Plasma nitrate+nitrite levels are regulated by ovarian steroids but do not correlate with trabecular bone mineral density in rats

R L van Bezooijen¹, I Que¹, A G H Ederveen²,
H J Kloosterboer², S E Papapoulos¹ and C W G M Löwik¹

¹Department of Endocrinology and Metabolic Diseases, Leiden University Medical Center, PO Box 9600, 2300 RC, Leiden, The Netherlands and ²Department of Endocrinology, Scientific Development Group, NV Organon, PO Box 20, 5340 BH, Oss, The Netherlands

(Requests for offprints should be addressed to R L van Bezooijen, Department of Endocrinology, Bldg1 C4-R86, Leiden University Medical Center, PO Box 9600, 2300 RC, Leiden, The Netherlands)

Abstract

Nitric oxide (NO) is a mediator of bone metabolism and its production is under the control of gender hormones in several cell types or tissues. Changes in endogenous NO production, measured as plasma nitrate+nitrite levels, may therefore contribute to ovariectomy (OVX)-induced bone loss. We studied plasma nitrate+nitrite levels and trabecular bone mineral density (TBMD) 4 weeks after sham-operation or OVX in rats receiving various hormonal treatments. OVX decreased plasma nitrate+nitrite levels significantly and this was accompanied by a significant decrease in TBMD. Treatment with oral ethinyl oestradiol (EE) and subcutaneous 17 β -oestradiol dose-dependently prevented the decrease in plasma nitrate+nitrite levels after OVX, but treatment with oral 17 β -oestradiol did not. Oestrogen treatment, 17 β -oestradiol (s.c. or orally) or EE (orally), prevented the OVX-induced decrease in TBMD. Treatment of sham-operated rats with the anti-oestrogen ICI164,384 induced a significant decrease in TBMD that

corresponded to 54% of the decrease observed after OVX, but did not affect plasma nitrate+nitrite levels. Treatment of ovariectomized rats with Org 2058, a pure progestagen, did not prevent bone loss, but prevented the decrease in plasma nitrate+nitrite levels dose-dependently. Treatment with tibolone, a synthetic steroid with combined weak oestrogenic, progestagenic, and androgenic properties, or with progestagen in combination with EE completely prevented bone loss after OVX. These treatments, however, only partly prevented the OVX-induced decrease in plasma nitrate+nitrite levels. In conclusion, OVX decreased both TBMD and plasma nitrate+nitrite levels. Although plasma nitrate+nitrite levels were under the control of both oestrogen and progesterone, TBMD was affected by oestrogen only. Decreased systemic production of NO is, therefore, not involved in OVX-induced bone loss in rats.

Journal of Endocrinology (1998) **159**, 27–34

Introduction

Loss of ovarian function is associated with bone loss as a consequence of an imbalance between bone resorption and formation in favour of the first. This can be prevented by oestrogen replacement. Progesterone may also be involved, as some progestagens have been shown to reduce postmenopausal bone loss (reviewed by Prior 1990). The mechanisms by which these ovarian steroids may exert their protective effect on bone are not well understood.

Nitric oxide (NO) may be involved in ovariectomy (OVX)-induced bone loss, as it modulates both osteoclastic bone resorption (MacIntyre *et al.* 1991, Stern & Diamond 1992, Löwik *et al.* 1994, Ralston *et al.* 1995, Ralston & Grabowski 1996) and osteoblast proliferation and activity (Damoulis & Hauschka 1994, Ralston *et al.* 1994, Hukkanen *et al.* 1995, Riancho *et al.* 1995) *in vitro* and its production is regulated by gender-steroids in several cell types and tissues (Hayashi *et al.* 1992, 1995,

Weiner *et al.* 1994, Yallampalli *et al.* 1994). Administration of the NO-synthase (NOS) inhibitor aminoguanidine to rats induced bone loss (Kasten *et al.* 1994, Tsukahara *et al.* 1996) and reduced bone formation rate (Turner *et al.* 1997). The NOS inhibitors N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) prevented the increase in cancellous bone formation by mechanical stimulation in rats (Fox *et al.* 1996, Turner *et al.* 1996). Treatment with the NO donor nitroglycerine prevented OVX-induced bone loss and administration of L-NAME completely blocked the preventive effect of oestrogen on OVX-induced bone loss (Wimalawansa *et al.* 1996). Nitrate+nitrite (NO₃+NO₂) plasma levels, which reflect endogenous NO production (Hibbs *et al.* 1992, Shultz & Raji 1992), have been found to correlate positively with serum 17 β -oestradiol levels (Rosselli *et al.* 1994, 1995, Ramsay *et al.* 1995, Cicinelli *et al.* 1996, Kawano *et al.* 1996, Imthurn *et al.* 1997). On the other hand, progesterone

may have a down-regulating effect on $\text{NO}_3 + \text{NO}_2$ plasma levels in the presence of oestrogen (Rosselli *et al.* 1994, 1995, Cicinelli *et al.* 1996, Kawano *et al.* 1996, Imthurn *et al.* 1997).

