Expression of Heme Oxygenase-1 in Human Vascular Cells Is Regulated by Peroxisome Proliferator-Activated Receptors

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Objective—Activation of peroxisome proliferator-activated receptors (PPARs) by lipid-lowering fibrates and insulin-sensitizing thiazolidinediones inhibits vascular inflammation, atherosclerosis, and restenosis. Here we investigate if the vasculoprotective and anti-inflammatory enzyme heme oxygenase-1 (HO-1) is regulated by PPAR ligands in vascular cells.

Methods and Results—We show that treatment of human vascular endothelial and smooth muscle cells with PPAR ligands leads to expression of HO-1. Analysis of the human HO-1 promoter in transient transfection experiments together with mutational analysis and gel shift assays revealed a direct transcriptional regulation of HO-1 by PPARα and PPARγ via 2 PPAR responsive elements. We demonstrate that a clinically relevant polymorphism within the HO-1 promoter critically influences its transcriptional activation by both PPAR isoforms. Moreover, inhibition of HO-1 enzymatic activity reversed PPAR ligand-mediated inhibition of cell proliferation and expression of cyclooxygenase-2 in vascular smooth muscle cells.

Conclusion—We demonstrate that HO-1 expression is transcriptionally regulated by PPARα and PPARγ, indicating a mechanism of anti-inflammatory and antiproliferative action of PPAR ligands via upregulation of HO-1. Identification of HO-1 as a target gene for PPARs provides new strategies for therapy of cardiovascular diseases and a rationale for the use of PPAR ligands in the treatment of other chronic inflammatory diseases. (Arterioscler Thromb Vasc Biol. 2007;27:1276-1282.)

Key Words: atherosclerosis • heme oxygenase • inflammation • PPAR

Hyperlipidemia and diabetes type II are major causes for vascular disorders including atherosclerosis. Among the drugs that are used to treat these metabolic diseases, both the lipid-lowering fibrates and the insulin-sensitizing thiazolidinediones exert their effects via activation of peroxisome proliferator-activated receptors (PPARs).1 PPARs are ligand-activated transcription factors that bind to specific PPAR-responsive elements (PPREs) as heterodimers together with the retinoid X receptor and govern the expression of genes involved in the regulation of lipid and glucose metabolism.1,2 The 3 PPAR isoforms PPARα, PPARβ/δ, and PPARγ show unique tissue distribution and ligand specificity. Natural PPAR ligands include various fatty acids and fatty acid-derived compounds such as eicosanoids.1,2 Treatment with synthetic PPARα and PPARγ ligands such as fibrates and thiazolidinediones,3 respectively, has been shown to potently inhibit the development of atherosclerosis4-6 and restenosis.7,8 Increasing evidence suggests that the beneficial effects of PPAR ligands on the vascular wall are not only caused by changes in systemic metabolic parameters9 but additionally involve local anti-atherogenic effects,6 such as inhibition of inflammation10-12 and vascular smooth muscle cell (VSMC) proliferation.8 The beneficial effects of synthetic PPAR agonists on cardiovascular disease outcome have been demonstrated in major clinical trials that have reported cardiovascular risk reduction in patients with dyslipidemia. However, the favorable alterations in plasma lipids can only partially explain the reduction in cardiovascular events in these studies. Therefore, many beneficial effects of PPARs have been attributed to their anti-inflammatory activity.

Despite the large number of known target genes for PPARs, little is known about induction of expression of anti-inflammatory and antiproliferative genes by PPARs. We hypothesized that the local upregulation of anti-atherogenic genes by PPAR ligands contributes to their beneficial effects on the vascular wall. Recently, the presence of PPREs in the
antioxidant enzymes catalase and manganese superoxide dismutase have been reported. Numerous studies demonstrated a protective role for heme oxygenase-1 (HO-1) during atherogenesis, restenosis, and other inflammatory vascular disorders. HO-1 is the rate-limiting enzyme of heme catabolism, catalyzing the breakdown of heme into iron, biliverdin, and carbon monoxide. Both biliverdin and carbon monoxide have been shown to act as anti-inflammatory agents and to inhibit VSMC growth. It has been shown that the length of a GT-repeat within the proximal region of the human HO-1 promoter is highly polymorphic, which seems to influence the transcription of the HO-1 gene, rendering people possessing longer (>29 repeats) GT fragments more susceptible to inflammatory disorders such as coronary atherosclerosis.

