Induction of Heme Oxygenase 1 Attenuates Placental Ischemia–Induced Hypertension

Eric M. George, Kathy Cockrell, Marietta Aranay, Eva Csongradi, David E. Stec, Joey P. Granger

Abstract—Recent in vitro studies have reported that heme oxygenase 1 (HO-1) downregulates the angiostatic protein soluble fms-like tyrosine kinase 1 from placental villous explants and that the HO-1 metabolites CO and bilirubin negatively regulate endothelin 1 and reactive oxygen species. Although soluble fms-like tyrosine kinase 1, endothelin 1, and reactive oxygen species have been implicated in the pathophysiology of hypertension during preeclampsia and in response to placental ischemia in pregnant rats, it is unknown whether chronic induction of HO-1 alters the hypertensive response to placental ischemia. The present study examined the hypothesis that HO-1 induction in a rat model of placental ischemia would beneficially affect blood pressure, angiogenic balance, superoxide, and endothelin 1 production in the ischemic placenta. To achieve this goal we examined the effects of cobalt protoporphyrin, an HO-1 inducer, in the reduced uterine perfusion pressure (RUPP) placental ischemia model and in normal pregnant rats. In response to RUPP treatment, mean arterial pressure increases 29 mm Hg (136±7 versus 106±5 mm Hg), which is significantly attenuated by cobalt protoporphyrin (118±5 mm Hg). Although RUPP treatment causes placental soluble fms-like tyrosine kinase 1/vascular endothelial growth factor ratios to alter significantly to an angiostatic balance (1.00±0.10 versus 1.27±0.20), treatment with cobalt protoporphyrin causes a significant shift in the ratio to an angiogenic balance (0.68±0.10). Placental superoxide increased in RUPP (952.5±278.8 versus 243.9±70.5 relative light units/min per milligram) but was significantly attenuated by HO-1 induction (482.7±117.4 relative light units/min per milligram). Also, the preproendothelin message was significantly increased in RUPP, which was prevented by cobalt protoporphyrin. These data indicate that HO-1, or its metabolites, is a potential therapeutic for the treatment of preeclampsia. (Hypertension. 2011;57:00-00.)

Key Words: placental ischemia ■ soluble VEGF R1 ■ preeclampsia ■ reduced uterine perfusion pressure ■ ET-1

Preeclampsia, a gestation-dependent hypertensive disorder, presents with proteinuria and vascular dysfunction after the twentieth week of pregnancy.1,2 It is relatively common, contributing to ≤15% of preterm births, and is a leading cause of maternal- and fetal morbidity worldwide, additionally increasing the risk of later cardiovascular disease to the offspring.3–5 The exact cause of the disease is unknown, although it is thought to be a result of reduced placental perfusion, possibly through failure of fetal derived trophoblasts to adequately invade and remodel the maternal spiral arteries.6

As a consequence of inadequate placental perfusion, the placenta becomes increasingly hypoxic, as is indicated by the induction of hypoxia-responsive genes.7,8 The ischemic/hypoxic placenta has been shown in experimental animal models to be the source of many of the underlying physiological factors in the etiology of the disease, including generation of placental reactive oxygen species and imbalance of angiogenic and angiostatic factors, specifically vascular endothelial growth factor (VEGF) and soluble fms-like tyrosine kinase 1 (sFlt-1).9,10 These studies into animal models of placental ischemia have provided an important experimental system for the investigation of the pathophysiology of the disorder. Recently, there has been substantial interest in the use of heme oxygenase 1 (HO-1) as a therapeutic agent in multiple forms of cardiovascular disease and hypertension (for thorough reviews, see References 11 and 12). HO-1 functions in the heme salvage pathway, removing the pro-oxidant free heme by converting it to bilirubin, a known antioxidant molecule, and CO, a potent vasodilator.13–15 HO-1 induction has been shown to be beneficial in models of renovascular hypertension, pulmonary hypertension, angiotensin II–dependent hypertension, and the spontaneously hypertensive rat model.11,16–18 It has been reported that induction of HO-1 in the placenta protects isolated villi from tumor necrosis factor-α induced cellular damage and promotes vascular relaxation.19 Further studies indicated that, in vitro, HO-1

