Abstract—Apoptosis is a mode of cell death in which the cell participates in its own demise. We studied whether endothelium-derived relaxing factor, nitric oxide (NO), and natriuretic peptides affect apoptosis of rat vascular endothelial cells via a cGMP-dependent pathway and whether such effects are antagonized by an endothelium-derived vasoconstrictor, endothelin-1 (ET-1). Three natriuretic peptides (atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide) induced endothelial apoptosis as demonstrated by nucleosomal laddering on agarose gel electrophoresis and by the terminal deoxynucleotidyl transferase–mediated dUTP biotin nick end labeling method. This dose-dependent relation was assessed by quantifying the fragmented and intact DNA contents by the diphenylamine method. The atrial natriuretic peptide–induced endothelial apoptosis was completely blocked by a guanylate cyclase–coupled receptor antagonist (HS-142-1) and an inhibitor of cGMP-dependent protein kinase (KT5823). An NO donor, NOR3 \((\pm)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide; FK409) also induced endothelial apoptosis; the effect of this compound was abrogated by KT5823 and an inhibitor of soluble guanylate cyclase, ODQ \((1H-)[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one). A cGMP derivative, 8-bromo-cGMP, but not the cAMP derivative 8-bromo-cAMP, caused endothelial apoptosis; the effect of ODQ was also abrogated by KT5823. Endothelial apoptosis induced by ANP, NOR3, and 8-bromo-cGMP was similarly antagonized by ET-1. ANP, NOR3, and 8-bromo-cGMP caused marked accumulations of the tumor suppressor gene product p53 but not of bcl-2, as determined by Western blot analysis. These results demonstrate for the first time that endothelium-derived NO and natriuretic peptides are proapoptotic factors for endothelial cells, whereas the endothelium-derived vasoconstrictor ET-1 is an antiapoptotic factor, suggesting that the countervailing balance between these vasodilators and vasoconstrictors, in addition to regulation of vascular tone, may contribute to endothelial cell integrity. (Arterioscler Thromb Vasc Biol. 1999;19:140-146.)

Key Words: natriuretic peptides ■ apoptosis ■ nitric oxide ■ endothelial cells

Vascular endothelial cells (ECs), a monolayer of cells lining the intima of blood vessels, play important roles in a variety of functions, including coagulation, vascular permeability, vascular tonus, and remodeling. ECs release a variety of vasodilators, such as nitric oxide (NO) and prostacyclin, and vasoconstrictors, such as endothelin-1 (ET-1) and angiotensin II (Ang II).\(^1\) These endothelium-derived vasoactive factors not only regulate regional blood flow but also affect proliferation and/or hypertrophy of vascular smooth muscle cells (VSMCs). Regulation of EC death and survival contributes to vascular development and angiogenesis and to vascular pathology, such as occurs in inflammation, atherosclerosis, vascular prosthesis, and angioplasty. We have very recently found that endothelium-derived ET-1 acts as a survival factor in serum deprivation–induced apoptosis in rat ECs\(^2\) and fibroblasts.\(^3\)

Apoptosis, a ubiquitous, genetically programmed cell death, is involved in the regulation of cell numbers under physiological and certain pathological conditions.\(^4\) Apoptosis is associated with distinctive morphological and biological events, such as cellular shrinkage, membrane blebbing, and chromatin condensation and fragmentation.\(^5\) Diverse stimuli such as serum deprivation, radiation, chemotherapeutic agents, and antioxidants induce apoptosis in many cell types,\(^6\) whereas certain growth factors and cytokines are known to inhibit apoptosis.\(^7\) Although ECs have been demonstrated to undergo apoptosis,\(^8\)–\(^10\) its regulation in normal cellular physiology as well as in pathophysiological conditions remains largely unknown.

Three natriuretic peptides have been identified thus far: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP).\(^11\) ANP and BNP are cardiac hormones with potent natriuretic/diuretic and vasodilator properties, whereas CNP is a neuropeptide synthesized by and released from ECs as well.\(^12\) NO, a potent endothelium-derived relaxing factor, is synthesized not only
by endothelial NO synthase (eNOS) but also by cytokine-stimulated inducible NO synthase in VSMCs. It has been reported that ANP and NO inhibit proliferation of VSMCs. NO has been shown to be proapoptotic in many cell types but antiapoptotic in lymphocytes and in human umbilical ECs.