Here we demonstrate induction of HO-1 expression by PPARγ and PPARα ligands in cultured vascular cells and we show that HO-1 enzymatic activity mediates anti-inflammatory and antiproliferative effects exerted by PPAR ligands. Moreover, we show that HO-1 is a direct PPAR target gene and that a clinically relevant (GT)n dinucleotide length polymorphism within the human HO-1 promoter significantly influences the transcriptional regulation of HO-1 by both PPARα and PPARγ.

Materials and Methods
Expression of HO-1 and PPAR isoforms was studied using real-time polymerase chain reaction or Western blotting. The constructs (4.9 kb to 0.3 kb) used for human HO-1 promoter analysis were described previously. Site-directed mutagenesis of the HO-1 promoter was performed by a polymerase chain reaction-based technique using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Calif). Electromobility shift assays were performed using the TNT reticulocyte lysate kit (Promega). For more details, please see the supplemental Materials available online at http://atvb.ahajournals.org.

Results
Ligands for PPARγ and PPARα Induce HO-1 Expression in Human Vascular Cells
Activation of both PPARγ and PPARα was shown to inhibit the inflammatory response in endothelial cells, VSMCs, and macrophages, and ligands for both receptors have been shown to reduce atherosclerosis in mice and humans. Therefore we determined the influence of ligands specific for either PPARα (Wy-14,643 and fenofibrate) or PPARγ (rosiglitazone and troglitazone) on HO-1 expression in vascular cells. Western blot analysis (Figure 1A) and immunofluorescence (Figure 1B) demonstrated that both PPARα and PPARγ ligands induced HO-1 expression in human umbilical venous endothelial cells (human umbilical venous endothelial cells) as well as in human VSMCs.

We found a maximum expression of HO-1 protein at 18 to 20 hours after incubation with PPAR ligands, and dose-response experiments revealed a concentration-dependent increase of HO-1 protein (Figure 2A) and mRNA (Figure 2B and 2C) after stimulation with the PPARα ligand Wy-14,643 or the PPARγ ligand rosiglitazone in both human umbilical venous endothelial cells and VSMC. Quantification of PPARα and PPARγ mRNA levels showed that PPARα was the predominant PPAR isoform in both human umbilical venous endothelial cells and VSMC (Figure 2D). Accordingly, measurement of HO-1 mRNA levels in human umbilical venous endothelial cells and VSMC by quantitative polymerase chain reaction showed a marked increase in HO-1 mRNA after activation of PPARα by Wy-14,643, whereas treatment with the PPARγ-specific ligand rosiglitazone induced HO-1 mRNA to a lesser extent (Figure 2B and 2C). Together, these data suggest that depending on the expression levels of the respective PPAR isoforms, PPARα and PPARγ ligands induce HO-1 in vascular cells.
HO-1 Is a Direct PPAR Target Gene

To determine if HO-1 was a direct PPAR target gene, we analyzed the activity of a full-length (4.9 kb) human HO-1 promoter construct that had been previously cloned in our laboratory. To cotransfect expression vectors for PPARα and PPARγ together with a plasmid encoding for retinoid X receptor-α in HEK293 cells followed by treatment with the respective PPAR ligand. Analysis of deletion mutants demonstrated that PPARα or PPARγ-induced HO-1 promoter activity was dependent on a fragment located between −1.4 and −2.2 kb upstream of the transcription start site (Figure 3A). To narrow down the responsive promoter elements we created a second series of deletion mutants, which enabled us to locate the PPAR-responsive promoter region between −1740 kb and −1826 kb (Figure 3B). Bioinformatic analysis identified 2 putative PPAR-responsive elements (PPRE1 and PPRE2 GGGA-CAAAGGTTG and AGGTGAAAGGCCG) in DR1 configuration located within the 86-bp region.

