Angiotensin II Stimulates Endothelial Vascular Cell Adhesion Molecule-1 via Nuclear Factor-κB Activation Induced by Intracellular Oxidative Stress

Maria E. Pueyo, Walter Gonzalez, Antonino Nicoletti, Françoise Savoie, Jean-François Arnal, Jean-Baptiste Michel

Abstract—The recruitment of monocytes via the endothelial expression of vascular cell adhesion molecule-1 (VCAM-1) is a key step in the formation of the initial lesion in atherosclerosis. Because angiotensin (Ang) II may be involved in this process, we investigated its role on the signaling cascade leading to VCAM-1 expression in endothelial cells. Ang II stimulates mRNA and protein expression of VCAM-1 in these cells via the AT1 receptor. This effect was enhanced by N\textsuperscript{\textregistered}-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, and blocked by pyrrolidine dithiocarbamate, an antioxidant molecule. Ang II activated the redox-sensitive transcription factor nuclear factor-κB and stimulated the degradation of both inhibitor of κB (IκB)α and IκBβ with different kinetics. The degradation of IκBαs induced by Ang II was not modified by incubation with exogenous superoxide dismutase and catalase, suggesting that this effect was not mediated by the extracellular production of O\textsubscript{2}\. In contrast, rotenone and antimycin, 2 inhibitors of the mitochondrial respiratory chain, inhibited the Ang II–induced IκB degradation, showing that generation of reactive oxygen species in the mitochondria is involved on Ang II action. BXT-51702, a glutathione peroxidase mimic, inhibited the effect of Ang II, and aminotriazole, an inhibitor of catalase, enhanced it, suggesting a role for H\textsubscript{2}O\textsubscript{2} in IκB degradation. This is confirmed by experiments showing that Ang II stimulates the intracellular production of H\textsubscript{2}O\textsubscript{2} in endothelial cells. These results demonstrate that Ang II induced an intracellular oxidative stress in endothelial cells, which stimulates IκB degradation and nuclear factor-κB activation. This activation enhances the expression of VCAM-1 and probably other genes involved in the early stages of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2000;20:645-651.)

Key Words: endothelium [ ] nuclear factor-κB [ ] IκB [ ] superoxide anions [ ] hydrogen peroxide

One of the earliest detectable cellular responses in the formation of atherosclerotic lesions is the focal recruitment of leukocytes by intact endothelium. This localized accumulation of leukocytes is mediated by the endothelial expression of specific adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1, or platelet–endothelial cell adhesion molecule-1. VCAM-1, which is focally expressed by endothelial cells in atherosclerotic lesions, appears to be correlated with increased accumulation of mononuclear cells. The expression of VCAM-1 in endothelial cells is enhanced by inflammatory mediators or cytokines. The regulation of VCAM-1 expression occurs at the transcriptional level and is mediated, at least in part, via the redox-sensitive transcription factor nuclear factor-κB (NF-κB). Activated NF-κB is involved in the expression of several proinflammatory genes and is present in endothelial cells in the early lesions of atherosclerosis. In unstimulated endothelial cells, the predominant form of NF-κB is present in the cytoplasm as a heterodimer of p50 and p65 subunits complexed with inhibitor of κB (IκB) proteins. After cell stimulation, IκB is degraded, releasing NF-κB and thus allowing its translocation to the nucleus. Among IκBαs, IκBα and IκBβ play a major role in the regulation of NF-κB. These 2 molecules are structurally similar, but their degradation and their patterns of expression differ. Inducers of NF-κB activation, such as tumor necrosis factor-α (TNF-α), phorbol 12-myristate 13-acetate, or UV radiation cause oxidative stress, suggesting that the induction of radical oxygen species (ROS) is a common signal to a wide variety of NF-κB–inducing conditions.