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induction and CO alone were capable of downregulating the angiotensin protein sFlt-1 production from placental villous explants and that CO negatively regulates endothelin 1 (ET-1) at the message level in endothelial cells.\(^{20,21}\) In addition, it has been reported that heterozygous knockout of HO-1 in mice results in inhibited placental development, elevated plasma sFlt-1, and increased maternal diastolic pressure.\(^{22}\)

Although acute induction of HO-1 has been reported to negatively regulate many of the factors that are involved in the pathophysiology of hypertension in response to placental ischemia, it is not known whether chronic HO-1 induction alters the hypertensive response to placental ischemia during pregnancy. Therefore, the present study was undertaken to examine the hypothesis that HO-1 induction in a rat model of placental hypoxia/ischemia would have beneficial effects on blood pressure, angiogenic balance, and superoxide production in the ischemic placenta.

### Methods

#### Animals

Timed pregnant Sprague-Dawley rats (Harlan, Inc, Indianapolis, IN) were received on day 10 or 11 of gestation. All of the protocols were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Rats were maintained on a 12:12 hour light:dark cycle, at 23°C constant temperature, and were provided food and water ad libitum.

#### Reduced Uterine Perfusion Pressure Procedure and Cobalt Protoporphyrin Treatment

On gestational day 14, reduced uterine perfusion pressure (RUPP)–treated animals were subjected to aortic and bilateral ovarian artery constriction as described previously.\(^{23}\) Briefly, animals were anesthetized by controlled 3% isoflurane (Webster), and a midline abdominal incision was made. After exteriorization of both uterine horns, 1 single 0.203-mm silver surgical clip was placed on the lower abdominal aorta above the iliac bifurcation. One 0.100-mm silver surgical clip was placed on both the left and right ovarian arteries, which supply the uteri to prevent compensatory flow. Rats absorbing all of the pups as a result of the procedure were excluded from the study. For HO-1 induction, Cobalt (III) protoporphyrin IX chloride (CoPP; Frontier Scientific, Logan, UT) was injected iP at a dose of 5 mg/kg on day 14 of gestation, at doses described previously.\(^{24,25}\)

#### Measurement of Mean Arterial Pressure

On gestational day 18, rats were anesthetized as above and implanted with indwelling carotid catheters consisting of V-3 tubing (SCI). Catheters were tunneled subcutaneously and exteriorized at the back of the neck. The following day, rats were placed in individual restraining cages and acclimatized. Mean arterial pressure was measured continuously for 1 hour via Cobe III pressure transducers (CDX Sema). Each experimental group had 10 to 13 rats.

#### Tissue Harvest

Rats were anesthetized as above. After ventral midline incision, the uterus was externalized, and blood was collected by needle tapping of the abdominal aorta. The number of viable and reabsorbed pups was recorded in the uterus, and individual pups and placentas were weighed and recorded. Samples of the largest and smallest placentas from each horn, the thoracic aorta, and the liver were saved and flash frozen in liquid nitrogen before storage at −80°C for later analysis.

#### Measurement of VEGF and sFlt-1

Measurements of VEGF and sFlt-1 were made by sandwich ELISA (R&D Systems) in duplicate with internal standardization via supplied control protein. For placental measurements, the largest frozen placenta from an individual uterine horn was ground in a mortar and pestle while submerged in liquid nitrogen. Resulting particles were resuspended and homogenized in radioimmunoassay buffer with a multiprotease inhibitor mixture, PMSF, and sodium orthovanadate (Santa Cruz Biotechnology). Lysates were spun at 12 000g at 4°C for 20 minutes to remove cellular debris. Total protein concentration was measured by the bicinchoninic acid method (Pierce Biotechnology). VEGF and sFlt-1 were then measured and normalized to total protein concentration. Plasma obtained from the above harvests was assayed for VEGF concentration with the same assay kit. Each group had >6 rats. All of the samples were run in duplicate, with VEGF intra-assay and interassay variability of 6% and 7%, respectively, and sFlt-1 intra-assay and interassay variability of 5% and 7%, respectively.

