p38 Mitogen-activated Protein Kinase Down-regulates Nitric Oxide and Up-regulates Prostaglandin E₂ Biosynthesis Stimulated by Interleukin-1β*

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The inflammatory cytokine interleukin 1β (IL-1β) induces both cyclooxygenase-2 (Cox-2) and the inducible nitric-oxide synthase (iNOS) with increases in the release of prostaglandins (PGs) and nitric oxide (NO) from glomerular mesangial cells. However, the intracellular signaling mechanisms by which IL-1β induces iNOS and Cox-2 expression are obscure. Our current studies demonstrate that IL-1β produces a rapid increase in p38 mitogen-activated protein kinase (MAPK) phosphorylation and activation. Serum starvation and SC68376, a drug which selectively inhibits p38 MAPK in mesangial cells, were used to investigate whether p38 MAPK contributes to the signaling mechanism of IL-1β induction of NO and PG synthesis. Serum starvation and SC68376 selectively inhibited IL-1β-induced activation of p38 MAPK. Both SC68376 and serum starvation enhanced NO biosynthesis by increasing iNOS mRNA expression, protein expression, and nitrite production. In contrast, both SC68376 and serum starvation suppressed PG release by inhibiting Cox-2 mRNA, protein expression, and PGE₂ synthesis. These data demonstrate that IL-1β phosphorylates and activates p38 MAPK in mesangial cells. The activation of p38 MAPK may provide a crucial signaling mechanism, which mediates the up-regulation of PG synthesis and the down-regulation of NO biosynthesis induced by IL-1β.

Interleukin 1 (IL-1) is a potent immunoregulatory and proinflammatory cytokine secreted by a variety of cells in response to infection, activated lymphocyte products, microbial toxins, and inflammatory and other stimuli (1). IL-1 induces both Cox-2 and inducible nitric-oxide synthase (iNOS) with increases in the release of PGs and NO from mesangial cells (2).

The iNOS found in several cell types including macrophages, (3–5) vascular smooth muscle cells (6, 7), and renal mesangial cells (8) is highly regulated by cytokines, which can facilitate or inhibit the induction of this enzyme. Stimulatory cytokines such as IL-1 and tumor necrosis factor increase iNOS mRNA by transcripational activation. Once iNOS is induced, it produces tremendous amounts of NO that can contribute to cell and tissue regulation and damage. However, iNOS gene expression, mRNA stability, and protein synthesis and degradation are all amenable to modification by cytokines or other agents such as growth factors. Transforming growth factor-β, for example, reduces cytokine-induced iNOS activity by inhibiting mRNA translation and increases iNOS protein degradation, whereas interleukin-4 interferes with iNOS transcription (9).

The cyclooxygenase is a ubiquitous enzyme that is involved in many inflammatory processes. Cyclooxygenase isoforms, Cox-1 and Cox-2, are the key enzymes that convert arachidonic acid to PGs. Cox-2 is normally undetectable in most tissues but can be rapidly induced in certain cell types by various proinflammatory or mitogenic stimuli (10). This inducible enzyme is thought to be involved in inflammation, cellular differentiation, and mitogenesis by releasing proinflammatory PGs. However, mice lacking Cox-2 have normal inflammatory responses but develop severe nephropathy as the animal ages, suggesting Cox-2 may be critical for normal kidney growth, differentiation, and function (11).

The intracellular signaling mechanisms triggered by IL-1 are not completely defined (12). IL-1 stimulation activates a family of protein kinases known as the mitogen-activated protein kinases (MAPKs) (13). At least four genetically distinct MAPK pathways, which are functionally independent and regulated by distinct protein cascades, have been identified in yeast (14). In mammalian cells, three subgroups of MAPK have been detected and include the extracellular signal-regulated kinase (ERKs), the c-jun amino-terminal kinases (JNKs) and p38 MAPKs (15). These kinases are activated by distinct upstream dual specificity kinases (MAPK kinase/MAPK kinase), which phosphorylate both threonine and tyrosine in a regulatory Thr-X-Tyr motif present in all MAPKs. Once activated, these MAPKs then phosphorylate and activate their specific substrates on serine and/or threonine residues and produce their effects on downstream targets (14, 15).