To confirm the functional relevance of the 2 identified PPREs, we introduced mutations in each of these elements, which abolished the PPARα- or PPARγ-induced activity of the full-length HO-1 promoter construct (Figure 4A). Electrophoretic mobility shift assays showed that both PPRE1 and PPRE2 bound to in vitro-translated PPARα/retinoid X receptor-α and PPARγ/retinoid X receptor-α heterodimers (Figure 4B), whereas we did not observe binding to retinoid X receptor-α homodimers (data not shown). The specificity of the bands was confirmed by competition with excess cold wild-type probes or cold probes containing point mutations.

Together, these data demonstrate that HO-1 is a direct PPARα or PPARγ target gene regulated via 2 PPREs.

A Polymorphism Within the HO-1 Promoter Critically Affects Its Regulation by PPARα or PPARγ

The HO-1 promoter is highly polymorphic within the human population in that the length of a proximal (GT)n dinucleotide repeat varies (Figure 5A). Longer repeats have been reported to negatively influence HO-1 expression and thus are associated with a higher risk for coronary artery disease and restenosis. To determine if the length of the GT-repeat affects the transcriptional regulation of the human HO-1 promoter by PPARs, we generated 3 full-length (4.9 kb) HO-1 promoter constructs differing in the length of their proximal GT-repeats (11, 24, and 29) by amplification of the
respective DNA fragments in samples isolated from genotyped individuals. Subsequent analysis revealed a strong negative correlation between the PPAR\(\gamma\)/H9251- or PPAR\(\gamma\)/H9253-induced promoter activity and the increasing length of the GT-repeat within the HO-1 promoter (Figure 5B).

**Figure 4.** Functional properties of identified PPREs. A, HEK293 cells were transfected with either the wild-type (wt) HO-1 promoter constructs or constructs with the indicated mutations of PPRE1 or PPRE2 and cotransfected with PPAR\(\alpha\) or PPAR\(\gamma\) and retinoid X receptor-\(\alpha\) expression plasmids. HO-1 promoter activity was analyzed measuring firefly luciferase activity normalized to renilla luciferase activity. Activity in the absence of PPAR expression vectors was set to 1. B, Binding of in vitro-translated PPAR/retinoid X receptor heterodimers to the identified PPREs of the human HO-1 promoter was determined by electrophoretic mobility shift assay. Competition was performed using a 100-fold excess of nonlabeled oligonucleotides.

**Figure 5.** Influence of an HO-1 promoter polymorphism on PPAR-induced HO-1 promoter activity. A, Structure of the human HO-1 promoter. B, Promoter activity of full-length (4.9 kb) HO-1 promoter constructs carrying 11, 24, or 29 GT-repeats after cotransfection with PPAR\(\alpha\), PPAR\(\gamma\), and retinoid X receptor-\(\alpha\) expression plasmids and PPAR ligand treatment (24 hours). HO-1 promoter activity was analyzed measuring firefly luciferase activity normalized to renilla luciferase activity. Activity in the absence of PPAR expression vectors was set to 1.

**HO-1 Enzymatic Activity Contributes to the Anti-Inflammatory and Antiproliferative Effects of PPAR\(\alpha\) in VSMCs**

Ligands for PPAR\(\alpha\) have been shown to inhibit the cytokine-induced expression of inflammatory genes in vascular cells.\(^{26,27}\) To examine the contribution of HO-1 to anti-inflammatory effects exerted by PPAR ligands, we analyzed tumor necrosis factor-induced cyclooxygenase-2 (COX-2) expression in VSMCs, which was inhibited by the PPAR\(\alpha\) ligand Wy14,643 in a concentration-dependent manner (Figure 6A). Addition of the specific HO-1 inhibitor tin protoporphyrin (SnPP) abrogated the inhibition of COX-2 expression by Wy-14,643 (Figure 6A), demonstrating the involvement of HO-1 as a mediator of anti-inflammatory effects of PPAR ligands in VSMCs. In endothelial cells, in contrast, expression of COX-2 was not inhibited by Wy-14,643 (Figure 6B).

Ligands for PPAR\(\alpha\) have also been shown to inhibit smooth muscle cell (SMC) proliferation.\(^{32}\) Therefore, we investigated the effect of PPAR-induced HO-1 on SMC proliferation. Inhibition of HO-1 by zinc protoporphyrin (SnPP) abrogated the inhibition of COX-2 expression by Wy-14,643 (Figure 6A), demonstrating the involvement of HO-1 as a mediator of anti-inflammatory effects of PPAR ligands in VSMCs. In endothelial cells, in contrast, expression of COX-2 was not inhibited by Wy-14,643 (Figure 6B).

