Susceptibility of podocytes to palmitic acid is regulated by fatty acid oxidation and inversely depends on acetyl-CoA carboxylases 1 and 2

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DIABETIC NEPHROPATHY (DN) has become the primary cause of end-stage renal disease (ESRD), and most affected patients have type 2 diabetes (1, 15). Podocyte injury and loss are critical events in the course of DN (31) and precede albuminuria (6, 17, 21). Type 2 diabetes mellitus is characterized by hyperglycemia and dyslipidemia with increased plasma levels of free fatty acids (FFAs) (24). In the kidneys of diabetic humans, intraglomerular lipid deposits were already described in 1936 by Kimmelstiel and Wilson (14). However, the potential role of FFAs and fatty acid metabolism in the pathogenesis of DN is only emerging.

Previously, we reported that podocytes are highly susceptible to the saturated FFA palmitic acid but not to monounsaturated FFAs (MUFAs), such as oleic acid, which attenuate palmitic acid-induced lipotoxicity (27). Mechanistically, palmitic acid-induced podocyte death is linked to endoplasmic reticulum (ER) stress involving the proapoptotic transcription factor C/EBP homologous protein (CHOP) (27). In addition, we reported that the gene expression of key enzymes of fatty acid metabolism is altered in glomeruli of type 2 diabetic patients with DN (29). Specifically, we found that stearoyl-CoA desaturases 1 (SCD-1), the enzyme converting saturated FFAs to MUFAs, is upregulated in podocytes. Functionally, stimulation of Scds by the liver X receptor (LXR) agonists TO901317 (TO) and GW3965 (GW) as well as overexpression of Scd-1 were shown to be protective for palmitic acid-induced podocyte death. Importantly, the previously reported changed glomerular gene expression pattern (29) also suggests disposition for increased fatty acid β-oxidation (FAO) as all three isoforms of carnitine palmitoyltransferase (CPT)-1, the rate-limiting enzyme for FAO, were upregulated and acetyl-CoA carboxylase (ACC) 2, which catalyzes the formation of the CPT-1 inhibitor malonyl-CoA, was downregulated (29).

In humans and rodents, there are two ACC isoforms, ACC1 (ACC α) and ACC2 (ACC β) (25), which share considerable sequence identity and the same domain structure responsible for enzyme activity (25). In contrast to ACC1, ACC2 has an extra N-terminal hydrophobic domain, which facilitates its localization to the mitochondrial membrane (2), where it preferentially regulates local malonyl-CoA levels and CPT-1 activity. In contrast, cytosolic ACC1 is classically thought to regulate malonyl-CoA synthesis for incorporation into fatty acids in lipogenic tissues. However, more recently this classical view has been challenged, and at least in some cell types, e.g., hepatocytes, both isoforms have been shown to regulate CPT-1 activity synergistically (25).

A key regulator of FAO is the low-energy sensor AMP-activated protein kinase (AMPK). Increased levels of AMP lead to AMPK activation, which finally triggers ATP production (10). AMPK directly targets and inactivates ACC by phosphorylation (18). The inactivation of ACC prevents the formation of malonyl-CoA (19) and thereby disinhibits CPT-1.

Importantly, two recent genome-wide association studies in type 2 diabetic patients found a polymorphism in a noncoding region of ACC2 with a strong association with proteinuria (16, 30). The DN-risk single nucleotide polymorphism of ACC2 results in a higher ACC2 expression (16) potentially leading to decreased FAO and accumulation of toxic FFAs and their deleterious metabolites.
The objective of the present study was to investigate the effect of FAO on the susceptibility of podocytes to palmitic acid. Stimulation of FAO by the AMPK agonist aminooimidazole-4-carboxamide-1β-D-ribosunanoside (AICAR) was shown to protect from palmitic acid-induced cell death whereas inhibition of FAO by the CPT-1 inhibitor etomoxir enhanced the toxicity of palmitic acid. In addition, the functional role of the AMPK-ACC-CPT-1 pathway was assessed by gene silencing of ACC1 and ACC2.

