Inhibition of ornithine decarboxylase restores hypoxic pulmonary vasoconstriction in endotoxemic mice

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Abstract
Endotoxemia impairs hypoxic pulmonary vasoconstriction which leads to systemic hypoxemia. This derogation is attributable to increased activity of nitric oxide synthase 2 and arginase metabolism. Gene expression analysis has shown increased expression of ornithine decarboxylase in lungs of endotoxemic mice, a downstream enzyme of arginase metabolism. The aim of this study was to investigate whether inhibition of ornithine decarboxylase increases hypoxic pulmonary vasoconstriction in lungs of endotoxemic mice. Mice received lipopolysaccharides or saline intraperitoneal, and hypoxic pulmonary vasoconstriction was measured using an isolated perfused mouse lung model. Additional mice with and without endotoxemia were pretreated with the ornithine decarboxylase-inhibitor difluoromethylornithine before examination of hypoxic pulmonary vasoconstriction. Hypoxic pulmonary vasoconstriction was defined as the difference of pulmonary arterial pressure between normoxic and hypoxic ventilation. In addition, lung tissue was analyzed using real-time quantitative polymerase chain reaction, Western blot and immunohistochemistry. Lipopolysaccharides caused an up-regulation of ornithine decarboxylase mRNA level (2.73 ± 0.19-fold increase, p < 0.05) as well as ornithine decarboxylase protein level (4.05 ± 0.37-fold increase, p < 0.05). Endotoxemia attenuated hypoxic pulmonary vasoconstriction when compared with untreated control mice (26.3 ± 9.7% vs. 67.0 ± 17.5%). Difluoromethylornithine (20, 100, 500 mg kg⁻¹ body weight intraperitoneal) restored hypoxic pulmonary vasoconstriction in lungs of endotoxemic mice in a dose-dependent way (25.8 ± 9.9%, 57.3 ± 17.2%, 62.3 ± 12.4%) and decreased hypoxic pulmonary vasoconstriction in control mice (53.6 ± 13.6%, 40.0 ± 14.9%, 35.9 ± 12.4%). These results show that endotoxemia induces ornithine decarboxylase expression and suggest that ornithine decarboxylase contributes to the endotoxemia-induced impairment of hypoxic pulmonary vasoconstriction. Inhibition of ornithine decarboxylase might be a target in the therapy of diseases with inflammation impaired hypoxic pulmonary vasoconstriction, like the sepsis-associated acute respiratory distress syndrome (ARDS).

Keywords
sepsis, mouse, hypoxic pulmonary vasoconstriction, ornithine decarboxylase

Although investigated for more than half a century, the mechanism of hypoxic pulmonary vasoconstriction (HPV) remains incompletely understood.¹–³ In contrast to the systemic vasculature, where hypoxemia leads to arterial vaso-dilatation, pulmonary vessels contract in response to low regional oxygen levels.⁴ This physiological reflex distributes pulmonary capillary blood flow to alveolar areas of high oxygen partial pressure and preserves systemic

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Impairment of HPV causes a mismatch of ventilation and perfusion, leading to intrapulmonary shunting and systemic hypoxemia, like in sepsis-associated acute respiratory distress syndrome (ARDS), for example.6,7 Experimental endotoxemia impairs HPV,8–10 which may be caused by increased production of nitric oxide (NO) via nitric oxide synthase 2 (NOS2) during endotoxemia. NOS2 knock out mice show a persisted HPV after endotoxin challenge,1 but acute nonspecific inhibition of endogenous NO synthesis by the administration of Nω-nitro-L-arginine methyl ester (L-NAME) improves – however, it does not restore – HPV.11 Petersen et al. recently demonstrated that arginase contributes to the endotoxin-induced impairment on HPV in mice.2 Like arginase, gene expression of ornithine decarboxylase (ODC) was increased in endotoxemic wild-type mice, while endotoxemic NOS2 knock out mice showed no increase of ODC (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130936). Arginase metabolizes L-arginine to urea and L-ornithine. L-ornithine, in turn, is decarboxylated by ODC to putrescine and CO2, representing the first step of polyamine synthesis.12

Impaired HPV during endotoxemia and simultaneous increase of ODC gene expression led us to the hypothesis that inhibition of ODC using difluoromethylornithine (DFMO) would result in enhanced HPV in lungs of endotoxemic mice. We confirmed that ODC expression is increased in endotoxemic mouse lungs and observed a dose-dependent increase of HPV after inhibition of ODC in lungs of endotoxemic mice.

