

# Acute effects of tibolone on cerebral vascular reactivity *in vitro*

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## ABSTRACT

**Objective** To evaluate the acute effects of tibolone and its metabolites on cerebral vascular reactivity *in vitro*.

**Methods** Ring segments of the posterior cerebral artery from female rabbits were mounted in myographs for isometric tension recordings. Concentration–response curves with tibolone, 3 $\alpha$ -OH-tibolone, 3 $\beta$ -OH-tibolone,  $\Delta^4$  isomer and 17 $\beta$ -estradiol were obtained before and after addition of the NO blocker N<sup>o</sup>-nitro-L-arginine methyl ester (L-NAME, 10<sup>−4</sup> mol/l) or the potassium-channel blocker tetraethylammonium chloride (TEA, 10<sup>−2</sup> mol/l). Additionally, the effects of the hormones on the concentration–response curves with calcium were examined.

**Results** Tibolone and its metabolites induced a concentration-dependent relaxation comparable to that of 17 $\beta$ -estradiol (area under the curve (AUC); tibolone vs. 17 $\beta$ -estradiol: 242 vs. 251;  $p < 0.05$ , analysis of variance). L-NAME increased the AUC for all substances compared with controls ( $p < 0.05$ , Student's  $t$  test), except for 17 $\beta$ -estradiol. Preincubation with TEA induced no changes. The concentration-dependent contraction curves with calcium were shifted rightward by all hormones.

**Conclusions** The study demonstrates that the acute relaxation induced by tibolone and its metabolites in cerebral arteries *in vitro* is comparable to that with 17 $\beta$ -estradiol, and seems to be mediated by inhibition of voltage-gated calcium channels and possibly partly by a nitric oxide-dependent mechanism.

## INTRODUCTION

During the reproductive years, the overall incidence of cerebrovascular disease is significantly lower in females than in males<sup>1</sup>. The importance of sex steroids in this relative protection is unclear, but the differences between female and male diminish with increasing age<sup>2</sup>. Although some observational studies indicate that hormone replacement therapy may reduce stroke risk, the existing data are inconsistent<sup>3</sup>, with two of the largest studies reporting either a neutral<sup>4</sup> or an increased risk<sup>5</sup>.

In contrast, a substantial number of biological studies in humans and animals indicate a beneficial long-term cerebrovascular effect of estrogen. Cerebral vessel flow resistance, measured by pulsatility index, increases with time after the menopause<sup>6</sup>, and is significantly reduced by estrogen replacement therapy<sup>7</sup>. 17 $\beta$ -Estradiol promotes vasodilatation in cerebral arteries by an enhanced basal<sup>8</sup>, as well as agonist-stimulated, nitric oxide (NO) release<sup>9</sup>. Estrogen may also promote ischemic neuroprotection, and has been

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shown to improve recovery of blood flow in experimental cerebral ischemia<sup>10,11</sup> and to decrease lesion size following stroke<sup>11</sup>.

The vasodilatory effects of estrogen are partly mediated by acute, non-genomic effects on vascular tone<sup>12</sup>, which have mainly been described in non-cerebral arteries. Several studies have indicated an endothelium-independent mechanism via inhibition of voltage-dependent calcium ion channels<sup>9,13–15</sup>, or via stimulation of K<sup>+</sup> efflux<sup>16</sup>. At the same time, a role of the endothelium and NO is supported by observations in animal studies<sup>17,18</sup>, where inhibition of nitric oxide synthesis or endothelial denudation reduces estradiol-induced relaxation.

Tibolone (Livial®, NV Organon, Oss, The Netherlands) is a synthetic steroid which is used, as an alternative to estrogen replacement, for the treatment of climacteric symptoms<sup>19</sup> and the prevention of postmenopausal osteoporosis<sup>20</sup>. Tibolone is quickly metabolized *in vivo* into its three main active metabolites, 3 $\alpha$ - and 3 $\beta$ -OH-tibolone and the  $\Delta^4$  isomer<sup>21</sup>. The 3-OH metabolites bind only to the estrogen receptor, while the  $\Delta^4$  isomer shows affinity only to the progesterone and androgen receptors. Tibolone itself binds to all three receptors<sup>21</sup>. The effects of tibolone on cerebral arteries have not yet been investigated, but acute administration of tibolone in ewes resulted in a dose-dependent increase in coronary blood flow combined with a decrease in coronary vascular resistance<sup>22</sup>.

The impact of two recently published clinical trials<sup>23,24</sup> highlights the importance of alternatives to conventional hormone replacement therapy. We have therefore investigated the effects of tibolone and its metabolites on cerebral vascular reactivity using an *in vitro* animal model.