Angiotensin (Ang) II, the main effector of the renin-angiotensin system, plays an essential role in the regulation of blood pressure but is also involved in remodeling of the arterial wall. It has recently been shown that antagonists of AT\textsubscript{1} Ang II receptors decrease the formation of the atheromatous plaque in several animal models of atherosclerosis and that Ang II increases leukocyte adhesion to the endothelium in vitro. Given that VCAM-1 expression in endothelial cells is inhibited by AT\textsubscript{1} receptor antagonists in vivo, we hypothesized that Ang II could directly modulate VCAM-1 expression in these cells. Indeed, endothelial cells express AT\textsubscript{1} receptors, which activate different intracellular path-
Regulation of VCAM-1 expression by Ang II in endothelial cells. A, Expression of VCAM-1 mRNA evaluated by RT-PCR: cells were incubated for 4 hours with different concentrations of Ang II. Total RNA was extracted, reverse-transcribed, and amplified by using specific oligonucleotides for VCAM-1 and for β-actin. Quantification of amplified fragments was determined by counting the incorporated radioactivity. Results are expressed as the ratio of VCAM-1 mRNA relative to the β-actin mRNA levels. Results are mean ± SEM of 5 independent experiments that used separate endothelial cell isolates. B, Protein levels of VCAM-1 evaluated by Western blot. Cells were incubated for 18 hours with different concentrations of Ang II (top) or with 10⁻⁷ mol/L Ang II at indicated times (bottom). Total protein extracts were separated by electrophoresis, transferred to a nitrocellulose membrane, and probed with an anti–VCAM-1 antibody. Figure is representative of 4 independent experiments.

Figure 1. Regulation of VCAM-1 expression by Ang II in endothelial cells. A, Expression of VCAM-1 mRNA evaluated by RT-PCR: cells were incubated for 4 hours with different concentrations of Ang II. Total RNA was extracted, reverse-transcribed, and amplified by using specific oligonucleotides for VCAM-1 and for β-actin. Quantification of amplified fragments was determined by counting the incorporated radioactivity. Results are expressed as the ratio of VCAM-1 mRNA relative to the β-actin mRNA levels. Results are mean ± SEM of 5 independent experiments that used separate endothelial cell isolates. B, Protein levels of VCAM-1 evaluated by Western blot. Cells were incubated for 18 hours with 10⁻⁷ mol/L Ang II in the presence or absence of 10⁻⁶ mol/L irbesartan (Irb) or 10⁻⁷ mol/L PD123177 (PD). Results are expressed as the ratio of VCAM-1 mRNA relative to the β-actin mRNA levels. Results are mean ± SEM of 4 independent experiments that used separate endothelial cell isolates. **P<0.01 vs control. B, Protein levels of VCAM-1 evaluated by Western blot. Cells were incubated for 18 hours with 10⁻⁷ mol/L Ang II in the presence or absence of 10⁻⁶ mol/L Irb or 10⁻⁷ mol/L PD. Figure is representative of 4 independent experiments.

Methods

Materials

DMEM, HEPES, horse serum, and trypsin/EDTA were obtained from Life Technologies Inc. Ang II, penicillin, streptomycin, superoxide dismutase (SOD), catalase, N⁵-nitro-L-arginine methyl ester (L-NAME), pyrroolidinedithiocarbamate (PDTC), and aminotriazole were from Sigma Chemical Co. Antibodies against VCAM-1, NF-κB, and IκB were purchased from Santa Cruz Biotechnologies. Irbesartan was kindly provided by Sanofi Recherche (Montpellier, France), and PD123177 was provided by Dr P. Janiak (Synthelabo, Paris, France). The glutathione peroxidase mimic, BXT-51702, was a gift from OXIS International (Bonneuil sur Marne, France).

Cell Culture

Rat aortic endothelial cells were isolated as previously described. This method provides pure cell preparations (>95% of endothelial cells), as shown by immunostaining with a specific antibody against rat endothelial cells. Cells from passages 1 to 3 were used in these studies.

Semiquantitative Analysis of VCAM-1 mRNA Expression by RT-PCR

Extraction of total RNA and reverse transcriptase (RT)—polymerase chain reaction (PCR) are described in detail elsewhere. Oligo primers for VCAM-1 included 5'-CAC CTC CCC CAA GAA TAC AGA-3' (antisense) and 5'-GCT CAT CCT CAA CAC CCA CAG-3' (antisense), which amplify a 476-bp fragment. The primers for the rat β-actin were 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' (antisense) and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3' (sense). VCAM-1 mRNA expression was normalized to the housekeeping gene β-actin mRNA expression.

