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# Cardiovascular and autonomic phenotype of *db/db* diabetic mice

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## SUMMARY

The *db/db* mice serve as a good model for type 2 diabetes, characterized by hyperinsulinemia and progressive hyperglycemia. There are limited and conflicting data on the cardiovascular changes in this model. The aim was to characterize the cardiovascular/autonomic phenotype of male *db/db* mice and evaluate the role of angiotensin (Ang) AT1 receptors. Radiotelemetry was used to monitor 24 hr blood pressure (BP) in mice for 8 weeks. Parameters measured were mean arterial pressure (MAP), heart rate (HR) and their variabilities. MAP and BP circadian rhythms were not altered in 8 wk *db/db* while HR and locomotor activity were decreased. With aging, MAP gradually increased in *db/db* mice and the 12-h light values did not dip significantly from the 12-h dark periods. In 14 wk mice, MAP was increased during light (101 ± 1 vs. 117 ± 2 mmHg, $p < 0.01$; Control vs. *db/db*) and dark phases (110 ± 1.7 vs. 121 ± 3.1 mmHg, $p<0.01$; Control vs. *db/db*). This increase in BP was associated with significant increase in plasma ACE activity and Ang II levels. Chronic treatment with losartan (10 mg/kg/day) blocked the increase in MAP in *db/db* with no effect in controls. Spectral analysis was used to monitor autonomic cardiovascular function. The circadian rhythm observed in SAP variance and its LF component in control mice was absent in *db/db*. There were no changes in HR variability and spontaneous baroreflex sensitivity between control and *db/db* mice. Results document an age related increase in MAP in *db*/*db* reduced by antagonism of Ang AT1 receptors and alterations in autonomic balance and components of the renin angiotensin system.

### Keywords

ACE; hypertension; renin; spectral analysis; losartan; insulin resistance

## INTRODUCTION

The prevalence of type 2 diabetes is rising to epidemic levels worldwide. The pathology of diabetes is characterized not only by hyperglycemia, but also by hypertension, dyslipidemia, microalbuminuria and vascular inflammation. Diabetes is associated with a reduced lifespan and much of the cardiovascular disease (CVD) is attributed to hypertension. Thus, aggressive management of high blood pressure (BP) is essential for a reduction in risks of cardiovascular events (Sowers, 2004). Aggressive treatment of hyperglycemia is also an important aspect of the management of the long term complications of diabetes. Earlier

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clinical trials showed a correlation between strict glycemic control in patients with type 2 diabetes and a reduction in microvascular complications, but not macrovascular disease (UKPDS,1998). There are questions as to whether lowering blood glucose with currently available medications effectively reduces CVD. Recent clinical trials, such as ACCORD (Action to Control Cardiovascular Risk in Diabetes) (Gerstein *et al.*, 2007) and ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation) (Patel *et al.*, 2008), were designed to determine the relationship between glycemic control and macrovascular events. The message from both clinical trials is that near normal glycemic control for a median of 3.5 to 5 years does not reduce macrovascular events (Dluhy & McMahon, 2008), suggesting that other factors are important in disease progression. However, the ADVANCE trial reconfirmed the predicted reduction in microvascular complications such as microalbuminuria and nephropathy (Dluhy & McMahon, 2008).

There are clinical data to document the effectiveness of ACE inhibitors (ACEi) and Ang receptor blockers (ARBs) in treatment of hypertensive diabetics (Gilbert *et al.*, 2003). There are even data that show long term treatment with losartan lowers the risk of developing diabetes (Lindholm *et al.*, 2002). A general role for RAS in diabetes-induced hypertension is supported by the depressor effects of ACEIs and ARBs in diabetic patients (Horio *et al.*, 2004). In a recent clinical trial, candesartan prevented the development of diabetes in heart failure patients, further connecting cardiovascular disease, RAS and metabolic dysfunction. There are data in animals which show that ACEI or ARBs reduce the cardiovascular complications in animal models of type 1 and type 2 diabetes (Amazonas & Lopes De Faria, 2006; Nielsen *et al.*, 2003). Our previous work documented the importance of Ang AT1 receptors in the development of cardiovascular dysfunction in chemical and dietary induced diabetic states (Farah *et al.*, 2007a; Wichi *et al.*, 2007). In the fructose loading model, the hypertension was reverted to a hypotension in mice lacking the Ang AT1a receptor (Farah *et al.*, 2007a) and blocked in mice treated with losartan (Senador *et al.*, 2006). The fructose diet itself produced glucose intolerance, increased Ang II levels and increased BP (Farah *et al.*, 2006; Farah *et al.*, 2007a). Recently, it has been shown that diet-induced obesity in mice is associated with resistance to the metabolic actions of leptin, but preservation of its BP raising effects (Rahmouni *et al.*, 2005). In addition, leptin deficiency in murine model of diabetes, as in the *ob/ob* mouse, leads to decreased arterial pressure, despite severe obesity (Mark *et al.*, 1999).