MATERIALS AND METHODS

Materials. Palmitic acid (P9767), fatty acid free-BSA (A8806), etomoxir (E1905), compound C (P5499), and β-actin antibody (A5441) were purchased from Sigma (St. Louis, MO). Recombinant murine IFN-γ (CTK-358-2PS) was from MoBiTec (Goettingen, Germany). Type 1 collagen was from BD Biosciences. Annexin V (A23204) and propidium iodide (PI; P3566) were from Invitrogen (Eugene, OR). AICAR (no. 9944) and pAMPK (no. 2531), AMPK (no. 2532), pACC (no. 3661), ACC (no. 3576), and immunoglobulin heavy chain binding protein (BiP; no. 3183) antibodies were from Cell Signaling Technology. CHOP (sc-7351) antibody was from Santa Cruz Biotechnology (Dallas, TX). Adiponectin (no. 4902-100) was purchased from BioVision (Milpitas, CA). The horseradish peroxidase-conjugated secondary antibodies for rabbit and mouse were from Dako. Tritium-labeled palmitic acid (NET043001MC) was from PerkinElmer (Schwerzenbach, Switzerland).

Cell culture, free fatty acid preparation, and apoptosis assay. Murine podocytes were cultured as described before (27). Podocytes were differentiated for at least 11 days before the start of experiments. All experiments were carried out in six-well plates except for isolating protein or RNA for which 10-cm dishes were used. Free fatty acid preparations were done as described previously (27). The palmitic acid concentration used in this study was 200 μM complexed to BSA (0.2%), which is within the reported physiological range of 120–340 μM (7–9). Endotoxin concentration was equal or less than 0.5 ng/ml, as determined by a kit (no. L00350) from Genscript (Piscataway, NJ). Annexin V and PI stainings were performed as reported earlier (27). Flow cytometry was carried out with a CyAn ADP Analyzer (Beckman Coulter), and 20,000 cells were counted. Data from flow cytometry were analyzed by the FLOWJO (Tree Star, Ashland, OR) software program. Annexin V-positive/PI-negative podocytes were considered apoptotic, whereas annexin V-positive/PI-positive podocytes were considered (late apoptotic) necrotic cells (27).

Plasmids, RNA-mediated interference, and viruses. ACC1 and ACC2 genes were silenced by employing the following short hairpin (sh) RNA sequences (12): ACC1, 5’-GCAGATTGCCAACATC-3’ and ACC2, 5’-GTGGTGACGGGACGAGCAA-3’.

![Diagram](https://example.com/diagram.png)

Fig. 1. Aminoimidazole-4-carboxamide-1β-D-ribosunanoside (AICAR) and adiponectin protect podocytes from palmitic acid-induced cell death. A: metabolic pathway activated by AICAR and adiponectin, which results in stimulation of fatty acid oxidation. CPT-1, carnitine palmitoyltransferase. B: immunoblot showing phosphorylation of AMPK and acetyl-CoA carboxylase (ACC) after incubation of podocytes with either (PBS) vehicle or 0.5 mM AICAR for 14 h. Total AMPK and total ACC served as loading controls. C: AICAR attenuated palmitic acid-induced cell death. Values are mean percentages ± SD of apoptotic and necrotic cells after 48 h (n = 3, *P < 0.05, **P < 0.01). D: 0.5 μg/ml adiponectin decreased palmitic acid-induced apoptosis and necrosis of podocytes. NS, not significant. Values are mean percentages ± SD of apoptotic and necrotic cells after 48 h (n = 3, *P < 0.05).
21-nt scrambled sequence (5'-GACCAGCGACTGCAGCGTCG-3') (29) served as a control. ACC1, ACC2, and scrambled shRNA sequences were cloned into a pSH-I lentiviral expression plasmid. A four-plasmid lentiviral system was used with following helper plasmids: pRSV-REV (Rev expression vector), pMDLg/pRRE (Gag-Pol expression vector), and pMD2.G (VSV-G expression vector). All four plasmids were transfected to HEK cells via the FuGene HD (Promega, Madison, WI) transfection agent, and the medium was changed after 12 h. Forty-eight hours posttransfection, the supernatant enriched with lentiviral particles was harvested, spun at 780 g for 5 min, and filtered through 0.45-µm filter. Transduction of podocytes was done by pretreating podocytes with 5 µg/ml polybrene (Sigma). All functional experiments were started after 4 days of transduction.

