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Vasodilating effect of norethisterone and its 5α metabolites: a novel nongenomic action

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Abstract

Estrogens are generally administered in hormone replacement therapy in combination with synthetic progestins. Studies of cardiovascular risk factors in postmenopausal women have shown a variety of responses according to the molecular structure of the progestin used in hormone replacement therapy schemes. The present study sets out to determine the vasoactive effects of norethisterone and its 5α-dihydro (5α-norethisterone) and -tetrahydro (3α,5α-norethisterone and 3β,5α-norethisterone) metabolites in isolated precontracted rat thoracic aorta. The addition of norethisterone and 3α,5α-norethisterone in rat aorta exhibited a potent, concentration–response inhibition of noradrenaline-induced contraction, while 5α- and 3β,5α-norethisterone had very little, if any, vasorelaxing effect. Relaxation to norethisterone and 3α,5α-norethisterone had very rapid time-courses and it was neither affected by the absence of endothelium nor by the inhibitor of nitric oxide synthase, N^{ω}-nitro-L-arginine methyl ester (L-NAME). The addition of specific anti-androgen, anti-progestin and anti-estrogen compounds and protein synthesis inhibitors did not preclude the vasorelaxing effect of norethisterone and its 3α,5α-reduced metabolite. The results strongly suggest that these effects are not mediated by nuclear sex steroid hormone receptors. The overall data document a novel nongenomic endothelium-independent vasorelaxing action of a 19-nor synthetic progestin and one of its A-ring-reduced derivatives.

Keywords: 19-Nor-steroid; Norethisterone; Progestin, synthetic; Vasorelaxation; Hormone replacement therapy

1. Introduction

Synthesis of the first oral active progestin, norethisterone, was made more than 50 years ago in Mexico City (Djerassi et al., 1952, 1954). Since then, this synthetic progestin has been widely used as an ingredient of a number of contraceptive formulations. In addition, norethisterone has been used in combined hormone replacement therapy in postmenopausal women. Currently, a variety of synthetic progestin molecules combined with estrogens are being used in hormone replacement therapy worldwide (reviewed by Sitruk-Ware, 2000).

It is now well documented that cardiovascular disease is the major cause of death in women, particularly in the postmenopausal stage. The cardiovascular effects of the progestin component of hormone replacement therapy formulations have remained as a controversial issue. Combined hormone replacement therapy consists of estrogens and synthetic progestins, which might protect against cardiovascular disease (Grady et al., 1992; Meade and Berra, 1992; Barret-Connor, 1998). In this respect, it has been reported that while estrogens induce a protective cardiovascular role, most of the synthetic progestin molecules may reduce the beneficial effects of estrogens, thus increasing the risk of cardiovascular disease (Hillard et al., 1992; Sullivan et al., 1995).

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The preventive effect of estrogens on cardiovascular risk factors improve the lipid profile (Stevenson, 1996), carbohydrate metabolism (Godsland et al., 1993), and coagulation and fibrinolytic system (Lindoff et al., 1996). The role of these and other factors and the possible effect of estrogen on them have been reviewed by Sitruk-Ware (2000); however, the two most relevant effects of estrogens in the cardiovascular system are modulation of vascular tone and inhibition of vascular growth (Dubey and Jackson, 2001). The vasoactive effect of naturally occurring 17β-estradiol has been well documented. This steroid hormone induces relaxing effects on the vasculature via both genomic and nongenomic mechanisms that generate vasodilator agents, such as nitric oxide, cGMP, cAMP, adenosine and prostacycline, as well as alterations in ion channel activity (Dubey and Jackson, 2001). On the contrary, little is known about the effect of progesterone and natural and synthetic progestins on blood vessels.

Recent studies have shown that medroxyprogesterone acetate, a synthetic derivative of 17-hydroxyprogesterone widely used in hormone replacement therapy schemes, induces vasoconstriction and reverses the estrogen-induced vasorelaxation (Williams et al., 1994; Miyagawa et al., 1997) in human and nonhuman primates. In contrast, both progesterone (Williams et al., 1994; Miyagawa et al., 1997) and nomegestrol acetate (Williams and Adams, 1997; Williams et al., 1998; Paris et al., 2000) failed to reverse the vasodilating effect of estrogens.

Moreover, transdermal sequential administration of estradiol and norethisterone, a 19-nor synthetic progestin, reduces the vascular resistance in uterine arteries and induces a self-limiting growth of the uterus and endometrium in postmenopausal women (Cagnacci et al., 2000). In addition, estradiol and norethisterone acetate decrease the level of endotheline-1 (Ylikorkala et al., 1998), a potent vasoconstrictor agent. Clearly, the different progestins may produce different results. Very recently, Herkert et al. (2000) reported that a series of synthetic progestins, such as levonorgestrel, 3-keto-desogestrel, gestodene and chlormadinone acetate, are capable of inducing concentration-dependent relaxations in rabbit jugular vein, since according to their molecular structure, progestins produce different effects on vasomotion and it is critical to understand the effects of progestins, alone and in combination with estrogens on the vascular reactivity.