Western Blot Analysis

Cells were serum-deprived for 24 hours and incubated with 10⁻⁷ Ang II during times indicated in Results. When used, antagonists were preincubated for 30 minutes before the addition of Ang II. After incubation, cells were lysed by use of 10 mmol/L Tris-HCl, pH 7.4, 1% Triton, 0.5% Nonidet P-40, 0.5 mmol/L sodium orthovanadate, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L NaF, 30 mmol/L pyrophosphate sodium, 1 μg/mL aprotinin, and 1 mmol/L serine protease inhibitor (Interchim). The cell lysates were centrifuged for 20 minutes at 14 000g. Protein concentrations were determined by the Bradford assay. Whole-cell lysates (25 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond, Amersham). Immunodetection was performed by using Renaissance reagents (Amersham).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay

After treatment of endothelial cells for 1 hour with 10⁻⁷ Ang II, nuclear proteins were prepared as previously described.22
Gel-shift assays were performed with a commercial kit according to the instructions of the manufacturer (Promega). The NF-κB oligonucleotide probe used (5’-AGT TGA GGG GAC TTT CCC AGG C-3’) was labeled with [γ-32P]ATP by use of T4 polynucleotide kinase. Nuclear proteins were incubated for 20 minutes with the labeled probe and migrated in a 4% polyacrylamide gel.

The specificity of the binding reaction was determined by coincubating duplicate samples with either 100-fold molar excess of unlabeled oligonucleotide probe or anti-NF-κB antibodies (anti-p65 and anti-p50).

**Determination of H_{2}O_{2} Production**

The generation of intracellular peroxides in rat aortic endothelial cells was monitored with a fluorescent dye, 2’,7’-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes). Serum-deprived cells were stimulated with 10^{-7} mol/L Ang II for 1 hour. Forty-five minutes before the end of the incubation, DCFH-DA was added at a final concentration of 10^{-3} mol/L. Cells were then scraped off into 1 mL of distilled water, sonicated, and centrifuged. The fluorescence of supernatants was measured with a spectrofluorometer (HITACHI F-2000) at 485-nm excitation and 525-nm emission. Data were expressed in arbitrary fluorescence units and normalized with respect to the protein content in each culture dish.

**Statistical Analysis**

Data are expressed as mean±SEM. Groups were compared by ANOVA. Differences were considered significant at P<0.05.

**Results**

**Ang II Stimulates Expression of VCAM-1 via AT_{1} Receptor and NF-κB Pathway**

VCAM-1 mRNA expression, evaluated by RT-PCR, was stimulated by Ang II in a dose-dependent manner (Figure 1A). Ang II also enhanced VCAM-1 protein expression, as evaluated by Western blot (Figure 1B). These effects were inhibited by the AT_{1}-specific antagonist irbesartan but not by the AT_{2} receptor antagonist PD123177 (Figure 2), indicating that Ang II action was mediated by the AT_{1} receptor.

Because nitric oxide (NO) and ROS are implicated in the regulation of VCAM-1 expression through the redox-sensitive transcription factor NF-κB, we evaluated the effect of the NO synthase inhibitor L-NAME and of the antioxidant PDTC on Ang II–induced VCAM-1 expression. Figure 3 shows that L-NAME enhanced basal and, to a lesser extent, Ang II–induced VCAM-1 expression. Conversely, PDTC significantly decreased both basal and Ang II–induced VCAM-1 expression. Because PDTC is an antioxidant and a metal chelator known to inhibit the transcription factor NF-κB, and the VCAM-1 promoter possesses specific DNA binding motifs for NF-κB, we hypothesized that the Ang II–induced VCAM-1 expression was mediated via this transcription factor. To confirm this, we tested whether Ang II was activating the transcription factor NF-κB by use of an electrophoretic mobility shift assay. Figure 4 shows that Ang II increased the nuclear translocation of NF-κB. This effect was abolished by an excess of cold probe, and preincubation with anti-p65 and anti-p50 antibodies induced a supershift.