## METHODS

Forty adult female non-pregnant New Zealand White rabbits, with a mean weight of 2.99 kg (range 2.7–3.3 kg), were sacrificed using an overdose of pentobarbital (150 mg/kg). The thorax was cut open and the inferior caval vein was transected just below the heart for exsanguination, and the central arterial system was perfused with approximately 300 ml 0.9% NaCl through a canula inserted into the left ventricle. The skull was opened with an oscillating saw and the brain was removed and immersed in ice-cold Krebs buffer, bubbled with 5% CO<sub>2</sub> + 95% O<sub>2</sub>, to reduce metabolism and anoxia. Throughout the subsequent dissection, the tissue was immersed in

cold Krebs buffer kept in a glass Petri dish placed on ice. The posterior cerebral arteries were micro-dissected and the connective tissue was carefully removed. Four arterial ring segments (1–2 mm in length) were prepared, two each from the right and left artery specimens. The vessels were mounted in myographs (Myo-Interface 500A; JP Trading, Aarhus, Denmark) for measurement of isometric tension. Following a 15-min equilibration period (37°C), the vessels were normalized, i.e. the internal circumference IC<sub>1</sub> was adjusted to 90% of IC<sub>100</sub>, where IC<sub>100</sub> is the internal circumference corresponding to a transmural pressure of 13.3 kPa (100 mmHg) of the relaxed vessel *in situ*<sup>25</sup>. After normalization, the vessels were subjected to a routine run-up procedure (4 × 5-min exposure to 124 mmol/l K<sup>+</sup> Krebs buffer) to check the mechanical condition of each vessel segment. Segments producing a response of less than 1 N/m were excluded. In all tissues, except the vessels used for calcium concentration responses, the presence of endothelium was verified by a relaxation response to carbamylcholine chloride or substance P. At the end of each protocol, the arterial ring preparations were washed with calcium-free Krebs buffer to define 100% relaxation. Experimental protocols and vessel segments were randomly assigned to the myographs. Sixteen vessel segments were excluded from analysis, owing to a defective mechanical condition, lack of endothelial function or protocol failure. The artery segments were subjected to one of the following experimental protocols:

- (1) Following precontraction with 30 mmol/l K<sup>+</sup> Krebs buffer, concentration–response curves with tibolone, its metabolites ( $3 \times 10^{-8}$ – $1 \times 10^{-4}$  mol/l), 17 $\beta$ -estradiol ( $3 \times 10^{-8}$ – $4.7 \times 10^{-5}$  mol/l) or vehicle control, using equivalent volumes of ethanol, were established in separate vessel segments.
- (2) As in protocol (1), but precontraction with 30 mmol/l K<sup>+</sup> Krebs buffer was preceded by a 20-min incubation with either N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME,  $10^{-4}$  mol/l, an inhibitor of NO synthesis in vascular endothelial cells), tetraethylammonium chloride (TEA,  $10^{-2}$  mol/l, a non-specific inhibitor of potassium channels) or ICI<sub>172780</sub> ( $10^{-6}$  mol/l, an unspecific estrogen receptor antagonist).
- (3) To establish the effect of the various hormones on the calcium concentration–response curves, arterial segments were washed twice (10-min interval) with calcium-free Krebs

buffer containing 0.5 mmol/l ethylene glycol-bis-[ $\beta$ -aminoethyl ether] $N,N,N',N'$ -tetra-acetic acid (EGTA). After the second wash, the segments were incubated with tibolone ( $10^{-5}$  mol/l),  $3\alpha$ -OH-tibolone ( $10^{-5}$  mol/l),  $3\beta$ -OH-tibolone ( $10^{-5}$  mol/l), the  $\Delta^4$  isomer ( $10^{-5}$  mol/l or  $3 \times 10^{-5}$  mol/l), estradiol ( $10^{-5}$  mol/l) or vehicle (ethanol) for 20 min. Two different concentrations of the  $\Delta^4$  isomer were evaluated, as preliminary experiments indicated a lower potency of this substance. Phentolamine (Regitin®)  $3 \times 10^{-6}$  mol/l was added to the bath to inhibit any interfering contractile effect of noradrenaline released from perivascular nerves following depolarization with potassium. Concentration–response curves with  $\text{CaCl}_2$  ( $10^{-6}$ – $10^{-2}$  mol/l) were then determined in calcium-free, 100 mmol/l  $\text{K}^+$  Krebs buffer.

The mean lumen diameter of the vessel segments was  $514 \pm 49 \mu\text{m}$  (range 359–721  $\mu\text{m}$ ;  $n = 144$ ) and was comparable in the different protocols ( $p > 0.05$ , analysis of variance (ANOVA)).