The present study was designed to analyze the vasorelaxing action of norethisterone and three of its 5α-reduced metabolic conversion products (5α-reduced metabolites: 5α-norethisterone, 3α,5α-norethisterone and 3β,5α-norethisterone) on the vascular reactivity of rat thoracic aorta. Accordingly, we have determined the steroid molecular structure-vasorelaxing response relationship, and investigated the role of vascular endothelium on this process. The overall results allowed to elucidate the possible involvement of genomic and nongenomic pathways in the mode action of 19-nor synthetic progestins and its metabolites.

2. Materials and methods

2.1. Animals

Experiments were carried out in male adult Wistar rats (220–250 g). The animals were maintained at a 12:12-h light–dark cycle, with light beginning at 0700 h. The rats were kept in a special room at constant temperature (21 ± 1 °C) and humidity (50 ± 5%), with food and water ad libitum. The present project was approved by our Animal Care Committee, and experiments were conducted in accordance with the published Guiding Principles in the Care and Use of Animals approved by the American Physiological Society.

2.2. General methods

Male rats were killed by cervical dislocation. The descending thoracic aorta was then quickly removed and placed in a Krebs–Ringer bicarbonate solution with the following composition, in mM: $NaHCO_3$ 24.9, NaCl 119, KCl 4.74, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 2.5 and glucose 12.0 (pH 7.4, 37 °C). The aorta was then carefully cleaned of blood and adhering adipose and connective tissues and the midthoracic region was cut into rings of ~ 1 cm in length. During preparation of the rings, care was taken to avoid stretching the tissue or touching the luminal surface to preserve endothelial integrity, which was evaluated functionally in all experiments (as described below).

To assess the role of the endothelium in the vascular response to norethisterone and its metabolites, some aortas were denuded before mounting by gently rubbing the luminal surface with a plaited nylon string. After preparation, each ring was attached horizontally between two L-shaped stainless steel hooks, placed in a 10-ml organ bath containing Krebs–Ringer bicarbonate solution (pH 7.4, 37 °C) and gassed continuously with a mixture of 95% O_2 and 5% CO_2. Each vascular ring was tied to an isometric force transducer (FTO3C, Grass Instruments, Quincy, MA) connected to a polygraph (79, Grass Instruments) for continuous recording of aortic tension. A passive resting tension of 10 mN (1.00 g) was maintained throughout the experiments, and the aortas were equilibrated for 60 min. After equilibration, the blood vessel rings were stabilized with a maximal contraction to noradrenaline (0.3 μM); subsequently, the response was recorded during 30 min and the rings were washed. When the tone reached the baseline, tissues were allowed to equilibrate for 60 min and a second addition of noradrenaline, at the same concentration (0.3 μM), was made. Five minutes after inducing the contraction to noradrenaline, the functional integrity of endothelium was assessed by inducing relaxation to acetylcholine (20 μM) and the effect was observed during 10 min. Only aortic rings in which the relaxation to acetylcholine was greater than 40% were used as preparations with intact endothelium. However, this relaxation was absent after

mechanical removal of endothelium (preparations without endothelium). At the end of the experiments, the removal of endothelium was also confirmed by light microscopy of the aorta histological section as reported previously (Perusquía et al., 1996).

2.3. Effects of norethisterone and its metabolites on vascular tone

Preparations with and without endothelium were prepared and determined as described above. The tissues were then washed with Krebs–Ringer bicarbonate solution to re-equilibrate for 60 min before inducing a third noradrenaline (0.3 μM)-induced contraction, which was recorded during a 30-min period (control responses to noradrenaline-induced contraction). The tissues were washed and re-equilibrated for 60 min, rings were then precontracted with a fourth noradrenaline-induced contraction. After a stable contractile tension was attained (~ 15 min), norethisterone and its 5α-reduced metabolites (5α-norethisterone, 3α,5α-norethisterone or 3β,5α-norethisterone) were added, separately, to the bath in noncumulative concentrations (18, 31, 56, 100 and 180 μM), which means a single concentration of a single drug. Solubility limitations prevented examination of concentrations in excess of 180 μM. The effect of each steroid at the different concentrations was recorded during 10 min. Finally, the rings were washed and a last contraction induced by 0.3 μM noradrenaline was observed during 30 min to check the tissue recovery. Vascular relaxation responses to norethisterone and its 5α-reduced metabolites were calculated as a percentage of the noradrenaline-induced contraction and the concentration–response curves were performed for each preparation (with and without endothelium).