**Ang II Activates NF-κB by Inducing Differential Degradation of IκBα and IκBβ**

The translocation of NF-κB to the nucleus requires the release of NF-κB from its cytoplasmic inhibitors, the IκB proteins. These proteins are degraded after stimulation of NF-κB–activating factors. We investigated the effect of Ang II on IκBα and IκBβ degradation on endothelial cells. Ang II treatment resulted in a rapid degradation of IκBα (within 15 minutes), with a maximal effect after 30 minutes of stimulation (Figure 5). IκBβ was synthesized de novo after 1 hour. Ang II also induced the degradation of IκBβ, but this degradation was observed after only 30 minutes, and IκBβ was not synthesized de novo after a 2-hour treatment with Ang II (Figure 5) and remained at low levels for 24 hours (data not shown).

**Degradation of IκBs Is Dependent on Mitochondrial ROS Release**

Having demonstrated that Ang II stimulates the disappearance of immunoreactive IκBs and given that the induction of ROS is a common signal to a wide variety of NF-κB–inducing conditions, we next determined whether the degradation of IκB was dependent on the production of ROS. For this purpose, we used PDTC, a nonspecific antioxidant. Our results show that PDTC inhibits the Ang II–induced IκB degradation (Figure 6A). To better understand this phenomenon, we used SOD, an enzyme that dismutates O_{2} into H_{2}O_{2} and catalase, which degrades H_{2}O_{2}. SOD does not modify the Ang II–induced IκB degradation, and no effect was observed when cells were incubated with SOD plus catalase (Figure 6B). During the short time of incubation (1 hour), both enzymes may remain in the extracellular compartment, and they do not affect intracellular ROS, so that
our results suggest that Ang II–induced IκB degradation was not dependent on oxidant stress occurring on the cell surface.

Thus, we next studied the involvement of intracellular ROS. Mitochondrial respiration constitutes one of the most important sources of ROS in the cell. Figure 6C shows that 2 inhibitors of the mitochondrial respiratory chain, rotenone and antimycin, inhibit Ang II–induced IκB degradation. This suggests that Ang II–induced degradation of IκBs was provoked by ROS, which were most likely formed within the mitochondria. As a consequence of the abundance of manganese SOD in the mitochondria, most of the O₂⁻ generated in this organelle is converted into H₂O₂. Thus, we investigated the role of intracellular H₂O₂ by interfering with its intracellular degradation, a role devoted to glutathione peroxidase and catalase. BXT-51702, a potent glutathione peroxidase mimic, which catalyzes the degradation of H₂O₂ by glutathione, inhibited the effect of Ang II on the degradation of IκBα and IκBβ. In contrast, aminotriazole, an inhibitor of catalase but also of glutathione peroxidase, which increases intracellular levels of H₂O₂, enhanced the IκB degradation induced by Ang II (Figure 6D).

Detection of Generation of ROS by Ang II
To further evaluate the Ang II–induced oxidative stress, we assessed the production of O₂⁻ after Ang II stimulation of endothelial cells with 2 different methods, lucigenin-enhanced chemiluminescence and electron spin resonance by using 5,5'-dimethyl-1-pyrroline N-oxide as a spin trap. We failed to detect any O₂⁻ production in response to Ang II in endothelial cells. In contrast, we have previously reported the production of O₂⁻ by bradykinin with the use of these 2 methods. Because these techniques mainly measured extracellular production of O₂⁻, we conclude that Ang II does not enhance extracellular O₂⁻ production. Next, we evaluate the intracellular production of H₂O₂ with DCFH-DA, a probe used to detect intracellular H₂O₂ in a variety of cells, including endothelial cells. Figure 7 shows that endothelial cells incubated with 10⁻⁷ mol/L Ang II exhibited an increase...

Figure 5. Time course of IκBα and IκBβ degradation induced by Ang II in endothelial cells. Cells were treated with 10⁻⁷ mol/L Ang II for the indicated times. Total protein extracts were separated by electrophoresis, transferred to a nitrocellulose membrane, and probed with antibodies to IκBα and IκBβ. Figure is representative of 5 different experiments.
in intracellular DCFH-DA fluorescence, indicating the production of intracellular H$_2$O$_2$ in response to Ang II. This production may be implicated in the Ang II–induced IκB degradation.