**Methods**

**Animal welfare**

Handling of the animals was in accordance with the European Community guidelines. In total, 90 male adult C57BL/6 mice (body weight 21.3 ± 1.3 g) were obtained from Charles River, Sulzfeld, Germany.

**Experimental groups**

Eight to ten weeks old male C57BL/6 mice received an intraperitoneal (i.p.) injection of endotoxin (lipopolysaccharides (LPS) group, n = 10; 45 mg kg⁻¹ body weight LPS from *Escherichia coli* 0111:B4, Sigma-Aldrich, St. Louis, MO, USA) 18 h before isolated lung perfusion experiments. Mice receiving a bolus of the same amount of 0.9% sodium chloride (n = 14) served as control. Mice in the treatment group with and without LPS received additional DFMO (Calbiochem, San Diego, CA, USA) in doses of 20, 100 and 500 mg kg⁻¹ body weight i.p. (n = 7, respectively) 1 h before isolation of the lung and HPV measurement. For analyses of gene- and protein expression as well as immunoenzymatic staining, extra animals received an i.p. injection of endotoxin (n = 9) or physiological saline (n = 9).

**Isolated perfused and ventilated mouse lung model**

Mice were sacrificed by an i.p. injection of pentobarbital sodium (300 mg kg⁻¹ body weight; Narcoren, Merial, Hallbergmoos, Germany), and lungs were explanted and buffer perfused as previously described.11 The perfusate was Hanks’ Balanced Salt Solution (Gibco Life Technologies, Paisley, UK), with addition of bovine serum albumin (5%; Serva Electrophoresis, Heidelberg, Germany) and dextran (5%; Fluka BioChemika, Sigma-Aldrich) to prevent pulmonary edema as previously described.11 Indomethacin (30 μM; Sigma-Aldrich) and 1 mM of the nonselective NO synthase inhibitor L-NAME (Sigma-Aldrich) were added to the perfusate to inhibit an endogenous prostaglandin synthesis and NO synthesis, respectively. Sodium bicarbonate was added to adjust the perfusate pH (7.34–7.43). Perfusate flow was measured using an in-line probe and flowmeter (Transonic Systems, Ithaca, NY, USA). Lungs only were included in this study if they had a homogeneous white appearance without signs of hemostasis or atelectasis and showed a stable pulmonary arterial pressure (PAP) of less than 10 mmHg during the second 5 min of an initial 10 min baseline perfusion period. Using these two criteria, a total of six lung preparations (approximately 6%) were discarded before study. PAP and left atrial pressure (LAP) were measured using saline-filled membrane pressure transducers (Medex Medical, Klein-Winterheim, Germany) connected to a side port of the cannulae placed in pulmonary artery (inflow) and left atrium via mitral valve (outflow). Pressure transducers were connected to a biomedical amplifier (Transbridge TBM 4M, World Precision Instruments, Berlin, Germany), and data were recorded at 150 Hz on a personal computer using an analogue-to-digital interface with a data acquisition system (DI-220, Dataq Instruments, Akron, OH, USA). The system was calibrated before each experiment. Lungs were ventilated with a constant tidal volume of 10 ml kg⁻¹ body weight and a breathing rate of 100 per min while a positive end-expiratory pressure was set at 2 cm H₂O to avoid atelectasis. LAP was maintained at 2 mmHg during the whole experiment. HPV was quantified as the difference of basal PAP (FiO₂ = 0.21) and PAP at the end of a 6-min period of ventilation with an inspiratory oxygen concentration of 0.01 in percent of the baseline PAP.

**Lung wet-to-dry weight ratio**

At the end of the experiments, both lungs of the studied animals, excluding their hilar structures, were excised and immediately weighed. Thereafter, lungs were dried in an oven at 100°C overnight and then reweighed.
Lung wet-to-dry weight ratios were calculated by dividing the wet weight by the dry weight as previously described.  