In order to study the potential progestin antagonism on estrogen-induced vasorelaxation, in some experiments, the vasorelaxing effect of 17β-estradiol at 60 μM (as previously reported; Perusquía and Villalón, 1999) was confirmed ($26.52 \pm 0.92\%$ of the vasorelaxation on the noradrenaline-induced contraction, $n = 20$) in preparations without endothelium. After 10 min of 17β-estradiol effect, the maximal relaxation (R_{max}) induced by both norethisterone and 3α,5α-norethisterone (which turned out to be the most potent vasorelaxing compounds) was observed during a 10-min period. 17β-Estradiol-induced vasorelaxation was evaluated in the presence and absence of both norethisterone and 3α,5α-norethisterone; likewise, the effect induced by them was also quantified with the estrogen preincubation.

After each noradrenaline-induced contraction, the Krebs–Ringer bicarbonate solution was changed three times (washout) and the tissues were allowed to equilibrate for 60 min. The vehicle of progestins (ethanol 0.1% for 18–100 μM and 0.2% for 180 μM only) and time-control experiments were also performed to check for potential effects of ethanol on vasorelaxation and to determine the stability of noradrenaline-induced contraction.

2.4. The role of nitric oxide in the vasorelaxing effect of norethisterone and 3α,5α-norethisterone

In order to investigate the involvement of nitric oxide in the most potent vasorelaxing compounds (norethisterone and 3α,5α-norethisterone), preparations with endothelium were incubated with the nitric oxide synthase inhibitor, N^{ϖ}-nitro-L-arginine methyl ester (L-NAME, 10 μM), for 30 min on noradrenaline contraction, before the relaxation induced by each hormone (180 μM) to elicit R_{max}. The vasorelaxing effect of each hormone was observed during 10 min. Their R_{max} in preparations with endothelium were compared with both R_{max}, namely, with L-NAME pretreatment and endothelium-denuded aortas.

2.5. Antihormones and inhibitors of protein synthesis and transcription

The involvement of steroid receptors in the acute vasorelaxation to norethisterone and 3α,5α-norethisterone were examined in denuded preparations pretreated with 10 μM flutamide (an androgen receptor antagonist) or 100 μM RU 486 (a progesterone receptor antagonist) to observe the norethisterone effect, and 1 μM ICI 182,780 (an estrogen receptor antagonist) to observe the 3α,5α-norethisterone effect. These protocols were designed on the basis that norethisterone possesses androgenic and progestational properties (Lemus et al., 1997; Pasapera et al., 1995), while 3α,5α-norethisterone presents estrogenic activity (Mendoza-Rodríguez et al., 1999; Chávez et al., 1985; Vilchis et al., 1986; Larrea et al., 2001). In each case, the appropriate antihormones were added to establish the desired concentrations and allowed to equilibrate for 30 min on noradrenaline-induced contraction. Then, the corresponding hormones were added to observe their R_{max} at 180 μM during a 10-min period. The comparison was made with the R_{max} of each hormone without any pretreatment.

In other experiments, the aortic rings without endothelium were preincubated with the protein synthesis inhibitor cycloheximide (40 μM) or the transcription inhibitor actinomycin D (10 μM) for 30 min on the noradrenaline-induced contraction. Norethisterone or 3α,5α-norethisterone were then added (at 180 μM each) and their effect (evaluated during a 10-min period) was compared in the presence and absence of those inhibitors.

2.6. Data presentation and statistical analysis

Each experiment was performed on the rings prepared from different rats. All data in the text and figures are expressed as mean ± S.E.M. ($n \geq 6$, where $n = 1$ represents one rat). The concentration for each substance is expressed as the final concentration in the organ bath. Changes in tension are shown as percentage of the inhibition of the contraction induced by 0.3 μM noradrenaline. To test the effect of the steroids, rings were precontracted with nor-

adrenaline and relaxed by addition of the hormone in a noncumulative manner and the concentration–response curves were performed. The concentration of the vasorelaxant giving the half-maximal relaxation (IC_{50}) was obtained from those concentration–responses curves (Litchfield and Wilcoxon, 1949). IC_{50} values could not be accurately determined and statistical significance was therefore determined at each concentration. The rank order of potency was also obtained from the values at the highest concentration tested (180 μM, R_{max}). The nonpaired Student's *t*-test was utilized to compare the responses between two groups and ANOVA was used for multiple comparisons. The accepted level of significance was $P < 0.05$.