## Drugs and solutions

The following drugs were used: TEA, L-NAME,  $17\beta$ -estradiol, substance P, carbamylcholine chloride (Sigma, Copenhagen, Denmark), calcium chloride (Hvidovre Hospital Pharmacy), EGTA (Hvidovre Hospital Pharmacy),  $\text{ICI}_{172780}$  (Tocris, Avonmouth, UK) and phentolamine (Regitin; Novartis, Copenhagen, Denmark). Tibolone and its metabolites were kindly provided by Dr H. J. Kloosterboer, NV Organon, The Netherlands. Stock solutions (all  $10^{-2}$  mol/l) of tibolone, its metabolites,  $17\beta$ -estradiol and  $\text{ICI}_{172780}$  were prepared with ethanol. Krebs buffer had the following composition (mmol/l): NaCl 119, KCl 4.6,  $\text{NaHCO}_3$  15,  $\text{CaCl}_2$  1.5,  $\text{MgCl}_2$  1.2,  $\text{NaH}_2\text{PO}_4$  1.2 and glucose 11. In calcium-free Krebs buffer,  $\text{CaCl}_2$  was omitted from the solution. In  $\text{K}^+$  Krebs buffers, NaCl was replaced by an equimolar amount of KCl.

## Data analysis

Data are expressed as mean  $\pm$  standard deviation (SD);  $n$  refers to the number of experiments. Changes in tension were measured as absolute changes in active wall tension (N/m). To adjust for differences in vessel size and reactivity, data were calculated as percentages of the precontraction induced by 30 mmol/l  $\text{K}^+$  (relaxation responses to the hormones) or as percentages of the maximal

contraction induced by 124 mmol/l  $\text{K}^+$  (contractile responses to  $\text{CaCl}_2$ ). Owing to poor solubility of the compounds, full sigmoidal concentration–response curves could rarely be obtained, and values of  $E_{\text{max}}$  (the theoretical maximal effect) and  $\text{EC}_{50}$  (the concentration of agonist required to produce a half-maximal response) could not be accurately determined. Instead, the area under the curve (AUC) was calculated for each experiment to obtain a measure of the cumulative drug effect. Differences between groups were analyzed using Student's unpaired  $t$  test or ANOVA (with Newman–Keuls multiple comparison test or Bonferroni's multiple comparison test), as appropriate, with  $p < 0.05$  as the level of statistical significance. All calculations and statistical analyses were performed with Prism Software, version 3.03 (GraphPad, San Diego, CA, USA).

## RESULTS

Tibolone, its metabolites and  $17\beta$ -estradiol induced significant concentration-dependent relaxation of the vessel segments precontracted with 30 mmol/l  $\text{K}^+$  (Figure 1). Stable responses were reached within 10–15 min after each addition. All substances induced nearly complete relaxation.

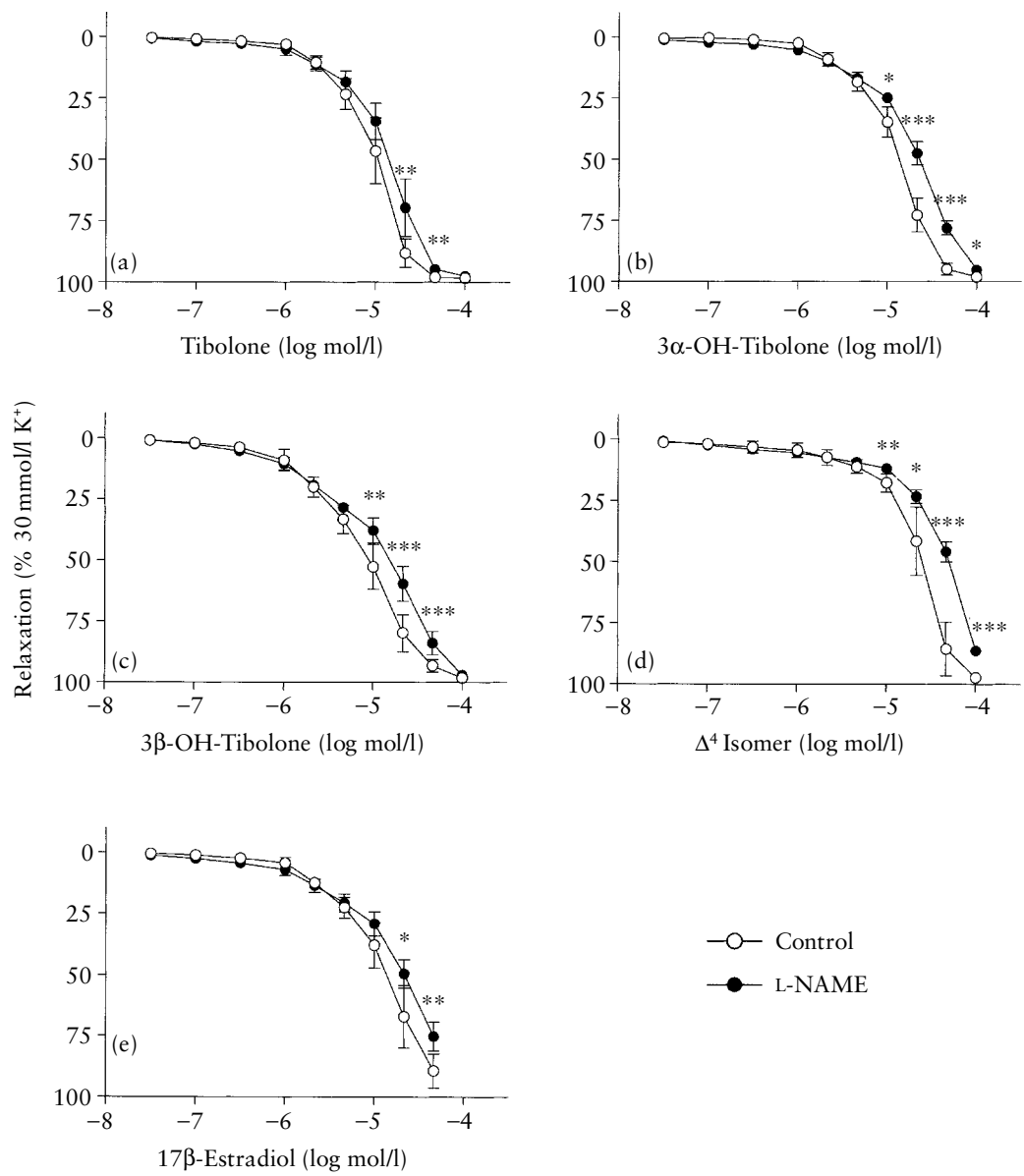
Estimated by AUC (Figure 2), the rank of potency of the substances was  $3\beta$ -OH-tibolone  $>$  tibolone  $\cong$   $17\beta$ -estradiol  $\cong$   $3\alpha$ -OH-tibolone  $>$   $\Delta^4$  isomer (ANOVA). Vehicle-only had no effect on vascular tone ( $n = 5$ ) (not shown).