An important issue for the study of diabetic pathologies and the role of the RAS is the availability of an animal model that simulates the human condition. We chose the *db/db* genetic mouse model that has a point mutation in the diabetes (*db*) gene encoding the leptin receptor gene (Chen *et al.*, 1996). At early ages, they serve as a good model for type 2 diabetes, characterized by hyperinsulinemia and progressive hyperglycemia (Coleman, 1983). With aging, there is a gradual loss of pancreatic function resulting in low insulin and extremely high glucose levels. The model bears similarities to the disease progression in humans in which the first stage is insulin resistance followed by a stage of pancreatic insufficiency (Coleman, 1978; Hummel *et al.*, 1966). Although there are reports of *in vitro* vascular hyperactivity in *db*/*db* (Kanie & Kamata, 2000; Guo *et al.*, 2005), there are limited and conflicting data on the *in vivo* cardiovascular changes in this model. Studies have employed the tail cuff measurement system and have found increased (Bagi *et al.*, 2005; Brezniceanu *et al.*, 2008), decreased (Moriyama *et al.*, 2004) or no BP change in *db*/*db* mice (Guo *et al.*, 2006; Kosugi *et al.*, 2006).

We designed a study to characterize the metabolic and cardiovascular phenotypes of the *db/db* diabetic model and to determine the cardiovascular effects of chronic blockade of Ang AT1 receptors. We examined age related changes in glucose, insulin, body weight, BP, HR

and cardiovascular autonomic function. A key aspect of the cardiovascular protocol was the use of radiotelemetry which allows for chronic BP measurements in freely moving mice. It is particularly useful for measurement of light/dark cardiovascular rhythms which are known to be altered in clinical and experimental and model of diabetes (Farah *et al.*, 2006; Nakano *et al.*, 1998).

## EXPERIMENTAL PROCEDURES

### Animals

Male BKS.Cgm +/+ *Lepr* $^{db}$/J (*db*/*db*, n=38) diabetic mice and their age-matchednondiabetic littermates (*db/m*, n=38) were used for the experiments. The animals were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed at 22°C under a 12-hr light/12-hr dark cycle with *ad libitum* access to water and standard mouse chow. All experimental protocols were approved by the WSU Animal Care and Use Committee.

### Measurement of Cardiovascular Parameters and Locomotor activity

Cardiovascular parameters were recorded by radiotelemetry. At 6–7 wks of age, mice were anesthetized with ketamine/xylazine (120:20 mg/kg, I.M.) and radiotelemetric catheters (model TA11PA-C10, Data Sciences International, St. Paul, MN) were inserted into the left common carotid artery. The transmitter body was positioned subcutaneously on the right flank (Farah *et al.*, 2007a; Wichi *et al.*, 2007). After 1 week recovery from surgery, continuous 24 h BP, HR and locomotor activity were monitored once per week, weekly for 8 weeks at 500 Hz. Cardiovascular parameters were analyzed and averaged over the entire 24 h period, the 12 h light phases (6:00 AM– 6:00 PM) and during the dark phases (6:00 PM to 6:00 AM). For spectral analysis, blood pressure (BP) was recorded at 5 kHz for 60 min during the light and dark phases. HR and pulse interval (PI) were calculated from the pressure signal. Data were stored and analyzed using Dataquest A.R.T. 4.0 software (Data Sciences International, St. Paul, MN).

### Variability and Spectral Analysis

Peak interval (PI) and systolic arterial pressure (SAP) variabilities (HRV and BPV, respectively) were assessed in time and frequency domains using autoregressive spectral analysis, as described before (Farah *et al.*, 2006). Briefly, PI and SAP series were divided in segments of 350 beats and overlapped by 50%. A spectrum was obtained for each of the segments via Levinson-Durbin recursion, with the model order chosen according to Akaike's criterion, ranging between 10 and 14. The oscillatory components were quantified in the low (LF: 0.1 to 1 Hz) and high (HF: 1 to 5.0 Hz) frequency ranges (Farah *et al.*, 2006). Spontaneous baroreflex sensitivity was calculated for each light/dark recording phase using sequence analysis technique(Farah *et al.*, 2006).

### Chronic treatment with losartan

To test the role of AT1 receptor, a separate group of db/*db* (n=6) and control mice (n=6) (6–7 wks of age) were given losartan in the drinking water (10 mg $kg^{-1}$ $day^{-1}$) for 8 wks. After 6 wks of administration of losartan, radiotelemetric probes were implanted. Mice were allowed to recover from surgery. At week 14, BP, HR and locomotor activity were sampled continuously day and night for a total period of 48 h at sampling rate of 500 Hz.

### Blood glucose measurements and glucose tolerance test (GTT)

Blood samples were taken from a cut made on the tip of the tail and glucose was determined using an Accu-Check Advantage Blood Glucose Monitor (Roche Diagnostic Corporation, Indianapolis, IN). GTT was performed in control and *db/db* mice, untreated and treated with

losartan (after 8 wks of treatment). Mice were fasted for 16 hours and blood samples were collected from a cut made at the tip of the tail at 0, 30, 60, 90 and 120 min after the glucose load (I.P. injection of glucose, 1.5 g/kg).