2.7. Drugs and chemicals

Norethisterone (NET; 17α-ethynyl-17β-hydroxy-19-nor-4-androstene-3-one), 5α-dihydronorethisterone (5α-NET; 17α-ethynyl-17β-hydroxy-19-nor-5α-androstane-3-one), 3α,5α-tetrahydronorethisterone (3α,5α-NET; 17α-ethynyl-3α,17β-dihydroxy-19-nor-5α-androstane-3-one) and 3β,5α-tetrahydronorethisterone (3β,5α-NET; 17α-ethynyl-3β,17β-dihydroxy-19-nor-5α-androstane-3-one) were all synthesized from norethisterone as previously described (Vilchis et al., 1986), dissolved in absolute ethanol and added to the organ bath in a final volume of 0.1% for 18, 31, 56 and 100 μM and 0.2% for 180 μM only. Furthermore, with the exception of the estrogen receptor antagonist, ICI 182,780 (7α-[9[(4,4,5,5,5-pentafluoropentyl)sulphinyl]nonyl]estra-1,3,5(10)-triene-3,17β-diol), which was obtained from a different source (Tocris Cookson, Ellisville, MO, USA), the remaining compounds were all purchased from Sigma (St. Louis, MO, USA), and included: 17β-estradiol (1,3,5(10)-estratriene-3,17β-diol), the androgen receptor antagonist, flutamide (2-methyl-*N*-[4-nitro-(3-trifluoromethyl)phenyl]propanamide), the progestin receptor antagonist, RU 486 (11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), the protein synthesis inhibitor, cycloheximide (3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide), the transcription inhibitor, actinomycin D (dactinomycin), the nitric oxide synthase inhibitor, N^{ϖ}-nitro-L-arginine methyl ester hydrochloride (L-NAME hydrochloride), acetylcholine chloride and noradrenaline hydrochoride. All compounds were dissolved in absolute ethanol and added to the organ bath in a final volume of 0.1%, except for L-NAME, acetylcholine and noradrenaline, which were dissolved in distilled water. Noradrenaline and actinomycin D were kept in the dark until use in order to avoid light-induced degradation.

3. Results

In isolated rat thoracic aorta rings, norethisterone and its three 5α-reduced metabolites showed the following results on the contraction induced by noradrenaline.

3α,5α-Norethisterone and norethisterone elicited an acute concentration-dependent vasorelaxing effect. In contrast, 5α- and 3β,5α-norethisterone were much weaker as they induced a vasorelaxation which was slightly, but significantly, greater ($P < 0.0005$) than ethanol (vehicle control; relaxed no more than an average of 1.77 ± 0.57%, $n = 6$) or noradrenaline alone (time control); this effect was not concentration-dependent (see Fig. 1) with an R_{max} >20% or >10%, respectively (Table 1). The vasorelaxing effect of all synthetic progestins was observed within 1 min. After washout, the amplitude of the next noradrenaline-induced contraction was totally recovered and the progestin action disappeared.

3α,5α-Norethisterone, the most potent compound, induces a nearly complete relaxation at the highest concentra-

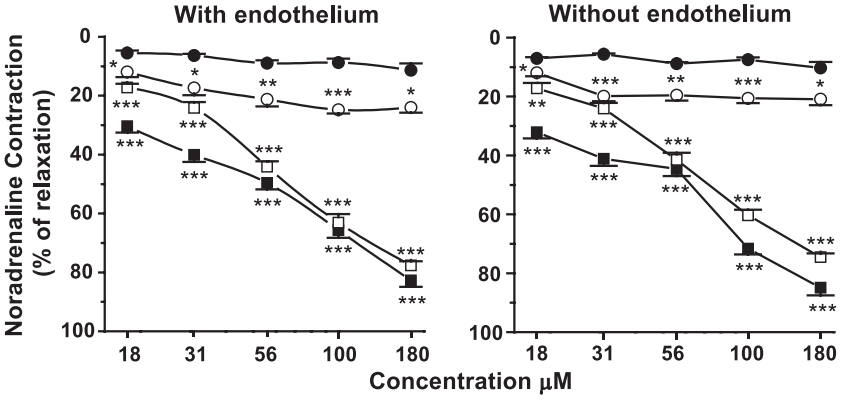

Fig. 1. Concentration–response curves of norethisterone (□), 3α,5α-norethisterone (■), 5α-norethisterone (○) and 3β,5α-norethisterone (●) on noradrenaline-induced contraction (0.3 μM) in paired, with and without endothelium thoracic aortas of male rats. Data points represent the mean ± S.E.M. of at least six experiments. All values were significantly different when compared to the vehicle control ($P < 0.005$). The curve of each compound obtained from rings with and without endothelium was not significantly different ($P > 0.1$). Statistical significances (*$P < 0.005$, **$P < 0.0005$, ***$P < 0.00005$) are indicated between each concentration. Norethisterone and 3α,5α-norethisterone curves were significantly different ($P < 0.00005$).