These observations led us to examine whether (1) natriuretic peptides and NO induce apoptosis in rat aortic ECs, (2) these effects involve the same cGMP-dependent mechanism, and (3) ET-1 affects such endothelial apoptosis.

Methods

Cell Culture

Rat ECs were prepared from 15-week-old male Wistar rat aortas by collagenase and elastase digestion, as described elsewhere. The endothelial origin was confirmed by the presence of factor VIII-related antigen by immunohistochemical detection. Subcultured ECs (eighth to 13th passage) from 2 independent isolates of ECs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and then used in the experiments.

Reagents

NOR3 {((-E)-4-ethyl-2-[(-E)-hydroxyiminio]-5-nitro-3-hexenamide; FK409} was purchased from Dojin Laboratory; synthetic rat ANP, BNP, CNP, and ET-1 were from the Peptide Institute Inc; 8-bromo-cGMP, 8-bromo-cAMP, and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were from Sigma Chemical Co; DMEM was from Life Technologies; FBS was from Hyclone Laboratories; and KT5823 was from Biomol Research Laboratories. HS-142-1 was kindly supplied by Dr Y. Matsuda, Kyowa Hakko Kogyo Research Laboratories, Tokyo, Japan. All other reagents were of analytical grade.

Fractionation of Nucleosomal Ladders

Rat ECs (5×10⁵) plated in 10-cm dishes in DMEM containing 10% FBS were incubated for 48 hours, then replaced in DMEM containing 1% FBS, and incubated further with or without the test compounds for the indicated times. For demonstration of nucleosomal ladders, apoptotic DNA fragments extracted with the NP-40 lysis method were fractionated as described. In brief, cells were treated with lysis buffer (50 mmol/L Tris, 20 mmol/L EDTA, and 1% NP-40), pH 7.5) and then centrifuged at 1600g for 5 minutes, and the supernatant was treated sequentially with RNase A and proteinase K. After ethanol precipitation, fragmented DNA was separated by agarose gel electrophoresis.

In Situ Detection of Apoptosis

Apoptotic cells were also detected by the terminal deoxynucleotidyl transferase-mediated deoxyUTP-biotin nick end labeling (TUNEL) method by using an in situ cell death detection kit (Takara Biochemicals). Cells grown on Lab-Tek chamber slides (Nalge Nunc International) were fixed for 15 minutes in 4% paraformaldehyde in PBS, blocked for 15 minutes with 0.3% H₂O₂ in methanol, washed, and permeabilized for 2 minutes with 0.1% sodium citrate in PBS. This procedure was followed by sequential exposure to the enzymatic reaction mixture for 60 minutes at 37°C, the anti–FITC–horseradish peroxidase conjugate for 30 minutes at 37°C, and 0.05% diamino benzidine in 1% NiSO₄ and 0.01% H₂O₂.

For quantitative analysis of TUNEL-positive cells, the percentage of TUNEL-positive cells in the total cell population was calculated by counting all cells from 5 random microscopic fields at a magnification of ×100.

Quantification of Fragmented DNA by Diphenylamine (DPA)

The harvested cells were lysed with 0.4 mL hypotonic lysis buffer (10 mmol/L Tris, 1 mmol/L EDTA, and 0.1% NP-40, pH 7.5) and centrifuged at 13 000g for 10 minutes to separate intact from fragmented chromatin. The supernatant containing fragmented DNA was placed in a separate Microfuge tube, and both pellet and supernatant were precipitated overnight at 4°C in 12.5% trichloroacetic acid. The precipitates were sedimented at 13 000g for 4 minutes. The DNA precipitates were hydrolyzed by heating to 90°C for 10 minutes in 5% trichloroacetic acid. For quantification of fragmented DNA, a modification of the DPA method of Burton was used. In brief, 0.16 mL of DPA reagent (0.15 g DPA, 0.15 mL H₂SO₄, and 0.05 mL acetaldehyde per 10 mL glacial acetic acid) was added to each tube, and the absorbance at 570 nm was measured after overnight color development (Beckman DU-50). “Percent fragmentation” refers to the ratio of DNA in the supernatant (“fragmented”) to the total DNA recovered in both supernatant and pellet (“fragmented plus intact”).

