard rat chow (Prolab RMH 1800, LabDiet). All Experiments were performed per guidelines by National Institutes of Health's *Guide for Care and Use of Laboratory Animals* and the American Physiological Society's *Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training.*

Implantation of blood pressure sensing transmitters

Under aseptic conditions and isoflurane anesthesia, a midline abdominal incision was performed and aorta was isolated. The tip of the transmitter catheter (Data Sciences International) was inserted rostral to the abdominal aorta distal to the kidneys. The catheter was secured with a piece of mesh and a small drop of cyanoacrylate adhesive and the aortic wall around the catheter was sealed. The catheter and its connected transmitter were sutured to the

abdominal wall using 4.0-prolene sutures. Following this the incision was closed and the rats were monitored until fully conscious. Cages of rats with transmitters were placed on a receiver (DSI) to measure blood pressure by telemetry. The AP and heart rate (HR) were measured for 20 seconds every 10 minutes.

RESULTS

Arterial pressure was recorded by telemetry and readings are 10-minute intervals over 7 days. Frequency distributions of AP readings showed a wider distribution with a lower peak in male OZR compared to LZR (Figure AI-1A). Accordingly, the standard deviation of the AP readings was higher in OZR than LZR (Figure AI-1B), as would be expected with impaired baroreflexes in adult male OZR. In contrast, frequency distributions of heart rate (HR) readings showed a narrower distribution in OZR than LZR (Figure AI-2A) that produced a lower standard deviation of HR in OZR than LZR (Figure AI-2B). These data corroborate the physiological impact of impaired baroreflex control of HR that have been reported in male OZR at this age.

Figure AI- 1. Frequency Distribution and Standard Deviation of Mean Arterial Pressure In Adult Male Zucker rats

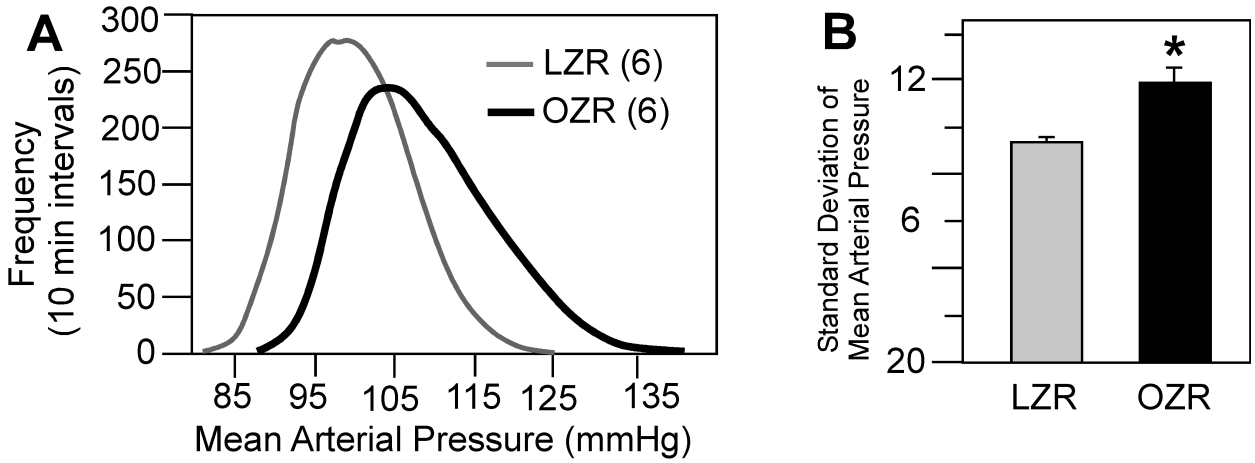

Figure 1. Frequency distribution and standard deviation of AP in adult male Zucker rats. The AP of conscious rats was measured by telemetry over the course of 7 days. A. Consecutive 10-minute readings plotted as a frequency distribution for lean Zucker rats (LZR) and obese Zucker rats (OZR), showing a higher peak in the LZR and a wider distribution in OZR. B. The standard deviation of AP derived from the AP readings shown in A. **P*<0.05, unpaired t-test.

