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Clustering of cardiovascular risk factors mimicking the human metabolic syndrome X in eNOS null mice

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Summary

Aims/hypothesis: The metabolic syndrome comprises a clustering of cardiovascular risk factors but the underlying mechanism is not known. Mice with targeted disruption of endothelial nitric oxide synthase (eNOS) are hypertensive and insulin resistant. We wondered, whether eNOS deficiency in mice is associated with a phenotype mimicking the human metabolic syndrome.

Methods and Results: In addition to arterial pressure and insulin sensitivity (euglycaemic hyperinsulinaemic clamp), we measured the plasma concentration of leptin, insulin, cholesterol, triglycerides, free fatty acids, fibrinogen and uric acid in 10 to 12 week old eNOS$^{-/-}$ and wild type mice. We also assessed glucose tolerance under basal conditions and following a metabolic stress with a high fat diet. As expected eNOS$^{-/-}$ mice were hypertensive and insulin resistant, as evidenced by fasting hyperinsulinaemia and a roughly 30 percent lower steady state glucose infusion rate during the clamp. eNOS$^{-/-}$ mice had a 1.5 to 2-fold elevation of the cholesterol, triglyceride and free fatty acid plasma concentration. Even though body weight was comparable, the leptin plasma level was 30% higher in eNOS$^{-/-}$ than in wild type mice. Finally, uric acid and fibrinogen were elevated in the eNOS$^{-/-}$ mice. Whereas under basal conditions, glucose tolerance was comparable in knock out and control mice, on a high fat diet, knock out mice became significantly more glucose intolerant than control mice.

Conclusions: A single gene defect, eNOS deficiency, causes a clustering of cardiovascular risk factors in young mice. We speculate that defective nitric oxide synthesis could trigger many of the abnormalities making up the metabolic syndrome in humans.

Key words: endothelial nitric oxide synthase; metabolic syndrome; arterial hypertension; insulin resistance; hyperlipidaemia; glucose intolerance

Introduction

Epidemiological studies demonstrate an association between insulin resistance, arterial hypertension and cardiovascular morbidity [1]. The hypertension-hyperglycaemia hyperuricaemia syndrome was first described by Kylin in 1923 [2]. Since then, dyslipidaemia, obesity, hyperleptinaemia and a hypercoagulable state have been added and the term metabolic syndrome was coined to describe this clustering of cardiovascular risk factors [3]. Moreover, subjects with the metabolic syndrome are often insulin resistant. While the exact underlying mechanism relating these disorders is not known, there is evidence in humans that a defect of nitric oxide synthesis could trigger several of the metabolic and cardiovascular abnormalities characteristic of insulin-resistant states [4]. Consistent with this hypothesis, preliminary evidence in mice with targeted disruption of the endothelial nitric oxide synthase (eNOS) gene suggests that in addition to being hypertensive, these mice are also insulin resistant [5]. We wondered whether eNOS deficiency in mice was associated with a phenotype resembling the metabolic syndrome X in humans. In addition to arterial pressure and insulin sensitivity (euglycaemic hyperinsulinaemic clamp), we therefore measured leptin, insulin, cholesterol, triglycerides, free fatty acid, uric acid and fibrinogen plasma concentration in eNOS$^{-/-}$ and wild type mice. We also assessed glucose tolerance under basal conditions and after a metabolic stress with a high-fat diet.

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Methods

All protocols were approved by the Institutional Animal Care and Use Committee. Homozygote eNOS$^{-/-}$ female C57BL6 mice and wild type littermates generated as previously described were studied [5]. Mice were housed with light on from 7 a.m. to 7 p.m. and had free access to water and normal chow diet (NC, UAR, Epinay sur Orge, France, energy content: 12% fat, 28% protein and 60% carbohydrate).

Measurement of arterial blood pressure

Arterial pressure was measured in conscious, partially restrained, 10–12 week-old mice (n = 7 for each group) using fluid filled PE-10 tubing connected to a pressure transducer. The catheter was inserted under halothane anaesthesia into the carotid artery and tunnelled subcutaneously to exit at the back of the neck 4 hours before the measurement.

Euglycaemic hyperinsulinaemic clamp studies

Glucose turnover during the glucose clamp was measured in freely moving mice after a 5 hour fast as described previously [5]. Briefly, on the day of the clamp, insulin (18 mU/kg per min) was infused into the femoral vein for 3 hours. Euglycaemia was maintained by periodically adjusting a variable infusion of 16.5% glucose. The glucose infusion rate was calculated as the mean of the values obtained every 10 minutes during the last hour of the 3-hour infusion. Mice showing variations of these parameters >15 percent were not included in the calculations. 9 eNOS$^{-/-}$ and 9 control mice were studied.