**Semi-quantitative real time polymerase chain reaction (RT-PCR)**

Mice were sacrificed with a lethal i.p. injection of pentobarbital sodium (300 mg kg\(^{-1}\) body weight) after 18 h of LPS exposure, and saline injected mice served as controls (n = 9, respectively). Lungs were perfused with iced physiological saline for 1 min at 50 ml kg\(^{-1}\) min\(^{-1}\) flow, dissected (excluding hilar structures), quick frozen and stored at −80°C. RNA was isolated from murine lungs using TRIzol™ reagent (Invitrogen, Karlsruhe, Germany) and complementary DNA was generated with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany). Quantitative real time polymerase chain reaction (RT-PCR) was performed with a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Munich, Germany) using specific primers for 18S (forward: TCAAGAACGAAGTCCGGAGG, reverse: GGCACTCTAGGGCATCA) and ODC (forward: GACGATTTTGACGCCACATC, reverse: CGCACAATAGAAGGCTCCTT) as well as the iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Munich, Germany). Postamplification dissociation curves were performed to verify the presence of a single amplification product in the absence of DNA contamination. Changes of expression of the gene of interest were determined using the ΔΔCt method with normalization to 18S ribosomal RNA.

**Immunoblotting**

Western blots were performed to assess protein levels of ODC as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In brief, lungs of mice with and without endotoxemia (n = 9 each) were homogenized at 4°C in Radio Immunno Precipitation Assay (RIPA) buffer (50 mmol l\(^{-1}\) Tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl) pH 7.5, 150 mmol l\(^{-1}\) sodium chloride, 1% Triton X-100, 0.1% SDS and cOmplete™ Mini Protease Inhibitor Mix, Roche Diagnostics, Mannheim, Germany) and centrifuged at 10,000g and 4°C for 10 min. Supernatant protein was subjected to electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated overnight with monoclonal Anti-ODC mouse IgG2b-isotype (1:1000; O1136, Clone ODC-29, Sigma-Aldrich) and Anti-GAPDH Antibody (1:10,000; MAB374, Merck Millipore, Darmstadt, Germany) and thereafter incubated with the secondary fluorescent antibodies (IRDye, 1:10,000; 926-68072, LI-COR, Lincoln, NE, USA). Proteins were visualized with an LI-COR infrared imager (Odyssey, LI-COR, Lincoln, NE, USA), quantitative densitometric analysis was performed by applying Odyssey version 1.2 infrared imaging software, and signals were normalized to GAPDH.

**Immunohistochemistry**

Lungs of mice with and without endotoxemia were fixed in 4% paraformaldehyde (n = 4 each group). Immunoenzyme stainings were performed on 2 μm paraffin-embedded sections using standard avidin-biotin anti-alkaline phosphatase technique (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. Tris-buffered saline supplemented with 0.2% bovine serum albumin (Biotrend, Cologne, Germany) was used as buffer. Primary antibody dilutions of polyclonal rabbit ODC (1:50; O1136, Sigma-Aldrich) and an isotype- and concentration-matched rabbit control Ig (1:20, X 0944, Dako, Glostrup, Denmark) were prepared in this buffer and incubated for 1 h at room temperature. A biotinylated donkey anti-rabbit IgG antibody (1:100; RPN1001, Amersham™, GE Healthcare, UK) was used as a secondary reagent (30 min at room temperature). Naphthol AS-BI-phosphate (Sigma-Aldrich) with New-fuchsin (Merck, Darmstadt, Germany) was used as the substrate for alkaline phosphatase. Slides were counterstained with hematoxylin (Sigma-Aldrich). Specimens were observed with an Olympus BX63 microscope (Olympus Scientific Solutions, Waltham, MA, USA) and analyzed with Olympus cellSens Dimension 1.17.