Ligands for PPAR\(\alpha\) have also been shown to inhibit smooth muscle cell (SMC) proliferation.\(^{32}\) Therefore, we investigated the effect of PPAR-induced HO-1 on SMC proliferation. Inhibition of HO-1 by zinc protoporphyrin dose-dependently increased SMC proliferation, induced by either platelet-derived growth factor or 15% fetal bovine serum. Moreover, treatment of SMCs with the PPAR\(\alpha\)-ligand Wy-14,643 inhibited fetal bovine serum-induced proliferation, while concomitant inhibition of HO-1 with zinc protoporphyrin reversed this effect (supplemental Figure 1, available online at http://atvb.ahajournals.com). Together these data demonstrate a HO-1–dependent and cell
type-specific modulation of COX-2 expression and VSMC proliferation by a PPARα ligand.

**Discussion**

In addition to their beneficial influence on glucose and lipid metabolism, pharmacological PPAR ligands have been shown to exert direct anti-inflammatory and antiproliferative effects on cells of the vascular wall and hence inhibit the development of atherosclerosis and reduce intimal growth. Nevertheless, the exact mechanisms behind these versatile qualities of PPARs are poorly understood. One important anti-inflammatory mechanism of PPARs is transcriptional downregulation of inflammatory genes, which involves both a direct interaction with other transcription factors and competition for coactivators. Recently, it was shown that PPARγ inhibits transcription of proinflammatory genes by SUMOylation. In addition, upregulation of protective genes by PPARs may significantly contribute to their anti-inflammatory properties. With HO-1 we have identified a novel PPAR target gene that has been shown to exert multiple beneficial effects on cells of the vascular wall and to inhibit the development of atherosclerosis and restenosis in vivo.

We show that ligands for PPARα or PPARγ upregulate HO-1 expression in cultured human endothelial cells and VSMCs. Although it has been shown that the vast majority of Wy-14,643–induced genes are regulated in a PPARα-dependent manner, Wy-14,643 can exert pan-PPAR agonistic activity. Therefore, we confirmed PPARα-dependent induction of HO-1 expression in endothelial cells and VSMCs after treatment with another PPARα activator, fenofibrate. PPARα was the predominant PPAR isotype expressed in both human umbilical venous endothelial cells and SMCs, and PPARα ligands seemed to be more effective than PPARγ ligands in inducing HO-1 expression.

PPAR ligands can also display receptor-independent effects, especially at high concentrations. Wy-14,643 is a fibrin acid derivative with an EC50 of 5 μmol/L for human PPARα. Although we demonstrate that Wy-14,643 at pharmacological doses significantly increases HO-1 expression, we also used higher doses (150 to 250 μmol/L), which likely have additional unspecific effects. However, the induction of HO-1 expression by receptor-specific concentrations of ligands as well as our promoter studies indicated a transcriptional regulation of HO-1 expression by PPARα or PPARγ. Detailed analysis of the human HO-1 promoter showed that HO-1 is indeed a direct PPAR-target gene, whose transcription is regulated by both PPARα and PPARγ via 2 PPREs.

Previous studies have highlighted the importance of stress-responsive elements (StREs) in the transcriptional regulation of HO-1. The repressor protein Bach1 constitutively binds to StREs and is displaced on activation of transcription factors such as Nrf2. 15-deoxy-delta 12,14 prostaglandin J2, which exerts multiple PPAR-dependent and PPAR-independent effects, has been shown to activate the Nrf2 regulatory pathway via Keap1 and to induce HO-1 expression via StREs in an PPAR-independent manner. However, prostaglandin J2-induced transcription of glutathione S-transferase involves synergistic activation of Nrf2 and PPARγ. In our present study the StRE-containing enhancer region of the human HO-1 promoter was dispensable for the PPAR-induced transcriptional regulation of the HO-1 promoter, because the 3.8-kb and 2.2-kb HO-1 promoter constructs, which lack this enhancer, but include the PPREs, were still PPAR-responsive. Moreover, mutation of the StREs did not affect PPAR-induced HO-1 promoter activity (data not shown). With the 2 identified PPREs located between the StREs and the basic HO-1 promoter, it will be nevertheless interesting to determine a possible influence of the Bach1/Nrf2 system on the PPAR-mediated transcriptional regulation of HO-1 and to analyze the accessibility of the 2 PPREs under various conditions of cellular stress.

