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The Influence of Nitric Oxide Synthase Inhibitor L-NAME on Bones of Male Rats with Streptozotocin-Induced Diabetes

P. D. BROULÍK, M. HALUZÍK[1], J. ŠKRHA

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

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Summary

The pathophysiological processes underlying the development of diabetic osteopenia has not hitherto been elucidated. Induction of streptozotocin diabetes leads in our experiments to decrease of bone density, ash, mineral content and to thinner cortical width compared to control male rats. In order to investigate the pathogenetic role of bone resorption by osteoclasts in streptozotocin-induced diabetes, we determined the circulating levels of tartrate-resistant acid phosphatase (TRAP), a biochemical marker for bone resorption. Plasma TRAP values in diabetic rats did not differ from their corresponding controls. Streptozotocin diabetes by itself did not have any effect on the weight of seminal vesicles which are highly testosterone-dependent. Low doses of nitric oxide cause bone resorption, but higher doses of NO inhibit bone resorbing activity. We examined the effect of L-NAME (inhibitor of nitric oxide production) after six weeks of administration to diabetic rats. There was no further significant loss of bone mineral density, ash and mineral content or tibia weight in diabetic rats treated with L-NAME. L-NAME itself did not decrease bone metabolism. In our study no evidence of an increased bone resorption was found. Our results have indicated that a predominance of bone resorption over bone formation is not involved in the pathogenesis of diabetes-associated osteopenia. Inhibition of NO neither increased osteoclastic activity (TRAP) nor induced osteopenia in L-NAME-treated rats. This suggests a possibility that NO is not involved in the pathogenesis of diabetic osteopenia.

Key words
Streptozotocin diabetes • Osteopenia • L-NAME • TRAP • Osteoclasts

Introduction

Although the loss of bone minerals is not generally regarded as one of the major complications of diabetes mellitus there is considerable evidence that diabetic patients have lower bone mass than normal subjects (Selby 1988, Thomas *et al.* 1997)

The pathophysiological process underlying the development of diabetic osteopenia has not hitherto been elucidated. There is some evidence that osteopenia may be a result of poor control of the metabolic abnormalities of diabetes and that the degree of bone loss is related to the extent of glycosuria, to fasting blood glucose concentration and to the glycosylated hemoglobin concentration (McNair *et al.* 1979) Much of the early histological and histomorphometric analysis of diabetic bone suggests that there is a low turnover with decreased bone resorption and formation (Silverberg 1986). However, there are papers suggesting that increased bone

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

resorption rather than decreased formation is the major defect in diabetic osteopenia (Verhaeghe *et al.* 1989)

Evidence suggests that L-arginine derived nitric oxide has a direct inhibitory effect on osteoclast-mediated bone resorption (Wimalawansa *et al.* 2000). Nitric oxide is a short-lived free radical involved in many biological processes. Nitric oxide is a potent inhibitor of bone resorption through its activity on osteoclast-mediated bone resorption by causing retraction of the cell (Kasten *et al.* 1994). It has also been reported that NO levels are significantly lower in postmenopausal women and amenorrheic women athletes with osteopenia (Stacy *et al.* 1998). The study of Wimalawansa (2000) agrees with the *in vitro* observation done by Kasten *et al.* (1994) that bone resorption was reduced in the presence of an nitric oxide donor. On the contrary, low doses of NO cause bone resorption (Lowik *et al.* 1994).

The aim of this study was to assess the effect of streptozotocin-induced diabetes on bone metabolism and to determine whether nitric oxide synthase inhibitor L-NAME affects the bone metabolism in streptozotocin diabetic adult male rats.

Methods

The experiments were performed on male rats of Wistar strain (Velaz, Prague) with initial body weight 200-230 g. The rats were kept at 22±2 ^{o}C in a 12:12 h light: dark regimen and fed a standard laboratory diet (Velaz, Prague 23 % protein, 19 % fat and 57 % carbohydrate) with water *ad libitum*. The rats were divided into four groups with 8 animals in each. Animals in group l served as controls, group 2 were diabetic rats (single dose of 70 mg/kg streptozotocin, Upjohn Co., Kalamazoo) intraperitoneally in 0.9 % NaCl after an overnight fasting, group 3 received L-NAME (Sigma USA) 100 mg/kg in the drinking water daily and the dose was reduced by 50 % in diabetic rats, group 4 consisted of diabetic animals simultaneously treated with L-NAME. Control rats which were matched for age and weight at the time of streptozotocin administration received an equal volume of saline. Rats were considered diabetic if the blood glucose was higher than 15 mmol/l, 72 h after the injection. Blood for glucose determination was obtained by tail vein puncture, blood glucose was measured by the glucose oxidase method using ESAT 6660-2 analyzer (Medingen Germany). Six weeks after the induction of streptozotocin-induced diabetes blood samples and the right femoral bone, both tibias and seminal vesicles were obtained and weighed. Blood samples were collected for determination of glycated hemoglobin, plasma insulin and plasma TRAP concentration.

Plasma insulin was measured by the RIA method using rat insulin (Amersham-Pharmacia Biotech Ltd., UK).

