

Insulin resistance in mice lacking neuronal nitric oxide synthase is related to an alpha-adrenergic mechanism

P. Turini^a, S. Thalmann^a, P.-Y. Jayet^a, S. Cook^a, C. Mathieu^a, R. Burcelin^b, P. Nicod^a, P. Vollenweider^a, C. Sartori^{a,1}, U. Scherrer^{a,1}

^a Department of Internal Medicine and Botnar Center for Clinical Research, CHUV, Lausanne, Switzerland

^b CHU Rangueil, Toulouse, France

Summary

Background: nitric oxide (NO) plays an important role in the regulation of cardiovascular and glucose homeostasis. Mice lacking the gene encoding the neuronal isoform of nitric oxide synthase (nNOS) are insulin-resistant, but the underlying mechanism is unknown. nNOS is expressed in skeletal muscle tissue where it may regulate glucose uptake. Alternatively, nNOS driven NO synthesis may facilitate skeletal muscle perfusion and substrate delivery. Finally, nNOS dependent NO in the central nervous system may facilitate glucose disposal by decreasing sympathetic nerve activity.

Methods: in nNOS null and control mice, we studied whole body glucose uptake and skeletal muscle blood flow during hyperinsulinaemic clamp studies in vivo and glucose uptake in skeletal muscle preparations in vitro. We also examined the effects of alpha-adrenergic blockade (phentolamine) on glucose uptake during the clamp studies.

Results: as expected, the glucose infusion rate during clamping was roughly 15 percent lower in nNOS null than in control mice (89 (17) *vs* 101 (12) [-22 to -2]). Insulin stimulation of muscle blood flow in vivo, and intrinsic muscle glucose uptake in vitro, were comparable in the two groups. Phentolamine, which had no effect in the wild-type mice, normalised the insulin sensitivity in the mice lacking the nNOS gene.

Conclusions: insulin resistance in nNOS null mice was not related to defective insulin stimulation of skeletal muscle perfusion and substrate delivery or insulin signaling in the skeletal muscle cell, but to a sympathetic alpha-adrenergic mechanism.

Key words: nitric oxide; neuronal nitric oxide synthase; insulin resistance; sympathetic nerve activity; muscle blood flow; intrinsic muscle glucose uptake

Introduction

Metabolic insulin resistance is a problem of major clinical importance. Epidemiological observations demonstrate an association between insulin resistance and cardiovascular disease [1-4], but the mechanism relating these disorders is not known. Nitric oxide (NO) plays an important role in the regulation of metabolic and cardiovascular homeostasis in experimental animal models and humans. In humans, altered NO synthesis and/or bioavailability is associated with cardiovascular disease [5-8] and insulin resistance [7, 9], and may represent a link between these two disease states. Studies in mice indicate that each of the three nitric oxide synthase (NOS) isoforms play a role in

the regulation of glucose homeostasis. Endothelial nitric oxide synthase (eNOS) null mice are hypertensive and insulin resistant [10, 11]. The latter appears to be caused by impaired insulin stimulation of blood flow and substrate delivery to skeletal muscle tissue, mitochondrial dysfunction [12, 13] and an intrinsic defect of muscle glucose uptake [11]. Partial deletion of the eNOS gene (eNOS^{+/-}) predisposes to exaggerated high-fat-diet-induced arterial hypertension and insulin resistance in mice, demonstrating an important interaction between environmental and genetic factors in the regulation of cardiovascular and glucose homeostasis [14]. iNOS null mice do not

1 C.S and U.S. contributed equally to this work.

This work was supported by grants from the Swiss National Science Foundation, the Fondation Romande pour la Recherche sur le Diabète, the Cloëtta Foundation, the Emma Muschamp Foundation, the CardioMet Foundation and the Placide Nicod Foundation.

develop high-fat-diet-induced insulin resistance, indicating that iNOS-driven NO synthesis contributes to insulin resistance in this model [15].

