17β-Estradiol reduces vasoconstriction in endothelium-denuded rat aortas through inducible NOS

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Binko, J., and H. Majewski. 17β-Estradiol reduces vasoconstriction in endothelium-denuded rat aortas through inducible NOS. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H853–H859, 1998.—Estrogen produces vasodilatation through the induction of nitric oxide synthase (NOS) in the endothelium, but there are many reports of endothelium-independent effects. In the present study, these processes were investigated in rat aortas isolated from ovariectomized rats. Long-term in vitro treatment with 17β-estradiol (10 nM for 24 h) in an organ culture system slightly reduced acetylcholine-mediated vasorelaxation in endothelium-intact aortic rings. 17β-Estradiol (1 and 10 nM for 24 h) also attenuated the phenylephrine-induced constriction in endothelium-denuded aortas, and this effect was inhibited by the NOS inhibitor L-N3-(1-iminoethyl)ornithine hydrochloride, as well as the estrogen receptor antagonist ICI-182,780. Furthermore, 17β-estradiol treatment (1 and 10 nM for 24 h) increased nitric oxide production as assessed by the conversion of [3H]arginine to [3H]citrulline in endothelium-denuded rat aortas. These effects were prevented by the protein synthesis inhibitor cycloheximide. 17β-Estradiol (10 nM for 24 h) treatment also induced the formation of inducible NOS (iNOS) protein in aortas. The results indicate that 17β-estradiol can attenuate the vasoconstrictor effect of phenylephrine by a process that involves induction of iNOS in nonendothelial cells of the aorta. We suggest that long-term estrogen therapy may induce a partial hyporesponsiveness in vascular smooth muscle via a small but sustained nitric oxide production.

Vascular smooth muscle: estrogen receptors

vascular smooth muscle is relaxed by 17β-estradiol both in vitro and in vivo in both animals and humans, and this may form part of the cardioprotective effect of hormone replacement therapy in postmenopausal women (30). There is an increasing body of evidence to suggest that the vasorelaxant properties of 17β-estradiol involve increased production of nitric oxide from vascular endothelial cells catalyzed by nitric oxide synthase (NOS). There are at least three distinct isoforms of NOS: eNOS (NOS I), which is found in endothelial cells and is a Ca2+-activated enzyme; nNOS (NOS III), which is found in neural cells and is Ca2+-activated; and iNOS (NOS II), which can be induced in various tissues by cytokines and is Ca2+-independent (21). In functional experiments, chronic in vivo or in vitro treatment with 17β-estradiol increased endothelium-dependent relaxation in monkey coronary arteries (34), rabbit aortas (23), rabbit femoral arteries (12), and pig coronary arteries (2). Furthermore, in sheep, the slowly developing estrogen-induced vasodilation is antagonized by NOS inhibition (31) and the protein synthesis inhibitor cycloheximide (20). This latter result suggests that estrogen is inducing formation of a protein to produce vasorelaxation, and it has been shown that chronic 17β-estradiol administration increases NOS mRNA for both endothelial and neuronal NOS isozymes in skeletal muscle in guinea pigs (33). Furthermore, pregnancy-induced and estrous cycle changes in NOS expression are correlated with changes in 17β-estradiol levels in animals (13, 33).

Acute 17β-estradiol administration increases endothelium-dependent relaxation in humans in a short time frame unlikely to be explained by genomic effects. For example, acute 17β-estradiol infusion (15–20 min) potentiates endothelium-dependent vasorelaxation in humans (11, 27), and endothelium-dependent relaxation is increased in vitro in rabbit coronary arteries treated acutely with 17β-estradiol (26). In contrast, some studies have shown that the acute vascular relaxation produced by 17β-estradiol is endothelium independent (17, 25). However, these latter studies used supraphysiological concentrations of 17β-estradiol (>3 µM).