Mammalian p38 MAPK, the homologue of the yeast HOG1, is activated through different receptors by multiple stimuli, such as hyperosmolarity, UV light, heat shock, arsenite, endotoxin, and cytokines (16). However the cellular consequences of this signaling pathway are incompletely understood. Recent studies have suggested that p38 MAPK activates MAPK-activated protein kinase-2, which, in turn, phosphorylates the small heat shock protein 27 (HSP27). The physiological role of this event is controversial but may help the cell to resist thermal stress (17–20). Several other investigators have provided evidence that p38 MAPK may be involved in the regulation of cytokine production (21), neuronal apoptosis (22) and platelet aggregation (23, 24).

We previously demonstrated that IL-1β activates JNK in...
renal mesenchelial cells. In this current study, we examined whether the p38 MAPK signaling pathway also responds to IL-1 activation in this cell type. Furthermore, we have investigated whether p38 MAPK may be involved in the regulation of Cox-2 and iNOS.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant IL-1β and restriction enzymes were purchased from Boehringer Mannheim. Myelin basic protein (MBP) and PGE₂, were from Sigma. SC68376, was kindly provided by Dr. Joe Portnova (G. D. Searle Corporation, St. Louis, MO), and its structure is shown in Fig. 1. Fetal bovine serum was purchased from Life Technologies, Inc. Polyclonal rabbit IgG antibodies against iNOS, Cox-2, p38, and phospho-specific p38 were from Transduction Laboratories (Lexington, KY), Cayman Chemical Co. Inc. (Ann Arbor, MI), Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and New England BioLabs (Beverly, MA), respectively. Murine cDNA probes ligated in Bluescript SK (pBSCOX-1) and Cox-2 (pBScox-2) were generous gifts of Dr. Karen Seibert (Monsanto Corporation, St. Louis, MO). Mouse iNOS and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were kindly provided by Dr. Maryann Gruda (Department of Molecular Biology, Bristol Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). His-c-jun (1–79) was generously provided by Dr. Maryann Gruda (Department of Molecular Biology, Bristol Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). His-c-jun (1–79) was expressed as a histidine-tagged fusion protein in Escherichia coli NovaLue (DE3) and purified by Hi-bind resin (Novagen) (25).

Cell Culture—Primary mesangial cell cultures were prepared from male Sprague-Dawley rats as described previously (25). Cells were grown in medium supplemented with 15% heat-inactivated fetal calf serum, 0.3 IU/ml insulin, 0.1 units/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, and 15 mM HEPES, pH 7.4. All experiments were performed with confluent cells grown in 25 or 75 cm² flasks before the experiment.

Western Blot Analysis—Confluent cells were incubated in RPMI 1640 media containing 5% fetal calf serum with or without other pharmacological agents for 3 h (for Cox-1 and Cox-2) or 12 h (for iNOS) and harvested the steady-state levels of GAPDH gene-related sequences and the nitric oxide production. All membranes were probed with GAPDH cDNA to determine the steady-state levels of GAPDH gene-related sequences and used to normalize the amount of Cox-1, Cox-2, and iNOS mRNA. The peak response of Cox-2 mRNA occurs at 3 h, while for iNOS mRNA, this time point is 12 h. Therefore, these times were chosen for Northern analysis.

PGE₂ Determination—PGE₂ in the culture media was measured by stable isotope gas chromatography-mass spectrometry as described previously (25). At the end of predetermined times, medium was removed and spiked with 25 ng of tetradecanate PGE₂ (d₄-PGE₂). The media was then acidified to pH 3.5, and PGE₂ was extracted with 1 ml of 100% octadecyl columns (Baker Co., Sanford, ME). Extracts were derivatized for gas chromatography-mass spectrometry analysis. The samples were analyzed as the pentfluorobenzyl ester methoxime trimethylsilyl ether by negative ion chemical ionization using methane as the reagent gas. Ions monitored were m/z 524 (d₄-PGE₂), and m/z 528 (d₄-PGE₂). Mass spectrometry was performed on a Hewlett-Packard 5989B spectrometer using a 25 μl Ultra 1 (Hewlett-Packard Co., Palo Alto, CA) capillary column, and data collection and analysis were performed using HP 2 (Teknivent, St. Louis, MO) software. PGE₂ production was normalized for protein as determined by the micro-bicinchoninic acid assay (29).

