Inhibition of Endothelial Vascular Cell Adhesion Molecule-1 Expression by Nitric Oxide Involves the Induction and Nuclear Translocation of IκBα*

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The induction of vascular cell adhesion molecule-1 (VCAM-1) expression by tumor necrosis factor (TNF)-α requires the activation of nuclear factor-κB (NF-κB) via a process involving the phosphorylation and degradation of its cytoplasmic inhibitor, IκBα. We have shown that nitric oxide (NO) decreases VCAM-1 expression via inhibition of NF-κB activation. To determine how NO inhibits NF-κB, we studied the fate of IκBα following TNF-α stimulation in the presence of NO donors S-nitrosoglutathione and sodium nitroprusside. Activation of NF-κB by TNF-α occurred within 15 min and coincided with rapid degradation of IκBα. Co-treatment with NO donors did not prevent IκBα phosphorylation or degradation. However, after 2 h of TNF-α stimulation, NO donors inhibited NF-κB activation and augmented IκBα resynthesis and nuclear translocation by 2.5- and 3-fold, respectively. This correlated with a 75% reduction in TNF-α-induced VCAM-1 expression. In a time-dependent manner, NO donors alone caused the nuclear translocation of IκBα. To confirm that NO donors have similar effects as endogenously derived NO, murine macrophage-like cells, RAW264.7, were co-cultured with endothelial cells. Induction of RAW264.7-derived NO inhibited lipopolysaccharide-induced endothelial VCAM-1 expression, which was reversed by the NO synthase inhibitor Nω-monomethyl-L-arginine. These findings indicate that NO inhibits NF-κB activation and VCAM-1 expression by increasing the expression and nuclear translocation of IκBα.

The adhesion of circulating leukocytes to the vessel wall is an initiating event in atherogenesis and vascular inflammation (1, 2). Under certain conditions, the “activated” endothelium expresses cell surface adhesion molecules which mediate specific interactions between the endothelium and circulating leukocytes (3, 4). Factors that affect the induction of endothelial cell adhesion molecules such as vascular cell adhesion molecule (VCAM-1)† therefore may be important in regulating vascular inflammatory processes. Recent studies suggest that the activation of the pletropic transcription factor nuclear factor-κB (NF-κB) is required for the transcriptional induction of endothelial cell adhesion molecules (5).

The activation of NF-κB involves the degradation of its cytoplasmic inhibitor, IκBα (6, 7). Presently, five distinct IκB proteins have been shown to functionally retain NF-κB in the cytoplasm and render it inactive (6). These IκB proteins contain ankyrin repeat motifs that mask the nuclear localization sequence of NF-κB subunits such as RelA (p65), c-Rel, and RelB (8, 9). Of the different IκB proteins, the best described is IκBα. Following cytokine stimulation, IκBα is phosphorylated by a novel ubiquitinated serine kinase (10). Phosphorylation of IκBα targets the IκBα for ubiquitination and rapid degradation by 26 S proteasomes (10, 11). The degradation of IκBα then allows the unbound NF-κB to translocate into the nucleus, where it can transactivate the enhancer elements of many proinflammatory genes (5).

Modulation of IκBα function and expression has been shown to regulate NF-κB activation. For example, the phosphorylation of IκBα is a key regulatory step in the activation of NF-κB (11, 12). Indeed, recent studies indicate that salicylates and antioxidants inhibit NF-κB and endothelial cell activation by preventing IκBα phosphorylation and subsequent degradation (13–16). Alternatively, the nuclear accumulation of IκBα resulting from overexpression of IκBα or following stimulation with tumor necrosis factor (TNF)-α has been shown to displace NF-κB from its cognate DNA and terminate NF-κB-mediated transcriptional activity (17, 18). Finally, some of the anti-inflammatory effects of glucocorticoids may be mediated through their inhibitory effects on NF-κB, since glucocorticoids are known to induce IκBα expression (19, 20).