In the present study, we hypothesized that loss of ovarian function causes a decrease in NO production, which in turn contributes to bone loss. To investigate this, sham-operated or ovariectomized rats were treated with various hormonal therapies and trabecular bone mineral density (TBMD) and $\text{NO}_3 + \text{NO}_2$ plasma levels were determined 4 weeks after surgery.

Materials and Methods

Animals and materials

Mature 3-month-old unmated female Wistar rats (strain Hsd/Cpd:Wu), weighing approximately 225 g, were obtained from Harlan, the Central Institute for Breeding of Laboratory Animals, Zeist, The Netherlands. The animals were housed under standard conditions and kept with a ratio of 14 h light:10 h darkness in an air conditioned room ($21^\circ\text{C} \pm 2^\circ\text{C}$). During the experiment the animals were housed individually, had free access to tap water and were pair-fed (16 g pelleted food per day (Hope Farms, Linschoten, The Netherlands)). All protocols were approved by the Animal Ethics Committee. Animals were divided into groups according to a randomized block design using body weight as the selection parameter. Rats were subjected to either OVX ($n=140$) or sham-operation ($n=72$) under general anaesthesia. From the day of operation onwards, the ovariectomized rats received various hormonal treatments (twice daily), consisting of vehicle (0.5% w/v gelatine and 5% w/v mannitol; $n=54$), ethinyl oestradiol (EE) (orally; $n=6$), 17β -oestradiol (orally; $n=4-10$, s.c.; $n=8$), (16 α)-16-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione (s.c.; Org 2058; $n=6$); a pure progestagen which exerts its effect via the progesterone receptor only, progestagen (s.c.) and EE (orally; $n=6$), or tibolone (orally; Org OD14: (7 α ,17 α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one; $n=12$) (de Visser *et al.* 1984). Sham-operated rats received control solution ($n=60$), the anti-oestrogen ICI164,384 (s.c., (7 α ,17 β)-N-butyl-3,17-dihydroxy-N-methylestra-1,3,5(10)-triene-7-undecanamide; $n=6$) (Wakeling & Bowler 1987) in arachis oil, or the anti-progestagen RU 486 (orally, 11 β -(4-(dimethyl-amino)phenyl)-17 β -hydroxy-17 α -(1-propynyl)-estra-4,9-dien-3-one; $n=6$) (Baulieu 1989). All preparations were made by the Department of Pharmaceutics, N.V. Organon, Oss, The Netherlands and were of analytical grade. After 4 weeks treatment and overnight fasting, blood was collected from the abdominal aorta under general anaesthesia. Heparin plasma samples were centrifuged (3000 g) for 10 min at 4°C , divided into small samples, and stored at -20°C . The right femora were dissected out for TBMD measurement at autopsy.

Plasma nitrate+nitrite analysis

Plasma samples were deproteinized with 3% (final concentration) ZnSO_4 for 15 min and centrifuged at 30 000 g for 15 min. NO_3 in supernatants was reduced to NO_2 with oxidized cadmium in 0.56% NH_4Cl and 0.15% Borax-buffer (Sigma, Zwijndrecht, The Netherlands) for 30 min and vortexed every 5 min. Cadmium was oxidized by two treatments with 2% HCl for 30 min under continuous shaking. Before oxidized cadmium was added to plasma supernatants, pH was neutralized by repeated washing with distilled water. NO_2 levels in the reduced plasma supernatants were determined with Griess reagent consisting of 0.5% sulfanilamide, 0.05% naphthylethelene-diamine-dihydrochloride, and 2.5% H_3PO_4 . Equal volumes (50 μl) of reduced samples and Griess reagent were mixed and incubated in a 96-well plate for 15 min at room temperature under continuous shaking. NO_2 concentration, proportional to OD_{550} , was determined using a microtiterplate reader (Thermomax, Molecular Devices, Menlo Park, CA, USA). Results are not differentiated into NO_3 and NO_2 but expressed as $\text{NO}_3 + \text{NO}_2$ levels, as very little or no NO_2 is present in plasma of humans (Green *et al.* 1982) and rats (own observation). The percentage of NO_3 that was reduced to NO_2 was extrapolated from comparing standard curves of serial dilutions of NaNO_3 and NaNO_2 which had undergone the same procedure as the plasma samples. $\text{NO}_3 + \text{NO}_2$ concentrations of the plasma samples were estimated from the NaNO_3 standard curve and corrected for the percentage NO_3 reduction to NO_2 . The mean percentage NO_3 reduction to NO_2 was $72.7\% \pm 2.2$ (range 64–79). The mean correlation coefficients of the NaNO_3 and NaNO_2 standard curves were 0.998 ± 0.001 (range 0.992–1.000) and 0.997 ± 0.001 (range 0.990–0.999) respectively.