Nitrite Determination—The conditioned incubation medium was collected, and nitrite content in the supernatant was measured by the addition of Griess reagent (1% sulfanilamide, 0.1% naphthylethylamine).
Statistical analysis was performed by using a paired or unpaired Student's t test. A difference with a value of 0.05 was considered statistically significant.

**RESULTS**

**IL-1β Activates p38 MAPK in Renal Mesangial Cells**—Previous studies in our laboratory have demonstrated that IL-1β activates several protein kinases, such as tyrosine kinases (31), JNK (25), and protein kinase C (32) in renal mesangial cells. To investigate whether the p38 MAPK pathway is involved in IL-1β signal transduction in glomerular mesangial cells, we examined whether the activation of p38 MAPK was induced by IL-1β. Cultured mesangial cells were treated with 100 units/ml IL-1β for 0–90 min. p38 was immunoprecipitated with p38 antibody and immune-complex p38 MAPK assay with MBP as substrate performed. Western blot assay with anti-phospho-p38 MAPK antibody as the primary antibody was performed to quantify phosphorylation of p38. Optical density for p38 MAPK phosphorylated MBP antibody was performed to quantitate phosphorylation. Western blot assay with anti-phospho-p38 MAPK antibody and immune-complex p38 MAPK assay with MBP as substrate performed. IL-1β was added to serum-starved or serum-replete mesangial cells, with or without serum stimulation. Western blot analysis using the anti-phospho-specific p38 MAPK antibody (which detected p38 MAPK only when phosphorylated on Thr-180) showed that IL-1β failed to significantly activate p38 MAPK in serum-replete mesangial cells when cells were serum starved for 24 h (data not shown). Western blot analysis using the anti-phospho-specific p38 MAPK antibody (which detected p38 MAPK only when phosphorylated on Thr-180) showed that IL-1β failed to significantly activate p38 MAPK in serum-replete mesangial cells when cells were serum starved for 24 h (data not shown).

**Effect of Serum on IL-1β-stimulated p38 MAPK Phosphorylation**—Mesangial cells were either serum starved for 24 h (open circles) or incubated with 5% fetal calf serum medium (filled circles) and then treated with 100 units/ml IL-1β for 0–24 h. Protein samples were subjected to Western blot assay.
These results demonstrate that, in primary mesangial cells, IL-1β-induced p38 MAPK activation and phosphorylation are dependent on serum.

**Effect of Serum on IL-1β-induced Nitric Oxide Biosynthesis**—Our laboratory previously reported that IL-1β increases in Cox-2 mRNA expression and PGE2 biosynthesis are dependent on serum (31). To investigate the effects of serum on IL-1β-induced nitric oxide synthesis, we measured the nitric oxide released in response to IL-1β as a function of time in serum-starved and serum-replete cells. As shown in Fig. 4, IL-1β increased nitric oxide secreted into the media both in the presence and absence of serum. Of note, however, was that in serum-starved cells, IL-1β produced significantly more NO than in serum-replete cells by 12 h of incubation. In previous experiments, we have demonstrated that IL-1β induced iNOS mRNA expression in serum-replete cells, which peaked at 12 h after IL-1β stimulation and declined to basal levels by 24 h (2). In contrast, when cells were serum starved for 24 h, iNOS mRNA expression was markedly enhanced. IL-1β significantly induced iNOS expression within 6 h of IL-1β stimulation, and remained at high levels for 24 h after exposure of cells to IL-1β (data not shown). Similar experiments carried out in serum-replete and serum-starved cells followed by immunoblot analysis of iNOS protein is demonstrated in Fig. 5. It demonstrates that in serum-replete media, iNOS is transiently expressed in response to IL-1β; whereas in serum-starved cells, iNOS protein expression is enhanced and sustained. Together, all of the above data suggest that, in rat mesangial cells, serum starvation inhibits IL-1β-induced PGE2 synthesis and, in contrast, increases IL-1β-induced nitric oxide biosynthesis, indicating that some additional "unknown factors" existing in the serum regulate IL-1β-induced nitric oxide and PGE2 production in reciprocal ways.