We and others have shown that nitric oxide can inhibit NF-κB and endothelial cell activation through non-cGMP-dependent mechanisms (21, 22). Although NO donors appear to “stabilize” the NF-κB-IκBα heterotrimeric complex (23), the possibility that newly synthesized IκBα could account for this stabilization has not been excluded. Furthermore, it is not known whether the induction of IκBα expression by NO donors could actually lead to an increase in cytoplasmic or nuclear IκBα protein levels. The purpose of this study, therefore, is to determine the mechanism(s) by which NO inhibits NF-κB activation and VCAM-1 expression in terms of its effects on IκBα phosphorylation, expression, and nuclear accumulation.

EXPERIMENTAL PROCEDURES

Materials—Medium 199 was purchased from Life Technologies, Inc. Fetal calf serum was purchased from Atlanta Biologicals (Norcross, GA). LPS, bacterial lipopolysaccharide; LNMA, Nω-monomethyl-L-arginine; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; NO, nitric oxide.

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†The abbreviations used are: VCAM-1, vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; mIFN-γ, murine interferon-γ; GSNO, S-nitrosoglutathione; IκBα, inhibitor κBα;

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Recombinant TNF-α was purchased from Endogen (Cambridge, MA). Heparin sulfate, sodium nitrite, alkaline phosphatase-conjugated secondary antibody, and p-nitrophenyl phosphate disodium were purchased from Sigma. Sodium nitroprusside (SNP) was purchased from Schwarz Pharma (Mannheim, Germany) and freshly prepared prior to each experiment. Sodium nitroprusside and nitric oxide donors were used as secondary control. The mammalian target of rapamycin (mTOR) was stimulated with the indicated agents, representative endothelial cell plates were fixed, and permeabilized with 0.1% gelatin in 6-well culture dishes (Falcon, Franklin Lakes, NY). Murine macrophage-like cells, RAW264.7, were grown on culture inserts above endothelial cells and identified by their typical morphological pattern (cobblestone morphology) and by immunostaining of representative plates for von Willebrandt factor antigen as described previously (25).

For the separated co-culture system, endothelial cells were grown on coverslips coated with 0.1% gelatin in 6-well culture dishes (Falcon, Franklin Lakes, NY). Murine macrophage-like cells, RAW264.7, were grown on inserts with 0.4-μm pore size (Falcon). These inserts were placed above the underlying endothelial cells and shared the same culture medium of Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 50 μg/ml endothelial cell growth factor. Treatment Conditions—Endothelial cells were stimulated with TNF-α (1000 units/ml) in the presence and absence of the indicated concentrations of NO donors. The preincubation period was 30 min for GSNO, 10 min for SNP, and 60 min for N-acetylcysteine (30 mM) before TNF-α stimulation. Degradation of phosphorylated iNOS was inhibited in the presence of a 26 S proteasome inhibitor, MG132 (10 μM), which was added 30 min prior to TNF-α stimulation. Following incubation with the indicated agents, representative endothelial cell plates were washed and fixed by trypan blue exclusion. Optiphot microscope. Photographic images were taken from each of the nitrate to nitrite before applying the Griess reagents. Preliminary studies indicate that the nitrate to nitrite ratio in all treatment conditions was always greater than 5:1.

Immunoprecipitation—Endothelial cells were harvested by scraping in ice-cold PBS. Nuclear extracts were prepared as described (23), and protein concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA). The rabbit polyclonal RelA and the affinity-purified rabbit IκBα (mouse IgG) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyvinylidene fluoride transfer membranes (Immobilon P) were purchased from Millipore (Bedford, MA). The micro-BCA protein assay was obtained from Pierce. Low molecular weight protein standards were purchased from Bio-Rad.

Cell Culture—Human saphenous vein endothelial cells of less than four passages were cultured in a growth medium containing M199, 5% fetal calf serum, 50 μg/ml endothelial cell growth factor (Pel-Freez Biological, Rogers, AK), and 100 μg/ml heparin sulfate. For cell surface immunosassays, endothelial cells were grown on gelatin-coated 96-well microtiter plates (Nurturing, Germany) and cultured at 37 °C with 5% CO₂ atmosphere. Endothelial cells were identified by their typical morphological pattern (cobblestone morphology) and by immunostaining of representative plates for von Willebrandt factor antigen as described previously (25).