Intraperitoneal glucose tolerance test

Groups of at least 7 controls and eNOS$^{-/-}$ each were fed either normal chow or carbohydrate free high fat diet (HFD, UAR, Epinay sur Orge, France, energy content: 72% fat [corn oil and lard], 28% protein, and <1% carbohydrate) for 6 weeks. On the day of the test, the mice were fasted for five hours before intraperitoneal injection of glucose (1 gram per kg body weight). Blood glucose concentration was measured with a glucometer (Glucotrend, Roche, Rotkreuz, Switzerland) 30 and 0 minutes before, and 30, 60, 90 and 120 minutes after glucose injection from a 3.5 µl drop of blood obtained from the tip of the tail vein.

Analytical methods

Glucose (Trinder kit 100, Sigma, St Louis, MO) and insulin (ELISA, Mercodia, Uppsala, Sweden) plasma concentrations were measured in conscious mice (n = 10 for each group), after a 6 hour fast. Total cholesterol, triglycerides and free fatty acids were measured after an 8 hour fast in 7 mice of each group by colorimetric enzymatic determination (Unimate 5 CHOL and 5 TRIG, Roche, NEFA-C, Wako). Leptin (Mouse Leptin RIA kit, Linco, n = 18 for each group) and uric acid (Uric acid plus kit, SYS 1 BM/Hitachi 917, Roche, n = 7 for each group) were measured in plasma samples obtained by retro-orbitary puncture after a 6 hour fast. Fibrinogen was determined by a clot-rate assay in plasma collected by cardiac puncture (n = 7 for each group).

Statistical analysis

Data were analysed with the JMP software package (SAS Institute Inc., Cary, North Carolina, USA). Statistical analysis was done with ANOVA for between group comparisons and with the 2-tailed t test for single comparisons. All data are presented as mean ± SE. A P value <0.05 was considered to indicate statistical significance.

Results

As expected, eNOS$^{-/-}$ mice were hypertensive (mean arterial pressure: 127 ± 6 vs. 99 ± 2 mm Hg, P = 0.001, Figure 1a) and insulin resistant, as evidenced by fasting hyperinsulinaemia (12.0 ± 1.4 vs. 7.1 ± 0.4 mU/ml, P <0.01, Figure 1b) and a roughly 30 percent lower steady state glucose infusion rate during the hyperinsulinaemic clamp (P <0.001, Figure 1c). eNOS$^{-/-}$ mice had a 1.5 to 2-fold elevation of the total cholesterol (1.84 ± 0.03 vs. 1.23 ± 0.06 mmol/l, P <0.01), triglyceride (0.85 ± 0.04 vs. 0.36 ± 0.03 mmol/l, P <0.01) and free fatty acid (1.9 ± 0.1 vs. 0.9 ± 01 mmol/l, P <0.001) plasma concentration (Figure 1d).

Body weight was comparable in both strains (18.1 ± 0.2 vs. 17.8 ± 0.3 grams, eNOS$^{-/-}$ vs. control), whereas fat pads (1.9 ± 0.2 vs. 1.3 ± 0.2% of body weight, eNOS$^{-/-}$ vs. eNOS$^{+/+}$, P <0.05) and the leptin plasma concentration were 30 to 40% higher in the eNOS$^{-/-}$ than in the control mice (2.7 ± 0.3 vs. 2.1 ± 0.2 ng/ml, P = 0.02, Figure 2a). Creatinine plasma concentration was comparable in eNOS$^{-/-}$ and control mice (33.2 ± 1.3 vs. 35.3 ± 1.6 µmol/litre). Finally, uric acid (69.1 ± 6.7 vs. 47.1 ± 7.9 mmol/l, P <0.05, Figure 2b) and fibrinogen (2.7 ± 0.1 vs. 2.2 ± 0.1 g/l, P <0.005, Figure 2c) plasma levels were elevated in the eNOS$^{-/-}$ mice.

Since glucose intolerance is often associated with the metabolic syndrome in humans, we assessed the glycaemic response to an intraperitoneal glucose administration in control and eNOS$^{-/-}$ mice fed a normal chow or high fat diet for 6 weeks. When fed a normal chow, no differences in glycaemic excursions were observed during the glucose tolerance test, whereas on high fat diet eNOS$^{-/-}$ mice became more glucose intolerant than control mice (Figure 3).