In preliminary experiments in aortas isolated from ovariectomized rats, we found that incubation with 17β-estradiol for 24 h in an organ culture system resulted in increased endothelium-dependent relaxation consistent with an induction of NOS in the vascular endothelium. However, when the endothelium was removed, the constrictor effects of the α-adrenoceptor agonist phenylephrine were attenuated, suggesting that the 17β-estradiol had an anticonstrictor effects independently of the endothelium. One candidate is the iNOS isoenzyme, which can be induced in vascular smooth muscle by cytokines and bacterial lipopolysaccharide (21). It was our starting hypothesis that iNOS induction in vascular smooth muscle may mediate the endothelium-independent actions of 17β-estradiol because vascular smooth muscle contains estrogen receptors (18) and in mouse uterus 17β-estradiol has been shown to induce iNOS (15). Furthermore, we found a 60% match of the estrogen response element (AGGTCA nnn TGACCT, where nnn = any base) (9) in the promoter region at base pairs 14–26 in human and rat iNOS genes (5, 10). This hypothesis was investigated in rat isolated aortas incubated with 17β-estradiol in an in vitro organ culture system.

METHODS

Ovariectomy. Female Sprague-Dawley rats (150–200 g) were anesthetized with methohexital sodium and amobarbital sodium (20 mg/kg ip and 60 mg/kg ip, respectively). The ovaries were ligated and then removed. Animals were then allowed to recover for 21 days with food and water ad libitum. At the time of removal of the aorta for further study (see Preparation of rat aortas), plasma 17β-estradiol was measured by a standard procedure (36) in 10 ovariectomized...
animals selected randomly, and in all animals it was below the detection limit (20 pM).

Preparation of rat aortas. Ovariectomized rats (250–350 g) were killed by decapitation. The thoracic aorta was removed under sterile conditions and placed in ice-cold physiological salt solution (PSS) that was bubbled with 95% O2-5% CO2. Each aorta was cut into either 6-mm rings (NOS assay) or 4-mm rings (contractile studies) or was left intact (Western blotting). The different tissue sizes were used only because of the different sensitivities of the methods. In some cases the endothelial cells were removed by gentle rubbing of the entire inner lumen with a stainless steel wire. In contractile studies, the removal of endothelium was verified by examining acetylcholine vasodilatation in phenylephrine-constricted aortas (see Vasoconstriction in rat aortic rings). In all cases acetylcholine had no vasodilator effect. In some aortas, removal of the endothelium was also verified histochemically using silver nitrate staining, and in all cases the removal of the endothelium was complete.

Rat aortic rings in 24-h organ culture. Rat thoracic aortas were isolated under sterile conditions from ovariectomized rats as in Preparation of rat aortas and were incubated with 3 ml PSS at 37°C in a sterile incubator with a 95% O2-5% CO2 atmosphere for 24 h with the drug under investigation. The bathing solution also contained penicillin (10 IU/ml), streptomycin (10 µg/ml), fungizone (25 ng/ml), and Dextran 70 (5% wt/vol, average mol weight = 70,000) to maintain oncotic pressure.

Vasoconstriction in rat aortic rings. Aortic rings (4 mm long) were placed in an organ bath containing 1 ml PSS at 37°C and bubbled with 95% O2-5% CO2. The rings were mounted between two stainless steel hooks that were passed through the lumen. Contractile force was measured by an isometric force displacement transducer. The rings were allowed to equilibrate for a period of 45 min with several washes of PSS, and the resting tension was adjusted to 2 g. When a steady baseline was obtained, the aortic rings were constricted with phenylephrine (100 nM), which produced ~80% of the maximal constriction. Tissues that did not constrict by at least 0.5 g were not used. At this stage the vasodilator effect of acetylcholine was tested to confirm removal of endothelial cells if endothelium denuded. In some experiments one or two cumulative phenylephrine concentration-response curves were then performed.

The completion of the 24-h incubation period the endothelium was removed and the rings were placed in bathing solution containing [3H]arginine (final concn 30 µM; 3.3 µCi/ml) for 30 min. The aortic ring was then chopped using a tissue chopper, homogenized in 500 µl of buffer [(in mM): 1 citrulline, 10 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 100 N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid, pH 5.5] and then centrifuged for 25 min at 10,000 g at 4°C. To separate [3H]arginine from [3H]citrulline, we placed 400 µl of the supernatant onto a previously prepared Dowex ion-exchange column (Na+ form). The eluate and the first water wash (2 ml) were collected and the radioactivity was measured by liquid scintillation counting. Recovery of [14C]citrulline was 80.9 ± 1.3% (n = 31), and cross-contamination with the [3H]arginine fraction was 2.0 ± 0.2% (n = 30). Corrections were made for counting efficiency by external standardization, as well as for column recovery and cross-contamination.