**Statistical analysis**

All data are expressed in mean±standard deviation if not mentioned otherwise. Normality distribution of data was checked using the Kolmogorov–Smirnov test with Lilliefors correction. To compare groups, a two-way Analysis of variance (ANOVA) was performed and when significant differences were detected, a Bonferroni post hoc least significant difference test for planned comparisons was used. For not normally distributed data, a Kruskal–Wallis ANOVA on ranks with a post-hoc Turkey test was used (IBM SPSS Statistics 24, International Business Corporation, Armonk, NY, USA). Statistical significance was assumed at a p-value of less than 0.05.

**Results**

Eighteen hours after i.p. injection of LPS (45 mg ml\(^{-1}\)), endotoxemic mice showed lethargy, piloerection and squaleness. Lung weight wet-to-dry ratios did not differ between the experimental groups (control 4.10±0.38 vs. LPS 4.25±0.68).

**Up-regulation of ODC mRNA and ODC protein expression**

Increased expression of ODC was measured in a gene array comparing lungs of endotoxemic mice with controls (https://...
To confirm these results on RNA and protein level, lungs of mice that received an i.p. injection of LPS or saline were explanted after 18 h and studied using RT-PCR and immunoblotting. Endotoxin challenge caused a 2.73±0.19-fold increase of ODC mRNA expression (p < 0.05) (Fig. 1a) and a 4.05±0.37-fold increase of ODC protein expression after 18 h compared to saline challenge (p < 0.05) (Fig. 1b). These results confirm that endotoxin provokes an induction of ODC gene and protein expression in mouse lungs.

In order to address the question which cell types undergo upregulation of ODC during endotoxemia, immunoenzyme stainings were performed. Fig. 2 shows four representative images of lungs with (b) and without endotoxemia (a) with (upper images) and without ODC immunoenzyme staining (lower images). Magenta positive area (corresponding to ODC positive staining) in the region of interest in controls was found predominantly in the bronchial epithelium (10.6±2.6%), less in the pulmonary arteriolar wall (1.9±0.6%) and connective tissue (0.5±0.6%).

**Fig. 1.** Endotoxemia increases ODC mRNA and protein expression in murine lungs. (a) RNA was extracted from whole mouse lungs of mice with and without endotoxemia. mRNA: messenger RNA; rRNA: ribosomal RNA; representative QPCR, n = 5, means ± SD, *p < 0.05 vs. control, control normalized on 1. (b) Immunoblots were prepared from extracts of whole mouse lungs with and without endotoxemia. Blots were reacted with antibodies directed against ODC and GAPDH. Endotoxemia led to increased expression of an approximately 56-kDa ODC-immunoreactive protein, while GAPDH expression was unchanged. Densitometric measurement of the ODC immunoreactive protein levels is shown. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; representative immunoblot, n = 3, means ± SD, *p < 0.05 vs. control, control normalized on 1.

**Fig. 2.** Endotoxemia-induced elevation of ODC expression was attributable to pulmonary arteriolar wall and bronchial epithelium. Immunoenzyme stainings were performed on 2 μm paraffin-embedded sections using polyclonal rabbit ODC antibody 1:50. Four representative images: (a) Lungs of control mice. Upper image with ODC antibody, lower image without ODC antibody. (b) Lungs of endotoxemic mice. Upper image with ODC antibody, lower image without ODC antibody.
Endotoxemic lung preparations showed an increase of ODC positive staining in the pulmonary arterial wall compared to controls (LPS vs. control: 11.8 ± 6.8% vs. 1.9 ± 0.6%, control normalized to 1: 6.36 ± 3.67% vs. 1.0 ± 0.31%, p < 0.05) and in the bronchial epithelium (LPS vs. control: 29.0 ± 9.1% vs. 10.6 ± 2.6%, control normalized to 1: 2.74 ± 0.86 vs. 1.0 ± 0.25, p < 0.05). Endotoxin did not change ODC expression in connective tissue (LPS vs. control: 1.1 ± 0.8% vs. 0.5 ± 0.6%, control normalized to 1: 2.33 ± 1.58 vs. 1.0 ± 1.22, no difference).

Taken together, endotoxiaemia-induced elevation of ODC expression was attributable to pulmonary arteriolar wall and bronchial epithelium.