Neuronal NOS (nNOS) dependent NO also appears to play a role in the regulation of insulin sensitivity, since nNOS null mice are insulin resistant [16], but the underlying mechanisms are unknown. nNOS could modulate insulin sensitivity in several ways. It is highly expressed in skeletal muscle tissue [17], where it may facilitate glucose uptake. Alternatively, nNOS derived NO could modulate insulin sensitivity by regulating skeletal muscle perfusion and substrate delivery, which are NO dependent [18, 19]. Finally, nNOS derived NO inhibits central [20–22] neural sympathetic outflow and counteracts sympathetic vas-

cular and metabolic effects in skeletal muscle tissue [23–25]. Sympathetic overactivity is associated with insulin resistance in animals and humans [2, 9]. Thus, nNOS derived NO could exert beneficial effects on insulin sensitivity by decreasing sympathetic activity.

To test these hypotheses, we measured, in nNOS null and control mice, insulin stimulated whole body glucose uptake and skeletal muscle blood flow during euglycaemic hyperinsulinaemic clamp studies *in vivo*, and basal and insulin stimulated glucose uptake in isolated skeletal muscle preparations *in vitro*. We also tested the effects of alpha-adrenergic blockade on whole body glucose uptake during clamp studies in these two groups of animals.

Methods

All protocols were approved by the Institutional Animal Care and Use Committee.

nNOS null C57BL6 mice, generated as previously described [16], were used. nNOS null and control mice were generated by mating heterozygous animals from our colony. Ten to 14 week old male and female mice of generation 9 to 12 were used for the studies.

Hyperinsulinaemic euglycaemic clamp studies

Euglycaemic hyperinsulinaemic clamps in freely moving mice were performed as described previously [11]. 16 nNOS null and 18 control mice were studied. Briefly, on the day of the clamp, after a 6-hour fast, insulin was infused (18 mU/kg per min, a dose known to suppress hepatic glucose production) [11, 14] into the femoral vein for 150 minutes. Throughout the clamp, blood samples were collected every 5 minutes from the tip of the tail to determine the blood glucose concentration. Euglycaemia (5.5 (SD 0.5) mmol/L) was maintained by periodic adjustment of a variable infusion of 15% glucose. The steady state glucose infusion rate was calculated as the

mean of the values obtained every 5 minutes during the last 30 minutes of the clamp.

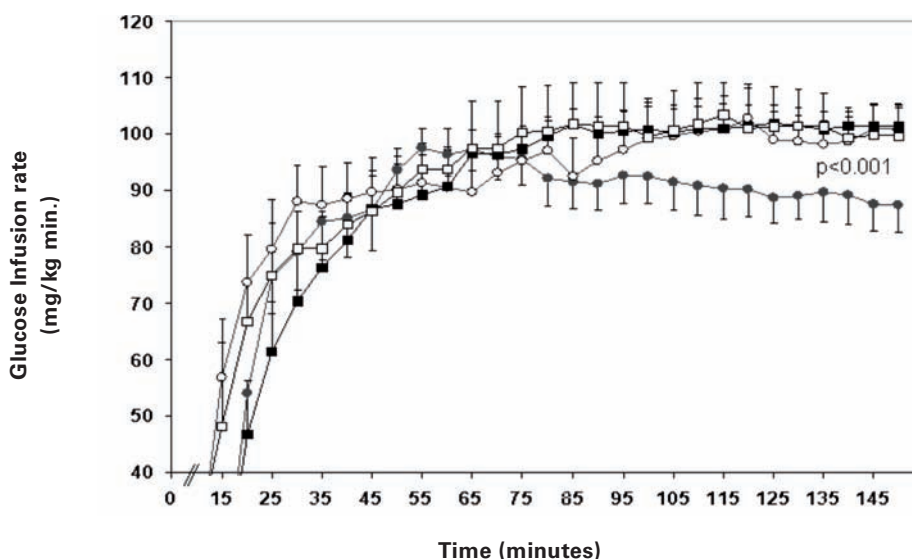
Identical studies were performed in 13 knockout and 5 control mice following the intraperitoneal injection of phentolamine (20 mg/kg, a dose known to prevent the increase in arterial blood pressure evoked by the α -adrenergic agonist phenylephrine) [26] 20 minutes before starting the clamp.

Muscle blood flow

Insulin stimulation of muscle blood flow was measured in anesthetized mice (4 nNOS null, 7 control mice) during a 90-minute glucose clamp with a laser Doppler probe (Perimed, Periflux System 5000, Probe #403, Sweden) inserted into the hindlimb skeletal musculature as described previously [11]. The body temperature was maintained at 37.5 ± 0.5 °C with a temperature control unit (Frederick Haer and Co., Bowdoinham, ME). The blood flow signal was recorded on a personal computer using specific data acquisition software (Powerlab 400, AD).