Table 1
Maximal relaxation and IC_{50} values on noradrenaline-induced contraction of male rat thoracic aortas in response to norethisterone and its 5α-reduced metabolites

Compound	*n*	Treatment	R_{max} (%)	IC_{50}	Potency
NET	6	Endo+	77.65 ± 1.42	66.78 μM	1.0
	6	Endo−	74.53 ± 1.25	72.03 μM	1.0
3α,5α-NET	6	Endo+	82.72 ± 2.21	47.83 μM	1.06
	6	Endo−	84.92 ± 2.58	45.73 μM	1.13
5α-NET	6	Endo+	24.01 ± 1.78	7.50 mM[a]	0.30
	6	Endo−	20.86 ± 2.07	21.63 mM[a]	0.27
3β,5α-NET	6	Endo+	11.33 ± 2.32	400 M[a]	0.14
	6	Endo−	10.27 ± 2.01	820 M[a]	0.13

Values are mean ± S.E.M.; *n* = number of animals. Endo+, endothelium intact; Endo−, endothelium denuded. Half-maximal relaxation (IC_{50}) was obtained from concentration–response curves. Maximal relaxation (R_{max}) at 180 μM. The potency was obtained by the formula R_{max} norethisterone (NET)/R_{max} 5α-reduced metabolite, assuming a value of 1.0 to NET. R_{max} were not significantly different between Endo+ and Endo−, $P>0.1$.
[a] Values estimated by extrapolation.

tion (180 μM), in contrast to precontracted preparations exposed to ethanol (1.77 ± 0.57% of relaxation, $n=6$) or noradrenaline alone. The sensitivity (IC_{50} value) of aortic rings to all compounds is expressed in Table 1. The importance of endothelium in mediating norethisterone-induced aorta relaxation was investigated by obtaining a series of complete concentration–response relationships. In these experiments, removal of the endothelium resulted not significantly different when compared to the norethisterone and its 5α metabolites response curves (Fig. 1). The relaxing potency was obtained from the R_{max} induced by each hormone (Table 1) in rings with and without endothelium, showing the same rank order of potency: 3α,5α-norethisterone ≥ norethisterone>5α-norethisterone>3β,5α-norethisterone.

The vasorelaxing effect with 60 μM 17β-estradiol was not reversed by the addition of norethisterone or 3α,5α-norethisterone. Moreover, pretreatment with 60 μM 17β-estradiol did not oppose norethisterone- and 3α,5α-norethisterone-induced vasorelaxation (Fig. 2), but significantly enhanced the maximal relaxation to norethisterone (R_{max} = 85.14 ± 1.31, $P<0.0005$, $n=6$). In equimolar concentrations (56 μM), 3α,5α-norethisterone and norethisterone resulted 1.59- and 1.48-fold more potent, respectively, than 17β-estradiol-induced vasorelaxation.

3.1. Effects of endothelial denudation and L-NAME on norethisterone and 3α,5α-norethisterone responses

As shown in Fig. 1, there was not significant difference between intact and denuded vessels. In addition, L-NAME (10 μM) totally inhibited acetylcholine-induced endothelium-dependent relaxation in the rings precontracted with noradrenaline (data not shown). However, the same concentration of L-NAME (10 μM) did not affect the R_{max} to norethisterone and 3α,5α-norethisterone. As the results show, L-NAME or removal of endothelium did not significantly inhibit the R_{max} to norethisterone (L-NAME: R_{max} = 74.1 ± 2.32%, $P>0.1$, $n=6$; endothelium-denuded: R_{max} = 74.53 ± 1.25%, $P>0.1$, $n=6$) and 3α,5α-norethisterone (L-NAME: R_{max} = 79.1 ± 1.31%, $P>0.1$, $n=6$; endothelium-denuded: R_{max} = 84.92 ± 2.58%, $P>0.5$, $n=6$).

3.2. Effects of the steroid antagonist and inhibitors of protein synthesis and transcription on norethisterone- and 3α,5α-norethisterone-induced vasorelaxation

Neither the norethisterone- nor the 3α,5α-norethisterone-induced R_{max} were, respectively, inhibited by the preincubation with flutamide or ICI 182,780 ($P>0.05$: see Fig. 3). Moreover, not only did RU 486 pretreatment fail to antagonize the R_{max} to norethisterone, but was even significantly augmented ($P<0.005$), due to the fact that RU 486 induces a vasorelaxing effect per se (11.64 ± 1.03%, $n=6$). Simi-