**Attenuation of HPV after LPS injection**

Murine lungs were examined using an isolated perfused lung model to measure the influence of LPS-induced endotoxiaemia and inhibition of ODC on HPV. At baseline, mean PAP did not differ between mice lungs of all treatment groups (Table 1). Lungs of LPS mice showed a reduced HPV compared to lungs of the control group (26.3 ± 9.7% vs. 67.0 ± 17.5%, p < 0.05). Fig. 3 shows the normalized increase of PAP during the 2 min of baseline perfusion and ventilation with an inspiratory oxygen concentration of 0.21 and the following 6 min of ventilation with an inspiratory oxygen concentration of 0.01. These results confirm a decrease of HPV in endotoxiaemia.

**Restoration of HPV after DFMO injection**

To investigate the effect of ODC inhibition, HPV was measured in lungs of mice with and without endotoxiaemia and with and without DFMO pretreatment. Inhibition of ODC with an i.p. single bolus of 100 (Fig. 4) or 500 (Figs 3 and 4) mg kg⁻¹ body weight led in endotoxiaemic mice to an HPV of 57.3 ± 17.2% or 62.3 ± 12.4%, respectively. This did not differ from values of untreated control mice.
(67.0 ± 17.5%) (Fig. 3). HPV in lungs of endotoxemic mice after 20 mg kg\(^{-1}\) body weight DFMO (25.8 ± 9.9%) were comparable to LPS-group (26.3 ± 9.7%) (Fig. 4). These results show that ODC inhibitor DFMO restores HPV in endotoxemic mice in a dose-dependent way.

In contrast, treatment with DFMO decreased HPV in control mice. Fig. 4 shows that a dose of 100 and 500 mg kg\(^{-1}\) body weight DFMO reduced HPV in lungs of healthy controls (40.0 ± 14.9% and 35.9 ± 12.4%).

**Discussion**

The present study investigated the role of ODC on HPV in a mouse model of endotoxemia. Lungs of healthy mice had intact HPV, while LPS pretreated lungs showed decreased HPV. Increase of ODC gene and protein expressions in endotoxemia were confirmed, and this was attributable to pulmonary arteriolar wall and bronchial epithelium. After inhibition of ODC with DFMO, a dose-dependent restoration of HPV in LPS-pretreated mice was observed, while DFMO decreased HPV in control mice.

To test the hypothesis that increased ODC expression contributes to loss of HPV in endotoxemic mice, we used an isolated, perfused lung model to study the dose-dependent effects of DFMO on pulmonary vasoreactivity in response to alveolar hypoxia in endotoxemic mice. Using this model, it was unlikely that hepatic metabolites were the reason for acute alterations of pulmonary vasoreactivity.

Increased levels of ODC mRNA and protein expression after LPS challenge observed in these experiments are in line with other studies. Salimuddin et al. analyzed time courses of the induction of ODC, NOS2 and arginase isoforms from whole mouse lungs after endotoxemia and found an increase of the gene and protein expression within 6 to 12 h, which persisted for 24 to 36 h.\(^{13}\) Immunohistochemical analysis revealed this induced ODC expression in the pulmonary arteriolar walls and the bronchial epithelium. HPV is regulated by pulmonary arterial smooth muscle cells but can be modified by extrinsic factors derived from neighboring cell types, like endothelial cells.\(^{3}\) Further studies could identify specific cell types in murine lung tissues with increased expression of ODC.

Inhibition of ODC in endotoxemic mice restored HPV in a dose-dependent way. Since challenge with DFMO had no effect on baseline PAP in LPS- or saline-pretreated mice, it solely impacts the vascular contractility during hypoxia, not the basal tone of the pulmonary vascular tissue in isolated perfused organs.

There are a number of hypothetical roles of ODC and ODC inhibition on HPV. One of them includes polyamines. ODC catalyzes the decarboxylation of L-ornithine into putrescine, which is the first step of polyamine biosynthesis. Polyamines are able to interact with ion channels; they can rectify ion channel conductance and alter ion conductance of cation channels which are present in pulmonary arterial smooth muscle cells.\(^{3,14}\) Increased ODC expression during endotoxemia might affect HPV through altered polyamine–ion channel interaction.

