Direct Effects of Nicotine on Contractility of the Uterine Artery in Pregnancy

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ABSTRACT

Recent studies indicate that smoking/nicotine increases maternal blood pressure and decrease in uterine blood flow in pregnancy. However, the mechanisms are not fully understood. The present study was designed to test the hypothesis that nicotine exposure decreases endothelium-dependent relaxation and increases vascular contractility of the uterine artery in pregnancy. Uterine arteries were isolated from near-term (~140 days gestation) pregnant ewes. Arteries were subjected to acute (20 min) or chronic (48 h) nicotine treatment, and agonist-induced contractions and relaxations were measured in tissue bath. Endothelial eNOS was detected by immunohistochemistry in situ in arteries and by Western blotting in isolated endothelial cells. Chronic nicotine treatment produced a concentration-dependent increase in α1-adrenoceptor agonist phenylephrine-induced contractions. In contrast, the acute treatment showed no effect. Inhibition of eNOS with Nω-nitro-L-arginine (L-NNA) significantly increased phenylephrine-induced contractions, which was abolished in uterine arteries after chronic nicotine treatment. In the presence of L-NNA, there was no significant difference in phenylephrine-induced contractions between control and nicotine-treated vessels. Chronic, but not acute, nicotine treatment significantly attenuated the calcium ionophore A23187-induced relaxations. Unlike A23187, the endothelium-independent relaxation mediated by sodium nitroprusside was not affected by nicotine. Endothelial eNOS protein levels and the phosphorylation levels of eNOSSer1177 were significantly decreased in nicotine-treated uterine arteries. The results suggest that nicotine impairs uterine vascular function in pregnancy, which may lead to an increased vascular resistance and a decrease in uterine blood flow.

Maternal cigarette smoking and/or prenatal nicotine exposure is the single most widespread prenatal insult in the world. Smoking has long been associated with adverse pregnancy outcomes, both for the mother, her fetus, and newborn. Maternal smoking produces at least two major different families of potential effects: direct actions of nicotine in the fetus and indirect actions on the maternal-fetal unit, which result in intrauterine growth retardation and sudden infant death syndrome (Naeye, 1992; Bell and Lau, 1995; Slotkin, 1998). Numerous studies have demonstrated that nicotine directly causes fetal damage (Navarro et al., 1988; Slotkin et al., 1997). On the other hand, the adverse effects of nicotine on fetal growth could be from indirect action of nicotine on the maternal-fetal unit and the uteroplacental circulation. Studies in humans and animal models have shown that nicotine reduces uterine blood flow by an average of 30 to 49%, resulting in an increase in perinatal mortality rate (Resnik et al., 1979; Suzuki et al., 1980; Clark and Irion, 1992; Lambers and Clark, 1996). However, the mechanisms underlying the nicotine-induced reduction of uterine blood flow in pregnancy are not fully understood.

During pregnancy, the development of uteroplacental circulation with low vascular tone accommodates a more than 20-fold increase in uterine blood flow in near-term pregnant sheep and in humans, which ensures normal fetal development. The adaptation of uterine artery contraction and relaxation mechanisms to pregnancy is complex. In addition to growth and remodeling of uterine vasculature, the decreased uterine artery resistance is accomplished by significantly increased endothelial nitric-oxide synthesis/release and decreased vascular contractility of the uterine artery (Sladek et al., 1997; Xiao et al., 1999, 2001a,b; Nelson et al., 2000; Bird et al., 2003). The effects of nicotine on vascular contractions are controversial and vary in different species, vessels, and experimental conditions. Nicotine can inhibit nitric oxide...
release and decrease endothelium-dependent vasorelaxation (Mayhan and Patel, 1997; Mayhan and Sharpe, 1999; Black et al., 2001; Conklin et al., 2001), In addition, both direct vasoconstriction and relaxation induced by nicotine have been reported previously (Waebcr et al., 1984; Toda et al., 1995; Zhang et al., 1998; Wang and Wang, 2000). Although clinical and in vivo studies have demonstrated that nicotine decreases uterine blood flow in pregnancy, the direct effect of nicotine on uterine artery contractility has not been studied. Herein, we present evidence in an ex vivo tissue culture model system that nicotine increased vascular contractility and decreased endothelium-dependent relaxation of the uterine artery in pregnancy.