Figure AI - 2. Frequency Distribution and Standard Deviation of Heart Rate in Adult Male Zucker rats

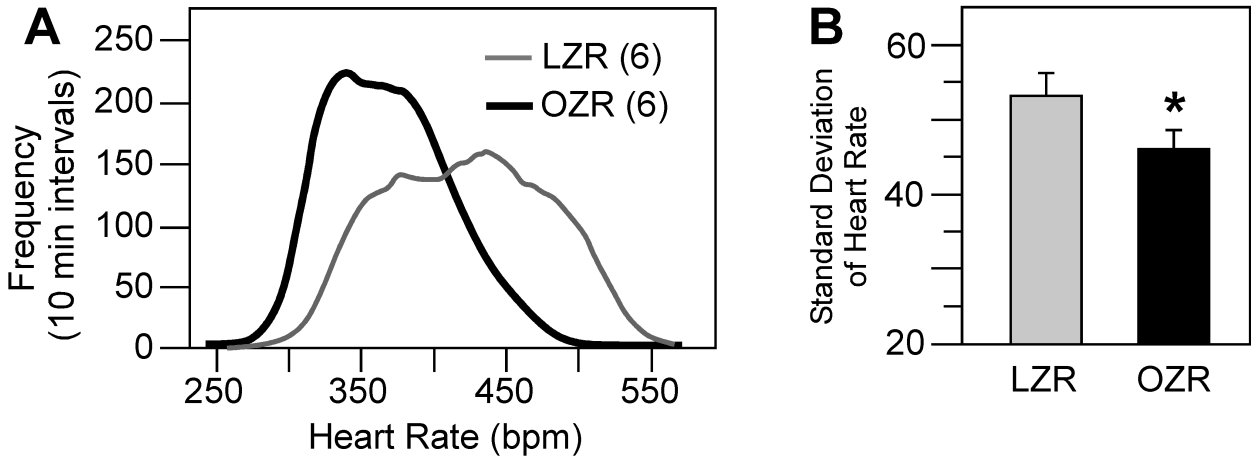

Figure 2. Frequency distribution and standard deviation of heart rate (HR) in adult male Zucker rats. The HR of conscious rats was measured by telemetry over the course of 7 days. A. Consecutive 10-minute readings plotted as a frequency distribution for lean Zucker rats (LZR) and obese Zucker rats (OZR), showing a higher peak in the OZR and a wider distribution in LZR. B. The standard deviation of HR derived from the HR readings shown in A. **P*<0.05, unpaired t-test.

DISCUSSION

As previously reported for obese humans and other animal models of obesity, the adult male OZR have a greater variability of AP and a reduced variability of HR compared to LZR. The modest hypertension observed in the adult male OZR may have exacerbated the impaired baroreflexes, several observations suggest hypertension is not the cause of this deficit. At 7 weeks of age juvenile OZR begin to show a deficit in their baroreflex control of sympathetic nerve activity with a reduced gain (37) weeks before the onset of hypertension at 12 weeks of age with AP measured by telemetry (28). In Sprague-Dawley rats made obese by a high fat diet, baroreflexes are impaired very early within 2 weeks of the diet with no change in AP (25), but they are hypertensive with 13 weeks on a high fat diet (38). Although variability of AP was not measured in these rats, impaired baroreflexes are strongly associated with increased AP variability (22). These data suggest that the baroreflex impairment occurs independent of hypertension, and that obesity fosters impaired baroreflexes before the onset of hypertension. In agreement, although hypertension is associated with a worsening of AP variability, people with controlled hypertension or a history of hypertension can still have increased variability of AP (16, 19, 41). Furthermore, AP variability is strongly associated with waist circumference and BMI but not AP levels (17), and in hypertensive people AP variability is augmented by obesity (39, 40).