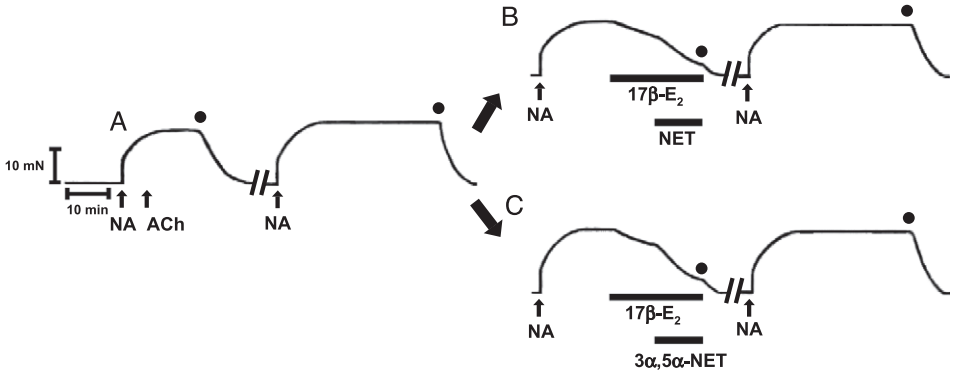

Fig. 2. Typical recordings of the noradrenaline-induced contraction (NA; 0.3 μM) in endothelium-denuded aortic rings of male rats; (A) loss of relaxing response to 20 μM acetylcholine (ACh) and time control of noradrenaline-induced contraction. The vasorelaxing effect induced by 17β-estradiol (17β-E_2; 60 μM) was not reverted by R_{max} to (B) norethisterone (NET; 180 μM) or (C) 3α,5α-norethisterone (3α,5α-NET; 180 μM). The solid black line indicates the incubation time with the steroid. The black circles represent the time of washout.

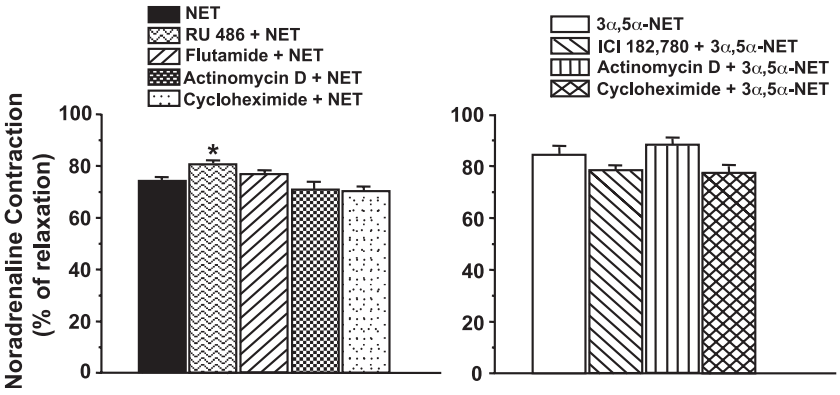

Fig. 3. Maximal relaxation to norethisterone (NET; 180 μM) and 3α,5α-norethisterone (3α,5α-NET; 180 μM) alone and in combination with different treatments in endothelium-denuded aortic rings precontracted by 0.3 μM noradrenaline: 100 μM RU 486 (progesterone receptor antagonist), 10 μM flutamide (androgen receptor antagonist), 1 μM ICI 182,780 (estrogen receptor antagonist), 40 μM cycloheximide (protein synthesis inhibitor) and 10 μM actinomycin D (transcription inhibitor). The bars represent the mean ± S.E.M. of at least six experiments. Statistical significance: $*P < 0.005$.

larly, pretreatment with 40 μM cycloheximide or 10 μM actinomycin D had no effect on the R_{max} to norethisterone and 3α,5α-norethisterone (Fig. 3).

4. Discussion

4.1. General

The results of the present study demonstrate that norethisterone and its 5α-dihydro (5α-norethisterone) and -tetrahydro (3α,5α-norethisterone and 3β,5α-norethisterone) metabolites are vasoactive substances by inducing vasorelaxation in blood vessels. Thus, these compounds cause a rapid vasorelaxation in the rat aorta precontracted with noradrenaline, with a specific rank order of potency. The relaxant responses to norethisterone and its metabolites were immediate in onset, occurring within 1 min, and reversible after washouts. With these findings, it is tempting to suggest that the vasorelaxation induced by these compounds could be due to nongenomic (membrane) mechanisms rather than the classical steroid modulation of nuclear transcription. This suggestion is strengthened by our findings showing that the vasorelaxing response to the above synthetic progestins was not modified by steroid receptor antagonists or inhibitors of protein synthesis and transcription.

Although numerous lines of evidence have demonstrated the direct cardiovascular effects of estrogens, there are few reports on the actions of natural (Jiang et al., 1992; Mukerji et al., 1995; Omar et al., 1995; Perusquía et al., 1996) and synthetic progestins (Herkert et al., 2000) as constituents of hormone replacement therapy on the vascular reactivity.

