Physiol. Res. 47: 337–341, 1998

The Influence of NO synthase Inhibitor and Free Oxygen Radicals Scavenger – Methylene Blue – on Streptozotocin-Induced Diabetes in Rats

M. HALUZÍK, J. NEDVÍDKOVÁ[1], J. ŠKRHA

Third Medical Department, First Faculty of Medicine, Charles University and [1]Institute of Endocrinology, Prague, Czech Republic

Received February 24, 1998
Accepted May 28, 1998

Summary
The excessive production of nitric oxide (NO) and the subsequent increase of local oxidative stress is suggested as one of the pathophysiological mechanisms of streptozotocin-induced diabetes. It was reported that the administration of NO synthase inhibitors partially attenuated the development of streptozotocin-induced diabetes and reduced hyperglycaemia. Here we have studied the influence of methylene blue, which combines the properties of NO synthase inhibitor with antioxidant effects. The experiments were performed on male rats divided into four groups: control, diabetic (single dose of 70 mg of streptozotocin/kg i.p.), methylene blue (50 mg/kg in the food) and diabetic simultaneously fed with methylene blue. After 45 days the experiments were discontinued by decapitation. Serum glycaemia, glycated haemoglobin and oxidative stress parameters (plasma malondialdehyde concentration and erythrocyte superoxide dismutase activity) were significantly higher in the diabetic group. Simultaneous methylene blue administration partially reduced glycaemia and glycated haemoglobin, but did not decrease oxidative stress. We conclude that NO synthase inhibitor methylene blue partially attenuates the development of streptozotocin-induced diabetes in male rats, but does not reduce the development of oxidative stress in the diabetic group.

Key words
Diabetes – Rat – Oxidative stress – Nitric oxide – Superoxide dismutase – Malondialdehyde

Introduction

Oxidative stress is suggested to play a role in the development of insulin-dependent diabetes mellitus and subsequent diabetic complications (Jennings *et al.* 1987, Wierusz-Wysocka *et al.* 1997). Cytokines released by activated T-lymphocytes and macrophages, in particular interleukin-1, have been implicated as immunological effector molecules that both inhibit insulin secretion from pancreatic β cells and induce β cell destruction (Mandrup-Poulsen *et al.* 1985). Series of findings have indicated that production of the free radical nitric oxide, resulting from the expression of the cytokine-inducible isoform of NO synthase mediates these effects (Corbett *et al.* 1992, 1993). The most commonly used experimental model of human insulin-dependent diabetes mellitus is streptozotocin-induced diabetes in rodents.

Fax +420 2 293 894
E-mail physres@sun1.biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

It has been reported that the development of streptozotocin-induced diabetes is partially inhibited by the administration of NO synthase inhibiting compounds (Catanzaro *et al.* 1994, Wu G. 1995). The most commonly used compound is aminoguanidine that has a relatively high selectivity to the inducible NO synthase. Wu (1995) found that neither aminoguanidine nor N^{ω}-nitro-L-arginine methyl ester administration were able to delay the onset of diabetes in spontaneously diabetic BB rats within 13–15 days and did not alter the incidence of diabetes. Catanzaro *et al.* (1994) reported that simultaneous N^{ω}-nitro-L-arginine methyl ester administration partially reduced the increased blood glucose concentration, diuresis and urinary protein excretion in male C57 BL/KS-mdb mice with streptozotocin-induced diabetes.

Methylene blue (MB) is a thiazine dye that has been used in human medicine for a long time as an antituberculotic and antiseptic agent and in the treatment of methaemoglobinaemia. MB was proposed to be a "selective inhibitor of guanylate cyclase" for a long time. It was recently found that the influence of MB on the guanylate cyclase pathway is at least in part due to the inhibition of NO synthase (Mayer *et al.* 1993).