Materials and Methods

Tissue Preparation. Time-dated pregnant (~140-day gestation) sheep were obtained from Nebeker Ranch (Lancaster, CA). Animals were anesthetized with thiamylal (10 mg/kg) administered via the external left jugular vein, and anesthesia was maintained with 1.5 to 2.0% halothane in oxygen throughout surgery. An abdominal incision was made to expose the uterus, and the uterine arteries were separated from surrounding tissues and cut into rings (2-mm in length). Arteries were mounted in 10-ml tissue baths, and arteries were perfused with Krebs’ solution (pH 7.4) of the following composition: 115.21 mM NaCl, 4.70 mM KCl, 1.80 mM CaCl2, 1.16 mM MgSO4. 1.18 mM KH2PO4, 22.14 mM NaHCO3, 0.03 mM EDTA, and 7.88 mM dextrose. The Krebs’ solution was oxygenated with a 95% O2-5% CO2 mixture. After tissues were removed, the animals were killed with euthanasia solution (T-61; Hoechst-Roussel, Somerville, NJ). All procedures and protocols used in this study were approved by the Animal Research Committee of Loma Linda University and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Contraction Studies. The fourth branches of the main uterine arteries were separated from surrounding tissues and cut into rings of 2-mm in length. Arteries were mounted in 10-ml tissue baths, and isometric tensions were measured in the Krebs’ solution at 37°C, as described previously (Xiao et al., 2002). After 60 min of equilibration, each ring was stretched to the optimal resting tension as determined by the tension developed in response to 120 mM KCl added at each stretch level. For acute treatment of nicotine, arteries were pretreated with or without nicotine for 20 min and then stimulated with increasing concentrations of phenylephrine. For the chronic treatment, arterial rings were maintained in Dulbecco’s modified Eagle’s medium (Cellgro; Mediatech, Herndon, VA) with 1% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin and were incubated at 37°C in a humidified incubator with 5% CO2-95% room air in the absence or presence of nicotine for 48 h, as described previously (Xiao et al., 2002). After the treatment, arteries were contracted with phenylephrine. In certain experiments, arteries were pretreated with nitric-oxide synthase inhibitor Nω-nitro-L-arginine (l-NNA) (100 μM, 20 min), as described in our previous study (Xiao et al., 2007), and then stimulated with increasing concentrations of phenylephrine. For relaxation studies, the tissues were precontracted with submaximal concentration of phenylephrine, followed by calcium ionophore A23187 and sodium nitroprusside (SNP), respectively, added in a cumulative manner. The concentrations of phenylephrine, calcium ionophore A23187, and SNP were chosen to produce full concentration-response curves in the arteries.

Immunoblotting. eNOS protein and phosphorylation levels were determined with Western blot analysis, as described previously (Cale et al., 2005; Xiao et al., 2007). Arteries were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin, pH 7.4. Homogenates were centrifuged at 4°C for 10 min at 10,000g, and supernatants were collected. Protein was quantified in the supernatant using the protein assay kit from Bio-Rad (Hercules, CA). Samples with equal protein were loaded on 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate and separated by electrophoresis at 100 V for 2 h. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites in the membranes were blocked by overnight incubation at 4°C in a Tris-buffered saline solution containing 5% dry milk. The membranes were then incubated with mouse eNOS monoclonal antibody and the antibody for eNOS phosphorylation at Ser1179 (p-eNOSSer1179), followed by a secondary horseradish peroxidase-conjugated goat anti-mouse antibody. Proteins were visualized with enhanced chemiluminescence reagents, and the blots were exposed to Hyperfilm (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software (Eastman Kodak, Rochester, NY).

