Nitric oxide donors, nitrosothiols and mitochondrial respiration inhibitors induce caspase activation by different mechanisms

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Abstract We investigated to what extent different types of NO donors induce caspase activation by opening of the mitochondrial permeability transition pore (PTP) or inhibition of mitochondrial respiration. We found that nitrosothiols can directly open the PTP in isolated mitochondria and cause cytochrome *c* release, whereas NONOate donors can not. In macrophages nitrosothiols cause caspase activation that is blocked by cyclosporin A or calcium chelation, both of which prevent PTP opening, whereas caspase activation caused by NONOates is much less sensitive to these agents. Inhibitors of mitochondrial respiration did not promote PTP opening in isolated mitochondria, and although they cause caspase activation in macrophages, this activation was slower than that caused by NO donors, and was relatively insensitive to cyclosporin and calcium chelators suggesting that PTP opening was not involved.

Key words: Nitric oxide; Apoptosis; Macrophage; Mitochondrion; Caspase

1. Introduction

Nitric oxide (NO) can induce caspase-mediated apoptosis in a variety of cell types, including macrophages, thymocytes, pancreatic islets and neurons (reviewed in [1,2]). NO-induced apoptosis may be important in macrophage-induced killing of tumour cells, beta-cell destruction in insulin-dependent diabetes, inflammatory diseases, neurodegenerative diseases and vascular disease (reviewed in [3,4]). The mechanisms by which NO induces caspase activation in cells are however unclear. Suggested mechanisms include the following. (A) NO (or peroxynitrite) induced DNA damage leading to the accumulation of p53 [5]. (B) Activation of guanylate cyclase, promoting apoptosis by unknown mechanisms [6]. (C) Direct activation of the mitochondrial permeability transition pore (PTP) [7,8], resulting in cytochrome *c* release and thus activation of caspases via cytosolic APAF-1. (D) Peroxidation and degradation of mitochondrial phospholipids leading to loss of cytochrome *c* from mitochondria into the cytosol [9]. (E) Inhibition of mitochondrial respiration, resulting in oxidant (including peroxynitrite) production and decreased membrane potential, promoting PTP opening and cytochrome *c* release [10–12]. In support of the later possibility we and others have shown that NO is a potent inhibitor of mitochondrial respiration [13], resulting in increased superoxide, hydrogen peroxide and peroxynitrite production [14,15], and these oxidants can open the PTP and cause cytochrome *c* release [16,17].

However, interpreting previous research is complicated by the fact that different types of NO donor molecule, with different chemical properties, have been used to supply NO to cells. The two most popular types of NO donors are: the NONOates, which are supposedly 'pure' NO donors, and the *S*-nitrosothiols, which can release NO, but also directly transfer the NO^+ group to a variety of recipients particularly reduced thiols (a process known as transnitrosylation) [18]. NONOates are adducts of NO with nucleophiles and they dissociate spontaneously generating NO and free nucleophile [18], thus, they are meant not to be involved in direct transnitrosylation reactions. Surprisingly most research on NO-induced apoptosis has been done using nitrosothiols (such as SNAP and nitrosoglutathione) as NO donors [7,8,19–24]. This complicates the interpretation of the work because nitrosothiols can transnitrosylate thiols in metabolites (such as glutathione - resulting in glutathione depletion) and accessible thiols in many if not most proteins (resulting in changes in protein function) [25].

In the work described here we sought to determine the extent to which two different types of NO donors, nitrosothiols and NONOates, induce caspase activation by opening of the PTP or inhibition of mitochondrial respiration. We show that nitrosothiols, NONOates and mitochondrial inhibitors activate caspases by different mechanisms, and this has immediate importance for understanding the means by which NO causes apoptosis.

2. Materials and methods

2.1. Materials

S-Nitroso-*N*-acetylpenicillamine (SNAP), spermine-NONOate, DEVD-7-amino-4-methyl-coumarin (DEVD-amc), IETD-amc were from Alexis; diethylenetriamine/NO adduct (NOC-18 or DETA-NONOate) - from RBI, cyclosporin A - from Calbiochem; *S*-nitrosoglutathione (GSNO), 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl)ester (BAPTA-AM) and other reagents were from Sigma.

