Diabetic Hypertensive Leptin Receptor–Deficient db/db Mice Develop Cardioregulatory Autonomic Dysfunction

Andrey C. da Costa Goncalves, Jens Tank, André Diedrich, Aline Hilzendeger, Ralph Plehm, Michael Bader, Friedrich C. Luft, Jens Jordan, Volkmar Gross

Abstract—Leptin receptor–deficient db/db mice develop human type 2 diabetes mellitus, hypertension, and obesity with disrupted circadian blood pressure (BP) rhythm. Whether leptin is the sole mechanism mediating autonomic imbalance and hypertension is unclear. To explore this notion further, we measured BP by radiotelemetry combined with fast Fourier transformation and assessed autonomic function pharmacologically before and after renin-angiotensin system blockade with enalapril. The resting period BP (117±3 versus 108±1.0 mm Hg) and heart rate (HR; 488±12 versus 436±8 bpm) were higher in db/db mice compared with db/+ mice. BP and HR amplitudes were lower in db/db mice compared with db/+ mice. BP response to trimetaphan (−43±5 versus −27±3 mm Hg) and HR response to metoprolol (−59±12 versus −5±4 bpm) were greater in db/db mice than in db/+ mice. The HR response to atropine was blunted in db/db mice (59±17 versus 144±24 bpm), as were baroreflex sensitivity and HR variability. Enalapril improved autonomic regulation in db/db mice. Stimulation of central α-2 adrenoreceptors enhanced both parasympathetic HR control and baroreflex sensitivity in db/db mice. We suggest that functional, rather than structural, α-2 adrenoceptor changes and the renin-angiotensin system are involved in the increased sympathetic and decreased parasympathetic tones in db/db mice. Our data suggest that db/db mice exhibit features found in humans with type 2 diabetic autonomic neuropathy and could serve as a model for this complication. **(*Hypertension*. 2009;53[part 2]:387-392.)**

Key Words: type 2 diabetes mellitus ■ obesity ■ hypertension ■ ACE inhibition ■ α-2 adrenoceptors ■ autonomic dysfunction

Almost all obese patients with type 2 diabetes mellitus have hypertension. The hypertension is mediated at least in part through sympathetic nervous system activation,[1,2] with concomitant reduction in cardiac parasympathetic tone.[3] In addition to the effect on blood pressure (BP), autonomic imbalance may predispose target organ damage and cardiac arrhythmias.[4,5] The close association between obesity and hypertension in most populations suggests involvement of an adipose tissue–derived signal. Leptin is the prime suspect, because it elicits sympathetic activation.[6–8] Indeed, circulating leptin concentrations are increased in patients with obesity and type 2 diabetes mellitus.[9,10] Leptin levels are also increased in nondiabetic patients with hypertension.[11] Apparently, many patients are resistant to leptin satiety and weight-reducing actions, whereas sympathoexcitatory actions are preserved, a phenomenon referred to as "selective leptin resistance."[12] Whether leptin is the sole mechanism mediating obesity-associated autonomic imbalance and hypertension is unclear. Therefore, we assessed BP and autonomic cardiovascular regulation in db/db mice. These animals develop obesity and type 2 diabetes mellitus at an early age because of mutation in the leptin receptor gene.[13] We hypothesized that BP and cardiovascular autonomic regulation should be normal in these animals, given the defect in leptin signaling.

Methods

Animals

We studied adult, 12-week-old, male db/db (C57BL/KsJ-db^{-}/db^{-}; n=14) mice and male db/+ (C57BL/KsJ-db^{+}/db^{-}; n=17) mice, purchased from the Jackson Laboratory (Bar Harbor, Maine). The genetically diabetic mouse (C57BL/KsJ-db/db) has a mutation on the chromosome 4 that inhibits the expression of the leptin receptor (long isoform), whereas heterozygous (control) mice cannot be distinguished morphologically or physiologically from normal mice.[14] The syndrome of type 2 diabetes mellitus in db/db mice is similar to type 2 diabetes mellitus in adult humans, which is also characterized by obesity, insulin resistance/hyperinsulinemia, and hyperglycemia. The animals were allowed free access to standard chow (0.25% sodium, SNIFF Spezialitäten GmbH) and drinking water ad libitum. The local council on animal care approved the