Obesity promotes a number of attributes that could influence baroreceptors, but a number of observations suggest hyperglycemia is a catalyst for increased fluctuations in AP. In humans, elevated AP variability is commonly observed in patients with impaired glucose tolerance independent of obesity (33). As seen with augmented AP variability, these same connections have been reported for impaired baroreflexes. Type 1 diabetics have hyperglycemia and impaired

baroreflexes in the absence of other MetS-associated traits (2, 10). Likewise, type 2 diabetic patients have a strong inverse association of blood glucose variability with baroreflex sensitivity (24). Moreover, db/db mice that have deficit leptin receptor also present with obesity and increased AP variability, and the AP variability is normalized by increasing peripheral insulin sensitivity by peripheral insulin sensitivity by protein tyrosine phosphatase1B (PTP1B) deletion (3).

Likewise, the amelioration of hyperglycemia in male OZR improves baroreflex efficacy and the brain's response to acute rises in AP, even with the persistence of hypertension, hyperinsulinemia, and dyslipidemia (Chapter II). In contrast, young adult female OZR are hypertensive with intact baroreflexes, and these rats also maintain glucose homeostasis (Chapter III). With the observation of increased AP variability in adult mal OZR (Figure AI-1), future studies will examine the impact of treatments that restore glucose homeostasis and baroreflex efficacy upon augmented AP variability in male OZR.

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APPENDIX II

NEUROPLASTICITY IN THE NTS OF MALE OBESE ZUCKER RATS

The comorbid expression of physiological abnormalities in MetS complicates the determination of which trait or traits are essential causative factors for a particular pathophysiology, such as impaired baroreflexes, and whether there are multiple contributors for its presentation and progression. One approach for unraveling the development of these pathologies is to examine the timelines for the emergence, progression, and potential reversal for individual traits in relation to the tracking of the pathophysiology under investigation. Comparisons between males and females can provide further insights by examining differences in timelines for the emergence of traits in relation to the pathophysiology of interest, in addition to an understanding of how sex-specific differences may protect or exacerbate these abnormalities. Although correlative, this approach is useful for ruling out potential causative factors and for providing supportive evidence of a relationship between variables that can be manipulated to determine whether they are co-regulated or causative.

The development of impaired baroreflexes in the setting of MetS has a time course that differs between males and females (10, 12). Furthermore, responses to rises in AP are more affected than those produced by decreasing AP (2, 7, 10, 11), suggesting the prominent defect is most obvious when baroreceptor afferent nerves and the NTS are activated to evoke physiological responses. In males the gain of baroreflex-mediated changes in SNA are apparent in juvenile OZR at 7 weeks of age with a later development of impaired range in baroreflex-

mediated decreases in SNA and HR in young adult OZR at 12-13 weeks of age (3, 10). In contrast, young adult female OZR have hypertension at this age with baroreflex-mediated bradycardia that is comparable to female LZR. Instead, female OZR develop blunted baroreflex-mediated changes in HR by 6 months of age. Data in Chapter II show that restoration of elevated blood glucose in young adult male OZR restores baroreflex function. Furthermore, in Chapter III the preserved baroreflex-mediated bradycardia in young adult female OZR is accompanied by maintenance of blood glucose throughout the day and night. These data suggest that hyperglycemia plays a significant role in the emergence of impaired baroreflexes corroborated by overlap in timelines and impact of intervention.

With the identification of overlapping timelines for the development of hyperglycemia and impaired baroreflexes and the positive impact of intervention for hyperglycemia upon baroreflex efficacy, pinpointing sites and mechanisms underlying these comorbidities becomes a next step toward understanding their interaction. Several observations suggest that the NTS is a critical site for changes that are likely to foster impaired baroreflexes. Specifically, physiological responses to activation of the NTS by baroreceptor afferent inputs and glutamate are reduced in young adult male OZR, but not young adult female OZR, coincident with the emergence of impaired baroreflexes in male OZR. Raising AP by infusion of phenylephrine produces less c-Fos expression in the NTS of young adult male OZR but not female OZR (Figure III-2). In agreement, electrical stimulation of baroreceptor afferent nerves evokes smaller decreases in SNA and AP in male OZR (4), and these responses are mimicked by microinjections of glutamate into the NTS in male OZR but not female OZR (3). Furthermore, glutamatergic activation of the caudal ventrolateral medulla, a critical target of NTS neurons for production of baroreflexes, is comparable in young adult male OZR and LZR (3). Likewise, in