**Figure 6.** Regulation of IκBα and IκBβ degradation induced by Ang II in endothelial cells. Cells were preincubated for 30 minutes with different compounds: 10$^{-5}$ mol/L PDTC (A), 100 U/mL SOD and 100 U/mL catalase (B), 10$^{-5}$ mol/L rotenone or 10$^{-5}$ mol/L antimycin (C), or 10$^{-5}$ mol/L BXT-51702 or 10$^{-5}$ mol/L aminotriazole (D). Cells were then treated with 10$^{-7}$ mol/L Ang II for 30 minutes in the presence or absence of drugs. Similar results were obtained in at least 3 independent experiments that used separate endothelial cell isolates.

**Discussion**

Upregulation of adhesion molecules, such as VCAM-1, occurs at sites of lesion formation in early atherosclerosis. Hypertension, one of the known clinical risk factors for atherosclerosis, increases endothelial VCAM-1 expression. In the present study, we demonstrated that Ang II directly stimulates VCAM-1 expression in endothelial cells, suggesting that the Ang II effect on VCAM-1 expression observed in vivo is not merely an indirect effect via its hypertensive action.

The Ang II action on VCAM-1 expression in endothelial cells was inhibited by irbesartan, an AT$_1$ antagonist, in agreement with studies showing that the AT$_1$ receptor–mediated signal is predominant in this cell type. These in vitro data are in accord with in vivo studies showing that AT$_1$ receptor antagonists decrease VCAM-1 expression in endothelial cells.

Because NO decreases the expression of VCAM-1, we evaluated the effect of L-NAME, a NO synthase inhibitor, on Ang II–induced VCAM-1 expression. NO blockade inhibited the basal expression of VCAM-1 in endothelial cells but produced only a discrete increase in this expression in Ang II–stimulated cells, suggesting that endogenous levels of NO in endothelial cells, at least in vitro, are not sufficient to limit Ang II–induced VCAM-1 expression, despite the simultaneous stimulation of NO production by Ang II.

A key component of the cytokine-inducible VCAM-1 expression is the redox-sensitive transcription factor NF-κB. In the present study, we demonstrate that Ang II activates NF-κB in endothelial cells, suggesting that the upregulation of VCAM-1 expression in response to Ang II is mediated by this transcription factor. However, the regulatory regions of the VCAM-1 gene are composed of multiple binding elements, which are recognized by a large number of transcription factors. The cytokine-induced transcriptional enhancer in the VCAM-1 promoter requires combinatorial interactions of NF-κB with other nuclear activators, such as stimulatory protein-1, interferon regulatory factor-1, and activator protein-1. Ang II activates the transcription factor, AP-1, but there are no data available about the effect of Ang II on Sp-1 or IRF-1. Further studies will be necessary to evaluate the role of these transcription activators in the modulation of Ang II–induced NF-κB transactivation of the VCAM-1 promoter.

Translocation of NF-κB to the nucleus requires the degradation of the cytoplasmic inhibitory proteins IκBα and IκBβ. Recent studies concluded that some NF-κB activators induce degradation of IκBα but not IκBβ, suggesting that these 2 proteins are regulated differentially. We demonstrated that Ang II stimulates the degradation of IκBα and IκBβ, but the kinetics of degradation were different: whereas IκBα degradation was rapid and transient, IκBβ degradation was slower and persisted for at least 24 hours. This differential regulation of the pools of IκBα and IκBβ may allow fine tuning of the activation/repression of NF-κB by Ang II. Indeed, some NF-κB inducers would cause a rapid but transient activation of NF-κB by primarily affecting IκBα complexes, whereas the others would cause persistent activation of NF-κB by...
affecting both IkBα and IkBβ complexes. In endothelial cells, TNF-α has been shown to activate NF-κB rapidly and persistently, in contrast to other agents, such as phorbol 12-myristate 13-acetate, which causes a slow and transient activation. In view of our results, it appears that the overall activation of NF-κB by Ang II consists of 2 overlapping phases, a rapid transient phase mediated through IkBα and a persistent phase mediated through IkBβ.