#### Determination of Placental Superoxide

Placental extracts were prepared and protein concentration determined as above. Lysates were incubated with lucigenin at 5-μmol/L final concentration. Samples were allowed to dark equilibrate for 3 minutes before measurement. Luminescence was recorded continuously for 15 minutes by luminometer (Berthold, Oak Ridge, TN). Values from lucigenin-only blanks were subtracted from final numbers. Levels are expressed as relative light units per minute per milligram of total protein in the lysate, with n=5 to 7 in each group.

#### Western Blotting and HO Activity Assays

Determination of total HO activity (n=5 in each group) and tissue levels was performed as described previously.\(^{26}\) Briefly, for HO activity, tissue lysates were prepared and measured as above. Reactions were performed in 1.2 mL consisting of 2 mmol/L of glucose-6-phosphate, 0.2 U of glucose-6-phosphatasedehydrogenase, 0.8 mmol/L of nicotinamide-adenine dinucleotide phosphate, 20.0 μmol/L of hemin, and 0.5 mg of protein from lysates. Incubations were allowed to proceed for 1 hour at 37°C. Resulting bilirubin was chloroform extracted, and concentration was determined by change in optical density at 464 to 530 nm, using an extinction coefficient of 40 mmol/L per centimeter. Activity was expressed as picomoles of bilirubin formed per hour per milligram of protein. Six samples were analyzed in each group.

For Western blots, 30 μg of protein were subjected to SDS-PAGE on 7.5% SDS-polyacrylamide gels (Bio-Rad). Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) for 2 hours at room temperature. Primary incubation was undertaken overnight at 4°C with a rabbit anti HO-1 polyclonal antibody (StressGen, Vancouver, British Columbia, Canada) at 1:2000 and a mouse anti–β-actin antibody (Gentest) at 1:5000. Secondary antibody incubation was done with Alexa Fluor 680 goat antirabbit (Molecular Probes) and IRDye 800 goat antimouse IgG (Rockland) for 1 hour at room temperature. Fluorescence was detected on an Odyssey infrared imager (LI-COR) for simultaneous detection of both species. Blot analysis was performed with the supplied Odyssey software, and HO-1 was normalized to β-actin, with 3 in each group.

#### Detection of Preproendothelin By Quantitative RT-PCR

Preproendothelin mRNA levels were determined by quantitative RT-PCR. Segments of the aorta above the kidneys were harvested from rats and quick frozen immediately in liquid nitrogen. For RNA isolation, the samples were ground in liquid nitrogen, and total RNA was isolated with the RNeasy mini kit (Qiagen) per the manufacturer’s instructions. cDNA was produced from 750 ng of total RNA by Superscript II in concert with oligo dT primers (Invitrogen). Measurements were made from 5 rats in each experimental group. Custom primers (Invitrogen) were used for both ET-1 and β-actin measurements. ET-1 primers used were forward (5′-CTAGGCTTAAGCGATCCCTTG-3′) and reverse (5′-TTC-TGTCTGCTTGCGC-3′). β-Actin primers used were forward (5′-
GCTCGTCGACAACGGCTCCGGC-3′ and reverse (5′-CAAAACAATGATCTGGGTATCTTCTCGCGG-3′). Real-time amplification was carried out with SYBR-Green Supermix (Bio-Rad) with a Bio-Rad iCycler undergoing 40 rounds of amplification. Relative fold change was determined by the 2^ΔΔCt method, with 6 rats in all of the groups.

**Statistical Analysis**
All figures display mean data ± SE. Data underwent Shapiro-Wilk tests for normal distribution, and comparisons between groups were performed by unpaired Student t test with a significance threshold value of P < 0.05. All of the statistical comparisons and graphs were generated with OriginPro 8 (Microcal).

**Results**

**CoPP Induces HO-1 Production in the Liver and Placenta of RUPP-Treated Rats**
To induce HO-1 production, CoPP was administered at 5 mg/kg IP on day 14 of gestation. As indicated by Western blotting in Figure 1A and 1B, on day 19 of gestation, production of HO-1 in the placentas of control animals was unaffected by CoPP. RUPP animals actually demonstrated a significant (P < 0.05) increase in HO-1 production of ~60% compared with control animals. Most dramatically, however, RUPP animals treated with CoPP showed a ~2.4-fold increase in HO-1 production versus normal pregnant controls. These findings were confirmed by analysis of total HO activity in the placenta of the 4 experimental groups. As shown in Figure 1C, despite increased expression of HO-1, total HO activity in the placenta of RUPP-treated rats is relatively unaffected by ischemia. In contrast, levels of HO activity in the placenta (Figure 1C) and liver (Figure 1D) of both control and RUPP rats were increased in response to CoPP treatment compared with normal pregnant rats, although, in the case of placental HO activity in RUPP rats, this failed to meet significance.