Western blot analysis of iNOS in rat aortic rings after 24-h organ culture. Endothelium-intact thoracic aortas isolated from ovariectomized rats were incubated for 24 h in a sterile incubator. The endothelial cells were removed after the incubation period. The aortas were then homogenized in extraction buffer [(in mM): 154 NaCl, 20 tris(hydroxymethyl)-aminomethane (Tris) base, 10 EDTA, and 10 sodium vanadate, as well as 2% sodium dodecyl sulfate (SDS), pH 7.4]. The homogenate was centrifuged for 20 min at 10,000 g at 4°C. The supernatant was removed and used for Western blot analysis. Protein content was determined using the Pierce bicinchoninic acid kit (Pierce, Rockford, IL) and proteins were adjusted with homogenizing buffer so that the protein load in each lane of the gel was equal. To this solution was added 0.5 volume of reducing buffer (10% bromphenol blue marker, 20% glycerol, 10% 2-mercaptoethanol, and 2% SDS). Samples were boiled at 100°C for 3 min before running on gels, and 15 µl of each sample (8–15 µg of protein) were loaded per well. Proteins and molecular weight markers were electrophoresed for 30–40 min at 200 V in running buffer (25 mM Tris base, 0.19 M glycine, and 1% SDS) on a 7.5% reducing SDS-polyacrylamide gel. The proteins were then transferred to nitrocellulose membrane in transfer buffer (25 mM Tris base, 0.19 M glycine, and 20% methanol, pH 8.1–8.4) at 4°C and 100 V for 75 min. Nonspecific binding sites on the membranes were blocked for 1 h with 5% bovine serum albumin (BSA) solution in Tris-buffered saline (TBS; 20 mM Tris base-137 mM NaCl). Membranes were then incubated at room temperature for 20–24 h with a polyclonal rabbit anti-mouse iNOS antibody (diluted 1:1,000 in 5% BSA). Membranes were then washed twice with TBS for 10 min, once with 0.1% Tween-20 in TBS for 15 min, then twice with TBS for 10 min. Membranes were then incubated for 1 h with swine anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; diluted 1:5,000 in TBS). The membranes were then washed again with the above procedure. They were then reacted with hydrogen peroxide and luminol [enhanced chemiluminescence (ECL) system; Amersham, UK], and iNOS corresponding to a single 130-kDa band was visualized by exposure of the membrane to Kodak chemiluminescence film. Molecular weight markers were run in parallel to all samples, and additional verification of iNOS was performed by comparing Western blot analysis of lipopolysaccharide (LPS)/ interferon-γ-stimulated macrophage lysate (Transduction Laboratories, Lexington, KY); and all Western blot reagents were from Transduction Laboratories.
stored at +4°C. On the day of the experiment it was further diluted in PSS. LPS was dissolved in sterile water to a concentration of 1 mg/ml to yield a final concentration of 10 µg/ml. A stock solution of L-NIO was made in sterile filtered water and stored frozen until the day of experiment. All other drugs were made up daily in fresh PSS. Vehicle control experiments for 17β-estradiol and LPS were carried out where applicable.

PSS, PSS was composed of (in mM) 118 NaCl, 4.7 KCl, 1.03 KH2PO4, 25 NaHCO3, 11.1 d-(+)-glucose, 1.2 MgSO4, 1.6 CaCl2, and 0.067 Na2EDTA.

Statistical analyses. All results are expressed as means ± SE, and n indicates the number of observations. The results were analyzed by one-, two-, or three-way analysis of variance (ANOVA) with repeated measures where appropriate. Individual means were compared with either Student’s t-test, Mann Whitney U-test, or Dunnetts test where appropriate. In all cases a value of P < 0.05 was taken as significant using the GB-STAT computer package (Dynamic Microsystems, Silver Spring, MD). Although multiple aortic rings were taken from each animal, only one type of experiment was conducted in each animal.

RESULTS

Effects of 10-min exposure of 17β-estradiol on vasoconstriction. In endothelium-denuded aortic rings freshly isolated from ovariectomized rats, phenylephrine produced a concentration-dependent vasoconstriction, and 10-min exposure to 17β-estradiol (10 nM) did not affect the vasoconstriction to phenylephrine (Fig. 1A).