Immunohistochemistry. In situ determination of eNOS was performed using anti-Ig horseradish peroxidase detection kit (BD Biosciences, San Diego, CA), as described previously (Xiao et al., 2007). Uterine artery rings were fixed in 10% neutral buffered formalin and embedded in paraffin. Cross-sections of arterial rings were incubated with monoclonal anti-eNOS primary antibodies (1:100) diluted in the antibody diluents for 60 min at room temperature. After rinsing the slides three times in phosphate-buffered saline for 30 min, the slides were incubated with biotinylated goat anti-mouse IgG (1:100) for 60 min at room temperature. The samples were then exposed to streptavidin-horseradish peroxidase and reacted with diaminobenzidine substrate solution according to the manufacturer’s recommendations and counterstained with hematoxylin. The negative control of eNOS staining was performed in the absence of eNOS antibody. The slides were viewed on an Olympus BH-2 microscope (Olympus, Tokyo, Japan). Images were captured with an attached SPOT digital camera imaging system (Diagnostic Instruments Inc., Sterling Heights, MI).

Materials. Phenylephrine, l-NNA, SNP, nicotine hydrogen tartrate, and other chemicals were obtained from Sigma (St. Louis, MO). eNOS monoclonal antibody was from Transduction Laboratories (Lexington, KY). p-eNOSSer1179 antibody was from Cell Signaling Technology (Danvers, MA). Electrophoresis and immunoblotting reagents were from Bio-Rad.

Data Analysis. Concentration-response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA) to obtain the values of pD2 (−logEC50) and the maximal response. Results were expressed as means ± S.E.M., and the differences were evaluated for statistical significance (P < 0.05) by one-way or two-way analysis of variance followed by Bonferroni’s post-tests.

Results

Effects of Nicotine on Phenylephrine-Induced Constrictions. Figure 1 shows that phenylephrine induced concentration-dependent contractions in both control and nicotine-treated uterine arteries. Chronic nicotine treatment resulted in a rightward shift of phenylephrine-induced concentration-contraction curves and produced a concentration-dependent increase in the pD2 values of phenylephrine-induced contractions (Fig. 1; Table 1). In contrast, the acute treatment of nicotine did not significantly affect phenylephrine-induced contractions of the uterine arteries (Table 1). 

Effect of Nicotine on Endothelium/Nitric Oxide-Mediated Relaxations. To test whether nicotine affects basal nitric oxide-mediated response, phenylephrine-induced contractions were determined in the absence or presence of the eNOS inhibitor l-NNA (100 μM, 20 min), respectively. In the control tissues, l-NNA shifted the phenylephrine-induced...
concentration-response curves to the left and significantly increased the $pD_2$ values of phenylephrine-induced contractions (Fig. 2, top; Table 2). In contrast, L-NNA did not significantly change the $pD_2$ values of phenylephrine-induced contractions in nicotine-treated arteries (Fig. 2, bottom). In the presence of L-NNA, there was no significant difference in phenylephrine-induced contractions between control and nicotine-treated uterine arteries (Table 2).

We further determined the effect of nicotine on endothelium-dependent relaxations induced by calcium ionophore A23187 in uterine arteries. Our previous studies demonstrated that calcium ionophore A23187-induced relaxation of the uterine artery was endothelium-dependent (Xiao et al., 2001b; Xiao and Zhang, 2004). Inhibition of eNOS with L-NNA blocked calcium ionophore A23187-induced relaxation of the uterine artery (data not shown). As shown in Fig. 3, calcium ionophore A23187 produced a concentration-dependent relaxation in both control and nicotine-treated arteries. Chronic nicotine treatment resulted in a significant decrease in the maximal relaxation induced by calcium ionophore A23187 (Fig. 3, top; Table 3). In contrast, the acute treatment of nicotine did not alter calcium ionophore A23187-mediated, endothelium-dependent relaxations of the uterine arteries (data not shown).