juvenile males and young adult females, glutamatergic activation of the NTS produces comparable decreases in SNA and AP in OZR and LZR coincident with intact baroreflexes (10) (Figure III-4). One step in determining mechanisms underlying these changes in the NTS is to identify which NTS neurons are affected in male OZR at this age (~ 7 weeks old and beyond), but not in female OZR.

Chronic activation of neurons within the central nervous system induces changes in gene expression patterns that contribute to long-term alterations in neuronal function (8). The expression of FosB and ΔFosB (a truncated splice variant of FosB) accumulates over time in response to a chronic stimulus(9). FosB and ΔFosB belongs to the Fos family of transcription factors that includes cFos, Fos-related antigen 1 (Fra1), and Fra2 (9). These transcription factors dimerize with Jun proteins to form an AP-1 (activator protein 1) transcription factor complex, which bind to specific AP-1 sites and alter expression of specific target genes in brain region (8, 9). Although these genes encoding Fos proteins have been identified as immediate early genes based on their expression with transient induction and short-lived response, the ΔFosB protein are relatively stable and are induced in response to wide range of chronic stimuli. Because ΔFosB protein persists for a relatively long period in the brain, this protein is thought to play an important role in the induction and maintenance of long-term plasticity (6). The expression of Fos-like proteins is increased in the NTS of dogs made obese by a high fat diet (5), suggesting a chronic change in the activity of NTS neurons in response to this diet that coincides with excess weight gain. However, the time course for changes in expression of Fos-like proteins in the NTS during the natural development and progression of MetS attributes in a model such as OZR has not been examined. Therefore, we sought to determine the pattern of ΔFosB expression in NTS of male Zucker rats using an age range that encompasses the development of impaired

baroreflexes. In addition, we sought to determine whether these changes in ΔFosB expression were absent in female OZR at these ages.

MATERIALS AND METHODS

Animals. Male OZR [Lepr (*fa*/*fa*)] and LZR [Lepr (+/+) and (+/*fa*)] from Charles River (Houston, TX) were examined at 4 age ranges: 1) 5 - 6 weeks: after weaning and before earliest observation of baroreflex impairment, 2) 7 - 8 weeks, juveniles with early signs of impaired baroreflex gain, 3) 11 -12 weeks, young adults with clear impairment of baroreflex range, and 4) 18 -19 weeks, adults with chronic baroreflex impairment but before pancreatic failure. Female OZR and LZR examined at 2 critical age ranges, 7 - 8 weeks and 11 - 12 weeks. Rats were allowed to acclimate for 1 week after arrival (shipped ages 4 – 18 weeks old). The rats were deeply anesthetized with urethane (5 g/kg from a 5 g/ml solution, i.p.) and perfused transcardially with phosphate-buffered saline (250 ml) followed by 4% paraformaldehyde (300 - 500 ml) was performed. The brain was then removed and stored in the same fixative for 48-72 hours at 4°C. The brain stem was sectioned in the coronal plane (30 μm) using a Vibratome, and sections were stored in cryoprotectant solution.