2.2. Measurement of mitochondrial swelling and cytochrome c release from isolated mitochondria

Rat heart mitochondria were prepared as described previously [17]. For measurement of mitochondrial swelling and cytochrome *c* release, mitochondria (1 mg protein per 1 ml incubation buffer) were incubated at 37°C in the medium containing 110 mM KCl, 10 mM Tris–HCl (pH 7.2), 5 mM KH_2PO_4, 50 mM creatine, 2.24 mM $MgCl_2$, 5 mM succinate, 1 μM rotenone, 20 μM ATP or ADP (where indicated), 0.1 mM $CaCl_2$ (in some experiments it was omitted, see figure legend). To stabilise free Ca^{2+} at 5 μM concentration, 5 mM nitrilotriacetic acid was added (free Ca^{2+} was calculated as described in [26]).

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After 2 min preincubation, NO donors at 1 mM concentration were added to mitochondria. Mitochondrial swelling was determined as decrease in absorbance at 540 nm during 15 min of incubation. Supernatants of mitochondria (13 000 rpm×3 min in an Eppendorf centrifuge) were obtained after 15 min incubation and the ascorbate-reduced-minus-ferricyanide-oxidised absorption difference spectra of supernatants were recorded with Hitachi U-2000 spectrophotometer. Cytochrome *c* content was estimated as described in [27].

2.3. Cell culture

Murine macrophage J774 cells were maintained in DMEM supplemented with 10% foetal calf serum and 1% streptomycin+1% penicillin at 37°C in a humidified atmosphere containing 5% CO_2. For the experiments, cells ($2–3\times10^7$) were suspended in 8 ml of DMEM with 5% foetal calf serum and incubated with indicated NO donors (at 1 mM concentration) or inhibitors of the respiratory chain: 2 mM azide, 2 μM rotenone or 2 μM myxothiazol. When cyclosporin A (3 μM), ascomycin (3 μM) or BAPTA-AM (6 μM) were used, cells were preincubated for 30 min with indicated compounds before NO donors or respiratory inhibitors were added.

NO levels and oxygen consumption were determined using specific electrodes, as described previously [13].

2.4. Measurement of caspase activity

Cells were sedimented at 700×*g*×3 min, washed with Krebs–HEPES buffer (pH 7.4) and resuspended in 200 μl of lysing buffer containing 100 mM HEPES (pH 7.4), 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT, 5 μl protease inhibitor cocktail (Sigma). After 20 min on ice, cells were disrupted by passing 15 times through 27G needle and centrifuged at 13 000 rpm×10 min. Supernatants (50–100 μg total protein) were incubated with 100 μM DEVD-amc or IETD-amc for 60 min. Substrate cleavage was determined fluorometrically (excitation at 380 nm, emission at 460 nm). DEVD-CHO, 1 μM, and IETD-CHO, 1 μM, were used to inhibit caspase activity and to ensure specificity of the reaction. 10–200 nM 7-amino-4-methyl-coumarin was used for calibration of fluorescence signal.

3. Results

3.1. Effect of NO donors on cytochrome c release from isolated mitochondria

We wanted to determine what types of NO donors caused PTP and cytochrome *c* release from isolated mitochondria and by what means. Fig. 1 shows that incubation of isolated heart mitochondria with the nitrosylating NO donors SNAP or GSNO in the presence of 5 μM free Ca^{2+} resulted in the release of cytochrome *c* from mitochondria. The release of cytochrome *c* was inhibitable by cyclosporin A, a specific inhibitor of the PTP, and was accompanied by mitochondrial swelling suggesting that it was related to opening of PTP. SNAP- and GSNO-induced mitochondrial swelling and release of cytochrome *c* was observed only in the presence of Ca^{2+} and low, 20 μM, concentrations of ATP or ADP whereas higher concentrations of ATP (100 μM) completely suppressed both swelling and cytochrome *c* release (not shown). The non-nitrosylating NO donor NOC-18 (DETA-NONOate) or a specific inhibitor of cytochrome oxidase azide, had little or no effect on swelling and cytochrome *c* release (Fig. 1). At the concentrations used all the NO donors almost completely inhibited respiration (data not shown), due to NO inhibition of cytochrome oxidase [9,11], but since a more specific inhibitor of cytochrome oxidase, azide, did not cause pore opening, the NO donors can not have caused pore opening and cytochrome *c* release due to respiratory inhibition. We also compared the levels and the time-course of NO release to the incubation medium by various NO donors. The NO concentration reached a steady state after 5–8 min for all NO donors. Since the steady state concentration of NO produced