Western blotting. Western blotting was done as described before (27). Antibodies against pAMPK, AMPK, pACC, ACC, CHOP, BiP, and β-actin were employed at 1:1,600 and 1:4,000 dilutions, respectively. Secondary antibodies for rabbit and mouse were employed at 1:500, and 1:100,000 dilutions, respectively. Secondary antibodies for rabbit and mouse were employed at 1:600 and 1:4,000 dilutions, respectively.

Measuring oxidation of palmitic acid (β-oxidation). For these experiments, podocytes were pretreated with AICAR for 1 h. Podocytes were incubated with 200 µM palmitic acid in a serum starvation medium (0.2% FBS, 5 mM glucose) supplemented with 0.5% FFA-free BSA along with 0.5 µCi/ml 3H-palmitic acid. Supernatants were collected followed by chloroform/methanol/5 N HCl (2:1:0.05, vol/vol) extraction. Four hundred microliters of the aqueous phase (containing 3H2O) was added to 2 ml of scintillation buffer before measurement of radioactivity.

RESULTS

**AICAR protects from palmitic acid-induced podocyte death.** To investigate whether stimulation of FAO plays a protective role in palmitic acid-treated podocytes, we took advantage of the AMPK activator AICAR. AICAR acts by phosphorylating AMPK, which in turn phosphorylates and inhibits ACC, resulting in disinhibition of CPT-1 (see Fig. 1A). Phosphorylation of AMPK and ACC by AICAR in podocytes was examined by Western immunoblotting (Fig. 1B). As shown in Fig. 1C, AICAR significantly prevented palmitic acid-induced podocyte death assessed by flow cytometry after staining for annexin V and PI. Specifically, AICAR reduced palmitic acid-induced apoptosis (annexin V single-positive cells) and necrosis (annexin V/PI double-positive cells) by 50.5 ± 1.5 (P < 0.01) and 42.5 ± 6.1% (P < 0.05), respectively. Similarly, the physiological AMPK agonist adiponectin (26) also reduced palmitic acid-induced podocyte death, although to a lesser extent. AICAR significantly prevented palmitic acid-induced podocyte death assessed by flow cytometry after staining for annexin V and PI. Specifically, AICAR reduced palmitic acid-induced apoptosis (annexin V single-positive cells) and necrosis (annexin V/PI double-positive cells) by 50.5 ± 1.5 (P < 0.01) and 42.5 ± 6.1% (P < 0.05), respectively. Similarly, the physiological AMPK agonist adiponectin (26) also reduced palmitic acid-induced podocyte death, although to a lesser extent.
extent than AICAR. Figure 1D shows that adiponectin decreased both apoptosis and necrosis by 14.1 ± 4.7 (P < 0.05) and 9.9 ± 6.3% (NS), respectively. To see the protective effect of adiponectin, podocytes were kept at a high glucose concentration of 22 mmol/l, which is known to reduce phosphorylation of AMPK (26) and increase the susceptibility of podocytes to AMPK activation (26).

**Compound C aggravates palmitic acid-induced podocyte death and partially reverses the protective AICAR effect.** To further explore the role of AMPK we used the AMPK inhibitor compound C. Compound C was used at a low concentration of 4 μM, and incubation time was limited to 24 h maximally, as higher concentrations and longer exposure times were toxic; i.e., podocyte death was markedly increased for BSA control (data not shown). The AICAR-induced ACC phosphorylation was significantly reduced by compound C (Fig. 2, A and B, P < 0.05). Compound C increased palmitic acid-induced apoptosis by 140.1 ± 20.1% (P < 0.01) and necrosis by 130.9 ± 14.0% (P < 0.01) (Fig. 2C). In agreement with the partial reduction of the AICAR-induced ACC phosphorylation, compound C compared with AICAR alone only moderately increased palmitic acid-induced podocyte death; i.e., apoptosis was increase by 128.2 ± 9.3 (NS) and necrosis by 176.7 ± 9.7% (P < 0.01), respectively.