Effects of 24-h exposure to 17β-estradiol on vasoconstriction. Rat aortic rings isolated from ovariectomized rats were treated with 17β-estradiol (1 and 10 nM for 24 h) in organ culture and then the endothelium was removed. The endothelium was removed after the 24-h incubation as endothelium removal before the 24-h incubation resulted in the induction of iNOS, making it difficult to assess the effects of 17β-estradiol (not shown). The constriction effect of phenylephrine in 24-h vehicle-treated controls was similar to that of freshly excised aortas (compare Fig. 1, A and B). The 17β-estradiol treatment attenuated the phenylephrine-induced constriction (Fig. 1B), and this was most apparent in the maximum constriction. The NOS inhibitor L-NIO (22) (100 µM for 24 h) abolished the inhibitory effect of 17β-estradiol (10 nM for 24 h) on the phenylephrine-induced constriction (Fig. 1C). Concomitant treatment with progesterone (30 nM for 24 h) did not affect the inhibitory effect of 17β-estradiol on the phenylephrine-induced constriction (Fig. 1D); however, it should be noted that progesterone alone decreased the phenylephrine constriction. The estrogen receptor antagonist ICI-182,780 (32) (100 nM for 24 h) reduced the phenylephrine constriction and also decreased the effect of 17β-estradiol (Fig. 1E).

Effects of 24-h exposure to 17β-estradiol on endothelium-dependent relaxation. After 24-h organ culture of the aortic rings, acetylcholine (0.001–10 µM) concentration dependently relaxed endothelium-intact aortic rings isolated from ovariectomized rats that were preconstricted with phenylephrine (100 nM) (Fig. 2). In aortas that were exposed to 17β-estradiol (10 nM) for 24 h, the relaxation induced by acetylcholine was slightly but significantly enhanced.

Whole tissue NOS activity. The aortas were incubated for 24 h with 17β-estradiol with the endothelium intact. After this the endothelium was removed and the conversion of [3H]arginine to [3H]citrulline was measured to assess NOS activity in the rings. 17β-Estradiol treatment (0.001–10 µM for 24 h) increased [3H]citrulline formation (Fig. 3), and this effect was blocked by concomitant (24 h) cycloheximide treatment (1 µM; Fig. 3). LPS (10 µg/ml) also significantly increased [3H]citrulline production (P < 0.05, Student’s t-test: control = 14.9 ± 3.4 pmol·min⁻¹·mg tissue⁻¹, n = 6; LPS = 680.2 ± 84.1 pmol·min⁻¹·mg tissue⁻¹, n = 4).

Western blot analysis of iNOS. The presence of iNOS protein was investigated using a specific iNOS antibody and Western blotting. Endothelium-intact aortas were incubated with drugs and the endothelium was removed just before protein extraction. Incubation of aortas isolated from ovariectomized rats with LPS (10 µg/ml) resulted in a single 130-kDa band corresponding to iNOS, which was inhibited by the concomitant treatment with cycloheximide (1 µM) (data not shown). Incubation for 24 h with 17β-estradiol (10 nM) also resulted in a band corresponding to iNOS, and this was prevented by concomitant treatment with ICI-182,780 (100 nM) but not progesterone (30 nM) (Fig. 4).

DISCUSSION

The effects of 17β-estradiol on aortas from ovariectomized rats were studied using an organ culture technique in which we were able to treat the aortas with drugs for 24 h in vitro. After this time the tissues maintained constrictor responses to phenylephrine that were equivalent to that in freshly excised aortas, as was the endothelium-dependent relaxation to acetylcholine.

In the present study, acute application of 17β-estradiol (10 nM) had no effect on the vasoconstriction produced by phenylephrine in freshly excised rat aortas from ovariectomized rats that were endothelium denuded. This is consistent with the majority of isolated blood vessel studies, in which acute vasorelaxant effects of 17β-estradiol, which are endothelium independent, are only seen in a much higher concentration range (3–10 µM) (25), with lower concentrations being ineffective (16, 17, 26). Plasma estradiol levels reach 1 nM in the midcycle of premenopausal women and 55 nM during the final stages of pregnancy (1, 29), which makes the physiological relevance of these effects of estrogen debatable. It should be noted, however, that in rabbit coronary arteries (6) acute 17β-estradiol-mediated relaxation has been reported at 10 nM.