### Plasma measurements: Insulin and Ang II

Plasma Ang II and insulin were measured in a separate group of 8 wk (33.9 ± 0.8 g) and 14 wk (35.8 ± 1.4 g) *db/db* mice and their age matched 8 wk (23.8 ± 0.7 g) and 14 wk (26.2 ± 0.4 g) controls (n=8). Mice were decapitated and trunk blood was collected in ice-chilled heparinized tubes. Plasma was immediately separated and stored frozen at −80°C. Plasma insulin levels were measured by radioimmunoassay in 20 μl aliquots of plasma using a commercial kit (Linco Research Inc., Missouri). For plasma Ang II, blood was collected as above in presence of 10 mM EDTA and 10 μM bestatin. Plasma Ang II concentrations were measured as described (Farah *et al.*, 2006). Briefly, 50 μl plasma aliquots were extracted using phenylsilylsilica columns and Ang II was measured in duplicates using an RIA kit (ALPCO Diagnostics, Windham, NH).

### Plasma ACE activity

Blood was collected as described above. ACE activity was measured using an assay kit purchased from ALPCO Diagnostic (Windham, NH, USA). Briefly, 10 μl plasma was incubated with 100 μl of HEPES buffer (pH 8) containing the synthetic substrate $H^3$ hippuryl glycine glycine ($H^3$ Hip-Gly-Gly) at 37° C. After 60 min incubation the reaction was terminated by adding 50 μl of 1 N hydrochloric acid. Liberated $H^3$-Hippuric acid due to ACE activity in samples was separated from unreacted substrate by addition of 1.5 ml of scintillating fluid and measured in a beta counter. ACE activity is expressed in units/μg protein used as previously described (Neels *et al.*, 1982).

### Statistics

Values were expressed as means ± SEM. Data were analyzed using analysis of variance, two-way or three-way ANOVA when appropriate, followed by Tukey test for multiple comparisons. Differences were considered to be statistically significant at $p<0.05$.

## RESULTS

At the early age (8 wk), *db/db* mice showed hyperglycemia, hyperinsulinemia and increased body weight (Table 1). With aging, there was a gradual loss of pancreatic β-cells resulting in lower plasma insulin and severe hyperglycemia in 14 wk compared to 8 wk *db/db* mice ($p<0.001$, Table 1). To study the age-dependent changes in the cardiovascular parameters in *db/db* mice, MAP, HR, pulse rate and locomotor activities were recorded for 24 hours and analyzed during the light and dark phases once per week for 8 weeks. At the young age (8–10 wk) there was no difference in MAP between *db/db* and controls (Fig 1 and Fig 2). However, at 8 wk, *db/db* mice showed a significant decrease in HR during the light and dark phases (table 4, $p < 0.05$, control vs. *db/db*). This decrease in HR was observed in normotensive and hypertensive *db/db* mice. There was a gradual increase in BP and by 11 wks old, MAP was significantly higher in *db/db* mice compared to controls. This could be observed when BP measurements were averaged over the entire 24 h period (Fig 2, $p < 0.005$ control vs. *db/db*) and during 12 h light phases (6:00 AM– 6:00 PM) and dark phases (6:00 PM to 6:00 AM) (Fig 1, $p < 0.05$ control vs. *db/db*). In addition, there was a significant increase in MAP in 14 wk *db/db* mice compared to 8 wk *db/db* mice (Fig 1 and Fig 2, $p < 0.05$ 8 wks vs. 14 wks). As expected, the circadian rhythm of BP was intact in control mice (Fig. 1). However, there was an age-dependent change in the circadian rhythm of BP in *db/db* mice. At early age (8–10 wk), when the *db/db* mice were normotensive there was a significant difference between the 12-h light and the 12-h dark MAP. However, in

hypertensive *db/db* mice (12–14 wk) the 12-h light period did not significantly "dip" compared to the 12-h dark period (Fig. 1). Locomotor activity during the light and dark phase was significantly reduced in *db/db* mice (table 4, $p < 0.05$, control vs. *db/db*).

Chronic treatment with losartan (10 mg/kg/day in drinking water for 8 weeks) blocked the increase in MAP in 14 wk *db/db* mice (Fig 3, $p < 0.01$, untreated vs. losartan). However, treatment with losartan did not correct the disrupted BP circadian rhythms "nondipping" of the light period observed in the *db/db* mice. Losartan treatment had no significant effect on MAP in controls (Fig 3). HR and locomotor activity were not affected by losartan treatment in controls or *db/db* (data not shown). Losartan had no effect on blood glucose in control (124.6 ± 3.5 vs. 131.2 ± 4 mg/dl, untreated vs. losartan) or *db/db* mice (549.2 ± 8.6 vs. 550.2 ± 5.8 mg/dl, untreated vs. losartan). In regard to glucose handling, losartan had no effect on glucose tolerance test in control mice (Area under the curve for GTT was 179 ± 9 vs 186 ± 12 mg/dl/min, untreated vs losartan). In addition, losartan did not improve the impaired glucose tolerance in *db/db* mice (area under the curve for GTT was 756 ± 29 vs 788 ± 25 mg/dl/min, untreated vs losartan)

To study autonomic modulation of the heart and vasculature in the *db/db* model, we applied autoregressive spectral analysis of the telemetric blood pressure signal (Table 2 and 3). ANOVA showed an interaction between strain, age and time (light/dark) for SAP variance and LF components ($p < 0.05$). Post-hoc tests showed a significant light/dark difference in the 8 and 14 wk controls for SAP variance and LF as an evidence for a circadian rhythm (Table 2). The light/dark rhythm for SAP variance and LF (Table 2) were absent in 8 and 14 wk *db/db* mice.