Trabecular bone mineral density

TBMD (mg/cc) was measured directly in the distal metaphyseal part of the right femur by peripheral quantitative computed tomography (pQCT) adapted for measurements in small animals (Stratec XCT-960A, Stratec, Birkenfeld, Germany). A 360° X-ray scan, which had a standard thickness of 1 mm was taken. The scan had a resolution of 0.148×0.148 mm and was taken at 5.5 mm from the distal end of the femur, where TBMD of the metaphyseal part was measured. Intra- and interassay variation for the measurements were 3%. The XCT-960A was calibrated with a standard of hydroxyapatite embedded in acrylic plastic.

Statistics

Statistical analysis was performed by one-way ANOVA for multiple comparison followed by Fisher's PLSD (least significant difference test) test. Values are expressed as

Table 1 Plasma oestradiol levels and uterine weights of sham-operated and ovariectomized rats receiving various hormonal treatments

Treatment	Dose	17 β -oestradiol plasma levels (pg/ml)	Uterine weight (mg)
SHAM and vehicle	–	18.3 $\pm$ 7.2	531.1 $\pm$ 21.1
OVX and vehicle	–	6.0 $\pm$ 0.7	133.5 $\pm$ 11.5*
OVX and EE	2 $\times$ 2.5	ND	380.2 $\pm$ 53.7*†
(μ g/rat per day, orally)	2 $\times$ 10	ND	443.7 $\pm$ 30.0*†
	2 $\times$ 40	ND	450.8 $\pm$ 22.6*†
OVX and 17 β -oestradiol	2 $\times$ 32	10.0 $\pm$ 3.7	205.9 $\pm$ 19.5*
(μ g/rat per day, orally)	2 $\times$ 125	11.8 $\pm$ 2.4	396.9 $\pm$ 28.0*†
	2 $\times$ 250	18.3 $\pm$ 0.6	378.2 $\pm$ 21.9*†
	2 $\times$ 500	37.1 $\pm$ 5.3*†	468.4 $\pm$ 28.5*†
OVX and 17 β -oestradiol	2 $\times$ 2.5	ND	514.7 $\pm$ 25.1†
(μ g/rat per day, s.c.)			
SHAM and ICI164,384	2 $\times$ 2	244 $\pm$ 102.0*	173.3 $\pm$ 7.3*
(mg/rat per day, s.c.)			
OVX and progestagen (Org 2058)	2 $\times$ 2.5	ND	150.0 $\pm$ 6.8*
(μ g/rat per day, s.c.)	2 $\times$ 10	ND	161.0 $\pm$ 12.2*
	2 $\times$ 40	ND	155.4 $\pm$ 12.5*
SHAM and RU 486	2 $\times$ 2	29.5 $\pm$ 11.5	530.3 $\pm$ 65.6
(mg/rat per day, orally)			
OVX and progestagen and EE	2 $\times$ 40 and	ND	404.2 $\pm$ 12.7*†
(μ g/rat per day, s.c. and orally respectively)	2 $\times$ 40		
OVX and tibolone	2 $\times$ 500	7.1 $\pm$ 2.4	379.9 $\pm$ 11.0*†
(μ g/rat per day, orally)			

*Significant versus SHAM ($P < 0.05$); †significant versus OVX ($P < 0.05$). ICI164,384, anti-oestrogen; RU 486, anti-progesterone; ND, not determined.

percentage $\pm$ S.E.M. of the mean value measured for the sham-operated group. Statistical analysis was performed for each group to the corresponding sham-operated or ovariectomized group within that experiment and values were considered to be significantly different if $P < 0.05$.

Results

In all experiments, OVX induced statistically significant decreases in uterine weight (Table 1), NO₃+NO₂ plasma levels (Figs 1A, 2A, 3A and 4A) and TBMD (Figs 1B, 2B, 3B and 4B). Four weeks after surgery, the mean plasma NO₃+NO₂ levels of the sham-operated and ovariectomized groups were 33.6 $\pm$ 1.8 μ M (range 26.1–40.0) and 23.1 $\pm$ 0.8 μ M (range 21.3–24.3) respectively. The mean TBMD values of the sham-operated and ovariectomized groups were 339.1 $\pm$ 28.4 mg/cc (range 256.1–461.7) and 166.5 $\pm$ 23.4 mg/cc (range 104.0–245.1) respectively.

Treatment of ovariectomized rats with EE prevented the decrease in uterine weight (Table 1), NO₃+NO₂ plasma levels and TBMD in a dose-dependent manner (Fig. 1). This effect was statistically significant with EE doses 2 $\times$ 10 μ g/rat/day or higher.

Treatment of ovariectomized rats with oral 17 β -oestradiol (up to 2 $\times$ 500 μ g/rat/day) was associated with a dose-dependent increase in plasma oestrogen level, uterine weight (Table 1), and TBMD (Fig. 2B). This treatment, however, did not prevent the decrease in

plasma NO₃+NO₂ levels induced by OVX (Fig. 2A). In contrast, administration of 17 β -oestradiol subcutaneously (2 $\times$ 2.5 μ g/rat/day) prevented the decrease in uterine weight (Table 1) and TBMD (Fig. 2B) in addition to the decrease in plasma NO₃+NO₂ levels in the ovariectomized rats (Fig. 2A).