Immunohistochemistry. Immunohistochemistry was performed for detection of ΔFosB proteins using 1:6 brainstem sections. After rinsing out of cryoprotectant solution, sections were incubated in hydrogen peroxide (1%; 1 hour) to block endogenous peroxidases. Then, sections were incubated for 3 minutes in 10% heat-inactivated horse serum to reduce background staining before incubation with goat anti-FosB primary antibody (48 hours, 1:6000 with 10% horse serum / 3 % Triton-X100; # sc-48-G, Santa Cruz Biotechnology). After rinsing, sections were incubated with biotinylated donkey anti- goat (overnight, 1:600, Jackson ImmunoResearch) followed by

ExtrAvidin-HRP (4 hours, 1:1,500; Cat # E-2886; Sigma). The DFosB/FosB+ neurons were revealed by glucose oxidase (5-10 minutes, 1:5,000) and 3, 3'-diaminobenzadine-nickel . The stained sections were mounted onto gelatin-coated slides and allowed to dry overnight. The next day, sections were dehydrated and delipidated using the series of alcohols and xylenes. Coverslips were applied with DPX mounting media (Sigma-Aldrich).

Quantification of DFosB/FosB+ neurons. The slides were viewed in brightfield with an Olympus BX60 microscope. The ΔFosB/FosB+ neurons were mapped and counted using Neurolucida software (Microbrightfield). Four rostro-caudal levels of the NTS were examined, and counts were performed bilaterally. The total counts of ΔFosB/FosB+ neurons/rat from the 4 levels were averaged and compared by 2-way ANOVA for rat phenotype and age range followed by Tukey post hoc tests for comparisons between LZR and OZR within age range and within rat phenotype across age ranges. Significance was set at $P<0.05$.

RESULTS

Male Zucker rats. At 5-6 weeks of age, staining was sparse, and the number of ΔFosB/FosB+ neurons was comparable between male OZR (6) and LZR (6) at all 4 rostro-caudal levels examined (Figure AII-1). In contrast, at 7-8 weeks of age OZR (5) exhibited a surge of ΔFosB/FosB+ neurons in the NTS compared to age-matched LZR (5). At 11-12 weeks of age, this surge of total NTS ΔFosB/FosB+ neurons in OZR (6) was reduced from the expression observed at 7-8 weeks of age, but the total counts were still significantly elevated compared to age-matched LZR (7). Likewise, at 18-19 weeks of age OZR (4) maintained elevated ΔFosB/FosB expression compared to age-matched LZR (4).

Female Zucker rats. In female Zucker rats, the total number of ΔFosB/FosB+ neurons in 4 rostro-caudal levels of NTS were comparable between OZR (5/age range) and age-matched LZR (5/age range) at both age ranges (7 – 8 weeks and 11 – 12 weeks). The number of ΔFosB/FosB+ neurons did not change by age range for either OZR or LZR.

Figure AII- 1. Expression of DFosB/FosB in the NTS of Male and Female OZR and LZR