For PI variability, ANOVA showed no main effects for strain, age or time (light/dark) and post-hoc analysis showed no significant differences in PI variability (Table 3). However, there was an interaction between the three factors for PI variance and the frequency components ($p < 0.05$). Spontaneous baroreflex sensitivity also showed no differences between control and *db/db* mice at either young or old ages (Table 4).

To further evaluate the RAS in this model, plasma Ang II and ACE activity were measured in normotensive (8 wk) and hypertensive (14 wk) db/*db* mice and their age matched controls. ANOVA showed a main effect of group and age on plasma ACE activity and Ang II (Fig. 4). Both plasma ACE activity and Ang II level were increased in *db/db* compared to controls ($P<0.05$, Fig. 4). There was a significant increase in plasma ACE activity and Ang II levels between 8wk and 14 wk *db/db* mice (Fig. 4, $p<0.05$).

## DISCUSSION

Additional information is needed to clarify the cardiovascular phenotype of db/*db* diabetic mice and the role of RAS. The present study tested the hypothesis that there are developmental changes in BP in *db/db* mice, and these changes are mediated through Ang AT1 receptor. We used radiotelemetry to measure BP, allowing for study of day/night patterns. The key findings that young (8–10 wk) *db/db* mice were normotensive and old (11–14 wk) *db/db* mice were hypertensive compared to their age-matched controls. In addition, our data showed the light/dark circadian rhythm of BP was absent that in the hypertensive *db/db* mice. In addition, the spectral data from older *db/db* mice also showed disruption in SAP variance and its LF component circadian rhythms. This is the first report to demonstrate increased plasma Ang II and ACE in *db/db* mice. RAS appears to play a role in the development of hypertension in the *db/db* diabetic mice since the BP increase was blocked by chronic losartan treatment. The same low dose of losartan (10 mg/kg/day) had no

effect on BP in normal mice, which agrees with previous studies in mice (Brochu *et al.*, 2002) and rats (Iyer & Katovich, 1996).

Some of the discrepancies in the literature regarding BP changes in the *db/db* may be due to differences in age, time of experiment as well as methodology for BP measurement. Bagi *et al* reported increased BP in 12 wk mice (Bagi *et al.*, 2005), while Kosugi *et al.* found no changes (Kosugi *et al.*, 2006). Both studies used the tail cuff method which is not accurate for determining true baseline levels. Discrepancies can also be found in radiotelemetry studies of *db/db* mice. Reports documented either no change (Park *et al.*, 2008; Belmadani *et al.*, 2008), decreased (Bodary *et al.*, 2007) or increased BP in *db/db* mice (Su *et al.*, 2008). One study showed no difference in BP between 18 wk *db/db* and control when data was averaged over 24 hours or during light and dark phases (Park *et al.*, 2008). Interestingly, in the same study BP measured between 12:00 PM and 3:00 PM was significantly higher in db/db mice compared to controls (Park *et al.*, 2008). Our group previously reported that BP is increased in fructose-fed mice only during the dark phase (Farah *et al.*, 2006; Farah *et al.*, 2007a). Here for the first time, we show an age-dependent increase in BP which was seen when BP data were averaged over 24 h and during 12h light phase and dark phase. The present investigation used *db/db* micefrom Jackson Laboratories on a C57BL/BKS background, whereas Bodary *et al.* (Bodary *et al.*, 2007) used Jackson *db/db* mice with a C57BL/6J background. Differences in background strain may account for this differencein *db/db* phenotype since the diabetic phenotype of *db/db* mice is markedly influencedby background strain (Coleman, 1983).

There is a growing consensus driven both by clinical and basic studies that Ang AT1 blockers improve β-cell function, glucose tolerance and delay onset of type 2 diabetes (Lindholm *et al.*, 2002; Chu *et al.*, 2006). Earlier clinical trials demonstrated that strict glycemic control in patients with type 2 diabetes correlates with a reduction in microvascular complications, but not macrovascular disease (UKPDS, 1998). However, many of the cardiovascular complications seen in type 2 diabetes cannot be completely prevented by lowering blood glucose. Epidemiological studies demonstrated that RAS blockade therapy reduces blood pressure and the onset of type 2 diabetes in patients with hypertension (Yusuf *et al.*, 2005). The positive effects of ARBs and ACEi on metabolism are thought to be due to improvement in insulin sensitivity (Fogari *et al.*, 1998; Moan *et al.*, 1996). However, our results showed that chronic losartan treatment reduced BP in *db/db* mice without attenuating the state of diabetes and glucose tolerance. The lack of a blood glucose lowering effect of losartan agrees with previous studies in *db/db* diabetic mice that initiated treatment after glucose had started to rise (Mathew *et al.*, 2005; Sugaru *et al.*, 2007; Shao *et al.*, 2006). The study by Chu *et al.* (Chu *et al.*, 2006) reported a marked decrease in glycemia in *db/db* mice could be due to losartan treatment started at an earlier stage of diabetes, at 4 wks of age. In addition, studies in STZ diabetic rats and *ob/ob* mice also documented a lack of improvement in blood glucose after chronic treatment with losartan (Erbe *et al.*, 2006; Raimondi *et al.*, 2004). Therefore, it is unlikely that the BP lowering effect of losartan in *db/db* diabetic mice was mediated through lowering of blood glucose and insulin sensitization.