The contractile response to potassium was unaffected by incubation with L-NAME. Addition of L-NAME resulted in a rightward shift of the concentration–response curves (Figure 1) and in significantly higher AUCs for all substances, except for  $17\beta$ -estradiol, compared with controls (Figure 2).

Incubation of the vessel segments with TEA had no significant influence on the  $\text{K}^+$  response or the concentration–response curves with any of the substances (data not shown). AUCs calculated from TEA curves were the same as those for controls (Figure 2).

A stable precontraction with 30 mmol/l  $\text{K}^+$  could not be obtained following incubation of the vessel segments with  $\text{ICI}_{172780}$ , as all ring segments relaxed spontaneously ( $n = 5$ ). Concentration–response curves therefore could not be obtained.

The maximal contraction with  $\text{K}^+$  124 mmol/l during the run-up procedure averaged  $2.91 \pm 0.97 \text{ mN/mm}$  (range 1.05–4.81 mN/mm;  $n = 45$ ) in the vessel segments used for calcium



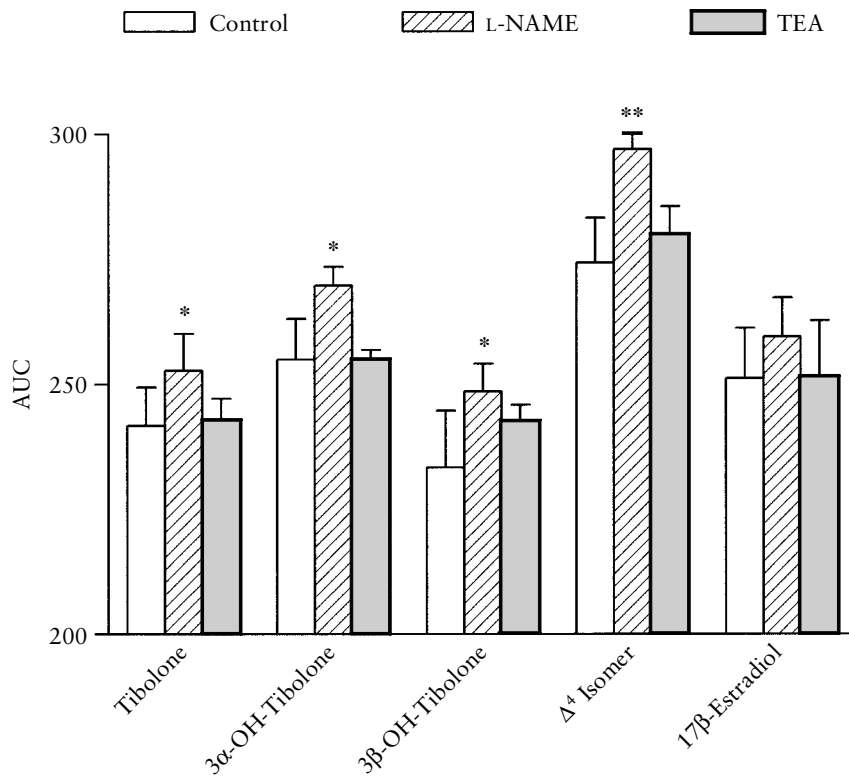
**Figure 1** Concentration-dependent relaxation by (a) tibolone, (b) 3α-OH-tibolone, (c) 3β-OH-tibolone, (d) Δ<sup>4</sup> isomer and (e) 17β-estradiol in rabbit posterior cerebral arteries with (filled circles) and without (open circles, control) addition of *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) (1 × 10<sup>-4</sup> mol/l). All data are expressed as percentage relaxation of contraction induced by K<sup>+</sup> 30 mmol/l and given as mean ± SD. Number of experiments ranged from five to seven. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 compared with corresponding control (Student's *t* test)