Figure 1

Glucose infusion rates during euglycaemic hyperinsulinaemic clamp studies in control (squares) and in nNOS null (circles) mice in the absence (filled symbols; n = 18 control and 16 nNOS null mice) and the presence (open symbols; n = 5 control and 13 nNOS null mice) of alpha-adrenergic blockade. Data are the mean with bars showing the standard error of the mean (P < 0.001, for group and time effects as well as for their interaction: ANOVA between nNOS null without phentolamine and both nNOS null mice with phentolamine and wild type mice).



Glucose utilisation in isolated skeletal muscle

After cervical dislocation, the soleus and EDL muscles were rapidly isolated, tied separately to silk threads by the tendons and immersed for 20 minutes in an incubation medium (Krebs-Ringer bicarbonate [pH 7.35] supplemented with 0.1% bovine serum albumin [Fraction V, pH 7.0] and 2 mM sodium pyruvate) [11]. Under an atmosphere containing 5% CO₂ and 95% O₂, the muscles were then incubated for an additional 60 minutes at 37 °C in this medium, in the presence or absence of 20 nM insulin. Thereafter, the muscles were immersed for 20 minutes in the incubation medium supplemented with ³H-2-deoxyglucose (0.1 mM, 1 µCi/ml). During this immersion the ³H-2-deoxyglucose is metabolised to ³H-2-deoxyglucose-6-phosphate. To stop the reaction, the muscles were immersed in ice-cold saline buffer, washed for 30 minutes and then dissolved in NaOH 1M at 60 °C for 60 minutes. An aliquot of the extract was spun down and the ³H-labeled radioactivity was counted in the presence of a scintillation buffer. Sample aliquots were used for protein determination. Soleus muscles of 8 nNOS null and 4 control mice, and EDL muscles of 4 nNOS null and 6 control mice, were studied.

Measurement of arterial blood pressure

Blood pressure was measured in awake, partially restricted mice (10 nNOS null and 6 control mice) with fluid filled PE-10 tubing connected to a pressure trans-

ducer, as described previously [11]. Blood pressure values were averaged over a 20 minute period.

Analytical methods

Fasting blood glucose and insulin (ELISA, Merckodia, Uppsala, Sweden) plasma concentrations were measured in conscious mice (n ≥ 9 for each group) after a 5-hour fast. Free fatty acids (n ≥ 8 for each group) were measured after a 5-hour fast by colorimetric enzymatic determination (NEFA-C, Wako).

Statistical analysis

Data were analysed with the JMP software package (SAS Institute Inc., Cary, North Carolina, USA), using the confidence interval for difference between means for single comparisons. Data are presented as mean (standard deviation) and compared using a 95% confidence interval for the difference between group means [95% confidence interval]. For the comparison of the glucose infusion rates during euglycaemic hyperinsulinaemic clamp studies (figure 1) we used a two-way analysis of variance (ANOVA) for repeated measures on one way (time) for assessing solution and time effects, as well as their interaction. Bonferroni correction was used to avoid bias due to multiple comparisons. Symmetrical distribution of all the data was confirmed using the Shapiro-Wilk test for normality.

Results

Figure 2

Insulin stimulation of muscle blood flow at the end of a 90-minute hyperinsulin-aemic euglycaemic clamp in 4 nNOS null and 7 control mice. Horizontal lines represent means.

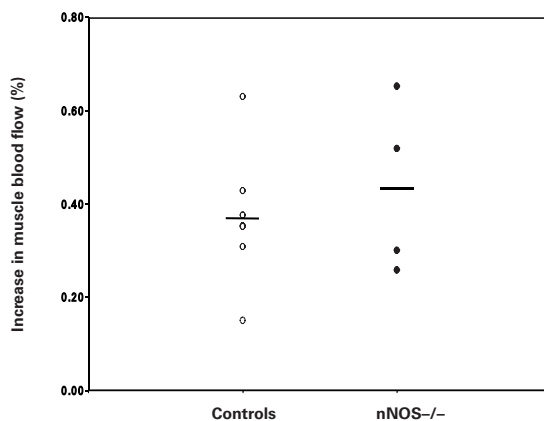
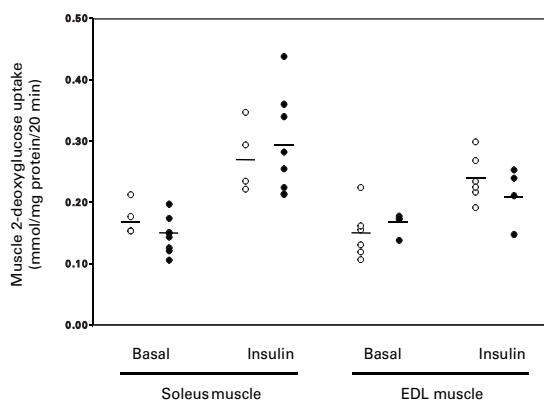