Glycated hemoglobin was assessed using an Abott analyzer (Abott Laboratories, USA). The activity of plasma TRAP (EC 3.l.3.2) was determined with 4-nitrophenyl phosphate as substrate (7.7 mmol/l) in a citrate buffer (sodium citrate 170 mmol/l, sodium tartrate 20 mmol/l, sodium chloride 120 mmol/l, pH 5.5) at 37 ^{o}C (Štěpán *et al.* 1983).

The tibias were isolated, cleaned of soft tissues, marrow elements were flushed out with cold saline through a needle inserted into the marrow cavity and finally the bone was weighed and placed in an unstoppered glass vial filled with deionized water which was then placed in a desicator. The bones were suspended on a fine wire mesh and weighed in air and in water to an accuracy of 0.1 mg. The volume and bone density of the tibias were calculated from the mass in air and water by Archimedes principle (Kalu *et al.* 1994). The bones were then dried to constant weight and incinerated for 24 h at 600 ^{o}C to white ash which was weighed. Bone ashes were then dissolved in hydrochloric acid before determination of the calcium and phosphorus content. Bone calcium was measured by the method of Gitelman (1967) and bone phosphorus according to Kraml (1966).

Standardized roentgenograms of dry rat femur were made using Philips Mamo Diagnost 3000 X-ray machine at controlled exposures of 26 kV at 5.5 mAS. Morphometric measurements were performed directly on the X rays after magnification with a fine caliper. On the roentgenograms at 40 % distance of the total bone length starting from the distal end the external bone diameter, inner bone diameter and cortical width were measured after magnification with a fine caliper by the method of Beall *et al.* (1984) and Vanderschueren *et al.* (1992).

The means ± 95 % confidence intervals were computed and the significance of differences between the means was evaluated by the analysis of variance and Duncan´s test (Duncan 1955).

Results

After six weeks of experiment body weight was significantly decreased in diabetic rats and in diabetic rats simultaneously treated with L-NAME in comparison with the control group. L-NAME did not affect the body

weight of animals when administered alone. (Table 2). Streptozotocin administration led to a significant increase of blood glucose and glycated hemoglobin. The simultaneous administration of L-NAME to diabetic rats significantly suppressed the increase of glycated hemoglobin, but did not affect blood glucose at the end of the experiment (Table 1). Streptozotocin administration significantly decreased blood insulin in comparison with the control and L-NAME group.

The weight of seminal vesicles confirmed that streptozotocin diabetes and L-NAME by themselves did not have an effect on seminal vesicles weight which is highly testosterone-dependent (Table 1).

Table 1. Blood glucose, glycated hemoglobin, insulin, TRAP and weight of seminal vesicles in control and diabetic (DM) as well as in L-NAME-treated control and diabetic animals.

	Intact n = 8	**L-NAME n = 8**	**DM n = 8**	**DM + L-NAME n = 8**
Blood glucose (mmol/l)	5.42±0.36	4.76±0.45	23.15±2.0*	21.1±2.12*
Glycated Hb (%)	7.26±0.47	7.05±0.71	17.4±1.89*	15.6±1.15*
Insulin (ng/ml)	2.06±0.3	2.01 ±0.4	0.27±0.05*	0.31±0.04*
TRAP (U/l)	15.0±1.5	14.7±1.9	16.2±2.1	16.4±1.9
Seminal vesicles (mg/100 g bw)	190±6	187±6	185±6	184±6

*Data are means ± S.D., * p<0.01 vs control animals*

Table 2. Body weight, bone density and the bone mineral content in control and diabetic (DM) as well as in L-NAME-treated control and diabetic animals.

	Intact n = 8	**L-NAME n = 8**	**DM n = 8**	**DM + L-NAME n = 8**
Initial body weight (g)	219±4	229±8	223±7	217±5
Final body weight (g)	363±29	351±31	266±44*	243±46*
Tibia dry weight (mg)	400±33	371±22	291±16*	296±28*
Tibia volume (µl)	318±15	315±18	299±27	300±20
Density of tibia (g/ml)	1.61±0.03	1.60±0.04	1.50±0.03*	1.50±0.04*
Tibia ash content (g/ml)	0.74±0.07	0.70±0.08	0.54±0.08*	0.55±0.06*
Tibia calcium (mg/ml)	282±12	262±16	205±10*	205±4*
Tibia phosphate (mg/ml)	124±4.2	115±5.2	90±3.9*	90±2.8*

*Data are means ± S.D., * p<0.01 vs control animals*

Bone density, ash and mineral content of the tibia were significantly decreased in streptozotocin diabetic rats compared with intact animals. Daily administration of L-NAME did not further decrease the bone density, ash and mineral content of the tibia in streptozotocin diabetic rats. L-NAME itself did not decrease bone metabolism in intact rats (Table 2).

Plasma tartrate-resistant acid phosphatase (TRAP) in diabetic rats was 16.2±2.1 U/l and this value was not significantly increased compared to that of 15.0±1.5 U/l in the corresponding controls. L-NAME alone had no effect on plasma TRAP in intact or diabetic rats (Table 1). Femoral length and outer diameter were not significantly different between the groups after six weeks of treatment. At this time, however rats with streptozotocin diabetes had significantly thinner cortical width. This decrease in cortical width was not further potentiated by the administration of L-NAME (Table 3).

