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Protective effect of endothelial nitric oxide synthase against induction of chemically-induced diabetes in mice

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Abstract

Since activation of endothelial nitric oxide synthase has been shown to exert protective effects against the metabolic syndrome, while endothelial nitric oxide synthase knockout mice develop hyperinsulinemia and glucose intolerance, we hypothesised that endothelial nitric oxide might play a protective role against induction of diabetes. The role of endothelial nitric oxide in the development of chemically-induced diabetes has been determined using mice in which the bioavailability of endothelial nitric oxide was either increased, through upregulation of endothelial nitric oxide synthase, or absent, through deletion of endothelial nitric oxide synthase gene. Diabetes was induced intraperitoneally with either a single dose of alloxan, streptozotocin, or multiple low doses of streptozotocin and blood glucose monitored twice a week. The role of cyclic guanosine monophosphate was investigated in wildtype mice by treatment with the phosphodiesterase inhibitor, tadalafil, during diabetes induction. Results showed that the incidence of diabetes was markedly decreased in mice overexpressing endothelial nitric oxide synthase, compared to wildtype or endothelial nitric oxide synthase knockout mice, regardless of the method of diabetes induction. Under normal physiological conditions, or during diabetes induction with alloxan or multiple low doses of streptozotocin, blood glucose was significantly lower in mice overexpressing endothelial nitric oxide synthase compared to wildtype or knockout mice. Treatment with tadalafil had no effect on the incidence or severity of diabetes in wildtype mice. We conclude that upregulation of endothelial nitric oxide synthase exerts a protective action against diabetes induction through a direct effect of nitric oxide, independently of cyclic guanosine monophosphate.

Keywords: eNOS overexpression; Alloxan; Streptozotocin; Type I diabetes

NO derived from endothelial nitric oxide synthase (eNOS) has been reported to exert protective effects against the metabolic syndrome which is characterised by abnormal plasma lipid profiles, high blood pressure and high fasting blood glucose [1] while the absence of eNOS expression in genetically modified mice was associated with hyperinsulinemia, hyperlipidemia, hypertension and impaired glucose tolerance [2]. Previous research has also shown that nitric oxide (NO) facilitates glucose uptake by skeletal muscle and adipose tissues [3–5] and increases body glucose disposal via insulin-dependent and independent pathways [6,7]. In addition, accumulation of the downstream effector of NO, cyclic guanosine monophosphate (cGMP), following chronic inhibition of phosphodiesterase has been shown to exert protective effects against ischemia/reperfusion induced cardiomyocyte necrosis and apoptosis [8] and hyperalgesia associated with diabetic neuropathy [9].

Given the link between eNOS and the metabolic syndrome, and the decreased bioavailability of endothelial NO during diabetes [10], we hypothesised that endothelial NO may exert a protective effect against induction of diabetes. We therefore aimed to clarify the role of eNOS in the development of chemically induced diabetes, by using mice in which the eNOS gene was either overexpressed

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or absent through deletion of the eNOS gene, as well as determining the role of elevated cGMP by treatment of wildtype mice with phosphodiesterase inhibitor.

Experimental procedures

Experiments were approved by the Animal Experimentation Ethics Committee of the Australian National University. eNOS transgenic (eNOSTg) mice overexpressed the bovine eNOS gene in vascular endothelial cells under control of the preproendothelin promoter [11]. Mice were genotyped by PCR using a forward primer located in the 3′ end of the preproendothelin promoter and reverse primer in the eNOS gene. eNOS knockout (eNOSKO) mice were developed by targeted disruption of the eNOS gene and previously characterised extensively [12,13]. Controls were age and sex-matched vehicle-injected eNOSTg negative littermates and wildtype C57BL/6 mice, as our preliminary data showed no differences between the two groups.

Diabetes was induced in 8–10 week male mice with either a single injection of streptozotocin (STZ, 2-deoxy(3-methyl-3-nitrosoureido)-D-glucopyranose, Calbiochem, Darmstadt, Germany, 200 mg kg^{-1}, i.p., 0.2 M acetate buffered saline, pH 4.4), multiple low doses of STZ (40 mg kg^{-1} day^{-1}, i.p., 5 days), or a single injection of alloxan (ALX, 5,6-dioxyuracil monohydrate, Sigma–Aldrich Co., St. Louis, USA, 175 mg kg^{-1}, i.p., saline). Capillary blood glucose concentration was assessed using a Medisense blood glucose electrode sensor (Abbott Laboratories, Victoria, Australia). Nonfasting blood glucose concentration was monitored twice a week after established diabetes as described before [14]. Diabetes was defined as blood glucose >13.0 mmol/l on two consecutive days, being a level higher than the mean + 3 SD of the control mouse groups. Intraperitoneal saline supplement was given if body weight loss exceeded 1 g/day.