Another possible mechanism involves NOS-uncoupling with higher amounts of reactive oxygen species, which can impair HPV.\(^{2,15}\) Inhibition of ODC might provide enough L-arginine as necessary substrate for NOS to maintain the
L-arginine – L-citrulline pathway attenuating NOS uncoupling.

Unexpectedly, inhibition of ODC in control mice was associated with decreased HPV. This can either be due to an accumulation of a vasodilatory substance upstream of ODC or a reduction of a vasoconstrictive substance downstream of ODC. It is conceivable that increased L-arginine levels lead to higher NO production and might therefore be a reason for an impaired HPV. An alternative explanation for the observed reduced HPV in DFMO challenged healthy mice is that DFMO forwards another vasodilating substance by suppressing rectification of potassium channels in pulmonary arteries.16

Petersen et al. showed that arginase inhibition by nor-NOHA restored HPV in endotoxemic mice.7 Although DFMO has some arginase inhibiting properties, it is unlikely that increased HPV in endotoxemic mice 1 h after DFMO is due to arginase inhibition, since concentrations of DFMO to inhibit arginase activity are very high (10 mM), and plasma levels after 1000 mg kg−1 body weight DFMO i.p. are reported to be around 10 nmol ml−1.17,18 Increased HPV in the present study was observed at a dose of 100 mg kg−1 body weight DFMO; thus, 100-fold lower than the described inhibiting dose of arginase.19

We used LPS-induced endotoxemia as an established murine model of sepsis associated lung injury.1,11 I.p. administration of LPS induces endotoxemia, which impairs HPV in several animal models similar to the decreased HPV in critically ill patients during sepsis.7,11 LPS is a key mediator of inflammation in gram negative sepsis. The advantage compared to other models is the high reproducibility and the absence of an initial operation, like it is necessary in a cecal ligation and puncture model.20 Like all other inflammatory models, LPS i.p. does not reproduce the full clinical characteristics of human sepsis-associated ARDS.

To study the response of pulmonary vasculature to alveolar hypoxia during endotoxemia, an isolated perfused and ventilated mouse lung model was used. Compared to isolated cells, the investigation of the whole lung includes physiological cell–cell interactions. It offers a reproducible model for elucidation of the HPV mechanisms when standardized proceedings are utilized.21 But it has to be noticed that it also excludes global circulatory effects, specifically if blood is replaced by buffer solution. In order to exclude acute cyclooxygenase dependent interaction with HPV by producing prostaglandins that are able to regulate vascular tone, the cyclooxygenase inhibitor indomethacin was added to the perfusate. The role of cyclooxygenase inhibitors in modulating HPV is not clear. Indomethacin was shown to increase HPV in a canine model of oleic acid-induced acute lung injury but decreased HPV in sheep pulmonary veins and had no effect in a murine model of HPV.22–24 By adding the nonselective NOS inhibitor L-NAME to the perfusion buffer, acute NO-dependent effects on HPV were excluded. Excessive NO production during septic shock causes systemic hypotension.25 Mice with a congenital deficiency of NOS2 show a preserved HPV after endotoxin-challenge, whereas treatment of wild-type mice with L-NAME improved but could not restore HPV during LPS-induced endotoxemia.1,11 Following these results, there are additional mechanisms than pulmonary vasodilation due to endotoxemia-induced NO overproduction, leading to impaired HPV.

Summary

Taken together, the results of this study reveal a critical role for ODC in the impairment of HPV during endotoxemia in mice. Inhibition of ODC restored impaired HPV in endotoxemic mouse lungs, offering a target to prevent systemic hypoxia.

Authors’ contributions

PHG and CJB: study conception and design, acquisition of data, analysis and interpretation of data, drafting of the article; MT: interpretation of data, drafting of the article; FL: immunohistochemistry, interpretation of data, critical revision of the article. MAW: interpretation of data, critical revision of the article. All authors read and approved the final article.

Guarantor

CJB.

Ethical approval

This study was approved by the Subcommittee on Research Animal Care (Regierungspräsidium Karlsruhe, Germany, AZ 35-9185.81/G-71/13).

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Conflict of interest

The author(s) declare that there is no conflict of interest.

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