With this background and to the best of our knowledge, the present study reports, for the first time, a nongenomic relaxing action of norethisterone and its metabolites on vascular tone, an effect that may contribute to the cardiovascular protection afforded by hormone replacement therapy in postmenopausal women. The acute vasorelaxing effect of norethisterone is consistent with a previous report, which shows that norethisterone acetate induces vasorelaxation in precontracted rat thoracic aorta (Glusa et al., 1997); however, to our knowledge, no evidence had been shown on the action of norethisterone metabolites in blood vessels. It is also important to highlight that our findings show the vasorelaxing properties of norethisterone and its metabolites in male rats only. Obviously, further studies will be required to confirm whether female rats would respond in a similar manner to this synthetic progestin and its metabolites.

4.2. Studies on the mode of action of synthetic progestins

As previously implied, the vasorelaxing properties of norethisterone and its metabolites can be explained by a nongenomic action. Although endothelial factors might be involved, this does not seem to be the case in view that the aortic vasorelaxation to norethisterone and its 3α,5α-reduced metabolite remained unaltered after removal of endothelium or L-NAME pretreatment. Therefore, the vasorelaxation to the above compounds involve endothelium-independent pathways. Notwithstanding, it is noteworthy that both endothelium-dependent and -independent actions by estrogens have been confirmed in isolated vascular preparations (reviewed by Ding and Stallone, 2001).

Although the available data have suggested that estrogens cause vasorelaxation through multiple cellular mechanisms (Dubey and Jackson, 2001), the actual mode(s) of action in this process remain(s) to be elucidated. Numerous in vitro studies have provided evidence for a modulatory role of steroids upon calcium channel function; thus, it has been suggested that estradiol, progesterone, testosterone (Murphy and Khalil, 1999; Crews and Khalil, 1999) and 5β-dihydrotestosterone (Perusquía and Villalón, 1999) induce vasorelaxation by an inhibition of extracellular calcium influx, probably by inactivation of voltage-dependent calcium channels, In addition, estrogen (Zhang et al., 1994; Shan et al., 1994) and progesterone (Barbagallo et al., 2001)

inhibited calcium influx through the voltage-dependent calcium channels in smooth muscle cells isolated from various blood vessels.

With respect to synthetic progestins, the vasorelaxing effect elicited by norethisterone and 3α,5α-norethisterone on noradrenaline-induced contraction suggests an interaction with α-adrenoceptors; however, this possibility appears not to be supported by the fact that norethisterone acetate (0.01–10 μM) was found to suppress the contraction induced by KCl, $CaCl_2$ or phenylephrine (Glusa et al., 1997). Thus, these findings suggested that the vasorelaxing effect of norethisterone seem to be due to inhibition of calcium uptake through voltage- and/or receptor-operated calcium channels. Moreover, it has been reported that other synthetic progestin, levonorgestrel, induces inhibition of calcium entry and protein kinase C activation (Herkert et al., 2000). Additionally, it has also been shown that a combined therapy with estradiol and norethisterone acetate was capable of decreasing the levels of endothelin-1 (a potent endogenous vasoconstrictor agent) in women (Ylikorkala et al., 1998). However, the underlying mechanisms remain unclear.

4.3. Efficacy

Norethisterone and its 3α,5α-reduced metabolite presented a marked potency to induce vasorelaxation; however, its other metabolites (5α-norethisterone and 3β,5α-norethisterone) would appear neutral on the vascular tone. The analysis of these findings leads to the conclusion that the sensitivity of noradrenaline-induced contraction to norethisterone derivatives depends on their molecular conformation. Thereby; the α/*trans* configuration at C_5 fails to induce vasorelaxation or has only a weak potency. However, this 5α configuration is highly dependent on the subsequent 3α- or 3β-hydroxylation; thus, the 3α-hydroxylation may produce a very active molecule, while the 3β-hydroxylation produces an inactive compound. We were not surprised by the highly significant correlation between the vasorelaxing potency and structural conformation of steroids, since we have observed that a similar chemical structure-relaxing effect relationship applies for natural 5-reduced steroids; the high relaxing potency on spontaneous contraction induced by the 3α,5α configuration of progesterone (in contrast to the insignificant relaxation induced by the 5α-dihydroprogesterone) was also confirmed in the uterus of different species, including humans (Perusquía and Jasso-Kamel, 2001; Perusquía, 2001). In line with this observation, it has also been observed that 3α,5α androgens (androsterone and androstanediol) induce a marked uterine relaxing effect (reviewed by Perusquía, 2001). In this respect, it has been explained that the different efficacy between progesterone and its 3α,5α-reduced metabolite is a consequence of a high electronic density in the A-ring when the 5α-reduced metabolite is 3α-hydroxylated (Kubli-Garfias, 1998). On these bases, it is suggested that the nongenomic effect of 5α-reduced, natural or synthetic, steroids is a generalized phenomenon on smooth muscle contractility.