**Etomoxir aggravates palmitic acid-induced podocyte death and reverses the protective AICAR effect.** To further investigate the impact of FAO on palmitic acid-induced podocyte death, we made use of the CPT-1 inhibitor etomoxir (Fig. 3A). Etomoxir exacerbated palmitic acid-induced podocyte death (Fig. 3B). Specifically, apoptosis was increased by 184.3 ± 6.0 (P < 0.01) and necrosis by 185.1 ± 16.3% (P < 0.01), respectively. Moreover, etomoxir reversed the protective effect of AICAR (Fig. 3C). Of note, this effect could already be seen at a very low etomoxir concentration (10 μM), which by itself had no significant effect on palmitic acid-induced podocyte death (data not shown). Compared with podocytes treated with AICAR alone, the presence of 10 μM etomoxir increased palmitic acid-mediated apoptosis by 131.1 ± 5.0 (P < 0.05) and necrosis by 127.3 ± 10.7% (P < 0.05), respectively. At 200 μM, etomoxir completely reversed the protective effect of AICAR (Fig. 3C). The experiments using AICAR, compound C, and etomoxir suggest an important role of the AMPK-ACC-CPT-1 pathway for regulating the susceptibility of podocytes exposed to palmitic acid.

**AICAR increases and etomoxir inhibits oxidation of palmitic acid in podocytes.** To directly measure the effect of AICAR on palmitic acid oxidation, we treated podocytes with 200 μM palmitic acid along with 0.5 μCi/ml tritiated palmitic acid in

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**Fig. 3.** Etomoxir exacerbates palmitic acid-induced podocyte death and reverses the protective effect of AICAR. A: mechanism of action of etomoxir, which inhibits CPT-1 and thereby fatty acid oxidation. B: etomoxir aggravated palmitic acid-induced podocyte death after 48 h. Values are mean percentages ± SD of apoptotic and necrotic cells (n = 3, **P < 0.01). C: etomoxir reversed the protection of AICAR in palmitic acid-induced podocyte death. Values are mean percentages ± SD of apoptotic and necrotic cells after 48 h (n = 3, *P < 0.05, **P < 0.01).
the absence or presence of AICAR. As a direct readout of palmitic acid β-oxidation, we measured tritiated water released in the supernatants of podocytes. As expected, the release of tritiated water gradually increased from 1 to 3 h (Fig. 4A). The stimulation of podocytes with AICAR significantly increased the formation of tritiated water (146.6 ± 22.0%, P < 0.05, Fig. 4A), reflecting the stimulatory effect of AICAR on palmitic acid oxidation. Importantly, and as shown in Fig. 4B, the effect of AICAR could be completely prevented by etomoxir.

AICAR significantly reduces ER stress and upregulation of the proapoptotic transcription factor CHOP. As palmitic acid-induced podocyte death involves ER stress and as CHOP gene silencing attenuates palmitic acid-induced podocyte death (27), we next investigated the effect of AICAR on the ER chaperone BiP and the proapoptotic transcription factor CHOP. AICAR strongly suppressed the upregulation of BiP and CHOP (Fig. 5, A and B).

Combined gene silencing of ACC1 and ACC2 protects from palmitic acid-induced podocyte death. Two recent genome-wide association studies (16, 30) found a single nucleotide polymorphism in ACC2, leading to increased ACC2 expression (16), to be associated with proteinuria in type 2 diabetic patients. To investigate further the role of both ACC isoforms in podocytes, we generated cells deficient in ACC1, ACC2, or both by lentiviral infection using specific shRNAs. Knockdown of ACC1 but not ACC2 strongly reduced the band corresponding to both isoforms. The residual band seen in ACC1 single-knockdown podocytes was almost completely gone in ACC1/ACC2 double-knockdown cells. (Fig. 6A). These data suggest that the expression level of ACC1 is much higher than ACC2 in podocytes. Functionally, only double knockdown of both isoforms significantly reduced palmitic acid-induced podocyte death. Specifically, in ACC1/ACC2 double-knockdown podocytes, palmitic acid-induced apoptosis and necrosis were reduced by 57.4 ± 3.9 (P < 0.01) and 72.1 ± 7.5% (P < 0.05), respectively, compared with podocytes transfected with scrambled shRNA (Fig. 6B), whereas single knockdown of ACC1 or ACC2 had no significant effect (Fig. 6C). These results are in complete agreement with results in hepatocytes, which have shown that both ACC isoforms regulate FAO (25). Of note, a residual protective effect of AICAR was seen in ACC1/ACC2 double-silenced podocytes (Fig. 6B).