Figure 3

Basal and insulin stimulated 2-deoxyglucose uptake in soleus and EDL muscle of nNOS null (filled symbols) and control (open symbols) mice. Soleus muscles of 8 knockout and 4 control mice and EDL muscles of 4 knockout and 6 control mice were studied. 95% confidence interval for the difference between group means: [-0.01 to 0.07] (basal) and [-0.12 to 0.09] (insulin) for the soleus muscle. [-0.07 to 0.04] (basal) and [-0.04 to 0.09] (insulin) for the EDL muscle. Horizontal lines represent means.



Body weight (20.9 (2.9) *vs* 20.9 (1.9) g), fasting plasma glucose (5.6 (0.4) *vs* 5.5 (0.4) mmol/L), insulin (16 (8) *vs* 18 (7) µU/ml), and free fatty acid concentration (1.0 (0.3) and 0.9 (0.3) mmol/L) were comparable in nNOS null and control mice.

As expected, the nNOS null mice were insulin resistant (figure 1), as evidenced by a roughly 15 percent lower glucose infusion rate during the last 30 minutes of the clamp (89 (17) *vs* 101 (12) [-22 to -2]). During the clamp studies, the glucose (5.6 (0.4) *vs* 5.5 (0.4) mmol/L) and insulin plasma concentration (340 (120) *vs* 320 (50) µU/ml) were comparable in nNOS null and control mice.

To determine whether insulin resistance in the nNOS^{-/-} mice was related to a defect in insulin stimulation of skeletal muscle perfusion, which is NO dependent, [18] we measured skeletal muscle blood flow during clamp studies. We found that the increase in muscle blood flow at the end of the clamp was comparable in the nNOS null and the control mice (37 (14) *vs* 43 (19) [-29 to 16]) % (figure 2). Mean arterial blood pressure (109 (5) *vs* 107 (13) [-8 to 11] mm Hg) was also comparable in the nNOS null and control mice.

nNOS is highly expressed in skeletal muscle tissue. To test whether nNOS deficiency may impair skeletal muscle glucose uptake, we measured glucose uptake in isolated soleus and EDL muscle in vitro. The basal and insulin stimulated muscle deoxyglucose uptake were comparable in the nNOS null and the control mice (figure 3).

Loss of nNOS-dependent NO may result in increased central neural sympathetic outflow that could induce insulin resistance. Therefore, we examined the effects of alpha-adrenergic blockade on insulin-stimulated glucose uptake during clamp studies. Phentolamine significantly increased the glucose infusion rate in the nNOS null mice (from 89 (17) to 102 (14) [1 to 25]

mg/kg/min), whereas it had no detectable effect on the glucose infusion rate in the control mice (101 (12) *vs* 101 (13) [-13 to 12] mg/kg/min) (figure 1). After phentolamine, the glucose infusion rates were comparable in the nNOS null and control mice (102 (14) *vs* 101 (12) [-8 to 11] mg/kg/min).

Discussion

Over the past decade, evidence has accumulated indicating that nitric oxide generated by each of the three NOS isoforms plays an important role in the regulation of glucose homeostasis, but it is not known, whether this regulation involves isoform-specific mechanisms or not. Here we found that insulin resistance in nNOS null mice was related to a sympathetic mechanism, because alpha-adrenergic blockade normalised insulin sensitivity in the nNOS null mice, whereas it had no detectable effect in the control mice. These findings indicate that NOS isoforms regulate glucose homeostasis by different mechanisms.