**HO-1 Induction Attenuates RUPP-Induced Hypertension in Pregnant Rats**
We next wished to determine whether HO-1 induction was able to attenuate RUPP-induced hypertension. As demonstrated in Figure 2A, when the RUPP procedure is performed on day 14 of gestation, by gestational day 19, mean arterial pressure (MAP) is increased ~29 mm Hg over controls (136 ± 7 versus 106 ± 5 mm Hg; P < 0.05). CoPP administration to control animals had no statistical effect on MAP (104 ± 8 mm Hg). Administration of CoPP to RUPP-treated animals, however, significantly attenuated the increase in MAP, decreasing pressure levels by 18 mm Hg versus RUPP-treated animals (118 ± 5 versus 136 ± 7 mm Hg; P < 0.05). Together, these data indicate that HO-1 induction has a significant ability to attenuate the increase in MAP induced by the RUPP model of placental ischemia/hypoxia. It should be noted, however, that the induction of HO-1 expression had no effect on either pup or placental weight in control animals; it was also unable to significantly attenuate the RUPP-NP

Figure 1. CoPP induces HO-1 production in RUPP rats. Placental HO-1 was measured by Western blot (A), and quantitation was performed by comparison with β-actin levels (B). HO-1 was increased in the placentas of both RUPP and RUPP-treated animals. HO activity was assayed in placental and liver lysates (C and D). CoPP induced increased HO activity in the liver of control and RUPP animals, while significantly increasing activity in the placenta of control animals. Error bars demonstrate SE.
induced decreases in both pup weight and placental weight, as can be seen in Figure 2B and 2C. There were also no differences noted in the number of pups born to RUPP mothers in response to HO-1 induction (data not shown).

**Figure 2.** HO-1 induction attenuates RUPP-induced hypertension but has no significant effect on pup size. **A**, RUPP treatment led to a significant 29-mm Hg increase in MAP, which was significantly attenuated by HO-1 induction. **B** and **C**, HO-1 induction had no significant effect on either pup or placental mass. Error bars demonstrate SE.

**Figure 3.** HO-1 induction normalized placental angiogenic balance in RUPP animals and promotes circulating VEGF. **A**, Placental levels of sFlt-1 and VEGF were determined by ELISA and molar ratio determined. In RUPP animals, sFlt-1/VEGF was increased, indicating an angiostatic phenotype. In response to HO-1 induction, the sFlt-1/VEGF ratio decreased significantly, indicating a net angiogenic balance. **B**, Circulating levels of VEGF were decreased significantly in response to RUPP treatment but were fully restored by induction of HO-1 by CoPP. Error bars demonstrate SE.

**HO-1 Induction Normalizes Placental Angiogenic Balance in RUPP Animals and Increases Circulating Free VEGF**

To investigate the effect of HO-1 induction on RUPP-induced changes in VEGF expression, both plasma and placental extracts were examined by ELISA assay. Both VEGF and sFlt-1 are increased in response to RUPP treatment (23±5 versus 30±6 pg/mg for VEGF and 596±128 versus 997±245 pg/mg for sFlt-1). Although HO-1 induction had no effect on placental VEGF in normal pregnant rats (21±3 pg/mg), in RUPP rats VEGF levels were decreased to control levels (22±7 pg/mg). In contrast, sFlt-1 was decreased in both control (379±109 pg/mg) and RUPP (425±99 pg/mg) rats when HO-1 was induced by CoPP.

As shown in Figure 3A, in response to RUPP treatment, the ratio of placental sFlt-1/VEGF increases ~25% versus controls (1.00±0.10 versus 1.27±0.20; *P*<0.05), indicating a
shift in the balance of these 2 factors to a more angiostatic phenotype. In control rats, the ratio is decreased in rats treated with CoPP (0.68±0.10; P<0.05), indicating a significantly more angiogenic placental balance. Strikingly, in RUPP rats treated with CoPP, the ratio of sFlt-1/VEGF is decreased significantly from RUPP controls (0.79±0.10; P<0.05), indicating a net proangiogenic placental balance versus both normal pregnant and RUPP control rats.