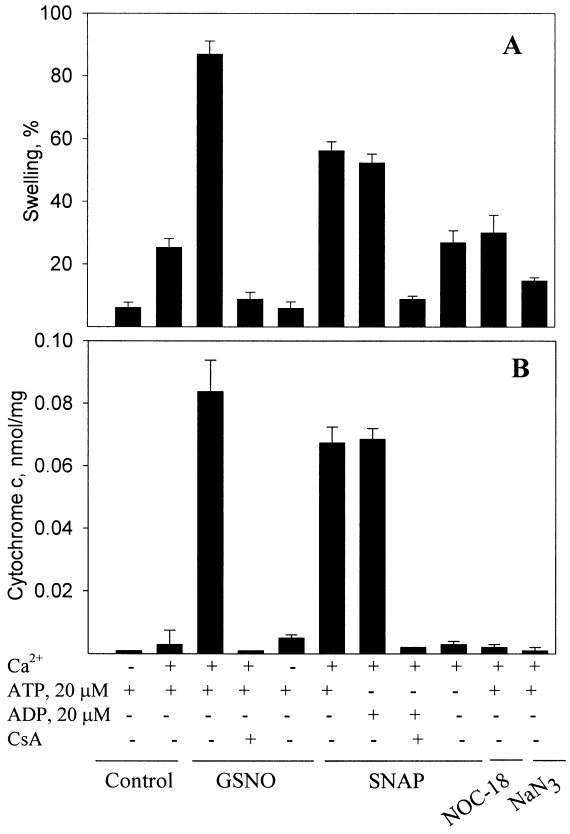

Fig. 1. Effect of NO donors on mitochondrial swelling (A) and release of cytochrome *c* (B). Isolated heart mitochondria were incubated with 1 mM GSNO, 1 mM SNAP, 1 mM NOC-18 or 2 mM azide in the presence of indicated compounds while assessing decrease in absorption at 540 nm. Mitochondrial swelling is expressed as decrease in absorption after 15 min incubation if compared to change in absorption caused by 0.1 mM $CaCl_2$ and 15 mM phosphate (100% swelling). Supernatants obtained after 15 min incubations were used for spectrophotometric determination of released cytochrome *c*.

over the incubation time by NOC-18 (0.89–1.5 μM) was similar to that produced by SNAP (0.66–1.34 μM) or GSNO (1.60–1.90 μM), but NOC-18 (and authentic NO [17]) failed to cause pore opening and cytochrome *c* release, these action of SNAP and GSNO can not be due to NO alone, but most likely involves their nitrosylating activity.

3.2. NO donors induce activation of caspases in macrophages

The release of cytochrome *c* from mitochondria can initiate activation of apoptotic proteases - caspases leading to cell death. We investigated whether NO donors can induce activation of caspases in the macrophage J774 cell line. Fig. 2 shows that exposure of cells to 1 mM GSNO, SNAP or NOC-18 resulted in activation of caspase-3-like-proteases (DEVD-cleaving activity) and caspase-3-processing proteases (IETD-cleaving activity). That was evident within 4 h of exposure and continued to increase through a further 4 h of incubation. The events leading to caspase activation require at least 1–2 h of incubation of cells with NO donors, as if SNAP or NOC-18 were removed from cells within that period, no significant increase in activity of caspases was observed during a further 4–5 h incubation (data not shown). Activation of caspases was also prevented by 1 mM oxyhemoglobin:

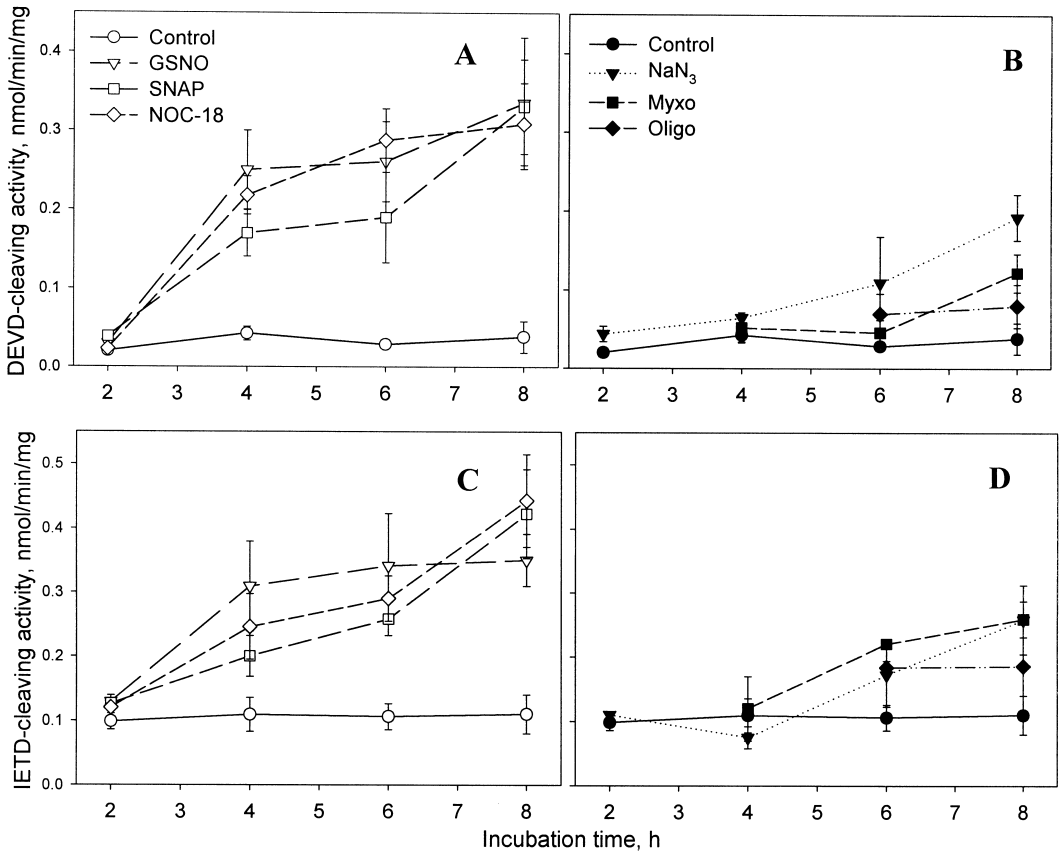

Fig. 2. Time-course of NO donors (A,C) and the respiratory inhibitors (B,D) induced activation of caspases in macrophages. Cells were treated for different periods of time with 1 mM GSNO, 1 mM SNAP, 1 mM NOC-18, 2 mM azide, 2 μM myxothiazol or 1 μg/ml oligomycin. Cell lysates were then prepared and incubated 60 min with fluorogenic peptides: DEVD-amc (A,B) or IETD-amc (C,D). The fluorescence of the product of reaction, amc, was measured in 1 ml of buffer containing 10 mM HEPES, 10% sucrose and 0.1% bovine serum albumin, pH 7.4.

DEVD-cleaving activity after 6 h incubation of cells with SNAP or NOC-18 and oxy-hemoglobin was 0.034 ± 0.003 and 0.042 ± 0.025 nmol/min/mg protein, respectively, and was not significantly different from control cells (see Fig. 2A), presumably due to the ability of oxyhemoglobin to scavenge NO or NO^+. Inhibitors of the mitochondrial respiratory chain, azide and myxothiazol, at concentrations that completely inhibited cell respiration, were less effective in caspase activation: increase in IETD- and DEVD-cleaving activity was observed only after 6 and 8 h of incubation, respectively. This suggests that the caspase activation induced by NO donors can not be due solely to respiratory inhibition. Oligomycin, an inhibitor of mitochondrial ATPase, did not induce significant activation of caspases even after 8 h of incubation.