concentration–response curves and did not differ significantly between groups (ANOVA), but was significantly related to vessel size (i.e. diameter; Pearson *r* = 0.83, *p* < 0.0001). All the substances tested induced a rightward shift of the calcium concentration–response curves, when expressed both as absolute values (data not shown) and as percentages of the maximal K<sup>+</sup> response of the individual vessel (Figure 3). Tibolone, 3α-OH-tibolone, 3β-OH-tibolone, 17β-estradiol (all 10<sup>-5</sup> mmol/l) and the Δ<sup>4</sup> isomer (3 × 10<sup>-5</sup> mol/l)

significantly diminished the AUC of the calcium concentration–response curves, compared with the vehicle control (Figure 4).

DISCUSSION

We found a vasorelaxant effect of tibolone comparable to that of 17β-estradiol in rabbit cerebral arteries. A vasodilating effect of estradiol has previously been shown in both human experiments and *in vitro* animal studies. The effects of



**Figure 2** Concentration–response results for tibolone, 3α-OH-tibolone 3β-OH-tibolone, Δ<sup>4</sup> isomer and 17β-estradiol in isolated rabbit posterior cerebral arteries incubated with either N<sup>w</sup>-nitro-L-arginine methyl ester 1 × 10<sup>−4</sup> mol/l (L-NAME), tetraethylammonium chloride 1 × 10<sup>−2</sup> mol/l (TEA) or no inhibitors (control). Data are calculated as area under the curve (AUC) and given as mean ± SD. Number of experiments ranged from five to eight. \**p* < 0.05, \*\**p* < 0.01, Student's *t* test versus control

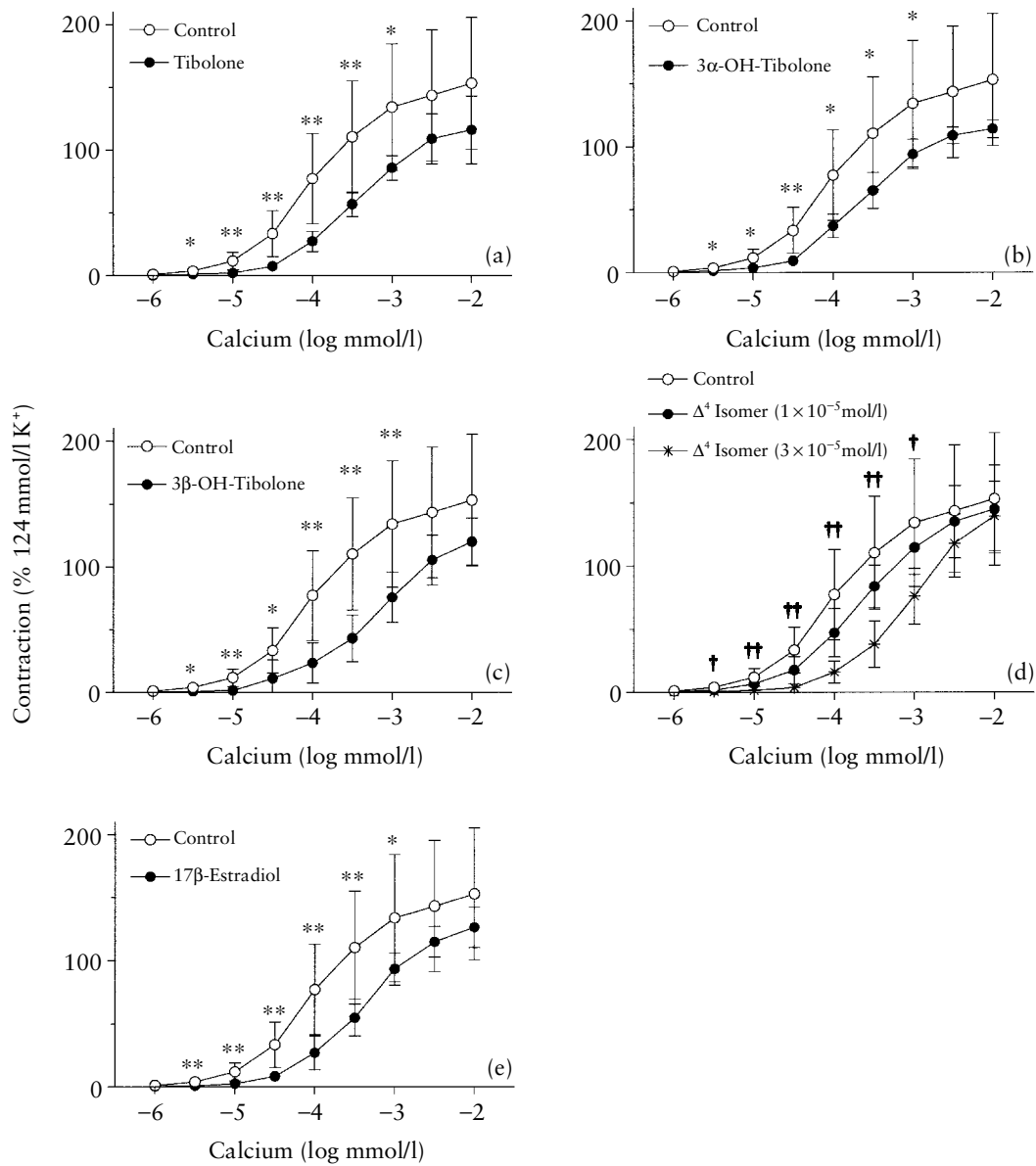
estrogen seem to be universal, occurring in both cerebral<sup>9,14,15</sup> and non-cerebral arteries<sup>13,17</sup>. The fast onset of relaxation for both estradiol and tibolone points to a non-genomic mechanism<sup>12</sup>.