Circulating plasma levels of VEGF were also examined. As can be seen in Figure 3B, RUPP treatment decreased circulating free VEGF levels versus normal pregnant controls (363.1±108.5 and 527.0±85.6 pg/mL, respectively; P<0.05). In response to CoPP induction of HO-1, circulating levels of free VEGF were unaffected in control rats. RUPP-treated rats, however, demonstrated normalization of circulating free VEGF levels in response to HO-1 induction.

**HO Induction Reduces Production of Placental Superoxide in RUPP Rats**

To investigate the effect of HO-1 induction on RUPP-induced superoxide production, placental samples from each control group were homogenized and subjected to lucigenin assays as described previously to measure superoxide levels in the tissue. As seen in Figure 4A, baseline levels of superoxide are increased in placental lysates as a result of RUPP treatment (952.5±278.8 versus 243.9±70.5 relative light units [RLU]/min per milligram; P<0.05). Although, with the induction of HO-1, there is a net increase in placental superoxide in control animals (617.4±225.3 RLU/min per milligram; P<0.05), in HO-1–induced animals, there is a net decrease in superoxide when compared with untreated RUPP animals (482.7±117.4 RLU/min per milligram; P<0.05). When activity of NADPH oxidase is examined in the same tissues by NADPH-dependent lucigenin luminescence, as shown in Figure 4B, NADPH oxidase activity is significantly elevated in RUPP placental samples versus normal pregnant rats (6600.8±2862.9 versus 2021.3±674.5 RLU/min per milligram; P<0.05). Placental NADPH activity is significantly increased in response to CoPP treatment (5525.6±2134.7 RLU/min per milligram; P<0.05), but again, in RUPP rats treated with CoPP, there is a decrease in NADPH activity versus untreated RUPP controls 3652.6±1288.9 RLU/min per milligram; P<0.05).

**HO-1 Induction Reduces Placental Ischemia–Induced Increases in Vascular Preproendothelin**

We next investigated the role of HO-1 induction in mediating vascular ET-1. To that end, we monitored preproendothelin message levels in the thoracic aorta of all experimental groups. As shown in Figure 5, in response to RUPP treatment, preproendothelin mRNA increased significantly in the aorta by about 235% (1.06- versus 3.99-fold of control; P<0.05). With administration of CoPP, however, RUPP treatment only induced an about 160% increase in vascular preproendothelin (1.8- versus 2.9-fold of control; P=0.31), half the increase in control rats, and failed to meet statistical significance. HO-1 induction then attenuated placental ischemia–induced preproendothelin at the message level.

**Discussion**

Currently the treatment options for preeclampsia are extremely limited, namely, induction of labor and delivery of the fetus and placenta. The present study raises interesting prospects for a therapeutic intervention for preeclampsia. Here we have once again demonstrated the importance of placental hypoxia and ischemia to the pathophysiology of preeclampsia. Furthermore, we have demonstrated that administration of CoPP, which has been shown to induce HO-1 expression at least partially through posttranscriptional manipulation of transcriptional repressor Bach1 and stabilization of transcription factor Nrf2,27,28 is able to induce expression and activity of HO-1 both systemically (eg, the liver) and specifically in the placenta of the pregnant rat. Although expression of the protein is clearly increased in RUPP rats treated with CoPP, the increase in activity is only mildly increased. This seeming incongruity is explained by the fact that HO-1 activity is inversely related to PO2, meaning that relatively minor changes in placental HO-1 activity are
powerful VEGF antagonist. Recent evidence has suggested that the ischemic placenta is a possible source of sFlt-1.

Previous in vitro studies have demonstrated that HO-1 induction in an in vivo model is able to induce an angiogenic balance in the RUPP placenta, suggesting one possible mechanism for the attenuation of hypertension in this context. Although technical constraints made accurate measurement of circulating sFlt-1 impossible in the present study, we do demonstrate that levels of circulating free VEGF are increased in RUPP animals treated with CoPP, suggesting the possibility that vascular endothelial function, a recognized factor in the symptomatic development of preeclampsia, might be improved in response to HO-1 induction through an increase in circulating bioavailable VEGF.