HR was reduced in 8 and 14 wks *db/db* mice without changes in spontaneous baroreflex. Isolated hearts from *db/db* mice showed cardiomyopathy, decrease in contractile function and altered metabolism (Aasum *et al.*, 2003). Echocardiography in *db/db* mice showed reduced heart function (Kosugi *et al.*, 2006), contractile function (Semeniuk *et al.*, 2002), cardiac output and heart weight (Bagi *et al.*, 2005). The changes in cardiac metabolism and contractile function observed previously in *db/db* mice are age-dependent (Aasum *et al.*, 2003) and could explain the decreased HR in the present study. Although previous studies reported no change in HR; the data could be compromised because of the use of the tail-cuff

method in which mice are restrained and stressed (Bagi *et al.*, 2005; Kosugi *et al.*, 2006). However, in agreement with our findings, a recent study using radiotelemetry demonstrated decreased HR in 18 wk *db/db* mice compared to controls (Park *et al.*, 2008).

In the present study *db/db* mice showed an absence in the circadian oscillation in SAP variance and its LF component. It is the LF component of SAP variance which is indicative of sympathetic input to the vasculature (Stauss, 2007). The changes in the BP rhythms were observed in *db/db* diabetic mice prior to any changes in BP itself. This agrees with previous data showing changed BPV as a marker for cardiovascular risk in experimental and human diabetes (Tamura *et al.*, 2007). Our data may suggest early occurrence of neuropathy in *db/db* mice as previously documented in these animals through nerve recording studies (Sullivan *et al.*, 2007). Furthermore, changes in BPV were not associated with alterations in spontaneous baroreflex sensitivity, which agree with our previous data on animal models of type 1 diabetes and glucose intolerance (Farah *et al.*, 2006; Wichi *et al.*, 2007; Farah *et al.*, 2007b). Decreased HRV is an early marker for diabetic neuropathy in humans (Vita *et al.*, 1999; Schroeder *et al.*, 2005). However, there is little evidence of decreased HRV in experimental models of type 1 diabetes (Wichi *et al.*, 2007; Farah *et al.*, 2007b) and high fructose fed mice (Farah *et al.*, 2006). The results of the present study agree with these findings, no significant changes in HRV in *db/db* mice. In fact, there were some reports of increased HRV after long-lasting diabetes in rats (Balbinott *et al.*, 2005).

There is much clinical interest in measurement of renin and ACE activity as biomarkers for disease states, such as hypertension and diabetes. In addition, there are considerable data to support the activation of plasma ACE levels in diabetes. However, in this emerging field of RAS there are few studies which address the activity of ACE in murine model of type 2 diabetes. Our results showed that *db/db* presented higher levels of plasma Ang II and ACE activity compared to their controls. To our knowledge, this is the first study to demonstrate increased plasma ACE activity in *db/db* diabetic mice. This increase in ACE activity and Ang II was significantly higher in 14 wk hypertensive mice compared to the 8 wks normotensive mice. The increase in plasma Ang II and ACE activity in 8 wk *db/db* mice was observed while these animals still have normal BP. Although, increases in ACE and/or Ang II levels are often associated with BP increases (Campbell *et al.*, 1995), the modulation of the RAS and its components' effects enclose a far more complex structure. For example, increased ACE activity in mice over expressing ACE gene copies is not associated with changes in BP (Krege *et al.*, 1997; Senador *et al.*, 2007). We have also shown previously increased renal ACE 2 activity in young normotensive *db/db* mice and decreased renal ACE 2 activity in older hypertensive *db/db* mice(Kanakamedala *et al.*, 2007). The down regulation of ACE and up regulation of ACE2, in the kidney is an intrigue finding and could explain the normal blood pressure levels observed in 8wk *db/db* mice in the face of increased plasma ACE activity. This finding also agrees with recent reports of a renoprotective effect of increased renal ACE 2 and decreased renal ACE activity combination in 8wk *db/db* mice (Wysocki *et al.*, 2006). It is tempting to speculate that there is an age-dependent change in the balance of ACE and ACE2 which could contribute to the regulation of BP in *db/db* mice.

In summary, the current study demonstrated that there was an age dependent increase in BP and disruption of its circadian rhythm during the progression of diabetes in *db/db* mice. In addition, our study documented a significant role of Ang AT1 in mediating hypertension in *db/db* mice since losartan blocked the progress in hypertension with no change in blood glucose. The spectral analysis of BP in *db*/*db* mice showed changes in BPV prior to changes in BP itself indicating a possible marker for cardiovascular risk and autonomic cardioneuropathy. The reduced HR observed in *db/db* mice could be related to contractile and metabolic dysfunctions of cardiac muscle since there were no changes in baroreflex

function. The significant increase in plasma ACE activity and increased Ang II in normotensive diabetic mice indicate that other factors such as ACE2 could be involve in the regulation of BP in this model. The present findings provide evidence for cardiovascular dysfunction in *db/db* mice.