DISCUSSION

The present study uncovered that regulation of FAO critically determines the susceptibility of podocytes exposed to palmitic acid. Our findings are of clinical interest, and they relevantly amend recent clinical and experimental studies indicating a potentially important role of FFAs and FFA metabolism in the pathogenesis of DN.

Several lines of evidence indicate that regulation of FAO and interference with the AMPK-ACC-CPT-1 pathway affects podocytes exposed to palmitic acid. Specifically, the AMPK agonist AICAR, which significantly stimulates FAO in podocytes, reduces palmitic acid-induced podocyte death. Conversely, the AMPK inhibitor compound C increased palmitic acid-induced cell death. Furthermore, the CPT-1 inhibitor etomoxir, which completely prevents the AICAR-induced increase in FAO in podocytes, potentiates the toxicity of palmitic acid and dose dependently reverses the protective effect of AICAR. Moreover, gene silencing of ACC1/ACC2 markedly reduced palmitic acid-induced cell death.

Adiponectin, a physiological activator of AMPK in podocytes (26), also reduced palmitic acid-induced podocyte death. Although its protective effect was relatively small compared with pharmacological activation by AICAR, the sustained action of adiponectin in vivo may still be relevant for the protection of podocytes from lipotoxicity. Activation of AMPK by adiponectin or AICAR is also reported to suppress oxidative stress and the NADPH oxidase Nox4 (26). As neither tempol, a membrane-permeable radical scavenger, nor the antioxidant N-acetylcysteine reduce palmitic acid-induced podocyte death (unpublished observations, Sieber J.), the modulation of oxidative stress through the AMPK pathway related to lipotoxicity needs further investigation.

To further address the role of AMPK in palmitic acid-induced podocyte death, we additionally used the AMPK inhibitor compound C, which increased the toxicity of palmitic acid. In addition, compound C reduced the AICAR-induced phosphorylation of ACC and partially prevented the protective AICAR effect. Together, these findings suggest that the susceptibility of podocytes exposed to palmitic acid can be greatly modulated by AMPK.

The present results indicating an important role of FAO and the AMPK-ACC-CPT-1 pathway in the susceptibility of podocytes exposed to toxic FFAs extend and potentially explain the
results of recent genome-wide associations studies which found a SNP in ACC2 with significant enhancer activity, resulting in increased ACC2 expression associated with proteinuria in type 2 diabetic patients (16, 30). Moreover, the results of this study suggest that the recently published observation of decreased expression of ACC2 and increased expression of all CPT-1 isoforms in glomerular extracts of type 2 diabetic patients (29) reflects an adaptive, protective mechanism against toxic FFAs in DN.

The differential role of ACC1 and ACC2 in the regulation of FAO is under debate (20). We found that only double knockdown of ACC1 and ACC2 has a protective effect on palmitic acid-induced cell death. This indicates that both isoforms contribute to the inhibition of CPT-1 in podocytes, as previously suggested for hepatocytes and skeletal muscle cells (20, 25).

Interestingly, AICAR showed a small residual protective effect in ACC1/ACC2 double-knockdown podocytes. This may be due to residual expression of ACC isoforms or an additional ACC-independent effect. Activation of AMPK stimulates peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α (11), for example, which has been shown to be important for mitochondrial function in podocytes (32). Finally, AMPK-independent off-target effects of AICAR cannot be excluded. Future studies are needed to confirm or refute this hypothesis.

The biguanide metformin is widely used to treat type 2 diabetes (23). Its mechanism of action is not fully established but is reported to involve indirect activation of the AMPK-ACC-CPT-1 pathway via inhibition of complex I of the respiratory chain and a consequent increase in the AMP:ATP ratio, which results in AMPK activation (33). Despite this potential mode of action, preliminary experiments showed that metformin from 0.5 to 2 mM displays no protection from palmitic acid-induced lipotoxicity in podocytes (data not shown). Previously, undesired effects of metformin leading to cell death have been reported for pancreatic β cells (13). Of interest, a potential beneficial effect of metformin was shown in podocytes exposed to a high glucose concentration of 30 mM by decreasing ROS production through reduction of NAD(P)H oxidase activity (22). Clearly, more studies are required to reassess the short- and long-term effects of metformin on podocytes.