Oral tibolone is rapidly absorbed and quickly metabolized into 3α- and 3β-OH-tibolone by 3α/β-hydroxysteroid dehydrogenase in the intestine and the liver<sup>21</sup>. The third metabolite, the Δ<sup>4</sup> isomer, is formed from 3β-OH-tibolone by 3β-hydroxysteroid dehydrogenase-isomerase, notably in the endometrium, and is found in the circulation for a short period of time. Both 3α- and 3β-OH-tibolone have weak estrogenic activity and circulate as sulfated compounds<sup>21</sup>. We find that not only tibolone, but also the major metabolites, have comparable actions on rabbit cerebrovascular function, with tibolone and 3β-OH-tibolone slightly more potent, 3α-OH-tibolone equal to and the Δ<sup>4</sup> isomer slightly less potent than 17β-estradiol.

The substantial evidence of a beneficial vasodilating effect of estrogen, obtained from *in vitro* studies and animal models, has been contradicted by the recent human intervention studies, finding

neither cardioprotective effects nor protection against stroke after estrogen–progestogen replacement in postmenopausal women<sup>23,24</sup>. However, the final outcome, i.e. a coronary or cerebral attack, is dependent on a variety of factors including genetic and hemostatic, and may depend on the state of the vessel before intervention.

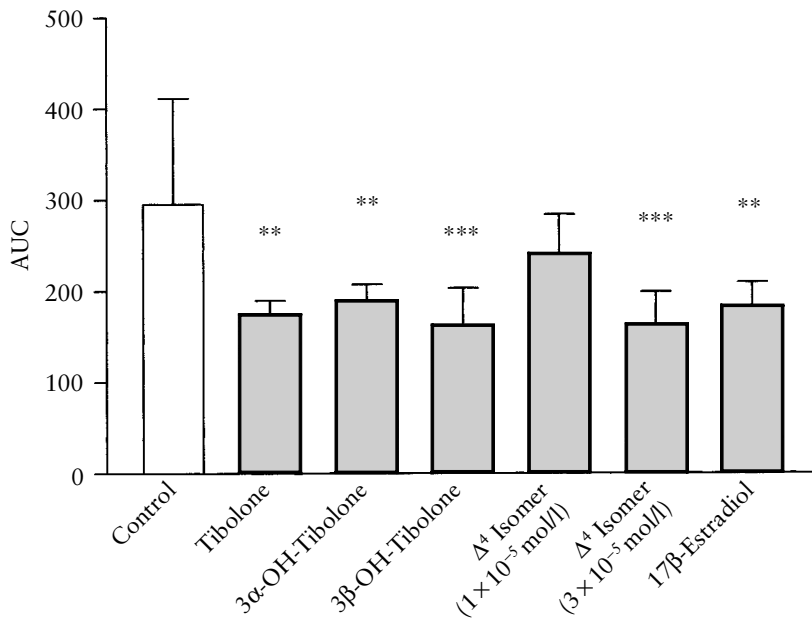
*In vitro* experiments are suitable for basic studies of vascular function, as the experimental conditions are easily manipulated. Obviously, *in vitro* studies are not easily performed in human arteries, for which reason animal studies serve as a model of vascular function in humans. The rabbit is a widely used model for studies of vascular effects of hormones in healthy<sup>13–15,26,27</sup> and in atherosclerotic subjects<sup>9,28</sup>. To investigate possible mechanisms of vascular action, we have chosen the healthy rabbit as a model. A major drawback is that rabbit cerebral arteries are difficult to investigate, since a stable pharmacologically induced precontraction is difficult to obtain. In our set-up, preliminary studies showed that precontraction induced by vasopressin or prostaglandin F<sub>2α</sub> was inconstant or characterized by