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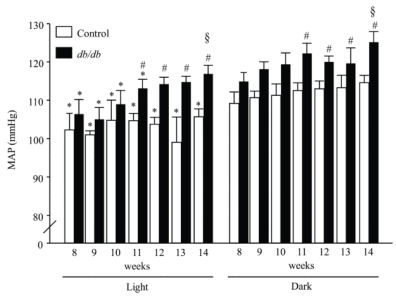


**Figure 1.**

Mean arterial pressure (MAP) in *db/db* diabetic mice and their age matched controls. Blood pressure was recorded continously for 24 hours (500 Hz) once per week, and analyzed during light (□) and dark (■) phases. Three-way ANOVA showed a main effect of group [F (1.176) = 27.26, $p < 0.0001$] and time [F (1.176) = 29.92, $p < 0.0001$]. * $p < 0.05$ light vs. dark. # $p < 0.05$ control vs. *db/db*. § $p < 0.05$ 14 vs. 8 wks, $n = 8$.

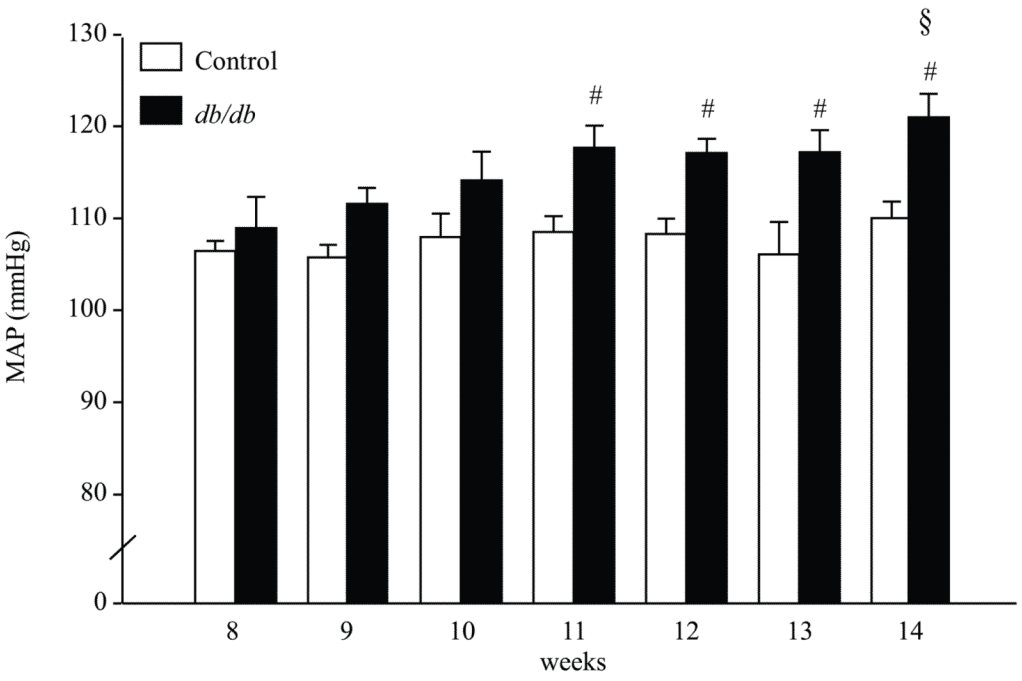


**Figure 2.**

24 hours mean arterial pressure (MAP) in *db/db* diabetic mice and their age matched controls. Blood pressure was recorded continously for 24 hours once per week at 500 Hz and averaged over entire 24 hours period. Two-way ANOVA showed a main effect of group [F (1.88) = 19.29, $p < 0.0001$]. # $p < 0.005$ control vs. *db/db*. $p < 0.05$ vs. 8 wks, $n = 8$.

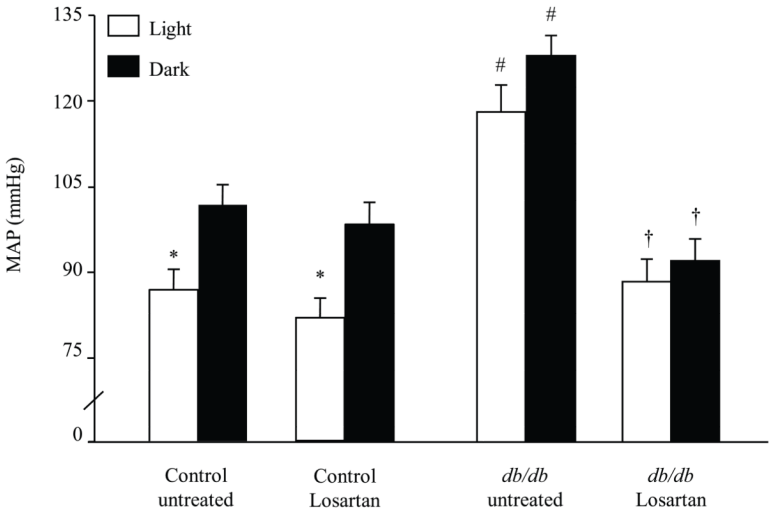


**Figure 3.**

Effect of chronic treatment (8 wks) with losartan (10 mg/kg/day in drinking water) on MAP in control and *db/db* mice. At 14 wks of age, blood pressure was recorded (500 Hz) continuously for 48 hours and analyzed during light (□) and dark (■) phases. Three-way ANOVA showed a main effect of treatment [F (1.40) = 42.81, $p < 0.0001$], group [F (1.40) = 7.48, $p < 0.01$] and time [F (1.40) = 17.89, $p < 0.0001$]. ANOVA also showed an interaction between treatment and group [F (1.40) = 7.61, $p < 0.01$]. * $p < 0.05$ light vs. dark. # $p < 0.03$ control vs. *db/db*. † $p < 0.03$ untreated vs. losartan, $n = 8$.