Discussion

The main new finding was that a single gene defect, eNOS deficiency, was associated with a clustering of cardiovascular risk factors in young mice. As expected [5], eNOS$^{-/-}$ mice were hypertensive, insulin resistant, and had dyslipidaemia. Here we show that in addition eNOS$^{-/-}$ mice also had elevated plasma levels of leptin, uric acid and fibrinogen and that they developed glucose intolerance when challenged with a metabolic stress.

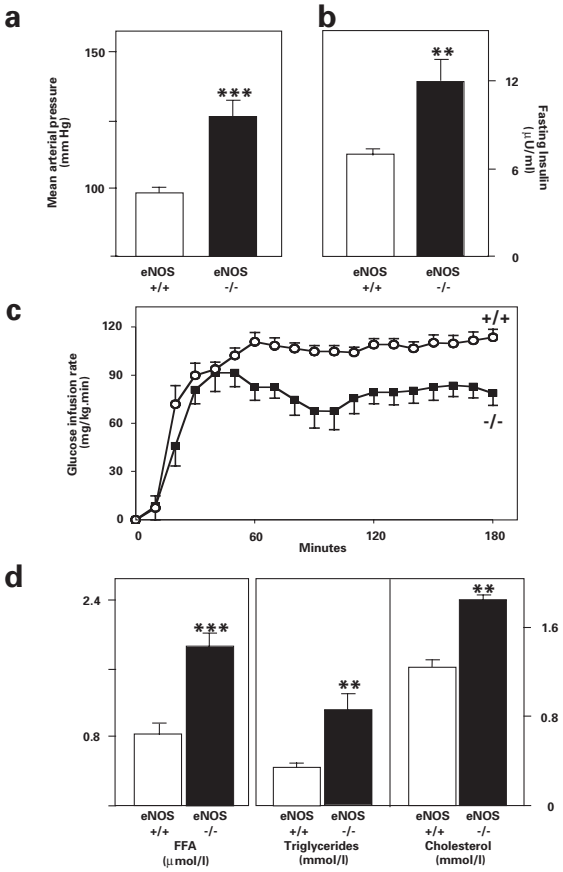

Figure 1

a: mean arterial pressure; b: fasting insulin plasma concentration; c: glucose infusion rate during hyperinsulinaemic euglycaemic clamp studies; d: fasting free fatty acid, triglyceride and total cholesterol plasma concentration, in control and knockout mice. Results are mean ± SEM for at least 7 mice in each group. *P <0.05, **P <0.01, ***P <0.001 vs. control mice

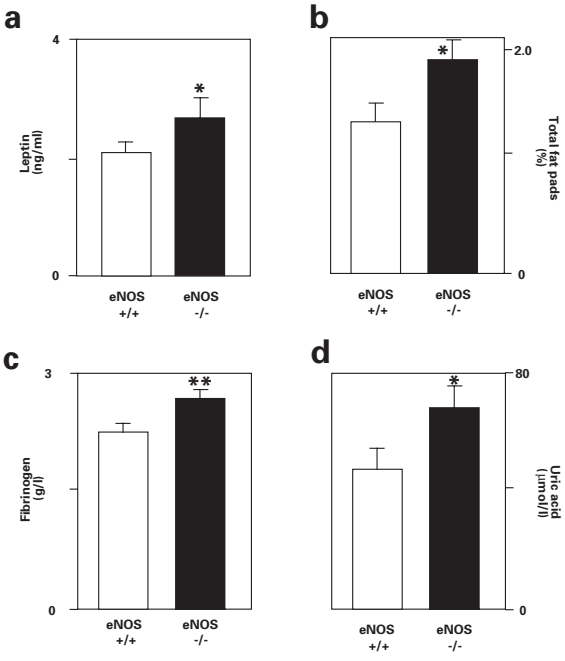

Figure 2

a: plasma concentration of leptin; b: total fat pads; c: fibrinogen; d: uric acid in control and knockout mice. Results are mean ± SEM for at least 7 mice in each group. *P <0.05, **P <0.01 vs. control mice.

Some components making up the human metabolic syndrome have already been found to be associated with defective NO synthesis in mice. NO plays a major role in the regulation of vascular tone, and as in the present study, eNOS$^{-/-}$ mice were found to have systemic arterial hypertension [5]. In human essential hypertension, endothelium-dependent vasodilatation is impaired [6] and eNOS gene polymorphism has been reported [7]. Consistent with our previous findings [5] eNOS$^{-/-}$ mice displayed hyperlipidaemia and insulin resistance, as evidenced by fasting hyperinsulinaemia and lower steady state glucose infusion rate during the euglycaemic hyperinsulinaemic clamp studies. In the human metabolic syndrome, insulin resistance is often associated with impaired glucose tolerance and eNOS gene polymorphism has been reported [8]. Here we found that insulin resistance in eNOS$^{-/-}$ mice when challenged with a metabolic stress (high fat diet), led to altered glucose homeostasis. Insulin resistance in eNOS$^{-/-}$ mice could be related to impaired insulin stimulation of skeletal muscle perfusion (and substrate delivery) and defects of insulin signalling in the skeletal muscle cell [5]. In insulin resistant humans similar mechanisms may play a role, since insulin stimulation of skeletal muscle blood flow, which is NO-dependent [9], is defective [4].