We have found that MB partially blocked the hypertrophic reaction of the anterior pituitary and the increase in blood prolactin levels after long-term oestrogen administration (Schreiber *et al.* 1993, Haluzík 1997, Haluzík *et al.* 1995). This effect of methylene blue is probably due to changes in hypothalamic dopamine synthesis and release (Nedvídková *et al.* unpublished results).

MB, when administered alone, increased the serum thyroxine concentration and decreased serum TSH levels. MB also partially inhibited the drop of serum thyroxine and the increase in thyroid weight and serum TSH levels when given simultaneously with the antithyroid agent carbimazole (Nedvídková *et al.* 1995, Haluzík *et al.* 1997). The effects of another NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester administration were similar (Haluzík *et al.* 1998).

The combination of free radical scavenging and NO synthase inhibiting properties of methylene blue made us to undertake the present study.

Materials and Methods

The experiments were performed on male Wistar rats (Velaz, Prague) with initial body weight 180–200 g. The rats were kept at 22±2 °C in a 12:12 h light:dark regimen and fed a standard laboratory diet (Larsen diet, Velaz, Prague) with water *ad libitum*. The rats were divided into four groups of 10 animals each. Animals in group 1 served as controls, group 2 were diabetic (single dose of 70 mg of streptozotocin in 0.9 % NaCl/kg i.p. after overnight fasting), group 3 received methylene blue (Fluka, Germany) 50 mg/kg in the food and group 4 consisted of diabetic animals simultaneously treated with methylene blue. The methylene blue treatment was started two days prior to streptozotocin administration. The protocol of the two experiments was identical. Blood for glycaemia determinations was obtained by tail vein puncture, glycaemia was measured by the glucose oxidase method using ESAT 6660-2 analyzer (Medingen, Germany). After 45 days, the experiments were terminated by decapitation, and blood was collected for determination of glycated haemoglobin, plasma malondialdehyde concentration and superoxide dismutase activity in erythrocytes.

Plasma concentration of malondialdehyde was measured by the thiobarbituric acid test of lipid peroxidation (Yagi 1976). The Cu,Zn-superoxide dismutase activity in erythrocytes was determined spectrophotometrically by a xanthine-xanthine oxidase system coupled with cytochrome c as previously described (McCord and Fridovich 1969). The percentage of glycated haemoglobin was assessed on Abbott analyzer (Abbott Laboratories, USA).

The SigmaStat statistical software (Jandel Scientific, USA) was used for statistical analysis. The means ± standard deviations were computed and the significance of differences between the groups was evaluated by analysis of variance and Dunnett's test.

Results

Streptozotocin administration significantly reduced body weight and increased glycaemia, glycated haemoglobin, superoxide dismutase activity in erythrocytes and plasma malondialdehyde concentration in comparison with the control group (Tables 1 and 2). In both experiments, the body weights in the group treated with methylene blue alone were slightly lower than those in the respective control groups (Tables 1 and 2). Glycated haemoglobin in the methylene blue group was significantly reduced in the first experiment (Table 1), while superoxide dismutase activity in erythrocytes and plasma malondialdehyde concentration were significantly higher than in the controls. There were no differences in glycaemia between the control and methylene blue group in both experiments, and in oxidative stress parameters in the second experiment. The simultaneous methylene blue administration to diabetic rats slightly reduced the loss of body weight, significantly decreased glycaemia and glycated haemoglobin, but did not clearly influence the parameters of oxidative stress (Tables 1 and 2).

Table 1. The initial and final body weight, glycaemia 7 days (glycaemia 1) or 30 days (glycaemia 2) after streptozotocin administration, glycaemia at the end of experiment (glycaemia 3), glycated haemoglobin (Hb), superoxide dismutase (SOD) activity and malondialdehyde (MDA) concentration in control (C), diabetic (D), methylene blue (MB) treated group and diabetic group treated simultaneously with methylene blue (D + MB) in experiment 1.