Table 3. Morphometric parameters of the femur midshaft in control and diabetic (DM) as well as in L-NAME-treated control and diabetic animals.

	Intact n = 8	**L-NAME n = 8**	**DM n = 8**	**DM + L-NAME n = 8**
Femur length (mm)	37.6±0.3	37.8±0.4	37.2±0.3	37.1±0.5
Outer diameter (mm)	4.20±0.08	4.21±0.06	4.18±0.07	4.19±0.07
Inner diameter (mm)	2.84±0.09	2.87±0.05	2.95±0.06*	2.90±0.09*
Cortical width (mm)	0.68±0.02	0.67±0.03	0.61±0.05*	0.60±0.06*

Data are means ± S.E., $p<0.01$ *vs control animals*

Discussion

The pathogenesis of diabetic osteopenia is not understood completely. Negative calcium balance and both accelerated and lowered bone turnover state, alterations in vitamin D metabolism and abnormal collagen metabolism have all been implicated as the pathogenetic mechanisms of diabetic osteopenia.(Seino and Ishida 2000). To explore the pathogenesis of osteopenia associated with diabetes we characterized the osteopenia in streptozotocin-diabetic rats biochemically and histomorphometrically.

The induction of streptozotocin diabetes in male rats decreased bone density, ash, mineral content and cortical width in our experiments. In order to investigate the pathogenetic role of bone resorption by osteoclasts in altered bone metabolism of rats with streptozotocin-induced diabetes, the circulating levels of tartrate-resistant acid phosphatase (TRAP), a biochemical marker for bone resorption, were determined. Plasma TRAP levels in diabetic rats did not differ from the corresponding controls.

Streptozotocin diabetes by itself had no effect on the weight of seminal vesicles which are highly testosterone-dependent. It is also evident that the changes in bone mineral density in rats with streptozotocin diabetes were not related to the testosterone level.

In our experiments the induction of streptozotocin diabetes was accompanied by significantly thinner cortical widths. The simultaneous L-NAME treatment of diabetic rats during a six-week period did not further decrease bone mineral density, ash, mineral content or tibia weight. Our studies have shown that diabetes-induced bone loss is not further aggravated by L-NAME. L-NAME administration did not have any effect on TRAP in control and diabetic animals.

L-NAME administration (NO synthase inhibition) did not cause osteopenia in control rats. TRAP levels were not increased in L-NAME treated control rats or in L-NAME treated diabetic rats. These results do not support the hypothesis that a decrease in NO levels increases bone resorption.

Several studies have revealed a wider role of NO in bone metabolism, suggesting that at lower concentrations NO enhances IL-1 induced bone resorption (Lowik *et al.* 1994), but higher NO concentrations inhibit bone resorption mediated by a variety of stimuli (Ralston *et al.* 1995).

Treatment with NO protects against ovariectomy-induced bone loss in adult female rats (Wimalawansa *et al.* 1996). Bone loss associated with diminished estrogen levels has been attributed to increased osteoclastic activity and bone resorption in postmenopausal women. Estrogen-induced increase in bone mineral density is dependent upon NO generation. L-NAME completely abolished the effects of estradiol (Wimalawansa *et al.* 1996).

Nitric oxide not only inhibits osteoclastic activity, but it modulates or enhances osteoblastic activity (Mancini *et al.* 2000). However, the effects of this gas radical on osteoblastic function are still not clear. A decrease in NO level not only leads to increase in osteoclastic activity but also decreases osteoblastic activity. It has been shown that NO has biphasic effect on bone metabolism. A slow and moderate release of nitric oxide stimulates the replication of primary rat osteoblasts and alkaline phosphatase activity while a rapid release and high concentration of NO inhibit proliferation and induce apoptosis (Mancini *et al.* 2000). Both cAMP and

cGMP have been proposed as mediators in bone remodeling (Wimalawansa *et al.* 2000). Mancini *et al.* (2000) demonstrated that both the stimulation and apoptosis-induced effects of NO on primary osteoblasts are mediated by the second messenger cGMP, since both are abolished by guanylate cyclase inhibitors 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one.

Our studies have shown that bone loss induced by streptozotocin diabetes is not further aggravated by L-NAME. During a six-week period there was no further significant loss of bone mineral density in diabetic rats treated with L-NAME. Inhibition of NO neither increased osteoclastic activity (TRAP) nor induced osteopenia in L-NAME-treated control rats. This raises the possibility that NO is not involved in the pathogenesis of diabetic osteopenia.

Thus regardless of the mechanisms involved in diabetic osteopenia it is tempting to propose that the effects of streptozotocin diabetes on bone are not fully dependent on the activation of osteoclasts. From our results it seems that the predominance of bone resorption over bone formation is not involved in the pathogenesis of diabetes associated osteopenia.

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Reprint requests

Prof. P.D. Broulík, Third Medical Department, First Faculty of Medicine, Charles University, U nemocnice 1, 128 21 Prague 2, Czech Republic. E-mail: pbrou@cuni.cz