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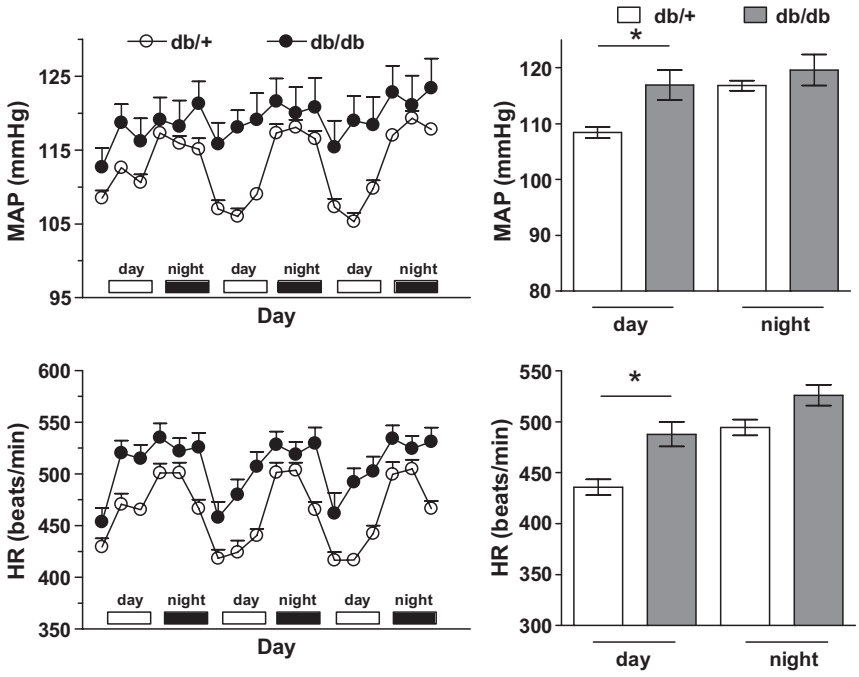

Figure 1. Circadian variation of MAP (top left) and HR (bottom left) for db/+ (n=10) and db/db (n=10) mice. Right panels show the 12-hour day/night average values of MAP (top) and HR (bottom). MAP and HR were higher in db/db during the day but not during the night vs db/+ mice (*$P<0.05$).

study according to requirements of the American Physiological Society. Our approaches to radiotelemetry, spectral analysis, and calculations of baroreflex sensitivity (BRS) have been described earlier.[15,16] We measured nonfasting blood glucose levels and body weights at weekly intervals. Blood was obtained from the tail, and glucose concentrations were determined using Accu-Check Compact glucose test strips (Roche Diagnostics GmbH).

Pharmacological Testing

To evaluate autonomic control of BP, the following drugs were applied: muscarinic blockade was obtained with atropine (4 mg/kg; db/+: n=10; db/db: n=10), β_1-adrenergic receptor blockade with metoprolol (8 mg/kg; db/+: n=10; db/db: n=10), ganglionic blockade with trimethaphan (120 mg/kg; db/+: n=10; db/db: n=10), blockade of α-2 adrenoreceptors with yohimbine (2 mg/kg; db/+: n=6; db/db: n=4), and stimulation of α-2 adrenoreceptors with clonidine (1 mg/kg; db/+: n=6; db/db: n=4). Furthermore, in 6 db/+ and 4 db/db mice, clonidine (1 mg/kg) and atropine (2 mg/kg) were given parallel. All of the substances were given intraperitoneally in the morning hours between 9 AM and 11 AM. Continuous beat-by-beat values of BP were recorded for 1 hour, after which the mice were briefly removed and drugs were applied. Thereafter, beat-by-beat values were recorded for 1 additional hour. As in our former study, the values (45th to 60th minute) after drug injection were used to characterize the respective responses to avoid the measurement of stress-induced BP and heart rate (HR) changes.[16] The protocols for the single injections were separated by ≥24 hours. Drug administrations were not randomized.

BP and HR were also measured under the angiotensin-converting enzyme (ACE) inhibitor enalapril (30 mg/L in drink water; db/+: n=8; db/db: n=7), which was given for 10 days. BP values also represent in this study means of 3 days.

Statistics

Data are presented as means±SEMs. Statistically significant differences in mean values were evaluated by ANOVA followed by Bonferroni posthoc test. For paired data we used the nonparametric Wilcoxon signed-rank test. $P<0.05$ was used to determine statistical significance.