**Figure 3** Effect of (a) tibolone  $1 \times 10^{-5}$  mol/l ( $n = 6$ ), (b)  $3\alpha$ -OH-tibolone  $1 \times 10^{-5}$  mol/l ( $n = 6$ ), (c)  $3\beta$ -OH-tibolone  $1 \times 10^{-5}$  mol/l ( $n = 6$ ), (d)  $\Delta^4$  isomer  $1 \times 10^{-5}$  mol/l ( $n = 6$ ) and  $3 \times 10^{-5}$  mol/l ( $n = 6$ ), and (e)  $17\beta$ -estradiol  $1 \times 10^{-5}$  mol/l ( $n = 6$ ) on concentration-dependent contraction responses to calcium compared with vehicle control ( $n = 9$ ) in isolated rabbit posterior cerebral arteries. Data are expressed as percentage of maximal contraction with K<sup>+</sup> 124 mmol/l obtained during run-up procedure (mean  $\pm$  SD). \* $p < 0.05$ , \*\* $p < 0.01$ , compared with vehicle control (Student's  $t$  test); † $p < 0.05$ , †† $p < 0.01$ ,  $\Delta^4$  isomer ( $3 \times 10^{-5}$  mol/l) vs. vehicle control (analysis of variance)

tachyphylaxia, while potassium induced a strong and stable contraction. Potassium, however, causes depolarization of all cells including the endothelium, the smooth muscle cells and the perivascular nerves, with the release of relaxant and contractile factors, for example nor-adrenaline. An adrenergic  $\alpha$  receptor-blocking substance, phentolamine, was therefore added in the calcium concentration-response experiments.

The force production in vascular smooth muscle is correlated to the distension of the smooth muscle cells and, hence, the distension of the vessel<sup>25</sup>. The normalization procedure ensured an identical distension (90% of IC<sub>100</sub>) of all the vessel segments, and a force production close to maximum in each segment<sup>28</sup>. Furthermore, to balance for differences in contractile response due to differences in vessel size, the calcium responses



**Figure 4** Effect of tibolone  $1 \times 10^{-5}$  mol/l ( $n = 6$ ),  $3\alpha$ -OH-tibolone  $1 \times 10^{-5}$  mol/l ( $n = 6$ ),  $3\beta$ -OH-tibolone  $1 \times 10^{-5}$  mol/l ( $n = 6$ ),  $\Delta^4$  isomer  $1 \times 10^{-5}$  mol/l ( $n = 6$ ) and  $3 \times 10^{-5}$  mol/l ( $n = 6$ ), and  $17\beta$ -estradiol  $1 \times 10^{-5}$  mol/l ( $n = 6$ ) on concentration-dependent contraction responses to calcium compared with vehicle control ( $n = 9$ ) in isolated posterior cerebral arteries. Data are calculated as percentage of maximal contraction to  $K^+$  124 mmol/l obtained during run-up procedure and expressed as area under the curve (AUC, mean  $\pm$  SD). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared with vehicle control (analysis of variance performed on log-transformed data)

were adjusted proportionally to the maximal response to 124 mmol/l  $K^+$  obtained in the same vessel segment during the run-up procedure.

Concentration-response experiments should ideally include comparisons of  $E_{\max}$  and  $\log EC_{50}$ . Owing to poor solubility of the substances, full sigmoidal concentration-response curves, which are necessary to estimate  $E_{\max}$  and  $\log EC_{50}$  reliably, could not be determined. However, the less ideal comparisons of AUCs and of responses at each individual concentration corresponded reasonably to the ideal model.