The anti-oestrogen ICI164,384 (2 $\times$ 2 mg/rat/day, s.c.) given to sham-operated rats reduced uterine weights (Table 1) and TBMD (Fig. 2B) compared with sham-operated rats treated with control solution, but had no effect on the plasma levels of NO₃+NO₂ (Fig. 2A). As expected, plasma oestrogen levels of rats treated with ICI164,384 increased by about 13-fold (Table 1).

A discrepancy between plasma NO₃+NO₂ levels and TBMD was also found after progestagen treatment. Progestagen (2 $\times$ 40 μ g/rat/day, s.c.) given to ovariectomized rats prevented the decrease in plasma NO₃+NO₂ levels in a dose-dependent manner (Fig. 3A), but had no effect on TBMD which remained low (Fig. 3B). As expected, progestagen treatment did not prevent the decrease in uterine weight after OVX (Table 1). Treatment of sham-operated rats with the anti-progestagen RU 486 (2 $\times$ 2 mg/rat/day, orally) had no effect on uterine weight (Table 1) and TBMD (Fig. 3B), but tended to decrease plasma NO₃+NO₂ levels (Fig. 3A).

Concurrent administration of effective doses of progestagen (2 $\times$ 40 μ g/rat/day, s.c.) and EE (2 $\times$ 40 μ g/rat/day, orally) partly prevented the decrease in plasma NO₃+NO₂ levels after OVX (Fig. 4A). This treatment,

Figure 1 Plasma NO₃+NO₂ levels and TBMD of sham-operated and ovariectomized rats receiving EE. Effect of EE treatment (µg/rat/day, orally) on (A) plasma NO₃+NO₂ levels and (B) TBMD 4 weeks after OVX (n=6). *Significant versus SHAM ($P<0.05$); #Significant versus OVX ($P<0.05$). SHAM, sham-operation.

however, prevented the decrease in uterine weight (Table 1) and TBMD (Fig. 4B) in ovariectomized rats. Treatment of ovariectomized rats with tibolone (2 × 500 µg/rat/day, orally), a synthetic steroid with combined weak oestrogenic, progestagenic, and androgenic properties, prevented partly the OVX-induced decrease in plasma NO₃+NO₂ levels (Fig. 4A) and totally the decrease in TBMD (Fig. 4B). Tibolone had a partial effect on uterine weight after OVX (Table 1).

The effects of the various hormonal treatments on both plasma NO₃+NO₂ levels and TBMD are summarized in Table 2.

Figure 2 Plasma NO₃+NO₂ levels and TBMD of sham-operated and ovariectomized rats receiving 17β-oestradiol or anti-oestrogen. Effect of 17β-oestradiol (orally or s.c., µg/rat/day) and anti-oestrogen (s.c., mg/rat/day) treatment on (A) plasma NO₃+NO₂ levels and (B) TBMD 4 weeks after surgery. SHAM, n=24; OVX, n=20; OVX and 17β-E2 (2 × 32), n=5; OVX and 17β-E2 (2 × 125), n=5; OVX and 17β-E2 (2 × 250), n=4; OVX and 17β-E2 (2 × 500) n=10; OVX and 17β-E2 (s.c., 2 × 2.5), n=8; SHAM and anti-oestrogen (ICI164,384, 2 × 2), n=6. *Significant versus SHAM ($P<0.05$); #Significant versus OVX ($P<0.05$). 17β-E2, 17β-oestradiol; SHAM, sham-operation; Data from four experiments.

Discussion

Previous studies in premenopausal women have shown that plasma NO₃+NO₂ levels increase during follicular

Figure 3 Plasma NO₃+NO₂ levels and TBMD of sham-operated and ovariectomized rats receiving progestagen or anti-progesterone. Effect of progestagen (µg/rat/day, s.c.) and anti-progesterone (RU 486, 2 × 2 mg/rat/day, orally) treatment on (A) plasma NO₃+NO₂ levels and (B) TBMD 4 weeks after surgery. SHAM, n=12; OVX, n=12; OVX and progestagen, n=6; SHAM and RU 486, n=6. *Significant versus SHAM (P<0.05); RU 486, anti-progesterone; SHAM, sham-operation. Data from two experiments.

development, peak at mid cycle and are correlated with plasma 17β-oestradiol levels (Rosselli *et al.* 1994, Cicinelli *et al.* 1996, Kawano *et al.* 1996). In addition, peak expiratory NO concentrations were found to be significantly higher at mid cycle than during menstruation (Kharitonov *et al.* 1994). Postmenopausal women, on the other hand, had lower plasma NO₃+NO₂ levels;

Figure 4 Plasma NO₃+NO₂ levels and TBMD of sham-operated and ovariectomized rats receiving progestagen in combination with EE or tibolone. Effect of treatment with progestagen (2 × 40 µg/rat/day, s.c.) in combination with EE (2 × 40 µg/rat/day, orally), or treatment with tibolone (2 × 500 µg/day/rat, orally) on (A) plasma NO₃+NO₂ levels and (B) TBMD 4 weeks after OVX. SHAM, n=18; OVX, n=16; OVX and progestagen and EE, n=6; OVX and tibolone, n=12. Significant versus SHAM (P<0.05); #Significant versus OVX (P<0.05). SHAM, sham-operation. Data from three experiments.