The behavior of agonists that activate NF-κB greatly depends on the cell type involved. It is of interest to point out that Ang II does not modify IkBβ levels in rat aortic smooth muscle cells, whereas it stimulates the degradation of IkBα in this cell type (data not shown). Moreover, others have demonstrated that TNF-α fails to modulate IkBβ but decreases IkBα levels in smooth muscle cells, and we have observed that TNF-α increases the degradation of IkBα and IkBβ in endothelial cells (M.E.P. et al, unpublished data, 1998). These data suggest that the regulation of IkBβ and possibly the nature of the NF-κB response (transient versus sustained) differ between smooth muscle and endothelial cells. The divergent NF-κB regulation in smooth muscle cells and endothelial cells may have potential functional consequences that are of particular interest in view of the proximity of these 2 cell types within the vessel wall.

Several lines of evidence indicate that ROS are implicated in the activation of NF-κB and the degradation of IkBs. Because it is known that Ang II stimulates oxidative stress in various cell types, including endothelial cells, we investigated whether the ROS generated in response to Ang II were implicated in the degradation of IkBs. This is shown by the inhibitory effect of PDTC on the Ang II–induced IkB degradation. Pharmacological studies that used aminotriazole or BXT-51702 suggest that H2O2 and/or derivatives mediate Ang II–induced IkBα degradation and agree with data showing that ROS, in particular, H2O2, act as second messengers in the activation of NF-κB. This hypothesis is further supported by our results showing that Ang II enhanced H2O2 production in endothelial cells. One can suppose that Ang II shares some of these ROS-dependent pathways leading to NF-κB activation with other activators, such as TNF-α. For example, BXT-51702, a potent glutathione peroxidase mimic that inhibits the Ang II–induced IkBα degradation, also decreases the VCAM-1 expression induced by TNF-α as well as other adhesion molecules activated by NF-κB. However, the critical steps in the signal transduction cascade of IkB degradation by ROS remain to be determined.

Recently, the understanding of the downstream pathways implicated in the regulation of IkB degradation has advanced to a great extent. Phosphorylation seems to be necessary for the degradation of IkBα, but not of IkBβ. Two IkB kinases, IKK-1 and IKK-2, have been identified as responsible for the phosphorylation of IkBα. These IKKs are activated by a NF-κB–inducing kinase, NIK, a member of the mitogen-activated protein kinase-3 family, which, in turn, is activated by TGFβ-activated kinase-1. Antioxidants specifically inhibit the IKK activity stimulated by TNF-α. However, their effect on NIK activation is unknown at present. Further studies will be necessary to determine the link between ROS and these signaling pathways and the role of Ang II in their modulation.

In conclusion, proinflammatory cytokines, via the induction of VCAM-1 expression, are thought to participate in monocyte accumulation in the vessel wall and contribute to atherosclerotic lesions. The present study demonstrates that Ang II, like cytokines, could participate in inducing these events through endothelial cells. The activation of the transcription factor NF-κB by Ang II, leading to the upregulation of proinflammatory genes, may explain, at least in part, the diminution of monocytes adhesion induced by Ang II receptor antagonists. Endothelial cells are the principal target of plasma Ang II, because little of the Ang II generated in the plasma passes to the media and vice versa. This chronic phenotypic modulation of endothelial cells by plasma Ang II could be involved in the development of atherosclerotic lesions and participate in the relation between the activation of the renin-angiotensin system and the risk of atherosclerosis.

Acknowledgments

This work was supported by INSERM and by a grant from SANOFI Recherche (Montpellier, France). M.E.P. was supported by a grant from SANOFI Recherche, and W.G. was supported by a grant from the Société de Secours des Amis des Sciences. We are indebted to Dr J. Chaudrière for providing the BXT-51702 and for helpful discussions.

References


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*Arterioscler Thromb Vasc Biol.* 2000;20:645-651
doi: 10.1161/01.ATV.20.3.645

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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