For the separated co-culture system, endothelial cells were grown on coverslips coated with 0.1% gelatin in 6-well culture dishes (Falcon, Franklin Lakes, NY). Murine macrophage-like cells, RAW264.7, were grown on inserts with 0.4-μm pore size (Falcon). These inserts were placed above the underlying endothelial cells and shared the same culture medium of Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 50 μg/ml endothelial cell growth factor.

Cell Surface Enzyme-linked Immunosorbent Assay—Enzyme immunoassays were performed on confluent human endothelial cell monolayers using a monoclonal VCAM-1 antibody. Nonbinding control antibodies (OX 6, against MHC class II antigen) were used in each experiment. The monolayers were washed with PBS. Following 4 h of stimulation with TNF-α, endothelial cells were fixed on 96-well microtiter plates with 1% paraformaldehyde for 45 min and then incubated with the indicated monoclonal antibodies (1:100 dilution in PBS). Following 45 min of incubation with the primary antibody at 37 °C, the alkaline phosphatase-conjugated secondary anti-mouse antibody was applied. Finally, the phosphatase substrate, p-nitrophenyl phosphate, was added, and absorbance was measured at 405 nm with a Millenia Microplate reader (DPC MKA 220, DPC, Los Angeles, CA).

Immunohistochemistry—Endothelial cells grown on coverslips in the separated co-culture system were fixed with 4% formaldehyde at 4 °C for 10 min and then incubated with VCAM-1 antibody for 1 h. Three after washing steps, in PBS, a biotinylated anti-mouse antibody (Bio Genex, San Ramon, CA) was added for 20 min. Following further washing steps, cells were incubated with streptavidin alkaline phosphatase-conjugated antibody and stained with fast red substrate (Dako, Carpinteria, CA). Immunostaining was visualized using a Nikon Optiphot-2 microscope.

Immunofluorescence—Endothelial cells were grown on gelatin-coated coverslips in 6-well plates. Following incubation with TNF-α or NO donors for the indicated time intervals, cells were fixed and permeabilized with acetone at −20 °C for 2 min (ReLA) or with 100% methanol at −20 °C for 7 min (iNOS). Blocking was performed with 3% normal goat serum for 20 min. Cells were incubated with a rabbit polyclonal antibody directed against the NF-κB subunit, RelA (p65), or with anti-iNOS for 1 h at room temperature. A biotinylated goat anti-rabbit antibody was used as secondary antibody. The secondary antibody, streptavidin-fluorescein isothiocyanate, was added for 45 min. Immunofluorescence was visualized using an Olympus BX 60F microscope. Photographic images were taken from four random fields.

Nitrile Assay—The NO production was determined by assaying for nitrite accumulation. Briefly, following stimulation of the separated co-culture system with the indicated agents, a small aliquot of medium (50 μl) was added to equal volumes (100 μl) of 0.5% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid (Griess reagents). Absorbance was measured at 543 nm, and nitrite concentration was determined using a standard curve of sodium nitroprusside from 1.6 to 200 nmol/ml. In some experiments, the medium was first treated with nitrate reductase (Sigma) to convert all of the nitrate to nitrite before applying the Griess reagents. Multiple comparisons were done by analysis of variance.

RESULTS

Effect of NO Donor on VCAM-1 Expression—The NO donor, GSNO, inhibited TNF-α-induced VCAM-1 expression in a concentration-dependent manner (IC₅₀ = −30 μM) (Fig. 1). Maximal 75% inhibition was achieved at a GSNO concentration of 200 μM. The highest concentration of GSNO used in our experiments (1 m) did not alter cell viability as assessed by cell number, cellular morphology, DNA content, and trypan blue exclusion.

Effect of Endogenous NO on VCAM-1 Expression—To confirm that the effects of the NO donors are comparable to the effects of endogenously derived NO, we developed a separated co-culture system where murine macrophage-like cells, RAW264.7, were grown on endothelial cell monolayers, but sharing the same culture medium (Fig. 2). In this separated co-culture system, no basal endothelial VCAM-1 expression or RAW264.7-derived NO production was observed (Fig. 2B and Table 1). Treatment with LPS (10 ng/ml) induced VCAM-1 expression in endothelial cells but not in RAW264.7 (Fig. 2D). Murine IFN-γ was inactive in human...
endothelial cells (i.e. no MHC class II expression) and, by itself, could not induce Type II NO synthase (iNOS) expression in RAW264.7 cells (data not shown). The combination of LPS and mIFN-γ induced iNOS expression and NO production in RAW264.7 but not in human endothelial cells.