Western Blot Analysis

Western blot analysis with mouse monoclonal antibodies against p53 and bcl-2 were performed as reported. Cells were homogenized in lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 10 mmol/L NaCl, 1.5 mmol/L MgCl₂, and 1% NP-40) and centrifuged. The supernatant was subjected to SDS–polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membranes (Hybond ECL, Amersham) by electroblotting and incubated with specific antibodies to p53 (1:2500 dilution) and bcl-2 (1:2500 dilution) for 24 hours at 4°C. After being washed, the membrane was incubated for 12 hours at 4°C with anti-mouse IgG conjugated to horseradish peroxidase and visualized using the ECL system (Amersham).

Statistical Analysis

Data are expressed as mean±SEM of at least 3 separate experiments. Statistical analysis was performed by ANOVA for paired data. A value of P<0.05 was considered statistically significant.

Results

Fractionation of extracted DNA from ECs incubated in the presence of 1% FBS during 24 hours revealed no nucleosomal laddering on agarose gel electrophoresis. By contrast, the 3 natriuretic peptides (ANP, BNP, and CNP; all 10⁻⁷ mol/L) similarly caused nucleosomal laddering, which was most evident after 4 hours (Figure 1A). Addition of a potent NO donor (NOR3) also elicited nucleosomal laddering in a dose-dependent manner (10⁻⁴ to 10⁻³ mol/L; Figure 1B). However, when ECs were incubated in medium to which NOR3 (10⁻³ mol/L) had been added and then preincubated for 24 hours to let the NO donor decay spontaneously, no distinct nucleosomal laddering was observed, showing that the effects were not attributed to any of its decomposition products, such as diketone. Because natriuretic peptides and NO activate particulate and soluble guanylate cyclase, respectively, to increase cGMP levels, we tested whether 8-bromo-cGMP, a membrane-permeable derivative, induces comparable effects. Addition of 8-bromo-cGMP (10⁻⁵ mol/L) resulted in the appearance of nucleosomal laddering in the same fashion as did natriuretic peptides and the NO donor (Figure 1C). These results demonstrate that natriuretic peptides and NO caused apoptotic EC death, possibly via cGMP-dependent mechanism.

Another hallmark of apoptosis with respect to morphology is DNA strand breakage caused by endonuclease, which can be detected in situ by the TUNEL method. In contrast to untreated cells, TUNEL-positive cells were easily visible in the nuclei of adherent cells treated for 4 hours with ANP (10⁻⁷ mol/L), NOR3 (10⁻⁴ mol/L), and 8-bromo-cGMP (10⁻³ mol/L; Figure 2); the numbers of TUNEL-positive cells were significantly (P<0.001) increased in cells treated with ANP (10.0±2.6%), NOR3 (14.8±4.6%), and 8-bromo-cGMP (8.0±2.2%) compared with untreated cells (1.6±1.2%).
These results verify the induction of endothelial apoptosis by ANP and NO as well as by cGMP.