**SC68376 Selectively Inhibits p38 MAPK Activity**—Recently, a group of pyridinyl imidazole compounds have been identified that bind to p38 MAPK and inhibit its activity (21). SC68376 was kindly provided to us by G. D. Searle and has similar properties. Therefore, in our experiments, we determined the specificity of this compound by assessing its ability to inhibit the MAP kinase family of enzymes. We first incubated differing concentrations of SC68376 directly with p38 MAPK, which was immunoprecipitated by either anti-p38 (A) or anti-phospho-specific p38 MAPK (B) antibody from whole mesangial cell extracts. Immune-complex kinase assay was performed with MBP as substrate. Position of p38 MAPK phosphorylated MBP is indicated.

![Figure 6](image6.png)

**Figure 6. Effect of SC68376 on p38 MAPK activity.** Cells were stimulated with or without 100 units/ml IL-1β for 15 min. Different concentrations of SC68376 (μM) were directly incubated with p38 MAPK, which was immunoprecipitated by either anti-p38 (A) or anti-phospho-specific p38 MAPK (B) antibody from whole mesangial cell extracts. Immune-complex kinase assay was performed with MBP as substrate. Position of p38 MAPK phosphorylated MBP is indicated.

![Figure 7](image7.png)

**Figure 7. Effects of SC68376 on nitrite and PGE2 formation as well as iNOS and Cox-2 protein synthesis.** Cells were stimulated with (plus IL-1β) or without (minus IL-1β) 100 units/ml IL-1β in the presence of different concentrations of SC68376 at the time points indicated. Nitrite (A) and PGE2 (C) in the culture medium were determined. Results are the mean ± S.E. (n = 3). iNOS (B) and Cox-2 (D) protein syntheses were analyzed by Western blot assay.
immunoprecipitated with anti-p38 MAPK antibody and tested the ability of the immune complex to phosphorylate myelin basic protein. Fig. 6A clearly shows that SC68376 dose dependently inhibited p38 MAPK activity, with an IC₅₀ of about 2–5 μM. Fig. 6B shows similar data except that the antibody used to immunoprecipitate was anti-phospho-p38 antibody. Mesangial cells were preincubated with various concentrations of SC68376 for 30 min, and then IL-1β was or was not added to stimulate the cells for another 30 min with the final concentration of 100 IU/ml of IL-1β. Western blot assays demonstrated that SC68376 did not significantly decrease IL-1β-stimulated p38 MAPK phosphorylation (data not shown). In-gel kinase assays using myelin basic protein or His-c-jun(1–79) as substrate showed that SC68376 failed to significantly inhibit either basal ERK or IL-1β-activated JNK activity, respectively. These results demonstrate that SC68376 directly inhibits p38 MAPK catalytic activity. Thus, this compound is a potentially useful pharmacological tool to explore the physiological and pathophysiological function of p38 MAPK.

Effects of SC68376 on IL-1β-induced Nitric Oxide and PGE₂ Biosynthesis—To determine whether the p38 MAPK signaling pathway is involved in the induction of PGE₂ and nitric oxide synthesis by IL-1β, we studied the effects of SC68376 on IL-1β-induced nitric oxide and PGE₂ biosynthesis in renal mesangial cells. SC68376, in the range 0.1–100 μM, dose dependently enhanced the effect of IL-1β on nitrite production (Fig. 7A), iNOS protein synthesis (Fig. 7B), and iNOS mRNA expression (Fig. 8). The experiments suggested an apparent discrepancy between the amount of protein detected by Western analysis and the amount of NO produced. We have no clear explanation for this; however, one may speculate that some of the NO produced is converted to nitrite, which we measured, and some further metabolized to nitrate, which is not measured in the assay. Furthermore, at high concentrations of NO the potential for covalent nitrosylation of mesangial proteins exists. In contrast, SC68376 markedly inhibited IL-1β-induced PGE₂ production (Fig. 7C), Cox-2 protein synthesis (Fig. 7D), and Cox-2 mRNA expression (Fig. 8). Quantitative densitometry of iNOS and Cox-2 protein expression seen in Fig. 8 is reported in Table I. In previous studies in our laboratory, we found that PGE₂ negatively modulates the induction of nitric oxide synthesis by IL-1β (1). To rule out the possibility that the effects of SC68376 on IL-1β-induced nitric oxide synthesis was secondary to the decrease of PGE₂ production, we treated cells with various concentrations of SC68376 with or without the addition of 25, 250, or 2500 ng/ml PGE₂. We found that exogenous PGE₂ did partially reverse the effect of SC68376 on IL-1β-stimulated nitrite production (Fig. 9), which was consistent with earlier observations (2). However, this reversal was only about 20% at the highest concentration of PGE₂, thus suggesting that SC68376 facilitates the induction of nitric oxide synthesis by IL-1β and that the up-regulation is not mediated by the decrease in PGE₂ production. These observations indicate that p38 MAPK inhibitor SC68376 negatively modulates the cyclooxygenase pathway and positively modulates the nitric-oxide synthase pathway.