Interestingly, AICAR significantly reduces the induction of CHOP in podocytes exposed to palmitic acid, which likely contributes to the protective AICAR effect, as gene silencing

![Fig. 5. AICAR mitigates palmitic acid-induced ER stress. A: AICAR attenuated palmitic acid-induced induction of C/EBP homologous protein (CHOP) and immunoglobulin heavy chain binding protein (BiP) after 24 h. CHOP and BiP levels were analyzed by Western immunoblotting. β-Actin served as a loading control. B: quantification of CHOP and BiP levels. BSA-treated controls were set to 100%. Values are mean expression levels ± SD (n = 3, **P < 0.01).](http://ajprenal.physiology.org/)

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of CHOP attenuates palmitic acid-induced cell death (27). The action of AICAR on the AMPK-ACC-CPT-1 pathway may indicate that increased FAO reduces palmitic acid-derived toxic metabolites and therefore suppresses the induction of ER stress. However, the basic unanswered question is how palmitic acid and its metabolites trigger ER stress. Some reports indicated that palmitic acid rapidly increases the saturated lipid content of the ER, leading to compromised ER morphology and integrity (4). In pancreatic β cells, palmitic acid depletes ER Ca\(^{2+}\) and slows ER Ca\(^{2+}\) uptake (5), which leads to accumulation of unfolded proteins. However, the detailed molecular mechanisms are not well known (3). Clearly, more experiments are needed to understand these principle mechanisms and to study whether FAO and ER stress are causatively related.

In conclusion, our results suggest that modulation of FFA metabolism and stimulation of FAO by activation of the AMPK-ACC-CPT-1 pathway critically influence the susceptibility of podocytes exposed to toxic FFAs. Together with our previous studies, the following working model is suggested (Fig. 7). Palmitic acid increases the generation of toxic metabolites, which leads to ER stress and podocyte death (27). Oleic acid or induction of Scd-1/-2 expression by the LXR-agonists TO and GW shift palmitic acid and its metabolites into a “safe lipid pool” containing triglycerides (TG), which reduces injurious metabolites and prevents podocyte death (29). In addition, activation of the AMPK-ACC-CPT-1 pathway by AICAR reduces palmitic acid-induced podocyte death by increasing FAO and reducing palmitic acid and its metabolites. Our findings may explain the results of recent genome-wide association studies indicating that modulation of FFA metabolism and specifically modulation of FAO directly affects the susceptibility to DN. In addition, our results have potentially important therapeutic implications for the prevention and treatment of DN in type 2 diabetic patients.

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**Fig. 6.** ACC1 or ACC2 single knockdown is not protective, whereas combined silencing of ACC1 and ACC2 protects from palmitic acid-induced podocyte death. A: ACC1, ACC2, or both were knocked down, and an immunoblot was done with an antibody recognizing both isoforms. β-Actin served as a loading control. B: combined knockdown of ACC1 and ACC2 protected podocytes from palmitic acid-induced cell death. Values are mean percentages ± SD of apoptotic or necrotic cells (n = 3, *P < 0.05, **P < 0.01). AICAR modestly further decreased podocyte death in ACC1/2 double-knockdown cells (***P < 0.01). C: silencing of either ACC1 or ACC2 was not protective for palmitic acid-induced cell death. Values are show mean percentages ± SD of apoptotic or necrotic cells (n = 3).
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.K., J.S., and A.W.J. provided conception and design of research; K.K. performed experiments; K.K., J.S., and A.W.J. analyzed data; K.K., J.S., and A.W.J. interpreted results of experiments; K.K. prepared figures; K.K., J.S., J.M.O., P.M., and A.W.J. prepared manuscript; K.K., J.S., J.M.O., P.M., and A.W.J. approved final version of manuscript; J.S., J.M.O., P.M., and A.W.J. edited and revised manuscript.

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