A more sustained application of 17β-estradiol may be more physiologically relevant because some vascular effects of 17β-estradiol are due to alterations in gene transcription, including enhanced synthesis of eNOS (13, 33), which takes a longer time frame to be apparent. In the present study, after 24-h treatment in vitro with 17β-estradiol (10 nM), the endothelium-dependent relaxation to acetylcholine in phenylephrine-
constricted aortas was significantly enhanced. This is consistent with results from prolonged in vivo treatment with estrogen, in which chronic treatment with 17β-estradiol potentiated the endothelium-dependent relaxation to acetylcholine in canine coronary arteries (24) and rabbit femoral arteries (23). In the endothelium-intact aortic rings used for the present acetylcholine study, the constrictor effect of phenylephrine had a tendency to be less after 17β-estradiol treatment (10 nM for 24 h). This was more clearly observed in endothelium-denuded aortic rings, in which higher phenylephrine concentrations were used, and in this case 17β-estradiol treatment (1 and 10 nM for 24 h) attenuated the phenylephrine constriction, particularly the maximal constriction. It is possible that this could be a manifestation of the direct nitric oxide-independent relaxation of 17β-estradiol seen previously on acute application at high concentrations (16, 17, 26), but the effect of 17β-estradiol was attenuated by the NOS synthesis inhibitor L-NIO, and, furthermore, acute administration of 17β-estradiol (10 nM) had no effect on the phenylephrine constriction. The involvement of NOS was not due to a residual endothelium in the aorta as in each individual ring the removal of the endothelium was confirmed by abolition of the relaxant response to acetylcholine and in randomly selected rings silver staining revealed that the endothelium had been completely removed. Thus it appears that the effect of 17β-estradiol on phenylephrine-induced constriction is mediated through a nitric oxide-dependent process. This was further examined by measuring NOS activity.

17β-Estradiol (10 nM for 24 h) increased basal NOS activity in the endothelium-denuded aortic rings isolated from ovariectomized rats. Symbols represent means ± SE. A: acute application of Est (10 nM); n = 4 for no Est and n = 5 for Est. B: chronic (24 h) treatment with Est (n = 10 for no Est, n = 4 for 1 nM Est, n = 12 for 10 nM Est). C: chronic (24 h) treatment with L-N5-(1-iminoethyl)ornithine hydrochloride (l-NIO) (100 µM) and Est (10 nM); n = 4 for no Est + l-NIO and n = 10 for Est (10 nM) + l-NIO. D: chronic (24 h) treatment with progesterone (Prog; 30 nM) and Est (10 nM); n = 4 for no Est + Prog, n = 8 for Est + 10 nM Prog. Prog alone decreased the Phe constriction (P < 0.05). *Significant difference from respective experiment in absence of estradiol (no Est) (P < 0.05, 2-way ANOVA with repeated measures). E: chronic (24 h) treatment with ICI-182,780 (100 nM) and Est (10 nM); n = 7 for no Est + Prog and n = 8 for Est (10 nM) + Prog. ICI-182,780 alone decreased the Phe constriction (P < 0.05).
These receptors mediate the induction of iNOS by vascular smooth muscle (4, 18), and we suggest that it is clear that further work needs to be carried out.

Our hypothesis. We can only speculate as to reasons for this discrepancy as there are considerable differences between these studies and our own in terms of treatment regimes, tissues examined, and assay conditions. It is clear that further work needs to be carried out.

Estrogen receptors have been previously detected in smooth muscle iNOS gene structure (5, 10) we found a 60% match of the estrogen response element (AGGTCA nnn TGACCT) (9) in the promoter region at base pairs 14–26. However, studies with chronic 17β-estradiol treatment in vivo did not observe any changes in Ca2+-independent NOS activity in broken cell assays (13, 33). iNOS is a Ca2+-independent enzyme (21) and should have been detected in these assays according to our hypothesis. We can only speculate as to reasons for this discrepancy as there are considerable differences between these studies and our own in terms of treatment regimes, tissues examined, and assay conditions. It is clear that further work needs to be carried out.