**TABLE I**

|          | iNOS/GAPDH | Cox-2/GAPDH |
|----------|------------|-------------|
| IL-1β    | 0.059      | 0.126       |
| IL-1β + 0.1 μM SC68376 | 0.082 | 0.081 |
| IL-1β + 1.0 μM SC68376 | 0.198 | 0.077 |
| IL-1β + 10 μM SC68376 | 0.347 | 0.036 |
| IL-1β + 100 μM SC68376 | 0.571 | 0.023 |

**DISCUSSION**

IL-1 is involved in several pathological processes of the renal glomerulus. The “activated” phenotype of mesangial cells stimulated by IL-1 could play vital roles in the further progression of glomerular inflammatory injury. In primary cultures of mesangial cells, we demonstrated previously that IL-1β induced iNOS and Cox-2 expression, which in turn, increased NO and PGE₂ production. However, the signaling pathways by which IL-1 induces NO and PG biosyntheses are not well understood (2, 28, 32). Recently, much effort has been directed at defining the signal transduction pathways utilized by IL-1. Cellular responses to IL-1 stimulation trigger a cascade of protein kinases that transmit signals from the cell surface to the nucleus and that ultimately regulate gene expression. The MAPKs are a family of serine/threonine kinases activated by dual phosphorylation of Thr and Tyr within a Thr-X-Tyr motif. There are at least three distinct types of MAPKs in mammalian cells, each of which has a distinct cascade of activation and distinct functions (33, 34). p38 MAPK is a member of the MAPK group of signal transduction kinases. It is activated by environmental stress and proinflammatory cytokines including IL-1β. In the present study, we have demonstrated the activation of p38 MAPK in IL-1β-treated rat mesangial cells by an immune-complex kinase assay. Our results indicate that IL-1β activates...
p38 MAPK in mesangial cells. An increase in p38 MAPK activity was detected 1 min after exposure of the cells to 100 units/ml IL-1β. The maximal activity occurred in 5–10 min, followed by a rapid decline in activity toward basal levels within 15 min. To assess whether the phosphorylation of Tyr-182 of p38 MAPK is also associated with its activation in mesangial cells, we examined the phosphorylation of p38 MAPK in response to IL-1β stimulation by using phosphospecific p38 MAPK (Tyr-182) antibody. Similar to the IL-1β-induced p38 MAPK activity, IL-1β significantly enhances p38 MAPK tyrosine phosphorylation. The increase of phosphorylated p38 MAPK was detectable 1 min after IL-1β stimulation, reached the peak at 5–10 min, and recovered to the basal level in 60 min. These data indicated that the activation of p38 MAPK signal cascade is an early event in mesangial cells in response to IL-1β stimulation. We therefore hypothesized that the activation of p38 MAPK signaling pathway may mediate IL-1β signal amplification and modulation that results in later events such as NO and PG production.