We demonstrate that PPAR-induced HO-1 promoter activity inversely correlates with the length of a polymorphic GT-repeat in the human HO-1 promoter. This polymorphism has been reported to affect hydrogen peroxide-induced HO-1 promoter activity as well as HO-1 mRNA expression and enzymatic activity in lymphoblastoid cells. It has been suggested that longer GT-repeats promote changes in DNA conformation, which in turn negatively affect transcriptional activity. Moreover, the length of the respective GT-repeat is associated with the susceptibility for various inflammatory diseases including coronary heart disease and restenosis.

It will be important to determine whether the vasculoprotec-
tive effects of PPAR ligands correlate with this HO-1 promoter polymorphism, which would provide a possibility to better assess the individual benefit of the treatment with fribates or thiazolidinediones.

Both PPAR ligands and HO-1 have been described to block the inflammatory response in vascular cells and to potently inhibit VSMC proliferation.5,8,17,21,26,32,48–50 Furthermore, inhibition of COX-2 expression and prostaglandin synthesis by HO-1 was reported.51–56 We determined the contribution of HO-1 to the described anti-inflammatory and antiproliferative effects of PPARα ligands in VSMCs57 and show that inhibition of HO-1 enzymatic activity by zinc protoporphyrin abrogated the inhibitory effect of PPARα ligands on cytokine-induced expression of COX-2 as well as on proliferation in VSMCs. Interestingly, Wy-14,643-induced HO-1 did not affect COX-2 expression in endothelial cells, and while the expression of vascular cell adhesion molecule-1 in endothelial cells was strongly inhibited by the PPARα ligand, inhibition of HO-1 did not reverse this effect (data not shown). Moreover, we show that treatment of SMCs with the PPARα-ligand Wy-14,643 inhibited platelet-derived growth factor and serum-induced proliferation, while concomitant inhibition of HO-1 reversed this effect. Interestingly, it has been shown that upregulation of HO-1 in endothelial cells increases proliferation.57 These data strongly indicate cell type specific mechanistic differences in the anti-inflammatory action of PPAR ligand-induced HO-1.

Since PPARs have been recognized as key transcription factors regulating the expression of genes involved in lipid and glucose metabolism, HO-1 represents a novel type of PPAR target gene. The finding that HO-1 expression is transcriptionally regulated by PPARα or PPARγ provides an important additional link between these ligand-activated transcription factors and their described anti-inflammatory and antiproliferative properties and thus contributes to our understanding of the pleiotropic beneficial effects exerted by PPAR ligands in inflammatory vascular disorders.

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Disclosures
None.

References


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Materials and Methods

Materials: Wy-14,643 was purchased from Alexis (Lausen, Switzerland), the antibody against actin, zinc and tin protoporphyrins (ZnPP, SnPP) were from Sigma-Aldrich (Vienna, Austria). Polyclonal antibodies against HO-1 were from Stressgen (BC, Canada) and antibodies against cyclooxygenase-2 (COX-2) were purchased from Santa Cruz Biotechnology (Santa Crz, CA). Peroxidase-conjugated secondary antibodies were purchased from Amersham Life Science (Amersham Place, Little Chalfont, UK).

Quantitative real-time RT-PCR: QPCR was performed using Sybrgreen. The expression of the target molecule was normalized to the expression of β-2 microglobulin (β2m) using a mathematical model by Pfaffl. Sequences of primers used in this study were: HO-1 forward, 5´-AAGATTGCCCAGAAAGCCCTGGAC-3´; HO-1 reverse, 5´-AACTGTCGCCACCAGAAAGCTGAG-3´; PPARα forward, 5´-TCTGGCCAAGAGAATCTACGA-3´; PPARα reverse, 5´-TCCTTGTTCTGGATGCCATT-3´; PPARγ forward, 5´-GAGCCCAAGTTTGAGTTTGC-3´; PPARγ reverse, 5´-CTGTGAGGACTCAGGGTGTG-3´.