To determine the potential effect of nicotine on endothelium-independent relaxations, SNP-induced relaxations of the uterine arteries were also examined in the present study. As shown in Fig. 3 (bottom), SNP-induced relaxations of the uterine arteries were not significantly changed by the nicotine treatment.

**Effect of Nicotine on eNOS and p-eNOSSer1179 Levels.** To determine whether eNOS expression in the uterine arteries was correlated with the alteration of endothelium-dependent relaxations after the chronic nicotine treatment, eNOS protein levels were determined with immunohistochemical staining and Western blot analyses. As shown in Fig. 4, eNOS immunoreactivity was exclusively detected in the endothelium of the uterine arteries, and there was an apparent decrease in eNOS levels in nicotine-treated arteries. This was confirmed by Western blot analyses in isolated endothelial cells, showing a concentration-dependent decrease in eNOS protein levels in nicotine-treated uterine arteries (Fig. 5A). This was accompanied by a significant decrease in p-eNOSSer1179 levels (Fig. 5B).

**Discussion**

The major findings of the present study are that, in ovine pregnant uterine artery, 1) nicotine did not affect $\alpha_1$-adrenoceptor-mediated contractions in an acute treatment but concentration-dependently increased the contractions after a chronic treatment; 2) inhibition of eNOS by L-NNA increased $\alpha_1$-adrenoceptor-mediated contractions in control arteries, which was abolished in nicotine-treated uterine arteries; 3)
chronic, but not acute, nicotine treatment significantly attenuated the calcium ionophore A23187-induced endothelium-dependent relaxations of the uterine arteries; 4) unlike A23187, the endothelium-independent relaxations mediated by SNP was not changed by nicotine; and 5) eNOS immuno-reactivity was only detected in the endothelium of the uterine arteries, and eNOS protein and phosphorylation levels were significantly decreased in nicotine-treated vessels.

The adverse effects of cigarette smoking/nicotine on cardiovascular system have been well documented (Pittilo, 1990; Zhu et al., 1994; Lambers and Clark, 1996; Benowitz, 1997; Tonnessen et al., 2000; Sener et al., 2004). Previous studies (Resnik et al., 1979; Suzuki et al., 1980; Lindblad et al., 1988; Clark and Irion, 1992) have demonstrated that systemic nicotine infusions decrease uterine blood flow and increase uterine vascular resistance and that these effects of nicotine can be blocked by pretreatment with the α-adrenoceptor blocker phentolamine. In the present study, we found no acute effect of nicotine on α₁-adrenoceptor-mediated contractions of pregnant uterine arteries in vitro. This finding suggests that nicotine has no short-term direct effect on uterine vascular resistance and that the decreased uterine blood flow after systemic nicotine infusion observed in the previous studies is probably due to a systemic release of catecholamines caused by nicotine. In agreement with our finding, a direct acute local infusion of nicotine into the uterine artery showed no effect on uterine blood flow (Resnik et al., 1979).