When the separated co-culture system was stimulated with the combination of LPS and mIFN-γ, NO production was increased significantly (0.6 ± 0.1 μM to 27.3 ± 4.5 μM, p < 0.01), while endothelial VCAM-1 expression was decreased by 76% compared with stimulation with LPS alone (Fig. 2g and Table I). However, addition of the iNOS inhibitor, LNMA (3 mM), to the separated co-culture system that had been stimulated with LPS and IFN-γ inhibited NO production by 91% and augmented endothelial VCAM-1 expression by 5-fold compared with the level of VCAM-1 expression stimulated with LPS and IFN-γ (Fig. 2f). Interestingly, treatment with LNMA alone resulted in mild endothelial VCAM-1 expression (12% of LPS-induced expression), suggesting that basal endothelial NO production can functionally and tonically inhibit VCAM-1 expression (Fig. 2c).

**Effect of NO on Cellular Localization of RelA—**Stimulation of endothelial adhesion molecule expression requires the activation of NF-κB (5). Since the activation of NF-κB involves the nuclear translocation of NF-κB subunits, we followed the intracellular localization of NF-κB subunit, RelA, by immunofluorescence following TNF-α stimulation in the presence or absence of GSNO. In unstimulated endothelial cells, RelA is predominantly localized to the cytoplasm with little, if any, present in the nucleus (Fig. 3a). Within 15 min following TNF-α stimulation, there is observable nuclear accumulation of RelA, which was sustained at 2 h (Fig. 3b and d).

Treatment with GSNO (200 μM) did not prevent the nuclear translocation of RelA after 15 min of TNF-α stimulation (Fig. 3c). After 2 h of TNF-α and GSNO, however, RelA was localized to both the cytoplasm and nucleus, suggesting partial inhibition of NF-κB activation and possible reverse nuclear to cytoplasmic translocation of NF-κB by GSNO (Fig. 3e). Treatment with GSNO alone did not activate NF-κB at the 2 h time point (Fig. 3f). Specificity was determined by the absence of RelA immunofluorescence when nonimmune serum was used instead of RelA antibody. These results indicate that the inhibitory effects of NO donors on NF-κB activation had a delayed onset (i.e. > 15 min) and suggest that NO does not prevent the initial degradation of IκBα following TNF-α stimulation.

**Effect of NO on IκBα Phosphorylation—**Since the activation of NF-κB involves the phosphorylation and subsequent degradation of its cytoplasmic inhibitor, IκBα, we followed the fate of IκBα after stimulation with TNF-α (1000 units/ml) in the presence and absence of GSNO (200 μM). Stimulation with TNF-α for 15 min caused an almost complete disappearance of IκBα, which was not prevented by co-treatment with GSNO (Fig. 4). Since phosphorylated IκBα has a very short half-life and is difficult to visualize by immunoblotting, we treated endothelial cells with a relatively specific IκBα 26S proteasome inhibitor,

![Fig. 1. Effects of GSNO on TNF-α (1000 units/ml)-induced endothelial VCAM-1 surface expression.](image)

![Fig. 2. VCAM-1 expression in endothelial cells grown in a separated co-culture system with murine macrophage-like cells, RAW264.7.](image)
TABLE I
Effect of macrophage NO on endothelial VCAM-1 expression

| Condition for co-culture | Nitrite accumulation (µM/24 h) | VCAM-1 expression (% of LPS) |
|--------------------------|-------------------------------|-----------------------------|
| Control                  | 0.6 ± 0.3                     | 1 ± 2%                      |
| LNMA (3 mM)              | 0.5 ± 0.2                     | 12 ± 4%                     |
| LPS (10 ng/ml)           | 0.9 ± 0.3                     | 100 ± 6%*                   |
| mIFN-γ (400 units/ml)    | 0.5 ± 0.3                     | 2 ± 3%                      |
| LPS + mIFN-γ             | 27.3 ± 4.5*                   | 24 ± 5%*                    |
| LPS + mIFN-γ + LNMA      | 2.4 ± 0.4***                  | 119 ± 9%***                 |

MG132 (10 µM), to “protect” phosphorylated IκBα. In the presence of MG132, stimulation with TNF-α for 15 min produced two bands corresponding to phosphorylated and nonphosphorylated IκBα.