Nitric oxide is synthesized in the endothelium from L-arginine by NO-synthase, and diffuses rapidly to the underlying smooth muscle, causing relaxation via stimulation of soluble guanylate cyclase and a subsequent increase in cyclic guanosine monophosphate (GMP). Blocking the endothelial NO synthesis with L-NAME significantly inhibited the relaxation induced by all the substances tested, suggesting that the relaxation induced by tibolone, its metabolites and  $17\beta$ -estradiol is partly mediated through release of NO (Figure 1). Considerable evidence has accumulated to support that  $17\beta$ -estradiol may acutely stimulate endothelial NO production and release, in cultured vascular endothelial cells<sup>29</sup>, in intact vascular preparations<sup>17,18</sup> and *in vivo*<sup>22,30</sup>, by

mechanisms independent of genomic expression. Whereas NO release is stimulated by physiological concentrations of  $17\beta$ -estradiol in isolated endothelial cells, concentrations several decades higher are required in intact vascular preparations<sup>17,18</sup>. The effect appears to be elicited mainly via a membrane-bound estrogen receptor  $\alpha$ <sup>31</sup>, but membrane-bound estrogen receptor  $\beta$  may also be involved<sup>32</sup>. Data regarding the significance of the estrogen receptor and NO release to the estradiol-induced acute relaxation in cerebral arteries are inconsistent, and restricted to studies in rabbits. In contrast with our findings, a recent study reports that  $17\beta$ -estradiol-induced relaxation in the middle cerebral artery was unchanged by incubation with L-NAME or endothelial denudation, perhaps reflecting an endothelial dysfunction caused by hypercholesterolemia<sup>9</sup>. Our observations regarding  $17\beta$ -estradiol find support in two studies reporting that the response is attenuated following endothelial denudation in the basilar artery<sup>15</sup> and is estrogen receptor-dependent in pial arterioles<sup>26</sup>.

Unlike estradiol, the acute vascular effects of tibolone have only been sparsely elucidated. In ewes, the tibolone-induced increase in coronary and uterine blood flow was almost completely abolished by concomitant administration of

L-NAME or the unspecific estrogen receptor antagonist, ICI<sub>172780</sub><sup>22</sup>, indicating an estrogen receptor-dependent mechanism elicited mainly via NO. The chemical structure and steroid receptor affinity differ significantly between estrogens, tibolone and its metabolites<sup>21</sup>. There was no difference in the response to the various compounds of the vessel segments incubated with L-NAME. As tibolone and its metabolites have low or no binding affinity to the classical cytosolic estrogen receptor<sup>21</sup>, the effects could be unspecific rather than mediated by estrogen receptors. Similarly, by use of ICI<sub>172780</sub>, relaxation with 17 $\beta$ -estradiol in the rabbit basilar artery has previously been shown to be independent of the estrogen receptor<sup>14</sup>.

Besides the NO-dependent effect, the direct actions on the vascular smooth muscle were elucidated. Voltage-gated calcium channels are activated by depolarization of the plasma membrane induced by high extracellular potassium concentration. The inhibition of extracellular Ca<sup>2+</sup> influx by 17 $\beta$ -estradiol, previously demonstrated in electrophysiological<sup>12</sup> and pharmacological studies<sup>9,14,15</sup>, is confirmed by the present results. Moreover, there is a close correlation between the estradiol concentration causing relaxation and the concentration producing inhibition of contraction. Calcium antagonistic properties also characterize other sex steroids, such as progesterone and testosterone<sup>33</sup>, and the selective estrogen receptor modulators (SERMs) raloxifene<sup>27</sup> and tamoxifen<sup>34</sup>. The present results raise functional evidence for a Ca<sup>2+</sup>-antagonistic effect of tibolone and its metabolites similar to that of 17 $\beta$ -estradiol.

Modulation of potassium channel activity in arterial smooth muscle membrane contributes to regulation of membrane potential and provides an important mechanism involved in the regulation of arterial tone<sup>35</sup>. Incubation with the non-specific potassium channel blocker TEA had no effect on the concentration–response curves with any of the hormones, confirming that modulation of

potassium-channel gating seems not to be involved in this relaxation<sup>9,14</sup>.

The circulating plasma concentration of tibolone achieved during postmenopausal hormone replacement therapy is in the order of nmol/l, which is several decades lower than the concentrations producing vasodilatation in our experiments. The present results may therefore reflect a pharmacological rather than a physiological effect of tibolone. However, the total plasma concentration of tibolone may not reflect the local concentration of the active lipophilic molecules in the vascular wall.

In the present study, we have focused on NO release, calcium influx and potassium efflux as possible mechanisms underlying the relaxant effect of tibolone and its metabolites. Factors involved in vascular tone are multiple and complex, and it is possible that steroids may exert their acute effects by several other mechanisms, for example release of vasodilatory prostaglandins or endothelium-derived hyperpolarizing factor.

Our data indicate that tibolone has an acute effect on cerebral arteries *in vitro* comparable to that of 17 $\beta$ -estradiol, and that the effects may be mediated by similar mechanisms.

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*Conflict of interest* NV Organon supplied tibolone and the metabolites but has not otherwise supported the study. NV Organon approved the final manuscript, but the study design and the interpretation of data are due solely to the authors and have not been influenced by NV Organon.

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