The present finding that chronic nicotine treatment enhanced contractions of the pregnant uterine artery in response to α₁-adrenoceptor agonist phenylephrine suggests that nicotine has long-term genomic effect on uterine artery contractility. Our knowledge, this is the first study showing the direct effects of chronic nicotine treatment on uterine artery contractility. Chronic nicotine exposure has been shown to cause mitogenic or cytotoxic effects in both smooth muscle cells and endothelial cells in culture at concentrations seen in the plasma of habitual smokers (Cucina et al., 2000a,b), which may change the ability of vascular smooth muscle cells to respond to stimulation and affect vascular contractility (Carty et al., 1997). Although the precise mechanisms underlying enhanced vascular contractility of the uterine arteries to chronic nicotine exposure are not clear at present, nicotine exposure may alter vascular reactivity through the nitric oxide signaling pathway. In the present study, we found that pretreatment of an eNOS inhibitor L-NNA significantly increased α₁-adrenoceptor-mediated contractions of the pregnant uterine arteries, which was completely abolished in the arteries after chronic nicotine treatment. These findings suggest at least two potential mechanisms of action. First, they suggest a significant component of basal eNOS activity in the regulation of vascular reactivity of the pregnant uterine arteries. This is consistent with our previous findings in ovine uterine arteries (Xiao et al., 1999). Second, they suggest that the enhanced α₁-adrenoceptor-mediated contractions of the pregnant uterine arteries after the chronic nicotine treatment are primarily due to the loss of the eNOS-mediated relaxation component, rather than increased α₁-adrenoceptor-mediated contractions per se. This is supported with the finding that, in the presence of L-NNA, there was no significant difference between the control and nicotine-treated uterine arteries. Previous studies have indicated that chronic cigarette smoking causes morphologic changes and functional alteration in endothelial cells (Davis et al., 1985; Powell, 1998). In addition, inhibition of eNOS with Nω-monomethyl-L-arginine produced a significantly greater decrement in forearm blood flow in smokers versus nonsmokers (McVeigh et al., 1996), suggesting that cigarette smoking altered endothelial function and increased vascular tone.

The present finding that chronic nicotine treatment attenuated calcium ionophore A23187-induced relaxations in the uterine artery reinforces the notion that the observed alterations in vascular reactivity are a consequence of primary changes in endothelium-mediated pathways. It has been well documented that calcium ionophore A23187 produces relaxation in blood vessels via an endothelium-dependent mecha-

### Table 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control pD₂</th>
<th>Control Eₘₐₓ</th>
<th>Nicotine pD₂</th>
<th>Nicotine Eₘₐₓ</th>
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<tr>
<td>−L-NNA</td>
<td>5.76 ± 0.07</td>
<td>154.8 ± 4.6</td>
<td>6.03 ± 0.08</td>
<td>161.1 ± 5.3</td>
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<tr>
<td>+L-NNA</td>
<td>6.20 ± 0.08*</td>
<td>162.4 ± 5.0</td>
<td>6.11 ± 0.10</td>
<td>176.6 ± 6.9</td>
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*Eₘₐₓ is the maximal response (% KCl response), n = 8 in each group.

TABLE 2

Effect of chronic nicotine exposure on phenylephrine-induced contractions of uterine arteries in the absence or presence of L-NNA

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**Fig. 3.** Effect of nicotine exposure on calcium ionophore A23187- and SNP-induced relaxations of the pregnant uterine arteries. Arterial rings were pretreated in the absence or presence of 0.1 μM phenylephrine for 48 h at 37°C. Arteries were precontracted with 3 μM phenylephrine, followed by a cumulative addition of calcium ionophore A23187 (top) or SNP (bottom). Data are means ± S.E.M. of tissues from seven animals. The pD₂ values and the Eₘₐₓ are presented in Table 3.
nism and a release of nitric oxide (Singer and Peach, 1982). Our previous studies demonstrated that calcium ionophore A23187-induced endothelium-dependent relaxations of the uterine artery were mediated by an increase in nitric oxide release (Xiao et al., 2001b; Xiao and Zhang, 2004). To determine whether the impaired relaxations of the uterine artery in nicotine-treated vessels were not related to a downstream signal of the endothelium, we examined the endothelium-independent relaxation of the uterine artery by SNP. The finding that SNP-induced relaxations of the uterine arteries were not significantly changed by nicotine further supports that the primary effect of nicotine is an inhibition of endothelial nitric oxide synthesis/release. The inhibited nitric oxide synthesis/release can result from a decrease in either eNOS activity or eNOS protein levels or both. Nicotinic acetylcholine receptors have been identified in vascular endothelial cells (Macklin et al., 1998), and nicotine can directly alter eNOS activity (Tonnessen et al., 2000).