	C	MB	D	D + MB
Body weight (g)				
Initial	193±5.4	191±8.1	193±7.9	191±6.6
Final	343±26.9	316±20.1*	249±13.7*	269±31.1*
Glycaemia (mmol.l^{-1})				
1	6.00±1.01	5.26±0.58	20.72±2.09*	17.23±4.94*$^{+}$
2	5.85±0.78	4.40±0.46	22.00±2.32*	9.17±3.98*$^{+}$
3	5.49±0.46	4.50±0.35	27.31±2.42*	16.80±6.32*$^{+}$
Glycated Hb (%)	6.79±0.38	4.91±0.31*	14.11±0.77*	7.67±1.48^{+}
SOD (IU)	0.86±0.17	1.23±0.28*	1.53±0.17*	1.39±0.36*
MDA (mmol.l^{-1})	1.93±0.31	2.82±0.57*	3.17±0.91*	3.86±1.15*

*The results are expressed as means±S.D. * significant difference ($p<0.05$) from control group, $^{+}$ significant difference ($p<0.05$) from diabetic group. (Computed by analysis of variance followed by Dunnett's test)*

Table 2. The initial and final body weight, glycaemia 7 days (glycaemia 1) or 30 days (glycaemia 2) after streptozotocin administration, glycaemia at the end of experiment (glycaemia 3), glycated haemoglobin (Hb), superoxide dismutase (SOD) activity and malondialdehyde (MDA) concentration in control (C), diabetic (D), methylene blue (MB) treated group and diabetic group treated simultaneously with methylene blue (D + MB) in experiment 2.

	C	MB	D	D + MB
Body weight (g)				
Initial	195±5.9	193±4.9	206±18.2	201±16.1
Final	363±27.6	343±22*	226±36.2*	261±80.9*
Glycaemia (mmol.l^{-1})				
1	4.98±0.61	5.25±1.30	25.02±2.51*	16.12±8.40*$^{+}$
2	5.02±0.58	4.86±0.74	28.42±3.20*	15.30±6.80*+
3	4.80±0.27	4.79±0.49	26.07±4.10*	23.67±9.20*
Glycated Hb (%)	6.79±0.58	6.52±0.50	16.84±0.92*	13.22±2.93*$^{+}$
SOD (IU)	1.48±0.46	1.77±0.44	2.05±0.16*	1.67±0.32^{+}
MDA (mmol.l^{-1})	3.36±0.41	3.41±0.48+	4.43±1.17*	4.96±1.34*

*The results are expressed as means±S.D. * significant difference ($p<0.05$) from control group, $^{+}$ significant difference ($p<0.05$) from diabetic group. (Computed by analysis of variance followed by Dunnett's test)*

Discussion

Oxidative stress is supposed to be one of the mechanisms taking part in the development of insulin-dependent diabetes mellitus and late diabetic complications (Wierusz-Wysocka *et al.* 1997). Several biochemical variables including malondialdehyde and scavenger enzymes have been used as markers of this process (Noberasco *et al.* 1991). While malondialdehyde levels indicate the global extent of oxidative stress, superoxide dismutase activity is commonly used as a measure of protective mechanisms.

Several papers have reported that the development of streptozotocin-induced diabetes is associated with the activation of immunocompetent cells, increased production of cytokines and overexpression of the inducible NO synthase isoform in macrophages and lymphocytes (Corbett *et al.* 1992, 1993). The activation of immunocompetent cells is accompanied with a strongly increased production of nitric oxide, that has chemical properties of free oxygen radical. The simultaneous administration of NO synthase inhibitors partially decreased NO production, reduced glycaemia, diuresis and urinary protein excretion in diabetic rats (Wu 1995, Catanzaro *et al.* 1994).

Here we have studied the influence of the thiazine dye, methylene blue, on the development of streptozotocin-induced diabetes and changes of oxidative stress parameters. Methylene blue is supposed to be a non-selective NO synthase inhibitor, i.e. that it acts on both constitutive NO synthase isoforms as well as on inducible NO synthase isoform (Mayer *et al.* 1993). Moreover, Salaris *et al.* (1991) found that methylene blue may represent a new class of antioxidant drugs that competitively inhibit the reduction of molecular oxygen to superoxide by acting as an alternative electron acceptor for tissue oxidases.

