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

THESE

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par

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## Rapport de synthèse

### **La résistance à l'insuline chez les souris déficientes en synthèse neuronale de monoxyde d'azote est due à un mécanisme alpha-adrénergique**

Le monoxyde d'azote (NO) joue un rôle important dans la régulation de l'homéostasie du système cardiovasculaire et du glucose. Les souris déficientes pour le gène codant l'isoforme neuronale de la synthase de monoxyde d'azote (nNOS) sont résistantes à l'insuline, mais les mécanismes sous-jacents sont inconnus. Le manque de NO produit par la nNOS pourrait être à l'origine d'une diminution de la perfusion du muscle squelettique et ainsi d'une diminution de l'apport de substrat. Alternativement, le déficit de nNOS normalement hautement exprimé dans le tissu musculaire squelettique pourrait directement y perturber la consommation de glucose. Finalement l'absence de l'action sympatholytique du NO neuronal pourrait diminuer la sensibilité à l'insuline.

Afin de tester ces hypothèses nous avons étudié, chez des souris déficientes en nNOS et des souris-contrôle, la consommation corporelle totale de glucose et le flux musculaire squelettique pendant des clamps hyperinsulinémiques euglycémiques in vivo, ainsi que la consommation de glucose dans le muscle squelettique in vitro. De plus nous avons analysé les effets d'une inhibition alpha-adrénergique sur la consommation de glucose pendant les clamps hyperinsulinémiques euglycémiques in vivo. Le taux de perfusion de glucose pendant les clamps était grossièrement 15 pourcent plus bas ( $P < 0.001$ ) chez les souris déficientes en nNOS que chez les souris-contrôle. Cette résistance à l'insuline chez les souris déficientes en nNOS n'était due ni à une stimulation déficiente du flux sanguin musculaire par l'insuline ni à un défaut intrinsèque de la consommation de glucose du muscle (qui étaient comparables dans les deux groupes), mais à un mécanisme alpha-adrénergique, car l'administration de phentolamine rétablissait la sensibilité à l'insuline chez les souris déficientes en nNOS.

Ces résultats suggèrent qu'une hyperactivité sympathique, potentiellement due à la perte de l'inhibition neuronale centrale du flux sympathique par le NO provenant de nNOS, contribue à la résistance à l'insuline des souris déficientes en nNOS. Par ailleurs ces résultats tendent à prouver qu'un défaut de production de NO provoquerait une résistance à l'insuline par des mécanismes différents selon l'isoforme de NO synthase déficiente (par exemple chez les souris déficientes pour la forme endothéliale de NO synthase, il a été montré que la résistance à l'insuline est due à un défaut de stimulation de la perfusion musculaire par l'insuline et à un défaut du signalling de l'insuline dans la cellule musculaire squelettique).

Chez l'être humain il est établi que les états de résistance à l'insuline sont associés à une synthèse défectueuse et/ou une mauvaise biodisponibilité du NO, ainsi qu'à une hyperactivité sympathique. Nous spéculons que la perte d'inhibition centrale du flux sympathique représente un mécanisme contribuant à la résistance à l'insuline et ses complications cardiovasculaires chez l'être humain.

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

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## 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.

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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.

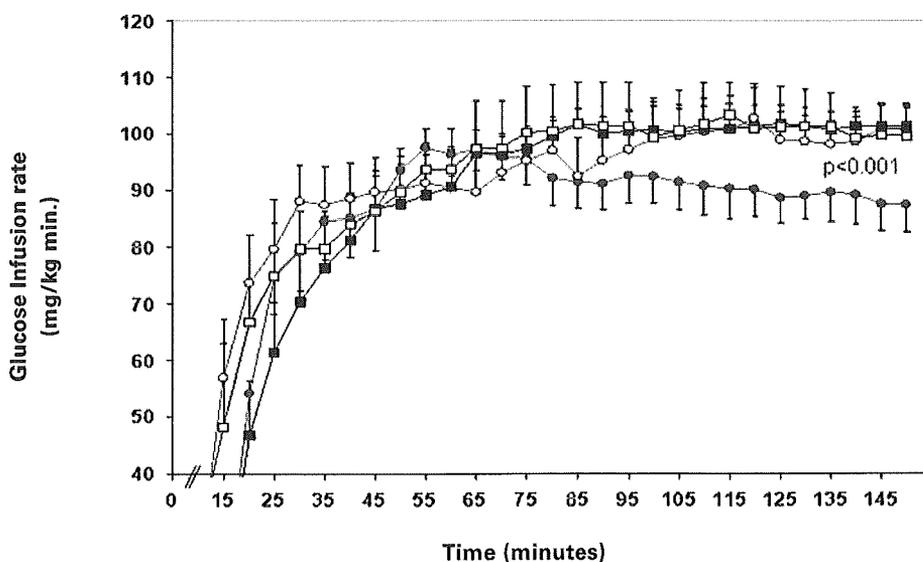
Identical studies were performed in 13 knockout and 5 control mice following the intraperitoneal injection of phentolamine (20 mg/kg, a dose known to prevent the increase in arterial blood pressure evoked by the  $\alpha$ -adrenergic agonist phenylephrine) [26] 20 minutes before starting the clamp.

### 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  $37.5 \pm 0.5$  °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% CO<sub>2</sub> and 95% O<sub>2</sub>, 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 <sup>3</sup>H-2-deoxyglucose (0.1 mM, 1 µCi/ml). During this immersion the <sup>3</sup>H-2-deoxyglucose is metabolised to <sup>3</sup>H-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 <sup>3</sup>H-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).

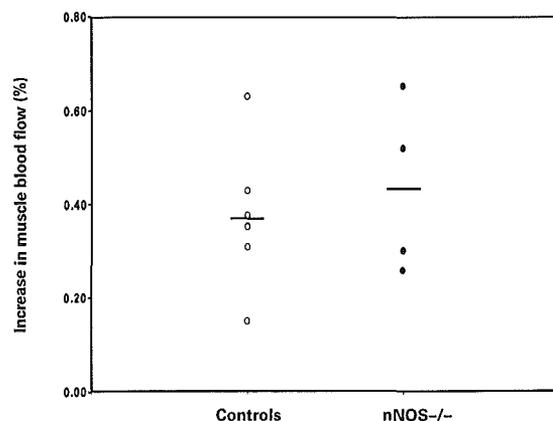
### Statistical analysis

Data were analysed with the JMP software package (SAS Institute Inc., Gary, 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

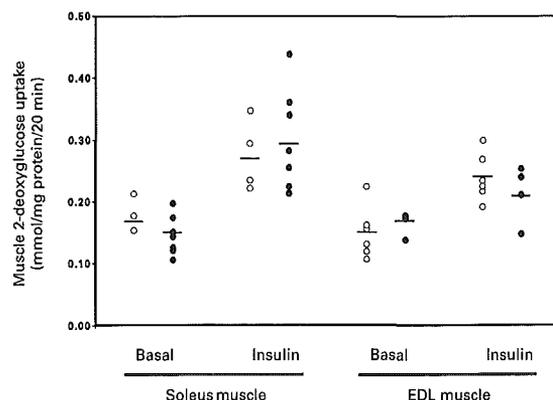
**Figure 2**

Insulin stimulation of muscle blood flow at the end of a 90-minute hyperinsulinaemic 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 iso-enzyme 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,  $\alpha$ -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.

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