In some experiments, wildtype mice were treated daily for 4 weeks with tadalafil (phosphodiesterase-5 inhibitor, 10 mg kg^{-1} i.p.; 1,3-benzodioxol hexa-hydro-2-methyl pyrazino pyridoindole-1,4-dione, Eli Lilly Australia, West Ryde, Australia). Mice in group 1 ($n = 6$) received tadalafil treatment and diabetes was induced with multiple low doses of STZ. Mice in group 2 ($n = 4$) received tadalafil treatment and the vehicle for STZ for 5 consecutive days. Treatment with tadalafil commenced 24 hours before STZ or vehicle.

Data were expressed as means ± SEM and analysed using one-way ANOVA with unpaired t tests and Bonferroni's correction for multiple comparisons. Statistical significance was set at $p < 0.05$. Blood glucose concentration for each diabetic mice was the average from the onset of diabetes to the end of the experimental period, or to the point of reversion to normal glucose levels. To test statistical significance of diabetes induction and reversion to normal blood glucose levels, mice were assigned a binary code of 0 for non-diabetic and 1 for diabetic, on each day of glucose testing. For each diabetic protocol, data were compared amongst the wildtype, eNOSTg and eNOSKO groups over the experimental time period using one-way ANOVA with unpaired t test using Bonferroni's correction for multiple comparisons.

Results

Nonfasting blood glucose levels (mmol/l) were significantly higher in eNOSKO mice (8.4 ± 0.16, $n = 67$) than in wildtype (8.0 ± 0.10, $n = 179$) or eNOSTg (7.6 ± 0.22, $n = 38$, $p < 0.05$). No significant difference in blood glucose levels was detected in eNOSTg mice compared to that in wildtype mice ($p > 0.05$).

Induction of diabetes with a single dose of STZ or ALX

For both drugs, the incidence of diabetes in eNOSTg mice was significantly less than that in wildtype mice (Figs. 1a and 2a, $p < 0.05$). Experiments using STZ were discontinued at 4 weeks due to the high mortality rate; only 35% (6/17) of wildtype diabetic mice and 40% (2/5) of eNOSTg diabetic mice surviving to this point (Fig. 1a, deaths indicated by crosses). None of the diabetic mice showed reversal to normal blood glucose levels.

In the case of ALX, the incidence of diabetes in wildtype mice was initially the same as that in eNOSKO mice (100%; Fig. 2a). However, over subsequent weeks, there was a sig-

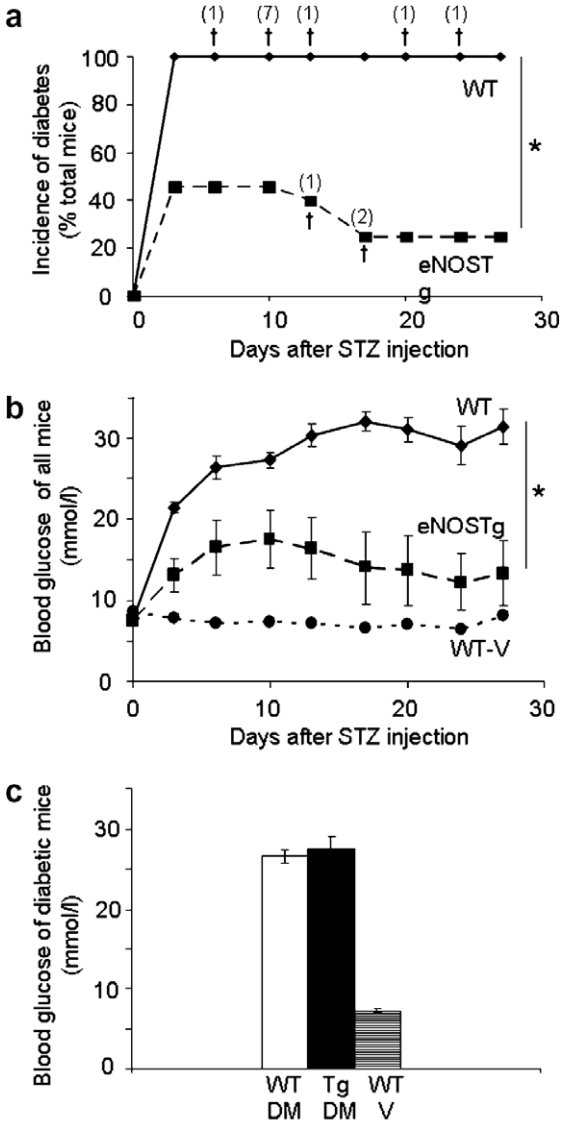

Fig. 1. Analysis of diabetes induced with a single dose of STZ in wildtype (WT), and eNOS transgenic (Tg) mice. (a) Cumulative incidence of diabetes against time after injection. Deaths are marked by crosses with the number of mice in parentheses. $^{*}p < 0.05$. (b) Average blood glucose levels in all mice injected with drug against the time after injection. $^{*}p < 0.05$. (c) The average blood glucose concentration of all diabetic mice in each group. Values represent average blood glucose levels from the onset of diabetes to the end of the experimental period, or to the point of reversion to normal glucose levels. WTDM, wildtype diabetic mice; TgDM, eNOSTg diabetic mice; WTV, wildtype vehicle injected mice. Wildtype $n = 17$, eNOSTg $n = 11$; wildtype/vehicle, WTV $n = 6$.