The insulin resistance of the nNOS null mice in the present studies was of similar magnitude to the level reported by Shankar and colleagues [16]. In eNOS null mice, insulin resistance is related, at least in part, to an intrinsic defect of skeletal muscle glucose uptake [11]. This mechanism does not appear to contribute to insulin resistance in nNOS null mice, because basal and insulin stimulated skeletal muscle glucose uptake *in vitro* were comparable in nNOS null and control mice. This surprising finding suggests that NO generated by nNOS, which is abundantly expressed in skeletal muscle tissue [17], does not appear to be essential for maintaining normal glucose homeostasis in this tissue. Impaired insulin stimulation of blood flow and substrate delivery to skeletal muscle tissue, which are NO-dependent [18], are additional mechanisms contributing to insulin resistance in eNOS null mice [11]. In the present study, we found that insulin stimulation of skeletal muscle blood flow during the clamp studies was comparable in the two groups, suggesting that vascular function is preserved in nNOS null mice. Consistent with this hypothesis, arterial blood pressure was also comparable in the two groups.

nNOS-derived NO inhibits central sympathetic outflow and counteracts sympathetic vasoconstriction in skeletal muscle tissue [20, 21, 23], and sympathetic overactivity is associated with insulin resistance in animals and humans [7, 27]. To test for the involvement of adrenergic mechanisms in the insulin resistance of nNOS null mice, we examined the effects of phentolamine on insulin stimulated whole body glucose uptake in the

two groups of mice. We found that alpha-adrenergic blockade normalised insulin sensitivity in the nNOS null mice, whereas it had no detectable effect in the control mice. This finding suggests that sympathetic overactivity, possibly related to the loss of central neural inhibition of sympathetic outflow by nNOS-derived NO, contributes to insulin resistance in nNOS null mice. In line with this hypothesis, intracerebroventricular injection of L-NMMA in rats causes insulin resistance by a central neural action [21].

Taken together with earlier observations, the present findings indicate that, in mice, defective NO synthesis causes insulin resistance by different mechanisms, depending on the NOS isoenzyme involved. Whereas in eNOS null mice, insulin resistance is related to defects in insulin stimulation of muscle perfusion and insulin signalling in the skeletal muscle cell [11], in nNOS null mice these mechanisms do not appear to play an important role, and sympathetic mechanisms appear to be the main culprit. Insulin resistant states in humans are associated with defective NO synthesis and/or impaired NO bioavailability, and with sympathetic over-activity [7, 9]. Moreover, α -adrenergic blockade has been shown to improve insulin sensitivity in insulin resistant subjects [28]. We speculate that loss of central neural inhibition of sympathetic outflow by NO may also represent a mechanism contributing to insulin resistance and its associated cardiovascular complications in humans.

We are indebted to Dr. Lorenz Hirt for letting us use his blood flow measurement equipment, to Mrs. Maria-Alba Guardia for technical help with some *in vitro* studies, and to Dr. Philippe Frascarolo for assistance with the statistical analysis.

Correspondence:

Urs Scherrer, MD

Department of Internal Medicine

BH 10.642

Centre Hospitalier Universitaire Vaudois

CH-1011 Lausanne

Switzerland

E-Mail: Urs.Scherrer@chuv.ch

References

- 1 Modan M, Halkin H. Hyperinsulinemia or increased sympathetic drive as links for obesity and hypertension. *Diabetes Care*. 1991;14:470–87.
- 2 Scherrer U, Sartori C. Insulin as a vascular and sympathoexcitatory hormone. Implications for blood pressure regulation, insulin sensitivity and cardiovascular morbidity. *Circulation*. 1997;96:4104–13.
- 3 Stern MP. Diabetes and cardiovascular disease. The “common soil” hypothesis. *Diabetes*. 1995;44:369–74.
- 4 Nash DT. Insulin resistance, ADMA levels, and cardiovascular disease. *Jama*. 2002;287(11):1451–2.
- 5 Petrie JR, Ueda S, Webb DJ, Elliott HL, Connell JMC. Endothelial nitric oxide production and insulin sensitivity. A physiological link with implications for pathogenesis of cardiovascular disease. *Circulation*. 1996;93:1331–3.
- 6 Scherrer U. Insulin and the regulation of the cardiovascular system: role of the l-arginine nitric oxide pathway and the sympathetic nervous system. In: Luscher TF, ed. *The Endothelium in Cardiovascular Disease*: Springer-Verlag 1995:108–28.
- 7 Scherrer U, Sartori C. Defective nitric oxide synthesis: a link between metabolic insulin resistance, sympathetic overactivity and cardiovascular morbidity. *Eur J Endocrinol*. 2000;142:315–23.
- 8 Boger RH, Zoccali C. ADMA: a novel risk factor that explains excess cardiovascular event rate in patients with end-stage renal disease. *Atheroscler Suppl*. 2003;4:23–8.
- 9 Sartori C, Scherrer U. Insulin, nitric oxide and the sympathetic nervous system: at the crossroads of metabolic and cardiovascular regulation. *J Hypertens*. 1999;17:1517–25.
- 10 Cook S, Hugli O, Egli M, Vollenweider P, Burcelin R, Nicod P, et al. Clustering of cardiovascular risk factors mimicking the human metabolic syndrome X in eNOS null mice. *Swiss Med Wkly*. 2003;133:360–3.
- 11 Duplain H, Burcelin R, Sartori C, Cook S, Egli M, Lepori M, et al. Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. *Circulation*. 2001;104:342–5.
- 12 Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, et al. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science*. 2003;299:896–9.
- 13 Le Gouill E, Binnert C, Jayet PY, Thalmann S, Nicod P, Scherrer U, et al. eNOS knock-out mice have defective mitochondrial beta-oxidation. *Diabetes*. 2007;56:2690–6.
- 14 Cook S, Hugli O, Egli M, Menard B, Thalmann S, Sartori C, et al. Partial gene deletion of endothelial nitric oxide synthase predisposes to exaggerated high-fat diet-induced insulin resistance and arterial hypertension. *Diabetes*. 2004;53:2067–72.
- 15 Perreault M, Marette A. Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med*. 2001;7:1138–43.
- 16 Shankar RR, Wu Y, Shen HQ, Zhu JS, Baron AD. Mice with gene disruption of both endothelial and neuronal nitric oxide synthase exhibit insulin resistance. *Diabetes*. 2000;49:684–7.
- 17 Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. *Physiol Rev*. 2001;81:209–37.
- 18 Scherrer U, Randin D, Vollenweider P, Vollenweider L, Nicod P. Nitric oxide release accounts for insulin’s vascular effects in humans. *J Clin Invest*. 1994;94:2511–5.
- 19 Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J Clin Invest*. 1994;94:1172–9.
- 20 Nishida Y, Chen QH, Tandai-Hiruma M, Terada S, Horiuchi J. Neuronal nitric oxide strongly suppresses sympathetic outflow in high-salt Dahl rats. *J Hypertens*. 2001;19:627–34.
- 21 Uemura K, Tamagawa T, Chen Y, Maeda N, Yoshioka S, Itoh K, et al. NG-methyl-L-arginine, an inhibitor of nitric oxide synthase, affects the central nervous system to produce peripheral hyperglycemia in conscious rats. *Neuroendocrinology*. 1997;66:136–44.
- 22 Shankar R, Zhu JS, Ladd B, Henry D, Shen HQ, Baron AD. Central nervous system nitric oxide synthase activity regulates insulin secretion and insulin action. *J Clin Invest*. 1998;102:1403–12.
- 23 Zanzinger J, Czachurski J, Seller H. Inhibition of sympathetic vasoconstriction is a major principle of vasodilation by nitric oxide in vivo. *Circulation Research*. 1994;75:1073–7.
- 24 Lepori M, Sartori C, Duplain H, Nicod P, Scherrer U. Sympathectomy potentiates the vasoconstrictor response to nitric oxide synthase inhibition in humans. *Cardiovascular Research*. 1999;43:739–43.
- 25 Thomas GD, Sander M, Lau KS, Huang PL, Stull JT, Victor RG. Impaired metabolic modulation of alpha-adrenergic vasoconstriction in dystrophin-deficient skeletal muscle. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95:15090–5.
- 26 Masuki S, Nose H. Arterial baroreflex control of muscle blood flow at the onset of voluntary locomotion in mice. *J Physiol*. 2003;553:191–201.
- 27 Julius S, Gudbrandsson T. Early association of sympathetic overactivity, hypertension, insulin resistance, and coronary risk. *J Cardiovasc Pharmacol*. 1992;20:S40–S8.
- 28 Pollare T, Lithell H, Selinus I, Berne C. Application of prazosin is associated with an increase of insulin sensitivity in obese patients with hypertension. *Diabetologia*. 1988;31:415–20.