Results

Nondipping Arterial Hypertension in db/db Mice

At the time of study, db/db mice weighed 46±1 g compared with 26±0.3 g of db/+ mice, and the blood glucose concentrations leveled in db/db at 510±24 and in db/+ at 156±3 mg/dL.

Figure 1 shows day and night mean arterial pressure (MAP) and HR values in db/+ and db/db mice. Daytime MAP was 117±3 mm Hg in db/db and 108±1 mm Hg in db/+ mice ($P<0.05$). Daytime HR was 488±12 bpm in db/db and 436±8 bpm in db/+ mice ($P<0.05$). In contrast, nighttime BP and HR were similar in both strains. Thus, db/db mice showed an attenuated diurnal BP and HR variation. In patients, this pattern is referred to as "nondipping" BP.

BRS Is Reduced in db/db Mice

The sequence method and cross-spectral analysis are established in the characterization of baroreflex HR regulation in patients and in animals. We underscored the validity of spontaneous BP and HR changes to characterize BRS in conscious mice,[15] as shown previously in humans.[17] BRS calculated by the sequence method and by cross-spectral analysis were substantially decreased in db/db compared with db/+ mice ($P<0.05$ for both), as shown in Figure 2 (left and middle panels).

Autonomic Imbalance in db/db Mice

Low-frequency systolic BP (LF-SBP) oscillations are produced by sympathetic modulation of vascular tone. The measurement increases when sympathetic drive to the vasculature is excessive.[18] LF-SBP oscillations were 4.2±0.7 mm Hg^2 in db/db and 2.2±0.3 mm Hg^2 in db/+ mice ($P<0.05$), as shown in Figure 2 (right).

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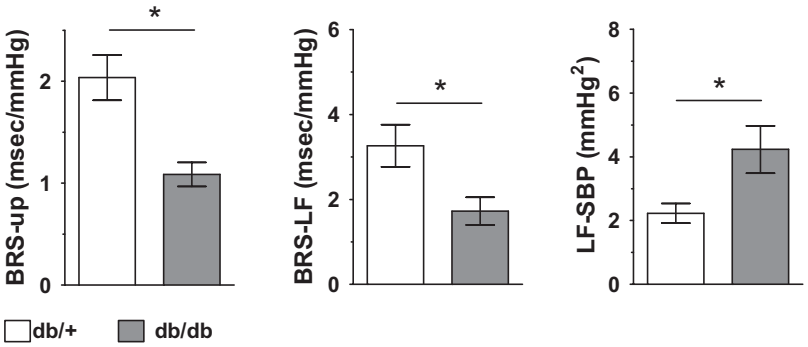

Figure 2. BRS calculated with the sequence method (up-sequences; BRS-up, left), as mean value of the transfer function between SBP and pulse intervals in the low frequency band (BRS-LF, middle), LF-SBP (right) in db/+ (n=14) and db/db (n=12) mice. BRS was decreased and sympathetic outflow described as LF-SBP increased in db/db vs db/+ mice (*P<0.05).

In mice, low-frequency HR oscillations are mediated through parasympathetic mechanisms.[19] In contrast, in human subjects, low-frequency HR oscillations result from sympathetic and parasympathetic activation. Low-frequency HR oscillation was strongly attenuated in db/db mice (4.0±1.0 versus 16.3±2.3 ms^2). SD and root mean square of successive differences, time domain parameters describing HRV, were also reduced in db/db mice.

Figure 3 shows BP responses to pharmacological testing with trimethaphane and HR responses to pharmacological testing with metoprolol and atropine. Complete interruption of efferent autonomic traffic with trimethaphane reduced HR (ΔHR: 106±7 bpm in db/db and 62±4 bpm in db/+ mice) and BP (ΔMAP: 43±5 mm Hg in db/db and 27±3 mm Hg in db/+ mice; both P<0.05). Metoprolol reduced HR stronger in db/db (ΔHR: 59±12 bpm) than in db/+ (ΔHR: 5±4 bpm; P<0.05) mice. Obesity-associated hypertension in db/db mice results from sympathetic activation, a conclusion supported by the BP and HR decreases in response to trimetaphane and metoprolol. In contrast, the HR response to atropine was attenuated in db/db (ΔHR: 59±17 bpm) compared with db/+ (ΔHR: 144±24 bpm; P<0.05) mice. The same pattern was found in changes of HRV-LF after atropine (ΔLF-HRV: db/db: 3.8±0.9 ms^2; ΔLF-HRV: db/+: 15.0±2.3).