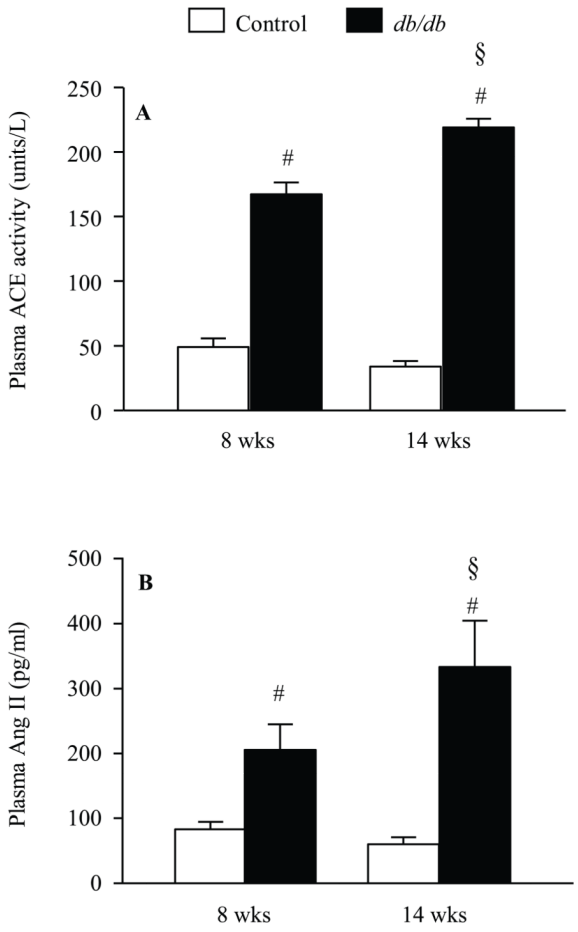


**Figure 4.**

Plasma ACE activity (A) and plasma Ang II (B) in young (8 wk) and older (14 wk) *db/db* diabetic mice and their age matched controls. ACE activity was determined using the synthetic substrate $H^3$ hippuryl glycine glycine purchased from ALPCO Diagnostic. Ang II was determined using RIA kit purchased from ALPCO Diagnostic. For ACE, two-way ANOVA showed a main effect of group [F (1.22) = 7.7, $p < 0.03$] and age [F (1.22) = 52.1, $p < 0.0001$] and an interaction between group and age [F (1.22) = 25.3, $p < 0.0001$]. For Ang II, two-way ANOVA showed a main effect of group [F (1.31) = 46.7, $p < 0.0001$] and an interaction between group and age [F (1.31) = 7.2, $p < 0.01$]. # $p < 0.005$ control vs. *db/db*. § $p < 0.005$ 14 wks vs. 8 wks, $n = 9$.

## Table 1

Body weight and metabolic parameters in control and *db/db* diabetic mice.

| **Age** | **8 weeks** | | **14 weeks** | |
|---|---|---|---|---|
| **Strain** | **Control** | ***db/db*** | **Control** | ***db/db*** |
| Weight (g) | 23.1 ± 0.3 | 33.5 ± 1.1* | 25.4 ± 0.3 | 35.5 ± 1.2* |
| Blood glucose (mg/mL) | 116.1 ± 0.3 | 315.1 ± 27.1* | 117.1± 0.5 | 550.1 ± 25.1*† |
| Insulin (ng/mL) | 0.9 ± 0.2 | 27.2 ± 2.1* | 1.2 ± 0.3 | 4.5 ± 0.8*† |

Body weight (g), blood glucose (mg/dL) and plasma insulin (ng/mL) in young (8 weeks) and older (14 weeks) *db/db* diabetic mice and their age matched controls.

*$p < 0.001$ control vs. *db/db;*

†$p < 0.001$ 8 vs. 14 wk, $n = 6$.

**Table 2**

SAP variability in time and frequency domains in control and *db/db* diabetic mice

| **Strain** | | **Control** | | ***db/db*** | |
|---|---|---|---|---|---|
| **Age** | | **Light** | **Dark** | **Light** | **Dark** |
| 8 weeks | SAP var ($mmHg^2$) | 12.8 ± 0.6* | 24.3 ± 1.3 | 22.2 ± 11.6 | 21.7 ± 4.2 |
| | LF ($mmHg^2$) | 9.0 ± 0.5* | 19.1 ± 1.0 | 7.0 ± 1.7* | 16.9 ± 3.5 |
| 14 weeks | SAP var ($mmHg^2$) | 16.6 ± 3.2* | 32.0 ± 4.8 | 28.3 ± 4.9 | 21.8 ± 3.4 |
| | LF ($mmHg^2$) | 9.1 ± 2.3* | 19.2 ± 1.9 | 12.8 ± 4.3* | 19.2 ± 4.2 |

Systolic arterial pressure (SAP) variability in time (variance) and frequency domains (low frequency-LF) in young (8 wk) and older (14 wk) *db/db* diabetic mice and their age matched controls. BP and HR signals were recorded (5kHz) during the light and the dark phases and submitted to spectral analysis. Three-way ANOVA showed an interaction between strain and time (light/dark) for SAP variance [$F(1.29) = 4.9$, $p < 0.05$] and SAP-LF [$F(1.29) = 5.4$, $p < 0.03$].