The augmented leptin plasma levels in eNOS$^{-/-}$ mice are intriguing. The increase of this adipocyte-derived signalling factor was not associated with increased body weight and was not related to impaired clearance (as evidenced by the normal creatinine plasma concentration in the eNOS$^{-/-}$ mice). However, relative adipose tissue content was augmented in eNOS$^{-/-}$ mice and could have contributed to augmented leptin levels. Alternatively, it appears possible that the elevated leptin levels merely reflect the hyperinsulinaemia, since insulin is known to stimulate leptin production [10]. Consistent with this interpretation, it has been speculated that hyperleptinaemia associated with the human metabolic syndrome could be related to insulin [11]. Finally, leptin increases insulin sensitivity and has depressor actions [12]. It is tempting to speculate that the elevated leptin concentration may represent a counter-regulatory mechanism, opposing the insulin resistance and hypertension in eNOS$^{-/-}$ mice. In humans, fibrinogen is an independent risk factor for coronary artery disease and is part of the metabolic syndrome. The elevation of the fibrinogen plasma concentration in eNOS$^{-/-}$ mice was of a similar magnitude to that observed in the human metabolic syndrome. Finally, eNOS$^{-/-}$ mice had elevated uric acid plasma levels, one of the classical components of the human hypertension-hyperglycaemia-hyperuricaemia syndrome first des-

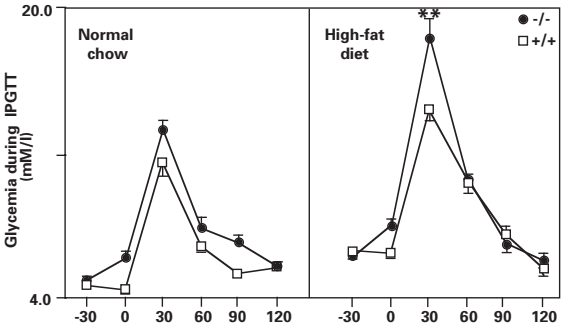

Figure 3

Plasma glucose concentration during intraperitoneal glucose tolerance tests (IPGTT). Results are mean ± SEM for at least 7 mice in each group.
**P <0.01 vs. control mice.

cribed by Kylin [2] and which has been incriminated as an independent risk factor for coronary heart disease, especially in women.

In conclusion, these findings demonstrate that a single gene defect, eNOS deficiency, causes an important clustering of cardiovascular risk factors in young mice. We speculate that a defect in nitric oxide synthesis may trigger many of the abnormalities making up the metabolic syndrome in humans, which thereby may represent a new target for pharmacological agents that deliver and/or modulate the bioavailability of endogenously produced nitric oxide [13].

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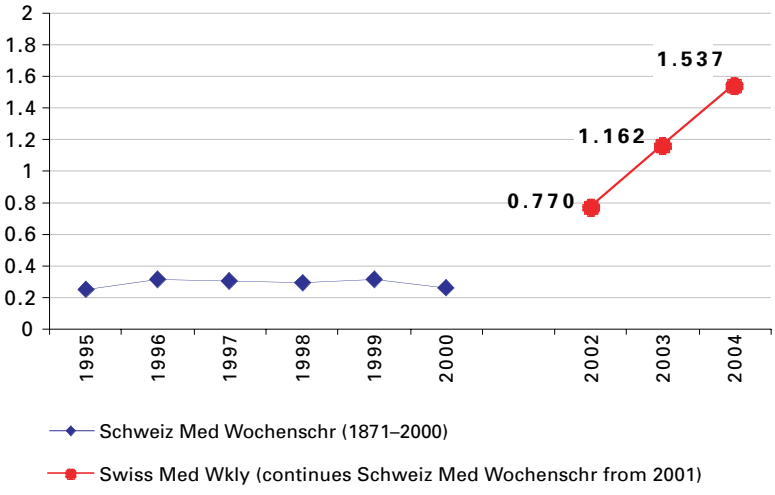

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