Treatment with GSNO (200 µM) did not prevent TNF-α-induced IκBα phosphorylation when compared with that of TNF-α alone (Fig. 4). Incubation with a higher concentration of GSNO (500 µM) or with a second NO donor, SNP (500 µM), also had no effect on TNF-α-stimulated IκBα phosphorylation (data not shown). Treatment with N-acetylcysteine (30 mM) stabilized IκBα and prevented its degradation following TNF-α stimulation. In contrast to MG132, a nonspecific proteasome inhibitor, ALL (10 µM), did not prevent IκBα degradation following IκBα phosphorylation.

Effect of NO on IκBα Expression—To investigate the effects of NO on IκBα expression, endothelial cells were incubated for longer time periods with GSNO. Stimulation with TNF-α (1000 units/ml) resulted in almost complete IκBα degradation by 15 min, followed by the reappearance or resynthesis of IκBα at 2 h (Fig. 5A). Co-treatment with GSNO (200 µM) did not prevent TNF-α-induced IκBα degradation at 15 min but did augment TNF-α-induced IκBα expression by 2.5-fold after 2 h compared to that of TNF-α alone. Treatment with GSNO alone produced a time-dependent 1.5- and 2.5-fold increase in IκBα levels above basal levels after 1 and 2 h, respectively (Fig. 5B).

Effect of NO on IκBα Nuclear Translocation—To determine whether NO can affect the cellular localization of IκBα, we performed immunofluorescence studies of IκBα following GSNO treatment. In unstimulated endothelial cells, IκBα is predominantly localized to the cytoplasm with little, if any, localized to the nucleus (Fig. 6A). In a time-dependent manner, treatment with GSNO (200 µM) caused a progressive nuclear accumulation of IκBα (Fig. 6, B–D). Incubation with nonspecific immune serum yielded no appreciable immunofluorescence (data not shown). The nuclear translocation of IκBα by NO, therefore, occurred in the absence of NF-κB activation, since GSNO alone does not activate NF-κB (Fig. 3F).

To determine whether the nuclear accumulation of IκBα induced by NO can terminate NF-κB signaling in the nucleus by binding to RelA, we studied the appearance of RelA-associated IκBα in the nucleus. Immunoblotting of nuclear extracts that have been immunoprecipitated with agarose-conjugated anti-RelA antibody demonstrated that RelA-associated IκBα begins to accumulate in the nuclear extracts of endothelial cells following 2 h of TNF-α (1000 units/ml) stimulation (Fig. 7). This time frame is consistent with the appearance of IκBα in the nucleus following GSNO treatment (Fig. 6, C and D).

Compared with TNF-α alone, co-treatment with GSNO (200 µM) caused a further increase in nuclear RelA-associated IκBα levels resulting in a 3-fold increase after 6 h (Fig. 7). Possible cytoplasmic contamination of the nuclear extracts was excluded by the absence of G-protein α_{1g} subunit in the nuclear extracts. In addition, IκBα, which is present in the cytoplasm of
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unstimulated endothelial cells, was not observed in the corresponding nuclear extracts.

**DISCUSSION**

The mechanism by which NO inhibits endothelial VCAM-1 expression occurs, in part, via the inhibition of NF-κB (21, 22). Since the activation of NF-κB is dependent upon its association with its inhibitor, IκBα, which not only binds and prevents the nuclear translocation of NF-κB (6, 7) but also may displace nuclear NF-κB from its cognate DNA (16), the level of IκBα in both the cytoplasm and nucleus may be important in determin-
ing the time of onset, duration, and magnitude of NF-κB activation. Regulation of NF-κB activation by IκBα therefore may ultimately determine the level of VCAM-1 expression in response to cytokines. We find that NO inhibits NF-κB activation and VCAM-1 expression by increasing cytoplasmic and nuclear levels of IκBα.