Parasympathetic HR Reserve

In mice, stimulation of central α-2 adrenoreceptors with clonidine decreases HR through activation of cardiac parasympathetic efferents,[19] which corresponds with results in humans in which α-2 adrenoreceptor stimulation augmented baroreflex-mediated bradycardia, most likely by parasympathetic activation.[20] Therefore, the clonidine HR response provides a measure of parasympathetic reserve. Clonidine profoundly decreased HR in both mice strains, as shown in Figure 4 (top). With clonidine, HR decreased 327±25 bpm in db/+ and 221±12 bpm in db/db mice. The response was abolished by atropine. Clonidine improved spontaneous BRS and HRV in both strains, illustrated also in Figure 4 (middle and bottom panels). Again, atropine abolished the clonidine responses. On the other hand, the inhibition of endogenous α-2 adrenoreceptor tone with yohimbine elicited a greater HR increase in db/+ than in db/db mice (131±18 versus 60±9 bpm; P<0.05).

ACE Inhibition Reverses Autonomic Imbalance

Enalapril given for 10 days, as shown in Figure 5, reduced MAP (Figure 5, left) 21±1.5 mm Hg in db/db and 15±2.2 mm Hg in db/+ mice (P<0.05), such that BP was identical in both strains (98±3 mm Hg in db/db, n=8; 98±4 mm Hg in db/+, n=7). Moreover, enalapril improved low-frequency BRS (Figure 5, middle) and LF-SBP (Figure 5, right) significantly.

Discussion

Given the important role of the leptinergic system in the pathogenesis of obesity-associated arterial hypertension, we expected to observe a dissociation between body weight and cardiovascular sympathetic activation in db/db mice. Instead, BP was increased in db/db compared with db/+ mice. BP failed to decrease during the inactive phase, thus resembling a nondipping BP pattern seen in patients. Moreover, db/db mice featured an increase in HR, reduction in HRV, and baroreflex dysfunction. Pharmacological testing revealed sympathetic activation together with reduced cardiac parasympathetic tone in db/db mice. Hence, db/db mice

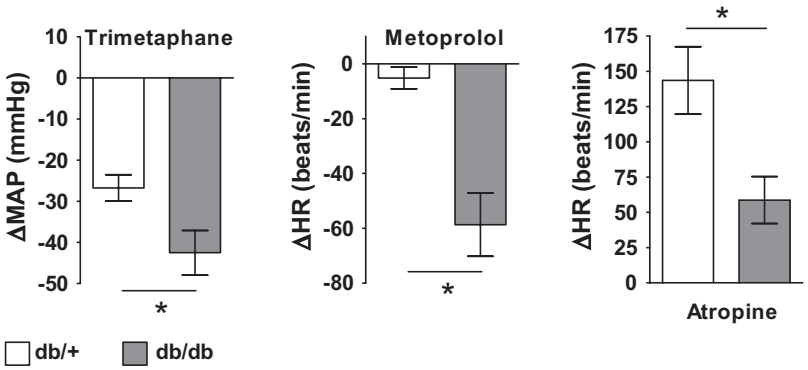

Figure 3. Effect of trimethaphane (120 mg/kg, left) on blood pressure (ΔMAP), and metoprolol (8 mg/kg, middle) and atropine (4 mg/kg, right) on HR (ΔHR) of db/+ (n=10) and db/db (n=10) mice. The greater response of HR and MAP after metoprolol and trimethaphane in db/db mice suggests a higher sympathetic tone, whereas the smaller response of HR after atropine points to a reduced parasympathetic tone in db/db mice (*P<0.05).

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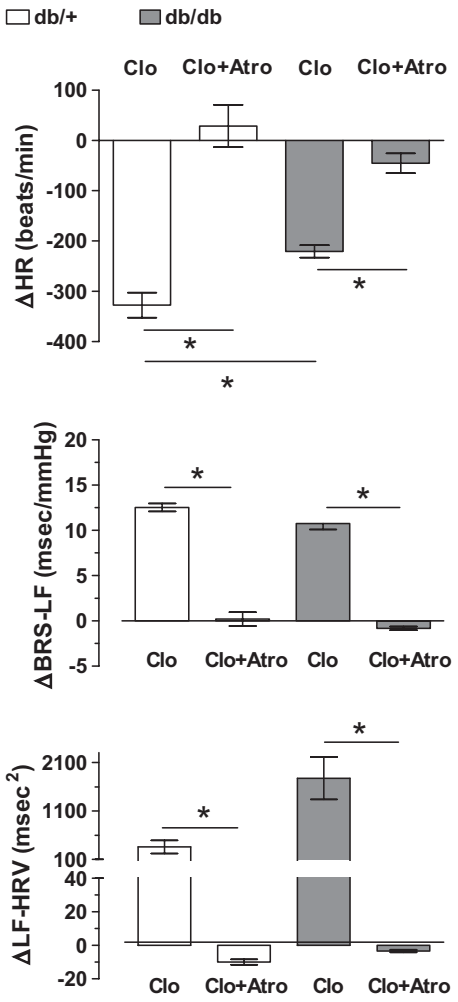