The results of our experiments (reduction of hyperglycaemia and glycated haemoglobin) support the hypothesis that NO synthase inhibitors partially suppress the development of streptozotocin-induced diabetes. However, the changes in oxidative stress parameters observed in our experiments are ambiguous. In the first experiment, methylene blue alone increased both superoxide dismutase activity as well as the concentration of malondialdehyde, the administration of methylene blue to diabetic rats in these experiments significantly decreased superoxide dismutase activity but did not affect malondialdehyde concentration. In the second experiment, methylene blue when administered either to intact or to diabetic rats did not significantly alter oxidative stress parameters.

The explanation of our results is thus rather difficult. It is possible that the effect of methylene blue is only transient, i.e. that its partial inhibitory influence on the development of this disease gradually decreases in long-term diabetes. The most significant differences in the glycaemic response between diabetic animals and the diabetic group treated with methylene blue were observed after 4 weeks of diabetes (Tables 1 and 2). At the end of the first experiment, the difference was less pronounced than after 4 weeks of diabetes and at the end of the second experiment it was not even significant. The suggested mechanism of "escape" of immunocompetent cells taking place in the destruction of β cells in pancreatic Langerhans islets from NO synthase inhibiting effect of methylene blue is, however, unknown. We can only speculate that after the initial phase of diabetes, which is probably to a great extent mediated by NO overproduction, the second phase appears in which the role of NO overproduction is less pronounced.

Our results concerning the antioxidant properties of methylene blue differ from those reported by Salaris *et al.* (1991). However, their experiments were performed *in vitro*. Our studies of *in vivo* effects of methylene blue could be influenced by the interaction of other redox systems. Moreover, we have studied only two parameters of oxidative stress status. It cannot therefore be excluded that oxidative parameters other than superoxide dismutase and malondialdehyde could exhibit different results.

It must also be taken into account that we have measured the activity of superoxide dismutase in erythrocytes and plasma malondialdehyde concentrations. Local oxidative stress in Langerhans islets of the pancreas could thus be different from that in plasma or erythrocytes. The local action of methylene blue could thus explain its antidiabetic effect. It should be stressed that streptozotocin was administered on day 1, when the animals were already fed methylene blue.

Another possibility is that the antioxidant effect of methylene blue is dose-dependent. The dose administrated in our experiments could be inappropriate for inducing the antioxidant effect. A similar situation was described after administration of vitamin E in a daily dose of 600 mg in patients with type II diabetes mellitus by Škrha *et al.* (1997). These authors have found that the above mentioned dose of vitamin E did not significantly decrease the global oxidative stress, although the lower doses of the same substance had a significant antioxidant effect. Moreover, vitamin E in a daily dose of 600 mg/daily diminished insulin sensitivity and fibrinolysis in this study.

We conclude that the NO synthase inhibitor and free radical scavenger methylene blue partially suppressed the development of streptozotocin-induced diabetes in rats, reduced glycaemia and glycated haemoglobin. However, the increased oxidative stress

status after 45-day-lasting diabetes was not significantly reduced by simultaneous methylene blue administration.

Further examination of the mechanism of methylene blue action on the development of streptozotocin-induced diabetes is thus necessary to elucidate the dynamic changes of oxidative stress during various phases of diabetes. This problem is being studied in our current investigations.

Acknowledgement
Supported by the grant GACR 311/98/P089.

References

CATANZARO O.L., MARINA-PRENDES M.G., HOPE S.I., ZUCCOLLO A., DOMINGUES A.: Streptozotocin induced hyperglycemia is decreased by nitric oxide inhibition. *Braz. J. Med. Biol. Res.* **27:** 2043–2047, 1994.