The NO donor, GSNO, has been shown to slowly release NO under physiological pH conditions (27). Furthermore, the findings of the separated co-culture system suggest that the effects of the NO donors is most likely due to biologically active NO and not some other metabolite. However, we cannot exclude possible interactions of NO with other reactive oxygen intermediates such as superoxide anion to form another NO derivative such as peroxynitrite (28). Neither the NO donors used in our experiments nor endogenously derived NO from RAW264.7 cells cause any cellular toxicity as determined by cell count, DNA content, cellular morphology, and trypan blue exclusion. Thus it is unlikely that changes in NF-κB activation or VCAM-1 expression were due to direct or indirect toxic effects of NO. Furthermore, we have previously shown that two constitutively expressed endothelial cell surface proteins, E1/1 and MHC class I, were not affected by similar treatment with these NO donors (21).

We find that the induction of IκBα by NO or TNF-α resulted in a delayed increase in IκBα protein levels (i.e. ≥2 h). Higher levels of IκBα protein in the cytoplasm could replace the initially degraded IκBα and help retain and “stabilize” the NF-κB/IκBα complex in the cytoplasm. The binding of newly synthesized IκBα to cytoplasmic NF-κB therefore may prevent further nuclear translocation of NF-κB subunits and limit VCAM-1 gene transcription. Treatment with NO donors, however, did not inhibit IκBα phosphorylation or degradation by 26 S proteasomes following TNF-α stimulation. This is in contrast to our previous study, which suggested that NO may stabilize IκBα (23). In the present study, a more detailed analysis using shorter stimulation periods indicates that stabilization of latent IκBα is not responsible for inhibition of NF-κB. Retention of NF-κB in the cytoplasm occurs later only after IκBα resynthesis.

An increase in IκBα protein level in the nucleus may also serve to terminate NF-κB-mediated VCAM-1 gene transcription by displacing NF-κB from its putative cis-acting element(s) (16). This is consistent with our finding that following treatment with NO, and to a lesser extent TNF-α, IκBα not only accumulates in the nucleus but also is bound to nuclear translocated RelA. Binding of RelA to IκBα may also serve to expel RelA from the nucleus into the cytoplasm. Thus, the observed increase in nuclear IκBα levels and possible reverse translocation of NF-κB may be additional mechanisms by which NO can rapidly terminate NF-κB-mediated gene transcription. It is not known, however, whether this increase in nuclear IκBα levels is due to enhanced nuclear translocation of IκBα or occurs.
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The activation of NF-κB following TNF-α stimulation leads to the transcriptional induction of IkBa via κB cis-acting elements in the IkBa promoter (29–31). The induction of IkBa by NF-κB, therefore, serves as an autoregulatory mechanism for terminating NF-κB-mediated gene transcription (15, 31). In our studies, the induction of IkBa by NO donors occurred independent of NF-κB activation in unstimulated human endothelial cells. The induction of IkBa by NO, however, is somewhat similar to the effect of glucocorticoids, which have also been shown to induce IkBa gene transcription (19, 20). Although the NO-responsive cis-acting element(s) has yet to be identified, analysis of the −2.1-kilobase porcine, −1.6-kilobase murine, and −385-base pair human IkBa promoter does not reveal site corresponding to putative glucocorticoid response elements (GRE) (29–31). Thus, it is interesting to speculate whether NO and glucocorticoids share a similar transcriptional signaling pathway leading to the transactivation of the IkBa gene.

In summary, NO inhibits VCAM-1 expression in cultured human vascular endothelial cells via a novel mechanism involving the induction and nuclear translocation of IkBa. These effects of NO appear to be distinct from that of antioxidants and salicylates, which prevent IkBa phosphorylation and degradation (13–16). Further investigation into how NO donors induce IkBa gene transcription and modulate NF-κB activity may provide greater insights into the role of NO in the vascular wall.

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