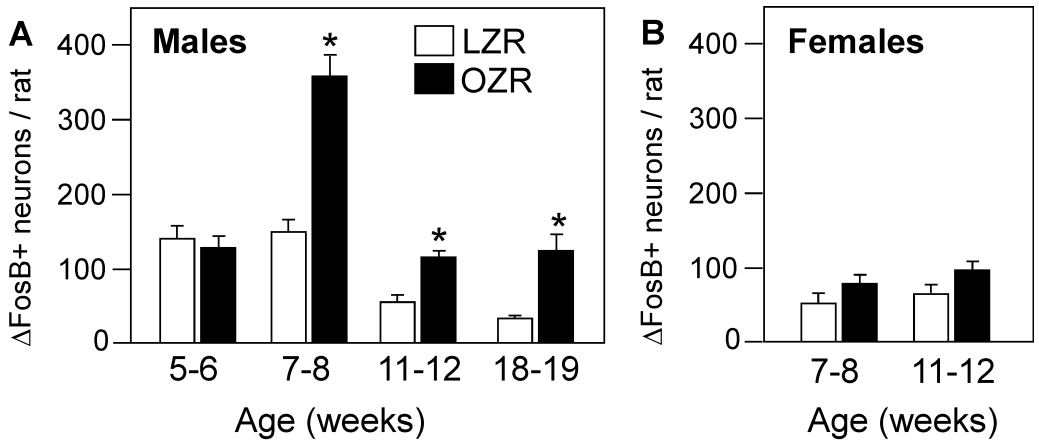

Figure 1. Expression of DFosB/FosB in the NTS of Male and Female OZR and LZR. A. Total bilateral counts of DFosB/FosB+ neurons from 4 rostro-caudal levels of NTS (-14.0, -13.8, -13.4, and -13.0 mm caudal to bregma) in age-matched groups of male OZR and LZR. *P<0.05 vs. LZR at that age. † vs. OZR at 7-8 weeks of age. B. A. Total bilateral counts of DFosB/FosB+ neurons from 4 rostro-caudal levels of the NTS (-14.0, -13.8, -13.4 and -13.0 mm caudal to bregma) in age-matched groups of female OZR and LZR. *N.S.* 2-way ANOVA for rat type and age range and Tukey post hoc tests.

DISCUSSION

This experiment examined whether neuroplasticity could be detected within the NTS at ages that corresponded with changes in attributes for MetS, such as hyperglycemia, and the development of impaired baroreflexes in young adult male OZR but not female OZR. At 7-8 weeks of age juvenile male OZR displayed a surge of ΔFosB/FosB expression in the NTS, and this difference persisted well into adulthood. In contrast, in female OZR of the same age range, very little expression of was detected, and there were no differences in ΔFosB/FosB expression between female OZR and LZR as juveniles or young adults. These data suggest a dramatic change in activity in NTS neurons of juvenile male OZR that corresponds roughly to the age when baroreflexes begin to show impairment characterized as a reduction in the gain of the reflex (10). Interestingly, the female rats at this age do not show significant changes in ΔFosB/FosB expression in the NTS, in agreement with an absence of changes in baroreflex efficacy in female OZR. Similarly, as young adults the female OZR have preserved baroreflexes and do not show a significant change in ΔFosB/FosB expression even at 11-12 weeks of age.

These observations support the notion that juvenile male OZR experience a significant change in inputs or response to inputs to the NTS or circulating factors that can be detected by NTS neurons. A major difference between young adult male and female OZR is that female OZR do not develop the chronic hyperglycemia seen in male OZR at this age (Figure III-7). It has been demonstrated that changes in circulating glucose can be detected within the NTS (14), and that increases in glucose can enhance release of glutamate from afferent nerve terminals in addition to sensitizing some NTS neurons to inputs (13, 14). Furthermore, with chronic hyperglycemia in the setting of type 1 diabetes, the NTS appears to become unresponsive to glucose (1). Changes in the transient phenylephrine-induced c-Fos expression in male OZR

corroborates the notion that the NTS is more sensitive to baroreceptor inputs in juvenile male OZR and later in young adulthood the male OZR become less responsive to acute rises in AP. Compared to age-matched LZR, juvenile OZR have a greater number of c-Fos+ neurons in the NTS after infusion of phenylephrine to raise AP by 40 mmHg (3). In contrast, young adult male OZR have fewer phenylephrine-induced c-Fos+ neurons in the NTS compared to age-matched LZR (3). In contrast, phenylephrine infusion produces a comparable number of c-Fos+ neurons in the NTS of young adult female OZR and LZR, coincident with intact glucose homeostasis and baroreflex-mediated bradycardia (Figure III-2).

A limitation of this experiment is that the phenotype and projections of the NTS neurons that express ΔFosB/FosB have not been identified. In addition, future studies will be needed to determine whether chronic changes in blood glucose underlie the changes in ΔFosB/FosB expression in the NTS. Although the timing of changes in blood glucose and ΔFosB/FosB expression coincide, other factors associated with MetS could also contribute. However, it is unlikely that elevated insulin or triglycerides are the mediators of ΔFosB/FosB expression in the NTS because they are already elevated during the first age range examined. A critical next step would be to determine whether prevention of hyperglycemia in male OZR with early metformin treatment would prevent the surge in ΔFosB/FosB expression in the NTS and changes in phenylephrine-induced c-Fos expression that occur in juvenile and young adult male OZR.

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