CORBETT J.A., MCDANIEL M.L.: Does nitric oxide mediate autoimmune destruction of β-cells? Possible therapeutic interventions in IDDM. *Diabetes* **41:** 897–903, 1992.

CORBETT J.A., WANG J.L., MISKO T.P., ZHAO W., HICKEY W.F., MCDANIEL M.L.: Nitric oxide mediates IL-1β-induced islet dysfunction and destruction: prevention by dexamethasone. *Autoimmunity* **15:** 145–153, 1993.

HALUZÍK M.: Methylene blue – the new effects of an old drug. *Thyroid Gland Clin. Exp.* **1:** 7–13, 1997.

HALUZÍK M., NEDVÍDKOVÁ J., SCHREIBER V.: Methylene blue – an endocrine modulator. *Sborník lék.* **96:** 319–322, 1995.

HALUZÍK M., NEDVÍDKOVÁ J., KOPSKÝ V., SCHREIBER V., JAHODOVÁ J., HOŘEJŠÍ B.: The changes of the thyroid function and serum testosterone levels after long term L-NAME treatment in male rats. *J. Endocrinol. Invest.* **21:** 234–238, 1998.

JENNINGS P.E., JONES A.F., FLORKOWSKI C.M., LUNEC J., BARNETT A.H.: Increased diene conjugates in diabetic subjects with microangiopathy. *Diabet. Med.* **4:** 452–56, 1987.

MANDRUP-POULSEN T., BENDTZEN K., NIELSEN H., BENDIXEN G., NERUP J.: Cytokines cause functional and structural damage to isolated islets of Langerhans. *Allergy* **40:** 424–429, 1985.

MAYER B., BRUNNER F., SCHMIDT K.: Novel actions of methylene blue. *Eur. Heart J.* **14** (Suppl. I): 22–26, 1993.

MCCORD J.M., FRIDOVICH I.: Superoxide dismutase. *J. Biol. Chem.* **244:** 6049–6055, 1969.

NEDVÍDKOVÁ J., ŠTERZL I., HALUZÍK M., SCHREIBER V.: An increase in the blood thyroxine level after methylene blue in rats: the interaction with carbimazole. *Endocrine Res.* **21:** 709–717, 1995.

NOBERASCO G., ODETTI P., BOERI D., MAIELLO M., ADEZATI L.: Malondialdehyde level in diabetic subjects. Relationship with blood glucose and glycosylated hemoglobin. *Biomed. Pharmacother.* **45:** 193–196, 1991.

SALARIS S.C., BABBS C.F., VORHEES W.D.: Methylene blue as an inhibitor of superoxide generation by xanthine oxidase. *Biochem. Pharmacol.* **42:** 171–174, 1991.

SCHREIBER V., NEDVÍDKOVÁ J., JAHODOVÁ J.: Anterior pituitary weight, cAMP, cGMP and prolactin levels after combined treatment with estradiol and methylene blue. *Physiol. Res.* **42:** 171–174, 1993.

ŠKRHA J., ŠINDELKA G., KVASNIČKA J., HILGERTOVÁ J.: High doses of vitamin E impair insulin action and fibrinolysis in obese type 2 diabetic patients. *Diabetes* **46** (Suppl.): 351A, 1997.

WIERUSZ-WYSOCKA B., WYSOCKI H., BYKS H., ZOZULINSKA D., WYKRETOWITZ A., KAZMIERCZAK M.: Metabolic control quality and free radical activity in diabetic patients. *Diabetes Res. Clin. Pract.* **27:** 193–197, 1997.

WU G.: Nitric oxide synthesis and the effect of aminoguanidine and N^G-monomethyl-L-arginine on the onset of diabetes in the spontaneously diabetic BB rat. *Diabetes* **44:** 360–364, 1995.

YAGI H.: A simple fluorimetric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15:** 212–216, 1976.

Reprint requests
M. Haluzík, M.D., Ph.D., Third Medical Department, First Faculty of Medicine, U nemocnice 1, Praha 2, 128 08 Prague 2, Czech Republic.