* $p < 0.01$ light vs. dark, $n = 6$.

**Table 3**

PI variability in time and frequency domains in control and *db/db* diabetic mice

| **Strain** | | **Control** | | ***db/db*** | |
|---|---|---|---|---|---|
| **Age** | | **Light** | **Dark** | **Light** | **Dark** |
| 8 weeks | PI var ($ms^2$) | 57.1 ± 14.8 | 46.4 ± 10.1 | 53.9 ± 9.1 | 33.7 ± 2.9 |
| | LF ($ms^2$) | 36.1 ± 18.1 | 21.1 ± 10.6 | 34.2 ± 17.1 | 12.1 ± 6.1 |
| | HF ($ms^2$) | 18.2 ± 9.1 | 20.3 ± 10.2 | 17.5 ± 8.7 | 13.9 ± 6.9 |
| 14 weeks | PI var ($ms^2$) | 67.6 ± 15.5 | 47.8 ± 3.5 | 24.3 ± 3.2 | 56.1 ± 7.1 |
| | LF ($ms^2$) | 20.6 ± 10.3 | 14.1 ± 7.2 | 5.2 ± 2.6 | 12.8 ± 6.4 |
| | HF ($ms^2$) | 36.8 ± 18.4 | 24.9 ± 12.5 | 17.4 ± 8.7 | 37.9 ± 18.9 |

Peak interval (PI) variability in time (variance) and frequency domains (low frequency-LF and high frequency-HF) in young (8 wk) and older (14 wk) *db/db* diabetic mice and their age matched controls. BP and HR signals were recorded (5kHz) during the light and the dark phases and submitted to spectral analysis. Three-way ANOVA showed no main effects for strain, age or time (light/dark) for PI variability. However, there was an interaction between the 3 factors for PI variance [$F(1.29) = 8.6$, $p < 0.05$], PI-LF [$F(1.29) = 7.8$, $p < 0.05$] and PI-HF [$F(1.29) = 8.5$, $p < 0.05$], $n = 6$.

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**Table 4**

Spontaneous baroreflex sensitivity (SBR), heart rate (HR), pulse pressure (PP) and locomotor activity in control and *db/db* diabetic mice.

| **Strain** | | **Control** | | ***db/db*** | |
|---|---|---|---|---|---|
| | **Age** | **Light** | **Dark** | **Light** | **Dark** |
| SBR (ms/mmHg) | 8 wks | 2.8 ± 0.4 | 2.3 ± 0.4 | 2.7 ± 0.8 | 2.2 ± 0.4 |
| | 14 wks | 2.4 ± 0.6 | 2.0 ± 0.3 | 2.1 ± 0.7 | 2.2 ± 0.4 |
| HR (bpm) | 8 wks | 515 ± 35$^{*}$ | 570 ± 38 | 396 ± 29$^{*\#}$ | 476 ± 41$^{\#}$ |
| | 14 wks | 501 ± 25$^{*}$ | 561 ± 16 | 422 ± 11$^{*\#}$ | 490 ± 14$^{\#}$ |
| PP (mmHg) | 8 wks | 32 ± 1.7 | 33 ± 1.6 | 25 ± 4.7 | 25 ± 3.7 |
| | 14 wks | 31 ± 2.6 | 30 ± 2.5 | 26 ± 2.1 | 27 ± 2.9 |
| Activity (counts/min) | 8 wks | 6 ± 0.7$^{*}$ | 11 ± 1.4 | 3 ± 0.9 | 5 ± 1.3$^{\#}$ |
| | 14 wks | 5 ± 0.2$^{*}$ | 12 ± 1.1 | 4 ± 0.6 | 5 ± 0.5$^{\#}$ |

SBR (ms/mmHg), HR (bpm), PP (mmHg) and locomotor activity (counts/min) in young (8 wk) and older (14 wk) *db/db* diabetic mice and their age matched controls. There were no changes in SBR and PP. Three-way ANOVA showed main effect of group [$F(1.29) = 33.2$, $p < 0.01$] and time (light/dark) [$F(1.29) = 16.8$, $p < 0.01$] for HR. For activity, Three-way ANOVA showed main effect of group [$F(1.43) = 40.2$, $p < 0.0001$], time (light/dark) [$F(1.43) = 27.8$, $p < 0.0001$] and an interaction between group and time [$F(1.43) = 43.6$, $p < 0.001$].

$^{*}p < 0.05$ light vs. dark;

$^{\#}p < 0.05$ control vs. *db/db*, $n = 6$.