Figure 4. ΔHR during clonidine (1 mg/kg) and during clonidine (1 mg/kg) with atropine (2 mg/kg) in db/+ (n=6) and db/db (n=4) mice (top). Changes in spontaneous baroreflex sensitivity (ΔBRS-LF, middle) and HR variability (ΔLF-HRV, bottom) during clonidine (1 mg/kg) and during clonidine (1 mg/kg) with atropine (2 mg/kg) in db/+ (n=8) and db/db (n=8) mice. Clonidine decreased HR more strongly in db/+ than in db/db mice. BRS and HRV were increased in db/+ and in db/db mice by clonidine. Atropine abolished the clonidine effects (*$P<0.05$).

showed all of the abnormalities in cardiovascular autonomic regulation that are commonly observed in obese patients with type 2 diabetes mellitus before the onset of overt autonomic neuropathy. Remarkably, chronic renin-angiotensin system blockade with enalapril and stimulation of central α-2 adrenergic receptors with clonidine reversed these abnormalities in db/db mice.

Chronic BP measurements in mice are by no means trivial. A previous study applying the tail-cuff method reported BPs of 145±6/106±2 mm Hg in 12- to 14-week-old db/db mice.[21] However, the tail-cuff method is unreliable and does not provide representative BP measurements.[22] Other investigators have also applied the telemetry technique.[23] Similar to our study, these authors observed changes in circadian BP regulation. In that study, the arterial catheter was placed in the carotid artery that may interfere with carotid baroreceptor function. Because our study focused on autonomic cardiovascular regulation, we implanted the arterial catheter in the femoral artery.

Concomitant increases in BP and HR in db/db mice strongly suggest an increase in sympathetic activity.[24] The BP increase was not attributable to changes in cardiac output as echocardiographic measurements showed (data not shown). The increase of BP in db/db mice may, therefore, be similar to diabetes in obese Zucker rats in which a mutation of the leptin receptor–encoding gene impairs the ability of leptin to suppress food intake and increases sympathetic nervous system activity.[25] Interestingly, leptin resistance in genetic and acquired murine obesity models is selective to metabolic actions of leptin, sparing its sympathetic pressure actions.[7] Indeed, in db/db mice, ganglionic and β-adrenoreceptor blockade caused an excessive decrease in BP and HR. Moreover, LF-SBP, which results from sympathetic modulation of vascular tone, was increased in db/db mice. Altogether, these changes point to an independent increase of sympathetic tone, so that we did not directly measure sympathetic nervous activity. Parasympathetic HR regulation was reduced in db/db mice, because atropine had a lesser effect on HR and HRV in these animals. Finally, we observed a reduction in BRS in these animals. Thus, db/db mice feature changes in cardiovascular autonomic regulation that are commonly observed in obese diabetic patients.[26]