Estrogen receptors have been previously detected in vascular smooth muscle (4, 18), and we suggest that these receptors mediate the induction of iNOS by 17β-estradiol because the anticonstrictor effect of 17β-estradiol (10 nM for 24 h) was blocked by ICI-182,780, a selective estrogen receptor antagonist (32). This result suggests that the anticonstrictor effect of 17β-estradiol is receptor mediated and rules out nonspecific physicochemical interactions of 17β-estradiol with nitric oxide signaling pathways or the possibility that the effect of 17β-estradiol is due to a contaminant such as endotoxin fragments. It should be noted, however, that ICI-182,780 treatment decreased the phenylephrine constriction by itself. The relevance of this finding is unclear and may involve other factors as ICI-182,780 did not induce iNOS immunoreactivity.

It has been suggested that estrogen has beneficial effects on the cardiovascular system (30), and it is possible that some of these effects may be related to an action involving iNOS. iNOS does not require Ca2+ activation and produces nitric oxide without additional stimulus (21). However, its reputation is far from positive, and nitric oxide formation from iNOS produces detrimental cardiovascular effects through excessive nitric oxide formation and the production of reactive chemical intermediates (7). However, these effects are most probably related to the amount of nitric oxide formed (which is high) and to the coactivation of other processes by the circumstances that induce iNOS (LPS, inflammation, or tissue damage), rather than the enzyme itself. It is important to point out that the nitric oxide synthesis induced by 1 nM 17β-estradiol in endothelium-denuded aortas is only a small percentage of that produced by LPS. We suggest that, if iNOS produces a low basal synthesis of nitric oxide, then this may well be beneficial to the circulation through attenuation of vasoconstrictor influences and platelet aggregation, as is proposed for endothelium-derived nitric oxide (see Ref. 7). Furthermore, induction of iNOS per se has been associated with protective cardiovascular effects, such as in preventing platelet aggregation at sites of vascular injury (14) and preventing ischemic damage in the myocardium (26).

Progesterone supplementation is now part of hormone replacement therapy (8), and there is an as yet unconcluded epidemiological debate as to whether pro-
gesterone may compromise the beneficial effects of estrogen on the cardiovascular system (35) because progesterone can interfere with estrogen at many sites, including downregulating the estrogen receptors (19). However, we found that progesterone cotreatment of rat aortas did not attenuate the endothelium-independent anticonstrictor effect of 17β-estradiol treatment (10 nM for 24 h). The concentration of progesterone used (30 nM) is within the physiological range (1). This suggests that cotherapy with progesterone may not limit the vascular effects of estrogen or at least those mediated through iNOS. At higher doses of progesterone, vascular endothelial effects may be seen. For example, progesterone in high doses equivalent to late-term pregnancy partially attenuated estrogen-induced uterine vasodilation in sheep (28), which is NOS dependent (31). Furthermore, progesterone treatment of dogs (plasma levels 50–120 nM) partially attenuated 17β-estradiol effects on endothelium-dependent relaxation in coronary artery rings (24). In the present study progesterone treatment by itself slightly reduced the phenylephrine constriction in endothelium-denuded aortas, but this is unrelated to iNOS inasmuch as progesterone did not induce iNOS immunoreactivity. In summary, we have shown that 17β-estradiol can increase nitric oxide production in endothelium-denuded rat aortas by inducing iNOS. The effect can be observed with 17β-estradiol concentrations in the physiological range and thus seems relevant to the long-term cardiovascular effects of 17β-estradiol. We suggest that long-term 17β-estradiol therapy may induce a sustained partial hyporesponsiveness in vascular smooth muscle via sustained nitric oxide production because iNOS is not a Ca2+-regulated enzyme and has inherent activity (21). Although we do not have evidence as to the cell type involved, we suggest that it may be vascular smooth muscle because both estrogen receptors (4, 18) and iNOS (5, 10) are present in this cell type. The present study used aortas, and whether the same effects can be observed in small resistance vessels remains to be investigated. Previous studies have revealed that 17β-estradiol can also induce NOS in endothelial cells (13, 33), which suggests that nitric oxide may participate at several sites in estrogen’s physiological actions.

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