In the present study, eNOS protein expression and local-

### Table 3

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<thead>
<tr>
<th></th>
<th>A23187</th>
<th>SNP</th>
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<tbody>
<tr>
<td>pD₂</td>
<td>Eₘₐₓ</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.18 ± 0.14</td>
<td>70.3 ± 3.2</td>
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<tr>
<td>Nicotine</td>
<td>8.14 ± 0.17</td>
<td>57.7 ± 3.2*</td>
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<thead>
<tr>
<th></th>
<th>pD₂</th>
<th>Eₘₐₓ</th>
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<tbody>
<tr>
<td>Control</td>
<td>6.85 ± 0.14</td>
<td>89.6 ± 3.7</td>
</tr>
<tr>
<td>Nicotine</td>
<td>6.93 ± 0.16</td>
<td>88.2 ± 4.3</td>
</tr>
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</table>

Eₘₐₓ: the maximal response (% relaxation). n = 7 in each group.
* P < 0.05, nicotine vs. control.

**Fig. 4.** Immunohistochemical express and localization of eNOS in the pregnant uterine arteries. Arterial rings were pretreated in the absence or presence of 0.1 μM nicotine for 48 h at 37°C. eNOS expression was detected by immunohistochemical staining and was seen in the endothelium in both control (A) and nicotine-treated (B) arteries. Arrows indicate dark brown staining of eNOS. Representatives of three animals from each group are shown. Original magnification, 400×.

**Fig. 5.** Effect of nicotine on eNOS protein and p-eNOS<sup>Ser1179</sup> levels in the pregnant uterine artery endothelium. Uterine arteries were pretreated with 0, 0.01, and 0.1 μM nicotine for 48 h at 37°C. Protein levels of eNOS and p-eNOS<sup>Ser1179</sup> were determined in freshly isolated endothelial cells of uterine arteries after treatment. Immunoblots illustrate eNOS and p-eNOS<sup>Ser1179</sup> bands detected by the monoclonal antibodies at the expected size of ~140 kDa. Data are expressed as fold of actin density for eNOS (A) and the ratio of p-eNOS<sup>Ser1179</sup>/eNOS (B) and are means ± S.E.M. of tissues from four animals in each group. * P < 0.05, versus control.
ization were examined in the uterine arteries. It is not a surprise that eNOS was detected only in the endothelium of the uterine artery, confirming an endothelial location of eNOS. The finding of significant decreases in eNOS protein levels, detected by both Western blot analyses and immuno-histochemistry in nicotine-treated uterine arteries in the present study, suggests that decreased endothelium-dependent relaxations of the uterine arteries after chronic nicotine treatment results primarily from a decrease in eNOS protein levels. These findings agree well with previous studies that showed a reduction in eNOS expression in porcine carotid artery and in pulmonary artery endothelial cells exposed to nicotine or cigarette smoke extracts (Su et al., 1998; Conklin et al., 2001). The mechanisms by which nicotine affects eNOS expression are not fully understood at present. Because nicotine can cross cell membranes, it is possible that nicotine can directly regulate eNOS expression. Indeed, previous studies have demonstrated that nicotine and its metabolic product cotinine significantly decreased eNOS mRNA and protein levels, resulting in impaired endothelial-dependent relaxations in the carotid artery rings after a 24-h treatment (Conklin et al., 2001). In addition to decreased eNOS protein levels, chronic nicotine treatment significantly attenuated eNOS phosphorylation at the site of Ser1179 that is involved in eNOS activation (Cale et al., 2005; Mount et al., 2007), suggesting a decrease in the function of the eNOS enzyme.

In summary, the present study demonstrates for the first time that chronic, but not acute, nicotine treatment has direct effects on contractility of the uterine artery in pregnancy and inhibits endothelium-dependent relaxations, resulting in an enhanced vasoconstriction of the pregnant uterine artery. Although it is not clear at present whether the inhibition of endothelial nitric oxide production and increased vasoconstriction could be a major reason for the reduced uterine blood flow observed with smoking/nicotine exposure during pregnancy, these findings provide a potential mechanism. Future studies are needed to treat the pregnant sheep with nicotine to obtain a chronic model and assess uterine artery function in vivo.

References

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