Cell Culture: Human umbilical vein endothelial cells (HUVEC) were cultured in medium 199 (M199) containing 20% fetal bovine serum (FBS), 1U/ml heparin and 50µg/ml bovine endothelial cell growth supplement (Technoclone, Vienna, Austria). Experiments were performed using cells at passage 4-6. Human umbilical artery or vein smooth muscle cells (SMC) were grown in M199 containing 10% FBS and used at passages 4-6. HEK 293 cells were cultivated in DMEM (10% FBS). HUVEC were stimulated in the presence of 1% FBS.
Plasmids: The human HO-1 promoter constructs (4.9kb-0.3kb) were described previously. The human HO-1 promoter constructs used to locate the PPAR responsive elements (PPREs) (2245bp-1435bp) were generated by PCR-amplification using the full length HO-1 promoter construct as a template and subsequent cloning into the PGL3 luciferase vector. To generate HO-1 promoter constructs varying in the length of their GT repeat, the proximal HO-1 promoter of genotyped individuals was amplified by PCR and cloned into the PGL-3 vector as previously described. Subsequently the distal part of the HO-1 promoter (Sacl-PstI fragment) was attached to the individual proximal PstI-XbaI promoter fragments, which contained the GT-repeats, to yield full-length (4.9kb) promoter constructs differing in their proximal GT-repeats.

Site-directed Mutagenesis: The PPREs of the HO-1 promoter were mutated by a PCR based technique using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the protocol provided by the manufacturer. For each mutation two complementary primers containing the mutation were synthesized.

Luciferase Assays: Luciferase activity of the cell lysates was determined using a Dual-Luciferase Reporter Assay system (Promega). Firefly activity was then normalized to SV40 renilla activity to yield relative Luciferase activity (presented as fold above control).

Electromobility shift assays (EMSA): RXR, PPARα and PPARγ were in vitro translated using the TNT reticulocyte lysate kit (Promega). EMSA was performed using 32P end-labelled oligonucleotides.
Western Blot Analysis: After stimulation, cells were lysed in Laemmli buffer and proteins separated by electrophoresis in 12% sodium dodecyl sulfate (SDS)- polyacrylamide gels. Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes, which were then incubated with the respective primary antibodies. Bound antibodies were detected by anti-IgG conjugated with peroxidase and subsequent chemiluminescent detection.

Proliferation assay: Human umbilical artery SMC were used in passage 4. Cells were plated into 96 well plates (2000 cells per well). After cells adhered, SMCs were starved in serum free medium for 24 hours. Proliferation was induced either with 20% FBS or with 5ng/ml PDGF-BB in the presence or absence of 100µM Wy, 14-643. HO-1 activity was inhibited with indicated concentrations of zinc protoporphyrin (ZnPP). After 72 hours cell proliferation was assessed colorimetrically (EZ4U proliferation and cytotoxicity assay, Biomedica, Austria). Briefly, cells were incubated with the substrate for the last 3 hours of the assay; absorbance was measured at 490nm with 620nm as a reference.

Statistical analysis: All data are presented as mean +/- standard deviations and are representative for at least two independent experiments. An unpaired (two-tailed) t test was used to determine significance. A value of p<0.05 was considered statistically significant.
Supplementary Figure I. Inhibition of SMC-proliferation by PPAR ligands is HO-1-dependent. PPAR-induced HO-1 inhibits smooth muscle cell proliferation. (A) SMCs were starved in serum-free medium overnight, proliferation was induced with either 5ng/ml PDGF-BB or 20% FBS either in the absence or in the presence of ZnPP (1, 5, 10µM). Proliferation was measured after 72 hours. (B) SMCs were starved and stimulated with 100µM Wy-14,643 for 18hours, then proliferation was induced by either 5ng/ml PDGF-BB or 20% FBS in the presence or absence of 10µM ZnPP. Proliferation was measured after 72 hours. *, $P < 0.05$. 
A

ZnPP

+ PDGF-BB

+ 20% FBS

B

Wy,14-643

+ PDGF-BB

+ 20% Serum

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