A second pathway affected by HO-1 induction in the present study is the presence of superoxide in the ischemic placenta. We reiterate earlier findings that reactive oxygen is increased in the ischemic placenta and demonstrate that HO-1 induction is able to reduce the basal level of placental superoxide. One of the proposed mechanisms by which HO-1 is believed to be cytoprotective and beneficial in hypertension is through the antioxidant properties of bilirubin. Indeed, bilirubin efficiently prevents plasma lipid peroxidation, and it has been shown to convey cardioprotection during reperfusion. Here we have demonstrated that, whereas CoPP in normal pregnant rats mildly increases both placent al superoxide and NADPH oxidase activity, it significantly decreases both superoxide and NADPH oxidase activity in RUPP-treated animals, suggesting the possibility that HO-1–derived bilirubin is functioning as an antioxidant buffer for excessive superoxide production in the RUPP model placenta. Whether the basal increases in superoxide seen here are an adverse effect of the CoPP treatment or HO-1 induction is not clear. Regardless, the net reduction of superoxide seen with HO-1 induction over RUPP control rats clearly demonstrates its overall beneficial effect. This provides a further possible mechanism by which HO-1 might function therapeutically in the treatment of preeclampsia.

We examined the production of vascular preproendothelin in response to HO-1 induction in the RUPP rat. Tissue production of preproendothelin mRNA has been shown to be elevated in the placentas of preeclamptic women. In addition, some studies have demonstrated elevated ET-1 in the maternal circulation during preeclampsia, although this effect was not seen universally. Animal studies looking at the role of tumor necrosis factor-α and sFlt-1, both believed to be derived from the ischemic placenta, have demonstrated induction of preproendothelin-1 in response to increased circulating maternal levels of the 2 factors. In our model of placental ischemia, we have demonstrated an important role for ET-1 in the etiology of placental ischemia–induced hypertension through its interaction with the endothelin A receptor.

Here we have demonstrated that HO-1 induction markedly attenuates the induction of vascular preproendothelin in response to placental ischemia, reducing the induction by approximately half. The precise mechanism by which this occurs is not clear, but as has been demonstrated, CO negatively regulates the preproendothelin message in endothelial cells. Further work is necessary to determine whether CO is responsible for this effect in the in vivo model.

Finally, CoPP treatment in our study did not totally normalize blood pressure in the RUPP rats. The reason for this is not clear but could be because of the dose of CoPP used. Alternatively, it is likely that HO-1 induction does not affect all of the physiological pathways that are responsible for the hypertensive response.
for the increase in blood pressure in response to placental ischemia. The dose that we chose was based on previous studies that indicated it produces maximum blood pressure reduction. Thus, we speculate that the lack of normalization of blood pressure in the RUPP rats is attributed to an inability of HO-1 to affect all of the physiological pathways that are responsible for the increase in blood pressure in response to placent al ischemia. We have reported previously that the blood pressure response to RUPP involves several pathways, including sFlt-1, reactive oxygen species, endothelin, and increases in tumor necrosis factor-α and angiotensin type 1 receptor autoantibodies. In this study we show that HO-1 induction attenuated the sFlt-1, reactive species, and endothelin responses to RUPP. Whether HO-1 induction attenuates the increase in tumor necrosis factor-α and angiotensin type 1 receptor autoantibodies is unknown.

**Perspectives**

Recent insights into the pathophysiology of preeclampsia have yielded insights into new potential therapeutic targets. Here we have demonstrated that HO-1 is capable of ameliorating the hypertension associated with placental ischemia/hypoxia. We further suggest 2 potential pathways through which HO-1 acts in this model, namely, return to normal angiogenic balance in the placenta and reduction in placental levels of oxidative stress. The ability to affect multiple pathological pathways makes HO-1 an intriguing potential approach for the treatment of preeclampsia. Further study into the mechanism of the attenuation of hypertension of HO-1 in this model, in addition to the role of its individual metabolites, CO and bilirubin, should provide an exciting new front in the treatment of preeclampsia.

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**Disclosures**

None.

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