Although p38 MAPK has been found to be activated by several forms of environmental stress and cytokines, the physiological and pathophysiological function of this kinase in mammalian cells is still unclear. Recently, a role for p38 MAPK in the regulation of HSP27, cytokine biosynthesis, platelet aggregation, and neural apoptosis has been reported (17–24). To demonstrate whether p38 MAPK pathway mediates and regulates IL-1β-induced NO and PG synthesis, SC68376 was used in our studies to inhibit p38 MAPK. We found that SC68376 selectively inhibited both basal and IL-1β-activated p38 MAPK activity in vitro but did not affect its phosphorylation in intact cells. In contrast, this agent failed to inhibit ERK and JNK activity. We therefore used this p38 MAPK inhibitor to explore the role of p38 MAPK in IL-1β-induced NO and PG production. Previous studies in our laboratory have found that, in mesangial cells, serum is crucial for the full induction of PGE₂ biosynthesis by IL-1β (31). We hypothesized that some “unknown serum factors” are necessary to trigger the IL-1β-induced signal transmitting mechanisms leading to the phosphorylation/dephosphorylation cascades. However, our early data suggested that IL-1β-activated JNK signaling pathways are independent of serum (25). Surprisingly, the present results show that in mesangial cells, serum starvation significantly reduces IL-1β-activated p38 MAPK phosphorylation and activation and clearly demonstrate that IL-1β activates p38 MAPK pathway in a serum-dependent manner. The unknown serum factors may converge on this cascade upstream of p38 MAPK. Thus, serum starvation is another experimental probe to determine the function of p38 MAPK in that serum starvation selectively inhibits this kinase response to IL-1β in the renal mesangial cell.

To evaluate whether the p38 MAPK pathway is important for the regulation of IL-1β-induced PGE₂ synthesis, we examined the effects of SC68376 in addition to serum starvation on IL-1β-induced Cox-2 gene and protein expression and PGE₂ production. In agreement with our previous results (2, 28, 31), in mesangial cells starved for 24 h of serum, the effects of IL-1β on Cox-2 mRNA expression, protein expression, and PGE₂ production were significantly reduced. Similar to the effects of serum starvation, SC68376 dose dependently inhibited Cox-2 mRNA and protein expression and PGE₂ formation. These results indicate that selective inhibition of p38 MAPK suppresses IL-1β-induced Cox-2 expression and PGE₂ production, suggesting that IL-1β-induced PGE₂ synthesis is up-regulated by the activation of p38 MAPK pathway.

IL-1β stimulates NO generation by enhancing iNOS expression with subsequent elevation of intracellular cyclic GMP. The increased formation of NO and cyclic GMP in mesangial cells may not only alter the contractile responses of cells but may also cause tissue injury and thus contribute to the pathogenesis of certain forms of glomerulonephritis (2, 28, 35). To test our hypothesis that p38 MAPK mediates and regulates IL-1β-induced NO synthesis, we evaluated the effects of SC68376 and serum starvation on IL-1β-induced NO formation in our current studies. In the presence of serum, IL-1β-induced Cox-2 and PGE₂ synthesis and also increases iNOS mRNA and protein expression and NO generation. The peak response of the mRNA for iNOS occurred at 12 h and returned toward basal levels by 24 h. However, in mesangial cells serum starved for 24 h, IL-1β markedly induced iNOS mRNA and protein expression as well as NO production. The mRNA and protein remained elevated at 24 h. As with serum starvation, SC68376 produced dose-dependent increases in IL-1β-induced iNOS expression and NO release in mesangial cells. The current studies demonstrate that the effects of SC68376 on IL-1β-induced NO production were not reversed by PGE₂. Thus, the effects of p38 MAPK inhibition on the amplification of IL-1β-induced NO generation are not secondary to its effects on the decrease of PGE₂ formation. Together, the above data demonstrate that inhibition of p38 MAPK promotes the IL-1β-induced iNOS expression and NO production in mesangial cells, suggesting that the signaling mechanism of IL-1β-induced NO synthesis is down-regulated by the activation of the p38 MAPK signal transmitting pathway.

In summary, our present studies demonstrate that IL-1β phosphorylates and activates p38 MAPK in mesangial cells. The activation of p38 MAPK provides a crucial signaling transduction mechanism, which may positively regulate IL-1β-induced PG synthesis but negatively regulate IL-1β-induced NO biosynthesis. These opposing actions of the p38 MAPK pathway may suggest the possibility that a p38 MAPK mechanism may play an important role in the regulation and balance of function of this important proinflammatory cytokine. However, because other mechanisms are involved in IL-1β signaling, the final response to IL-1β stimulation may be dependent on the integration of multiple signaling pathways.

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