20.2 ± 2.4 µM compared with 38 ± 3.0 µM in premenopausal women (Rosselli *et al.* 1995). The latter differences are very similar to those found in the present study between ovariectomized and sham-operated rats (23.1 ± 0.8 µM versus 33.6 ± 1.8 µM). Taken together,

Table 2 Effects of various hormonal treatments on NO₃+NO₂ plasma levels and TBMD in rats

Treatment	NO ₃ +NO ₂ plasma levels	TBMD
OVX and vehicle	↓↓	↓↓
OVX and EE	=	=
OVX and 17β-oestradiol (oral)	↓↓	=
OVX and 17β-oestradiol (s.c.)	=	=
SHAM and anti-oestrogen	=	↓
OVX and progestagen	=	↓↓
SHAM-operation and anti-progesterone	↓	=
OVX and progestagen and EE	↓	=
OVX and tibolone	↓	=

Compared to SHAM: no change (=); partially decreased (↓); decreased (↓↓).

these results strongly suggest that NO₃+NO₂ plasma levels are regulated by ovarian steroids. In addition, in all experiments OVX was associated with parallel decreases in plasma NO₃+NO₂ levels and in TBMD.

Plasma NO₃+NO₂ levels have been found to reflect endogenous NO production in humans receiving a low NO₃+NO₂ diet (Hibbs *et al.* 1992). In rats, lipopolysaccharide (LPS) treatment induced an increase in NO₃+NO₂ plasma levels that was prevented by L-NAME, suggesting that NO₃+NO₂ plasma levels reflect endogenous NO production (Shultz & Rajj 1992). The short half-life of approximately 5 h of NO₃ in plasma (Wagner *et al.* 1983) and the excretion of dietary NO₃ in the urine within 18 h of intake (Wasserman 1978), allowed us to determine the plasma NO₃+NO₂ levels after an overnight fasting period in rats receiving a regular diet. Indeed, the plasma NO₃+NO₂ levels (33.6 ± 1.8 µM) in sham-operated rats in the present study are in agreement with plasma concentrations (38 µM) in virgin rats receiving a low NO₃+NO₂ diet or a regular diet with sampling about 12 h after eating (Conrad *et al.* 1993).

Wimalawansa *et al.* (1996) postulated recently that a decrease in NO production contributes to bone loss following OVX in rats as treatment of ovariectomized rats with the NO donor nitroglycerine was shown to prevent bone loss. In addition, administration of the NOS inhibitor L-NAME abolished the preventive effect of 17β-oestradiol on bone loss, while it had no effect when given alone. Kasten *et al.* (1994), however, previously reported that inhibition of NOS by the relatively specific inducible NOS inhibitor aminoguanidine (Misko *et al.* 1993, Joly *et al.* 1994) decreased BMD regardless of oestrogen status in rats. In addition, administration of aminoguanidine to rats decreased BMD (Tsukahara *et al.* 1996) and reduced bone formation rate (Turner *et al.* 1997). The NOS inhibitors L-NMMA and L-NAME prevented the increase in cancellous bone formation by mechanical stimulation in rats (Fox *et al.* 1996, Turner *et al.* 1996). These findings raise the question whether the modulation in NO pro-

duction induced by ovarian steroids and the subsequent changes in BMD are related. In the present study, we addressed this issue by measuring plasma NO₃+NO₂ levels and TBMD in rats receiving various hormonal treatments.

Treatment with oral EE or subcutaneous 17β-oestradiol increased plasma NO₃+NO₂ levels following OVX in rats – a result that is similar to the increase in plasma NO₃+NO₂ levels reported after treatment of postmenopausal women with transdermal 17β-oestradiol (Rosselli *et al.* 1995) or premenopausal women with a non-specified oestrogen preparation (Ramsay *et al.* 1995). Both oral EE or subcutaneous 17β-oestradiol also prevented the decrease in TBMD following OVX in rats. However, treatment with oral 17β-oestradiol up to 2 × 500 µg/rat/day, while exerting all expected oestrogenic effects on uterine weight and TBMD, had no effect on plasma NO₃+NO₂ levels which remained at the OVX level. This may be due to differences in the activities of metabolites of 17β-oestradiol generated after passage of the liver on uterus and bone and on cells responsible for endogenous NO production. EE is relatively slowly transformed into inactive metabolites in the liver (Kuhl 1990) and may, therefore, be active on endogenous NO production after oral administration.