3.3. *Nitrosylating NO donors-induced activation of caspases is blocked by cyclosporin A and chelation of intracellular calcium*

In order to test whether the caspase activation caused by the NO donors and respiratory inhibitors was due to PTP, we tested whether this activation could be blocked by the PTP inhibitor cyclosporin A. As can be seen from Fig. 3, cyclo-

Table 1
Cyclosporin A and chelation of intracellular calcium does not prevent activation of caspases in macrophages induced by the respiratory chain inhibitors

Incubation conditions	DEVD-cleaving activity (nmol/min/mg)	IETD-cleaving activity (nmol/min/mg)
Control	0.031 ± 0.010	0.084 ± 0.022
Azide, 2 mM	0.154 ± 0.040	0.211 ± 0.043
+cyclosporin A, 3 μM	0.144 ± 0.034	0.114 ± 0.025
+BAPTA-AM, 6 μM	0.155 ± 0.039	0.165 ± 0.026
Myxothiazol, 2 μM	0.102 ± 0.017	0.239 ± 0.051
+cyclosporin A, 3 μM	0.066 ± 0.023	0.104 ± 0.024*
+BAPTA-AM, 6 μM	0.110 ± 0.013	0.170 ± 0.042
Rotenone, 2 μM	0.125 ± 0.019	0.192 ± 0.022
+cyclosporin A, 3 μM	0.168 ± 0.047	0.159 ± 0.011
+BAPTA-AM, 6 μM	0.163 ± 0.038	0.192 ± 0.046

Cells were incubated with the respiratory chain inhibitors for 8 h. Cyclosporin A and BAPTA-AM were added to the cells 30 min before the inhibitors. Data are means ± S.E.M. from at least three independent experiments. * - Significant difference from control ($P < 0.05$).

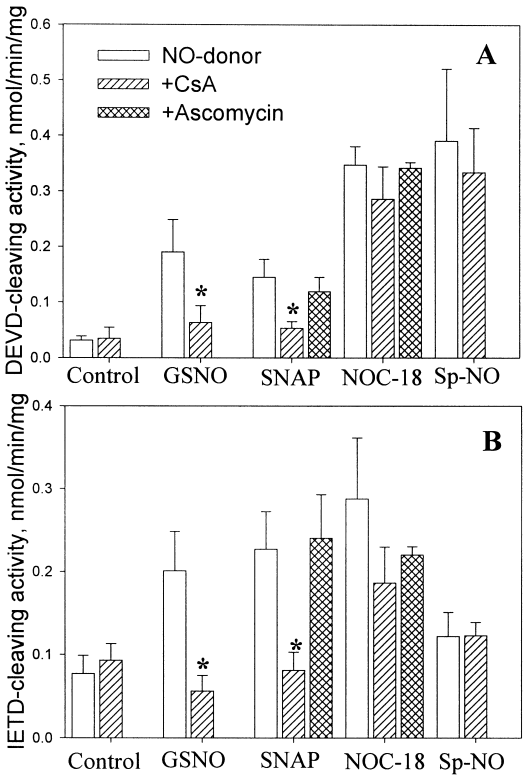

Fig. 3. Effect of cyclosporin A on activity of caspases in macrophages induced by NO donors. Cells were incubated 6 h with NO donors (all of them at 1 mM concentration) in the presence or absence of 3 μM cyclosporin A or 3 μM ascomycin. * - Statistically significant effect of cyclosporin A if compared to treatment with NO donor alone.