For quantification of apoptotic events, we measured fragmented DNA contents from total EC extracts by using the DPA method. The percentage of fragmented DNA to total DNA content in control cells varied from 5.8 ± 1.4% to 9.4 ± 1.1%. As shown in Figure 3, addition of ANP, BNP, and CNP significantly \( (P < 0.01) \) and dose-dependently (10\(^{-7}\) to 10\(^{-7}\) mol/L) increased the percentages of DNA fragmentation of total DNA to 25.7 ± 3.5% (10\(^{-7}\) mol/L ANP), 20.6 ± 5.8% (10\(^{-7}\) mol/L BNP), and 25.4 ± 2.5% (10\(^{-7}\) mol/L CNP). The 3 natriuretic peptides at a lower concentration (10\(^{-9}\) mol/L) did not cause significant changes. To determine whether ANP-induced apoptotic events involve a cGMP-dependent mechanism, the effect of a selective inhibitor of cGMP-dependent protein kinase (KT5823) was tested (Figure 4). Pretreatment with KT5823 (10\(^{-6}\) mol/L) completely blocked DNA fragmentation induced by ANP (10\(^{-7}\) mol/L). KT5823 added alone did not affect DNA fragmentation (data not shown). To determine whether ANP-induced DNA fragmentation was mediated via a specific ANP receptor, the effects of a guanylate cyclase–coupled receptor antagonist (HS-142-1)\(^{27}\) and an inhibitor of soluble guanylate cyclase (ODQ) were examined (Figure 4). Pretreatment with HS-142-1 (10 \(\mu\)g/mL) completely blocked DNA fragmentation induced by ANP, whereas ODQ (5 \(\times 10^{-6}\) mol/L) had no effect. Because ET-1 has been shown to exert an antiapoptotic effect on serum deprivation–induced endothelial apoptosis,\(^2\) the effect of ET-1 on apoptosis induced by ANP was examined (Figure 4). ET-1 (10\(^{-8}\) mol/L) completely abolished ANP-induced DNA fragmentation. These observations suggest that (1) endothelial apoptosis induced by ANP is mediated via a guanylate cyclase–coupled receptor, but not via a soluble guanylate cyclase; (2) a cGMP-dependent protein kinase is...
involved in endothelial apoptosis by ANP; and (3) ET-1 potently antagonizes these apoptotic events.

To determine whether NOR3-induced apoptosis was mediated by activation of soluble guanylate cyclase and a subsequent cGMP-dependent mechanism, the effects of ODQ and KT5823 were tested. As shown in Figure 5, both KT5823 (10^{-6} mol/L) and ODQ (5×10^{-6} mol/L) completely blocked DNA fragmentation induced by NOR3 (10^{-4} mol/L). HS-142-1 was without effect, whereas ET-1 (10^{-8} mol/L) also abolished NOR3-induced DNA fragmentation (Figure 5).

To ascertain that cGMP is actually involved in endothelial apoptosis, the effects of 8-bromo-cGMP and 8-bromo-cAMP were tested. As shown in Figure 6, addition of 8-bromo-cGMP (10^{-3} mol/L) resulted in a significant (P<0.01) increase in DNA fragmentation, whereas 8-bromo-cAMP (10^{-3} mol/L) was without effect. The apoptotic effect of 8-bromo-cGMP was abrogated by KT5823 (10^{-6} mol/L) and ET-1 (10^{-8} mol/L) but not by ODQ (5×10^{-6} mol/L). These results indicate an involvement of cGMP in endothelial apoptosis.

To determine whether such endothelial apoptosis involves a specific set of apoptosis-related genes, expression of the tumor suppressor gene product (p53) and B-cell leukemia/lymphoma gene product (bcl-2) was examined by Western blot analysis using specific antibodies for p53 and bcl-2, respectively. Pretreatment with ANP (10^{-7} mol/L), NOR3 (10^{-4} mol/L), and 8-bromo-cGMP (10^{-3} mol/L) caused...
marked accumulations of the nuclear phosphoprotein p53 (Figure 7, upper panel). The expression of bcl-2 protein, however, was unaffected by ANP, NOR3, 8-bromo-cGMP (Figure 7, lower panel), or ET-1 (data not shown).

**Discussion**

We have demonstrated here that the 3 natriuretic peptides (ANP, BNP, and CNP) as well as a potent NO donor (NOR3) induced marked apoptosis of rat ECs. Endothelial apoptosis induced by these vasodilators was substantiated by 3 lines of evidence. First, fragmented DNA extracted by the NP-40 lysis method from both floating and adherent cells showed nucleosomal laddering on agarose gel electrophoresis. Second, significant numbers of TUNEL-positive cells were observed in adherent cells treated with ANP and NOR3 compared with untreated cells. Third, quantitative analysis by the DPA method revealed that the percentages of fragmented DNA to total DNA content after treatment with ANP and NO donor were significantly greater than those of untreated cells.