Treatment with the anti-oestrogen ICI164,384 diminished TBMD to about half the decrease seen after OVX and decreased uterine weight to a level similar to that found in ovariectomized rats. Anti-oestrogen treatment, however, had no effect on plasma NO₃+NO₂ levels. These results also revealed a dissociation between circulating NO₃+NO₂ plasma levels and TBMD. While there was a clear relation between the changes in oestrogen plasma levels and TBMD, the present data suggest that another ovarian steroid besides oestrogen may be involved in the regulation of plasma NO₃+NO₂ levels. This may be progesterone, as progestagen treatment prevented the OVX-induced decrease in NO₃+NO₂ dose-dependently. In humans, progesterone serum levels did not correlate with NO₃+NO₂ plasma levels (Rosselli *et al.* 1994), but most of the progesterone plasma concentrations in that study were within a narrow range (1–3 nM). In later studies by the same group, treatment of postmenopausal women with both oestrogen and progesterone induced a slight elevation in plasma NO₃+NO₂ levels, while oestrogen alone increased these levels significantly (Rosselli *et al.* 1995, Imthurn *et al.* 1997). Plasma NO₃+NO₂ levels are lower in the secretory phase of the menstrual cycle than at the mid cycle and it has been suggested that this may be due to increased progesterone production during the secretory phase (Cicinelli *et al.* 1996, Kawano *et al.* 1996). These observations correspond well with the finding in the present study that concurrent administration of oestrogen and progestagen to ovariectomized rats partly prevented the decrease in plasma NO₃+NO₂ levels. In addition, tibolone, a synthetic steroid with weak oestrogenic, androgenic, and progestational activity (Tax *et al.* 1987, van der

Vies 1987), slightly elevated plasma $\text{NO}_3 + \text{NO}_2$ levels in ovariectomized rats but did not prevent the OVX-induced decrease. Taken together, these results suggest that the combination of oestrogen and progesterone decreases plasma $\text{NO}_3 + \text{NO}_2$ levels.

The effect of progesterone on bone is still controversial. Although short term positive effects of progesterone on bone have been reported in both humans (Abdalla *et al.* 1985, consensus report by Whitehead & Lobo 1988, reviewed by Prior 1990) and rodents (Barbagallo *et al.* 1989, Barengolts *et al.* 1990), other studies failed to demonstrate a positive effect of progesterone (Isserow *et al.* 1995). While progesterone has been reported to abolish the protective effect of oestrogen against bone mineral loss after OVX in rats (Barbagallo *et al.* 1989), no negative effect of progestagen on the bone sparing effect of oestrogen was observed in the present study. The available data in humans indicate that the addition of progestagen to oestrogen therapy does not influence the skeletal response (consensus report by Whitehead & Lobo 1988). Treatment with tibolone completely prevented bone loss after OVX in the present study. This protective effect of tibolone against bone loss has also been reported in postmenopausal women (Lindsay *et al.* 1980, Fogelman *et al.* 1981, Rymer *et al.* 1994, Lyritis *et al.* 1995, Bjarnason *et al.* 1996).

In conclusion, OVX decreased both TBMD and plasma $\text{NO}_3 + \text{NO}_2$ levels in rats. These two parameters, however, did not correlate with each other as they were differently affected by various hormonal treatments. While plasma $\text{NO}_3 + \text{NO}_2$ levels were regulated by both oestrogen and progesterone, TBMD was affected by changes in oestrogen levels only. Decreased systemic NO production, therefore, is not involved in bone loss after OVX. It is possible, however, that a reduction in local NO production within the bone microenvironment may be involved.

Acknowledgement

The authors thank H D Petronilia, M Scheepers, C Spanjers, and A Weekers for their technical assistance.