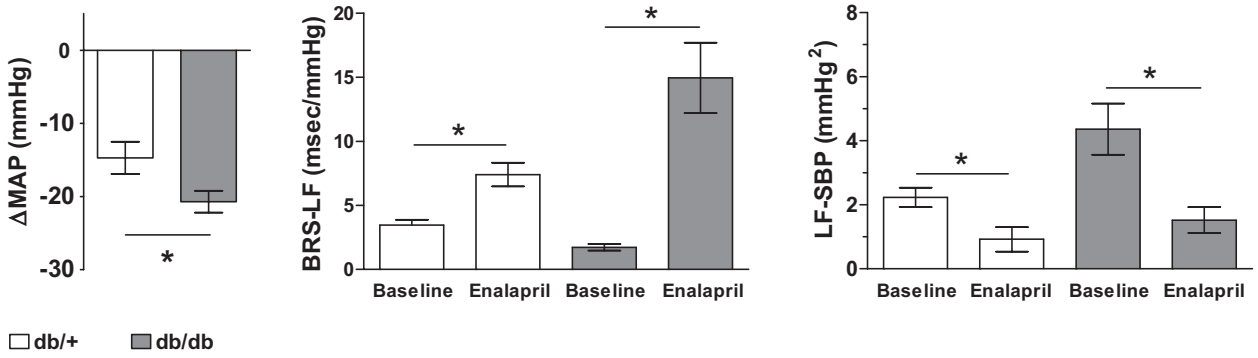

Figure 5. ΔMAP (left), response of BRS (BRS-LF, middle), and response of LF-SBP (right) after ACE inhibition with enalapril in db/+ (n=8) and db/db (n=7) mice. Enalapril reduced BP more strongly in db/db than in db/+ mice. Enalapril improved BRS and decrease sympathetic outflow to the periphery (LF-SBP), suggesting that the renin-angiotensin system is involved in autonomic dysregulation of db/db mice (*$P<0.05$).

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Theoretically, the imbalance between sympathetic and parasympathetic cardiovascular regulation in db/db mice could result from functional changes in brain areas regulating autonomic tone. An alternative explanation is that diabetes mellitus induced neuropathic changes in peripheral autonomic neurons. We applied clonidine to distinguish between functional and structural mechanisms. Clonidine, which stimulates central α-adrenergic receptors, profoundly increases parasympathetic outflow to the heart.[19] In db/db mice, HR decreased and HRV and BRS increased with clonidine, suggesting that parasympathetic efferents to the heart can be engaged. Our study suggests that imbalance between sympathetic and parasympathetic activity in db/db mice is explained by altered central autonomic regulation rather than diabetic autonomic neuropathy. This idea is supported by the observation that yohimbine had a differential effect on HR regulation in both strains. However, the slightly attenuated response of HR in db/db mice to clonidine may suggest reduction in parasympathetic reserve, which could be secondary to "subclinical" autonomic neuropathy.

The increase in sympathetic tone with parasympathetic inhibition in db/db mice suggests that the leptin system is not the sole mechanism driving obesity-associated arterial hypertension. One caveat is that the db/db mouse is not a complete knockout. The long form of the leptin receptor is not expressed in db/db mice. We cannot exclude completely that leptin via another mechanism or isoforms of its receptor elicited an effect on central autonomic regulation given the increased leptin levels in db/db mice and given that a selective leptin resistance for metabolic actions of leptin is discussed.[7] The renin-angiotensin system may be involved. Angiotensin II regulates BP through actions in peripheral tissues and in the brain. In the brain, angiotensin II activates the sympathetic nervous system. Furthermore, angiotensin II alters baroreflex HR regulation.[27] ACE inhibition normalized BP in db/db mice, in which plasma ACE activity is increased (unpublished data), suggesting that the increase in BP was at least in part mediated by the renin-angiotensin system. The associated improvement of LF-SBP and BRS by enalapril is consistent with a central mechanism.

The observation that nondipping hypertension and autonomic imbalance in obesity-associated type 2 diabetes mellitus may be mediated through the renin-angiotensin system may be clinically relevant. Indeed, altered autonomic nervous system regulation and reduced nocturnal BP reductions are described in diabetic patients.[28] Reduced circadian BP variation is associated with increased mortality.[29] Therefore, treatment in diabetes mellitus, especially in diabetic subjects known to have cardiac autonomic neuropathy, may have to consider the status of the autonomic nervous system.[30,31] We conclude that the obese diabetic db/db mice model autonomic cardiovascular abnormalities in patients. The leptin system is not the sole mechanism mediating sympathetic activation and parasympathetic withdrawal. The renin-angiotensin system, which, in contrast to the leptin system, is currently amenable to treatment, appears to be involved.

Perspectives

The study that we have reported has established db/db mice as a model showing abnormalities in cardiovascular autonomic regulation that are commonly observed in obese patients with type 2 diabetes mellitus before the onset of overt autonomic neuropathy. The db/db mice could serve as a reliable model to study cardiovascular changes in diabetic neuropathy and may open new avenues to look on pathophysiological mechanisms and therapeutic approaches to prevent autonomic neuropathy in type 2 diabetes mellitus with adipositas and hypertension.

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Disclosures

None.

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