The natriuretic peptide family consists of ANP, BNP, and CNP. Both ANP and BNP, which are mainly released from cardiac atria and ventricles, respectively, play important roles in the regulation of blood pressure and body fluids. Plasma concentrations of ANP and BNP increase in pathological conditions, such as congestive heart failure, myocardial infarction, hypertension, and chronic renal failure. These 2 natriuretic peptides have potent diuretic/natriuretic and vasodilatory effects and inhibit aldosterone production. CNP, a third member of the natriuretic peptide family, originally identified as a neuropeptide, was later shown to be synthesized by ECs as well. Plasma concentrations of CNP increase in endothelial dysfunction, such as sepsis. There exist 3 distinct receptor subtypes for the natriuretic peptide family: NPRA (GC-A), NPRB (GC-B), and the clearance (C) receptor. The biological actions of natriuretic peptides are initiated by binding to NPRA and NPRB to generate cGMP, which serves as a second messenger, whereas the C receptor functions for the clearance and metabolism of natriuretic peptides. ECs possess all 3 receptor subtypes. Although we have previously reported that ANP inhibits thrombin-stimulated ET-1 biosynthesis via a cGMP-dependent mechanism in rat ECs, the physiological function(s) of endothelial natriuretic peptide receptors remains largely unknown. The present study clearly demonstrates that ANP, BNP, and CNP equally and dose-dependently induce apoptosis of rat ECs and that ANP-induced apoptosis is blocked by a natriuretic peptide receptor antagonist (HS-142-1), which selectively antagonizes both NPRA and NPRB. Apoptosis was also blocked by a selective inhibitor of cGMP-dependent protein kinase (KT5823), but not by a selective inhibitor of soluble guanylate cyclase (ODQ). Furthermore, membrane-permeable 8-bromo-cGMP, but not 8-bromo-cAMP, also induced endothelial apoptosis; the effect of the former was abolished by KT5823. Taken together, these results suggest that natriuretic peptide–induced apoptosis of ECs is mediated by activation of particulate guanylate cyclase–coupled receptors (NPRA and/or NPRB), which involves cGMP as a signal for endothelial apoptosis. Very recently, it has been reported that ANP induces apoptosis in neonatal rat cardiomyocytes via a cGMP-dependent mechanism. These data are compatible with those of the present study in rat ECs. The physiological and pathophysiological relevance of our in vitro data to the in vivo situation remains uncertain because of the supraphysiological concentrations of natriuretic peptides used. However, the concentrations of natriuretic peptides used in the present in vitro study were comparable to those of many previous studies. Taking the autocrine/paracrine secretion of the natriuretic peptide family in the cardiovascular system into consideration, it is possible that the natriuretic peptide family at supraphysiological concentrations could act on the vascular endothelium under certain pathological conditions, such as congestive heart failure, myocardial infarction, and septic shock.

The present study clearly shows that a potent NO donor (NOR3) dose-dependently induced endothelial apoptosis. The mechanisms by which NO induces apoptosis appear complicated. Excess NO generation may lead to direct DNA damage through several mechanisms, including nitrosative deamination of deoxynucleotides, DNA strand breakage by NO2, and DNA modification by metabolically activated N-nitrosamines. NO also activates soluble guanylate cyclase to generate cGMP and subsequent activation of a cGMP-dependent protein kinase (PKG). In the present study, endothelial apoptosis by NOR3 was completely blocked by a PKG inhibitor (KT5823) as well as by a soluble guanylate cyclase inhibitor (ODQ), and 8-bromo-cGMP–induced apoptosis was blocked by KT5823. Thus, the data strongly suggest that cGMP plays a major role in NO-mediated apoptosis in rat ECs.

Our results are in contrast to those of Dimmeler et al, who showed that shear stress–mediated NO formation as well as addition of NO donors suppressed tumor necrosis factor–α–induced endothelial apoptosis via inhibition of caspase-3/caspase-1. The opposite effects of NO between our results and theirs may be accounted for by the species difference (rat aortic ECs versus human umbilical ECs) and/or the different experimental conditions. It should be noted that Dimmeler et al applied physiological shear stress (15 dynes/cm²) to stimulate NO production via eNOS and used a low concentration (10−5 mol/L) of conventional NO donors (sodium nitroprusside, 5-nitrooacetylpenicillamine), whereas we used higher concentrations (10−5 to 10−3 mol/L) of a novel and potent NO donor (NOR3). In fact, they observed that higher concentrations (>3×10−4 mol/L) of NO donors were proapoptotic for ECs. Thus, the contrasting effects of NO can be rationalized by the dose-dependent phenomenon, being antiapoptotic in lower doses and proapoptotic in higher doses. Such bifunctional roles of NO in endothelial demise may have physiological and pathological implications. Under normal condi-
tions, low levels of NO derived from eNOS may protect against endothelial apoptosis triggered by numerous insults, thereby contributing to EC integrity. In contrast, high levels of NO derived from inducible NOS in VSMCs and macrophages activated by bacterial endotoxin and certain cytokines under pathological conditions may induce endothelial apoptosis and cytotoxicity, thereby contributing to endothelial dysfunction and injury.