On the other hand, a possible influence of pharmacokinetic factors (e.g. metabolism) cannot be categorically excluded. It is important to consider that orally administered norethisterone is metabolized in vivo in the positions 5 and 3 to render 5α/β-dihydronorethisterone (into 5α- and 5β-norethisterone) and subsequently the corresponding 3α/β-tetrahydroderivatives (into 3α,5α-, 3β5α-, 3α,5β- and 3β,5β-norethisterone); this has been shown in uterus, vagina and aorta of rat (Blom et al., 2001), which are three target tissues for hormone replacement therapy ingredients. The in vivo bioconversion of norethisterone into its 3α,5α tetrahydro-reduced metabolite in women has been well documented (Stanczyk and Roy, 1990). After administration per os of [^{14}C]-labeled norethisterone at several doses (2–25 mg) in women on reproductive age, the overage plasma values as free and conjugated norethisterone and 3α,5α-norethisterone are in the order of 653 and 183 nM respectively (Braselton et al., 1977). Moreover, the in situ bioconversion of norethisterone into its tetrahydro-reduced metabolites in target tissues including aorta may contribute to the relevant intracrine effect of this synthetic progestin and its derivatives. Admittedly, further studies that fall beyond the scope of the present investigation will be required to document the role of norethisterone metabolism in the cardiovascular system.

4.4. Possible physiological and clinical implications

It has been reported that medroxyprogesterone acetate may reverse the vasorelaxing protective effect of estrogens (Williams et al., 1994; Miyagawa et al., 1997; Sitruk-Ware, 2000). In particular, medroxyprogesterone may antagonize the beneficial antiatherosclerotic estradiol effects on vasculature, whereas norethisterone may be neutral in this respect (Seeger et al., 2001). In contrast, progesterone (Williams et al., 1994; Miyagawa et al., 1997) or nomegestrol acetate (Williams and Adams, 1997; Williams et al., 1998; Paris et al., 2000) failed to reverse the vasorelaxing effect of estrogens. It is not known whether this discrepancy is due to a chronic administration of progestins; however, in our study, the nongenomic vasorelaxing effect of 17β-estradiol was not antagonized by norethisterone or 3α5α-norethisterone, since they also induced an acute, nongenomic, vasorelaxing effect. Remarkably, 17β-estradiol-induced vasorelaxation seems to be potentiated by these compounds. Interestingly, our results show that the relaxing potency of the most active progestins was higher than that induced by 17β-estradiol. The simplest interpretation of these findings is that norethisterone and its 3α,5α-tetrahydro metabolite do not antagonize the positive activity of estrogen on the vascular wall; hence, they might possess additive vasorelaxing effects in postmenopausal women and, consequently, a

hypotensive effect. In keeping with this suggestion, it has been reported that norethisterone acetate failed to inhibit the positive effects of 17β-estradiol on cardiac function and blood pressure in postmenopausal women (Alfie et al., 1997; Seeger et al., 1997). Likewise, a reduction in mean systolic and diastolic blood pressure was reported with combined regimens of transdermal estradiol and medroxyprogesterone acetate in normotensive postmenopausal women (Pang et al., 1993). In addition, norethisterone acetate combined with 17β-estradiol reduces uterine artery resistance and diastolic blood pressure in postmenopausal women (Cagnacci et al., 2000; Sorensen et al., 2000). Moreover, the possible hypotensive response of progestins is consistent with the fact that natural 5-reduced androgens may elicit a vasodepressor response in vivo (Perusquía and Villalón, 2002).

Recently, the Heart and Estrogen/progestin Replacement Study (HERS) research group showed that in postmenopausal women with established coronary heart disease, and hormone replacement therapy regimen with conjugate equine estrogens (CEE) plus medroxyprogesterone acetate (MPA) does not protect from further heart attacks (Hulley et al., 1998) and increased the rates of venous thromboembolism and biliary tract surgery (Hulley et al., 2002). In addition, The Women's Health Initiative found that the risk of hormone replacement therapy (CEE + MPA) exceeded its benefits in a large group of older postmenopausal women (Rossouw et al., 2002). Nevertheless, these studies did not consider the efficacy of hormone replacement therapy in relieving vasomotor symptoms. Notably, most of large trials have selected the same hormone replacement therapy regimen for their study design (CEE + MPA). Given the different responses by progestins, it would appear inappropriate to claim that progestins in general compromise the cardioprotective effects of estrogens, without specifying which progestins reverse these effects. Therefore, studies with other treatment regimens are needed and should consider the various steroids used in different countries. Our present findings reveal that norethisterone is an alternative to hormone replacement therapy, as a hormonal component with favourable effects on cardiovascular risk, specifically on vasomotion, but their impact on clinical outcomes remains to be determined.

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