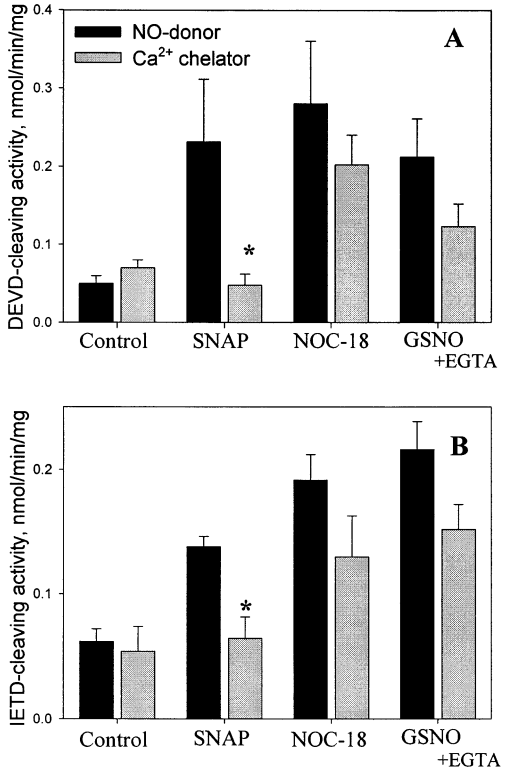

Fig. 4. Effect of Ca^{2+} chelators on NO donors-induced activation of caspases in macrophages. Cells were incubated 6 h in the presence of SNAP or NOC-18 in the presence or absence of 6 μM cell permeable calcium chelator BAPTA-AM. In the experiments where cells were treated with GSNO, 2 mM EGTA was used as chelator of extracellular calcium. * - Statistically significant effect of BAPTA-AM if compared to treatment with NO donor alone.

sporin A completely prevented DEVD- and IETD-cleaving activity in macrophages incubated with nitrosylating NO donors GSNO and SNAP, whereas it had no significant effect in the case of NOC-18 and spermine-NONOate. Preincubation of cells with ascomycin, which inhibits calcineurin but not PTP, had no effect on SNAP- or NOC-18-induced activation of caspases, indicating that calcineurin had no significant role in the activation of caspases. Cyclosporin A had no effect on DEVD-cleaving activity induced by the respiratory chain inhibitors, however it significantly reduced IETD-cleavage induced by myxothiazol (Table 1). Nitrosylating NO donors-induced activation of caspases also was completely prevented by BAPTA, a chelator of intracellular Ca^{2+}, and to some extent by EGTA which binds extracellular Ca^{2+} (Fig. 4). Chelation of intracellular Ca^{2+} had no significant effect in the case of NOC-18 and the respiratory chain inhibitors induced activation of caspases (Fig. 4 and Table 1). Altogether the data suggest that nitrosylating NO donors-induced activation of caspases is mediated by mitochondria via opening of PTP pore, whereas the caspase activation induced by 'pure' NO donors NOC-18 and spermine-NONOate and by the respiratory inhibitors involves the PTP only partially or not at all.

4. Discussion

In isolated mitochondria we found that the nitrosothiols SNAP and GSNO could induce cyclosporin A-sensitive PTP opening and cytochrome *c* release, in the presence of calcium, whereas NO and NOC-18 could not. This suggests that nitrosothiols may open the pore by means other than NO production alone. Hortelano et al. [7] reported that NO could induce pore opening in isolated mitochondria, using conditions similar to those used here. However, as NO donors they used SNAP and GSNO, which are nitrosothiols, and SIN-1, which is a donor of both NO and superoxide, and thus produces peroxynitrite (a known pore opener) [16]. Balakirev et al [8] used photolysis of a nitrosothiol (*S*-nitroso-*N*-acetylcysteine) to generate NO with isolated mitochondria, and found that this halved the lag-time before PTP induced by high calcium. However, it is difficult to know what other products might be generated by photolysis of nitrosothiols. We do not know how nitrosothiols induce PTP. Possibilities include: direct transnitrosylation of thiols known to be involved in controlling pore opening, or oxidation of intra-mitochondrial glutathione.

In macrophages all NO donors induced caspase activation to a similar extent and with a similar time course. However, cyclosporin A and calcium chelators, which block the PTP, prevented caspase activation by the nitrosothiols, but were much less efficient at preventing caspase activation induced by the NONOates. Hortelano et al. [7,24] found that cyclosporin A blocked apoptosis in thymocytes and caspase activation in macrophages, but this was induced by 0.2–1 mM GSNO. This is consistent with the nitrosothiols directly activating the pore, whereas NO itself does not.

The three different mitochondrial respiration inhibitors

caused caspase activation to a similar extent and with a similar time course, and this activation was relatively insensitive to cyclosporin A and calcium chelators. This suggests that mitochondrial inhibition induces caspase activation not primarily by PTP opening. Oligomycin, which inhibits the mitochondrial ATPase, did not cause any caspase activation, suggesting that the respiratory inhibitors did not cause caspase activation primarily by inhibition of ATP production. However, it is possible that respiratory inhibition might cause a greater fall in cellular ATP/ADP than inhibition of the ATP synthase, since the former will cause the ATP synthase to reverse hydrolysing ATP generated by glycolysis.

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