References

- Abdalla HI, McKay Hart D, Lindsay R, Leggate I & Hooke A 1985 Prevention of bone mineral loss in postmenopausal women by norethisterone. *Obstetrics and Gynecology* **66** 789–792.
- Baulieu EE 1989 Contraception and other clinical applications of RU 486, an antiprogesterone at the receptor. *Science* **245** 1351–1357.
- Barbagallo M, Carbognani A, Palummeri E, Chiavarini M, Pedrazzoni M, Bracchi PG & Passeri M 1989 The comparative effects of ovarian hormone administration on bone mineral status in oophorectomized rats. *Bone* **10** 113–116.
- Barengolts EI, Gajardo HF, Rosol TJ, D'anza JJ, Pena M, Botsis J & Kukreja SC 1990 Effects of progesterone on postovariectomy bone loss in aged rat. *Journal of Bone and Mineral Research* **5** 1143–1147.
- Bjarnason NH, Bjarnason K, Haarbo J, Rosenquist C & Christiansen C 1996 Tibolone: prevention of bone loss in late postmenopausal women. *Journal of Clinical Endocrinology and Metabolism* **81** 2419–2422.
- Cicinelli E, Ignarro LJ, Lograno M, Galantino P, Balzano G & Schonauer LM 1996 Circulating levels of nitric oxide in fertile women in relation to the menstrual cycle. *Fertility and Sterility* **66** 1036–1038.
- Conrad KP, Joffe GM, Kruszyna H, Kruszyna R, Rochelle LG, Smith RP, Chavez JE & Mosher MD 1993 Identification of increased nitric oxide biosynthesis during pregnancy in rats. *FASEB Journal* **7** 566–571.
- Damoulis PD & Hauschka PV 1994 Cytokines induce nitric oxide production in mouse osteoblasts. *Biochemical and Biophysical Research Communications* **201** 924–931.
- Fogelman I, Bessent RR, Smith ML, Hart DM & Lindsay R 1981 Assessment of synthetic steroid (Org OD14): effect on skeletal metabolism by 24 h whole-body retention of disphosphonate. *Maturitas* **3** 265–269.
- Fox SW, Chambers TJ & Chow JWM 1996 Nitric oxide is an early mediator of the increase in bone formation by mechanical stimulation. *American Journal of Physiology* **270** E955–E960.
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS & Tannanbaum SR 1982 Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Analytical Biochemistry* **126** 131–138.
- Hayashi T, Fukuto JM, Ignarro LJ & Chaudhuri G 1992 Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: implications for atherosclerosis. *Proceedings of the National Academy of Sciences of the USA* **89** 11259–11263.
- Hayashi T, Yamada K, Esaki T, Kuzuya M, Satake S, Ishikawa, Hidaka H & Iguchi A 1995 Oestrogen increases endothelial nitric oxide by a receptor-mediated system. *Biochemical and Biophysical Research Communications* **214** 847–855.
- Hibbs JB, Westenfelder C, Taintor R, Vavrin Z, Kablitz C, Baranowski RL, Ward JH, Menlove RL, McMurray MP, Kushner JP & Samlowski WE 1992 Evidence for cytokine-inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy. *Journal of Clinical Investigation* **89** 867–877.
- Hukkanen M, Hughes FJ, Buttery LDK, Gross SS, Evans TJ, Seddon S, Riveros-Moreno V, MacIntyre I & Polak JM 1995 Cytokine-stimulated expression of inducible nitric oxide synthase by mouse, rat, and human osteoblast-like cells and its functional role in osteoblast metabolic activity. *Endocrinology* **136** 5445–5453.
- Imthurn B, Rosselli M, Jaeger AW, Keller PJ & Dubey RK 1997 Differential effects of hormone-replacement therapy on endogenous nitric oxide (nitrite/nitrate) levels in postmenopausal women substituted with 17 β -oestradiol valerate and cyproterone acetate or medroxyprogesterone acetate. *Journal of Clinical Endocrinology and Metabolism* **82** 388–394.
- Isserow JA, Rucinski B, Romero DF, Mann GN, Liu CC & Epstein S 1995 The effect of medroxyprogesterone acetate on bone metabolism in the oophorectomized, tamoxifen-treated rat. *Endocrinology* **136** 713–719.
- Joly GA, Ayres M, Chelly F & Kilbourn RG 1994 Effects of N^G -methyl-L-arginine, N^G -nitro-L-arginine, and aminoguanidine on constitutive and inducible nitric oxide synthase in rat aorta. *Biochemical and Biophysical Research Communications* **199** 147–154.
- Kasten TP, Collin-Osdoby P, Patel N, Osdoby P, Krukowski M, Misko TP, Settle SL, Currie MG & Nickols GA 1994 Potentiation of osteoclast bone-resorption activity by inhibition of nitric oxide synthase. *Proceedings of the National Academy of Sciences of the USA* **91** 3569–3573.
- Kawano H, Motoyama K, Kugiyama K, Hirashima O, Ohgushi M, Yoshimura M, Ogawa H, Okumura K & Yasue H 1996 Menstrual cyclic variation of endothelium-dependent vasodilation of the brachial artery: possible role of oestrogen and nitric oxide. *Proceedings of the Association of American Physicians* **108** 473–480.