It has been shown that vasoconstrictor peptides such as ET-1 and Ang II stimulate cell proliferation and/or hypertrophy of VSMCs, whereas vasodilators such as NO and natriuretic peptides inhibit mitogenesis. It has very recently been reported that Ang II promotes cell survival, whereas NO increases susceptibility to apoptosis in rat VSMCs. We have very recently found that ET-1 acts as an autocrine/paracrine survival factor for rat ECs in serum deprivation–induced apoptosis and for a rat fibroblast cell line in c-myc–dependent apoptosis. The present results further demonstrate that ET-1 also rescues vasodilator-induced endothelial apoptosis. Such countervailing survival-versus-apoptosis regulation by Ang II and NO has been reported in rat VSMCs. Therefore, it is reasonable to speculate that the countervailing balance between endothelium-derived vasoconstrictors (ET-1) and vasodilators (NO) may contribute to EC integrity by regulating cell death and survival.

The cellular mechanisms by which cGMP induces endothelial DNA fragmentation and ET-1 has the opposing action as documented in this study remain unknown. However, a plausible explanation is possible from observations in recent studies. We have recently shown that ET-1 suppresses serum deprivation–induced apoptosis of a rat fibroblast cell line, possibly via mitogen–activated protein (MAP) kinase activation. Furthermore, ET-1 has been shown to stimulate proliferation of rat VSMCs and mesangial cells by activating the Raf-1/MAP kinase (MEK)/MAP kinase pathway. On the other hand, it has recently been shown that NO donors and 8-bromo-cGMP inhibit growth factor–stimulated proliferation of rat VSMCs by blocking the Raf-1/MEK/MAP kinase pathway via PKG-mediated phosphorylation of Raf-1. Moreover, it has been reported that ANP, NO donors, and 8-bromo-cGMP inhibit proliferation of rat mesangial cells by inducing MAP kinase phosphatase-1 (MKP-1), which selectively dephosphorylates MAP kinase. Collectively, one can speculate that NO and natriuretic peptides may cause endothelial apoptosis via a cGMP-dependent inhibition of the MAP kinase pathway by PKG-stimulated phosphorylation and/or MKP-1–stimulated dephosphorylation. Conversely, ET-1 may exert its antiapoptotic action by activation of the MAP kinase pathway, although the exact site(s) where the “survival” signal by ET-1 lies to counteract the cGMP-dependent apoptotic signal remains to be determined.

The tumor suppressor gene p53 has been shown to induce apoptosis in many cell types, and NO-induced apoptosis is accompanied by increased expression of p53 in macrophages and thymocytes. Conversely, bcl-2 has been demonstrated to prevent apoptosis induced by diverse stimuli. In the present study, we have demonstrated that ANP, NOR3, and 8-bromo-cGMP caused marked accumulations of p53 protein in rat ECs, whereas bcl-2 expression was not affected. These results suggest a possible involvement of cGMP in NO- and ANP-induced p53 expression and subsequent apoptotic events.

In summary, we have shown that vasodilators (NO and ANP) induce apoptosis and p53 accumulation in rat ECs, whose effects are mediated via a cGMP-dependent pathway, and that a vasoconstrictor (ET-1) antagonizes vasodilator–induced endothelial apoptosis. Thus, the countervailing balance between endothelium-derived vasodilators and vasoconstrictors may determine EC apoptosis and survival; an imbalance may contribute to the development and/or progression of vascular pathology, such as in atherosclerosis, angio- genesis, and vascular remodeling.

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