- Kharitonov SA, Logan-Sinclair RB, Busset CM & Shinebourne EA 1994 Peak expiratory nitric oxide differences in men and women: relation to the menstrual cycle. *British Heart Journal* **72** 243–245.
- Kuhl H 1990 Pharmacokinetics of oestrogens and progestogens. *Maturitas* **12** 171–197.
- Lindsay R, Hart DM & Kraszewski A 1980 Prospective double-blind trial of synthetic steroid (Org OD14) for preventing postmenopausal osteoporosis. *British Medical Journal* **280** 1207–1209.
- Löwik CWGM, Nibbering PH, van de Ruit M & Papapoulos SE 1994 Inducible production of nitric oxide in osteoblast-like cells and in fetal mouse bone explants is associated with suppression of osteoclastic bone resorption. *Journal of Clinical Investigation* **93** 1465–1472.
- Lyrakis GP, Karpathios S, Basdekis K, Grigoriou O, Katostaras T, Paspatis T, Stamatiopoulos T, Siampalioti G & Lyrakis PG 1995 Prevention of post-oophorectomy bone loss with tibolone. *Maturitas* **22** 247–253.
- MacIntyre I, Zaidi M, Alam ASMT, Datta HK, Moonga BS, Lidbury PS, Hecker M & Vane JR 1991 Osteoclastic inhibition: an action of nitric oxide not mediated by cyclic GMP. *Proceedings of the National Academy of Sciences of the USA* **88** 2936–2940.
- Misko TP, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, McDaniel ML, Williamson JR & Currie MG 1993 Selective inhibition of the inducible nitric oxide by aminoguanidine. *European Journal of Pharmacology* **233** 119–125.
- Prior JC 1990 Progesterone as a bone-tropic hormone. *Endocrine Reviews* **11** 386–398.
- Ralston SH & Grabowski PS 1996 Mechanisms of cytokine induced bone resorption: role of nitric oxide, cyclic guanosine monophosphate, and prostaglandins. *Bone* **19** 29–33.
- Ralston SH, Todd D, Helfrich M, Benjamin N & Grabowski PS 1994 Human osteoblast-like cells produce nitric oxide and express inducible nitric oxide synthase. *Endocrinology* **135** 330–336.
- Ralston SH, Ho LP, Helfrich MH, Grabowski PS, Johnston PW & Benjamin N 1995 Nitric oxide: a cytokine-induced regulator of bone-resorption. *Journal of Bone and Mineral Research* **10** 1040–1049.
- Ramsay B, Johnson MR, Leone AM & Steer PJ 1995 The effect of exogenous oestrogen on nitric oxide production in women: a placebo controlled crossover study. *British Journal of Obstetrics and Gynaecology* **102** 417–419.
- Riancho JA, Salas E, Zarrabeitia MT, Olmos JM, Amado JA, Fernández-Luna JL & González-Macías J 1995 Expression and functional role of nitric oxide synthase in osteoblast-like cells. *Journal of Bone and Mineral Research* **10** 439–446.
- Rosselli M, Imthurn B, Macas E, Keller PJ & Dubey RK 1994 Circulating nitrite/nitrate levels increase with follicular development: indirect evidence for oestradiol mediated NO release. *Biochemical and Biophysical Research Communications* **202** 1543–1552.
- Rosselli M, Imthurn B, Keller PJ, Jackson EK & Dubey RK 1995 Circulating nitric oxide (nitrite/nitrate) levels in postmenopausal women substituted with 17 β -oestradiol and norethisterone acetate: a two-year follow-up study. *Hypertension* **25** 848–853.
- Rymer J, Chapman MG & Fogelman I 1994 Effect of tibolone on postmenopausal bone loss. *Osteoporosis International* **4** 314–319.
- Shultz PJ & Raji L 1992 Endogenously synthesized nitric oxide prevents endotoxin-induced glomerular thrombosis. *Journal of Clinical Investigation* **90** 1718–1725.
- Stern PH & Diamond J 1992 Sodium nitroprusside increases cyclic GMP in fetal rat bone cells and inhibits resorption of fetal rat limb bones. *Research Communications in Chemical Pathology and Pharmacology* **75** 19–28.
- Tax L, Goorissen EM & Kicovic PM 1987 Clinical profile of Org OD14. *Maturitas* **9** 3–13.
- Tsukahara H, Miura M, Tsuchida S, Hata I, Hata K, Yamamoto K, Ishii Y, Muramatsu I & Sudo M 1996 Effect of nitric oxide inhibitors on bone metabolism in growing rats. *American Journal of Physiology* **270** E840–E845.
- Turner HC, Takano Y, Owan I & Murrell GAC 1996 Nitric oxide inhibitor L-NAME suppresses mechanically induced bone formation in rats. *American Journal of Physiology* **270** E634–E639.
- Turner HC, Owan I, Jacob DS, McClintock R & Peacock M 1997 Effects of nitric oxide synthase inhibitors on bone formation in rats. *Bone* **21** 487–490.
- Vies van der J 1987 Pharmacological studies with (7 α ,17 α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one (Org OD14). *Maturitas* (Suppl 1) 15–24.
- Visser de J, Coert A, Feenstra H & Vies van der J 1984 Endocrinological studies with (7 α ,17 α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one (Org OD14). *Arzneimittel Forschung/Drug Research* **34** 1010–1017.
- Wagner DA, Schultz DS, Deen WM, Young VR & Tannenbaum SR 1983 Metabolic fate of an oral dose of ¹⁵N-labeled nitrate in humans: effect of diet supplementation with ascorbic acid. *Cancer Research* **43** 1921–1925.
- Wakeling AE & Bowler J 1987 Steroidal pure antioestrogens. *Journal of Endocrinology* **112** R7–R10.
- Wasserman AE 1978 The nitrate–nitrosamine situation: a review. *Food Engineering* **50** 110–116.
- Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG & Moncada S 1994 Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proceedings of the National Academy of Sciences of the USA* **91** 5212–5216.
- Whitehead M & Lobo RA 1988 Progestagen use in postmenopausal women. *Lancet* **26** 1243–1244.
- Wimalawansa SJ, De Marco G, Gangula P & Yallampalli C 1996 Nitric oxide donor alleviates ovariectomy-induced bone loss. *Bone* **18** 301–304.
- Yallampalli C, Byam-Smith M, Nelson SO & Garfield RE 1994 Steroid hormones modulate the production of nitric oxide and cGMP in the rat uterus. *Endocrinology* **134** 1971–1974.

Received 26 January 1998

Accepted 30 April 1998