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Inducible nitric oxide synthase evoked nitric oxide counteracts capsaicin-induced airway smooth muscle contraction, but exacerbates plasma extravasation

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Abstract

The contribution of nitric oxide (NO) to capsaicin-evoked airway responses was investigated in rats. The measurement of plasma NO level, airway dynamics, airway smooth muscle electromyogram, and plasma extravasation by India ink and Evans blue leakage technique was adapted. Capsaicin-evoked hypotension, bronchoconstriction, trachea plasma extravasation as well as increases in plasma NO level in a dose-dependent manner. L-732138 (NK_1 receptor antagonist) or SR-48968 (NK_2 receptor antagonist) pretreatment reduced capsaicin-enhanced hypotension, bronchoconstriction, plasma extravasation, and plasma NO level. N^G-nitro-L-Arginine methyl ester (L-NAME, 10 mg/kg, i.v.), a non-selective NO synthase (NOS) inhibitor, or aminoguanidine (10 mg/kg, i.v.), a selective inducible NOS (iNOS) inhibitor, reduced capsaicin-induced increases in plasma NO level and protected against capsaicin-induced plasma extravasation, whereas L-arginine (150 mg/kg, i.v.), a NO precursor, enhanced capsaicin-evoked plasma NO level and plasma extravasation. L-Arginine pretreatment ameliorated capsaicin-induced bronchoconstriction, whereas L-NAME and aminoguanidine exaggerated capsaicin-induced bronchoconstriction. In summary, NK_1 and NK_2 receptors and iNOS play a role in NO formation and on capsaicin-induced bronchoconstriction and plasma extravasation. NO generated by iNOS counteracts tachykinin-mediated bronchoconstriction, but exacerbates tachykinin-mediated plasma extravasation.

Keywords: Bronchoconstriction; Plasma extravasation; Nitric oxide; Tachykinins; Inducible nitric oxide synthase

Although autonomic nervous system mainly innervates the airway [2,28], non-adrenergic and non-cholinergic (NANC) nervous system is important in the regulation of various airway responses via tachykinins and nitric oxide (NO) [1,21,25,28,30]. Tachykinins are neuropeptides that are released from lung C-fiber nerve endings when stimulated and that are known to induce neurogenic airway responses including bronchoconstriction and airway plasma exudation [15,20,25,26]. NO synthesis is mediated by three different types of nitric oxide synthases (NOS): neuronal, endothelial, and inducible [27]. The first two synthases are expressed constitutively (cNOS), whereas inducible NOS (iNOS) must be induced [27]. Endogenous NO generated by different NOS has been reported to have different effects on the airways including bronchodilation and airway plasma exudation [1,3,4,7]. Likewise, exogenous NO has also been demonstrated to have conflict results in regulation of airway responses [14,27,31]. Tachykinins can induce the synthesis and release of NO via neurokinin type 1 (NK_1) and type 2 (NK_2) receptor activation [19,26]. Inhibition or activation of NK receptors affected NOS activity, NO amounts, and airway responses [7,14–16,29]. However, the role of NO in the C-fiber-mediated neurogenic airway responses is not completely understood and whether NO generated by cNOS and iNOS may have different involvements in the C-fiber-mediated neurogenic airway responses is not clear.

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Capsaicin acts on vanilloid type 1 receptor to stimulate lung C-fiber nerve endings and cause a release of tachykinins [2]. The released tachykinins subsequently may activate NK_1 receptor mediated vascular permeability and plasma protein extravasation in the airway [6,13] and activate NK_2 receptor provoked bronchoconstriction [5,6]. In the present study, we used capsaicin to stimulate these nerve endings, to cause the release of tachykinins, and to produce the resultant neurogenic airway responses. We used a non-selective and selective iNOS inhibitors to investigate the role of NO generated from different NOS in the modulation of capsaicin-induced airway responses. We also investigated the effects of increased production of NO, which arises from exogenous administration of L-arginine, on capsaicin-induced airway responses.

On the day of experiment, L-arginine ($C_6H_{14}N_4O_2$), L-N^G-nitro-arginine methyl ester (L-NAME) (Sigma Chemical Co., USA), aminoguanidine hydrochloride (Cayman Chemical, Ann Arbor, MI, USA) were dissolved in normal saline. Capsaicin (8-methyl-*N*-vanillyl-nonenamide; Sigma) was dissolved in 1% ethanol and 1% Tween 80 solution at the concentration of 300 nmol/ml. The non-peptide NK_1 receptor antagonist, L-732138 (*N*-acetyl-L-tryptophan-3,5-bistrifluoromethyl benzyl ester, Sigma), and non-peptide NK_2 receptor antagonist, SR-48968 {(*S*)-*N*-methyl-*N*-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl] benzamide} (Sanofi Recherche, France) were dissolved in 10% dimethyl sulphoxide in 0.9% saline at the concentration of 1 μmol.

Male Sprague–Dawley rats (260–280 g) were purchased from National Laboratory Animal Center and were housed at the Experimental Animal Center, National Sun Yat-Sen University, at a constant temperature and with a consistent light cycle (light from 00:700 to 18:00 h). On the day of experiment, the rats were anesthetized with subcutaneous urethane (1.2 g/kg). The body temperature was kept at 36.5–37.0 °C by an infrared light and was monitored with a rectal thermometer. The study was conducted according to the *Guiding Principles in the Care and Use of Animals* of the American Physiological Society and was approved by the Animal Care and Use Committee of the National Sun Yat-Sen University. All efforts were made to minimize both animal suffering and the number of animals used throughout the experiment.

The trachea was cannulated caudal to the larynx (PE-200) and the animal breathed spontaneously through a Pneumotachometer (TSD 137C, Biopac Systems) connected to a flow transducer (TSD 160A, amplifier DA 100C, Biopac Systems) for monitoring airflow with a zero-flow method. Intratracheal pressure was measured by a pressure transducer (Model DP 103-24) connected to a manometer (Model CD-15-A-1-B-1, validyne). A fluid-filled PE-50 cannula was introduced into the esophagus to measure the esophageal pressure as an approximation of pleural pressure. The transpulmonary pressure (defined as the pressure difference between the intratracheal and the esophageal pressures) was measured with a manometer. Total pulmonary resistance (R_L) was calculated as previously described [12]. PE-50 catheters were placed in the left femoral artery for measurement of arterial blood pressure and in the left femoral vein for administration of test drugs. Arterial blood pressure was recorded on a polygraph (DA 100C, Biopac Systems, Inc., Goleta, CA, USA) with a transducer (TCI 100, Biopac Systems). Plasma NO concentration was examined with the NO chemiluminescent probe containing luminol 0.018 (Sigma), H_2O_2 10, desferrioxamine 0.15 (Sigma) and K_2CO_3 2 mmol/l (Sigma) and detected by a Chemiluminescence Analyzing System (CLD-110, Tohoku Electronic Inc. Co., Sendai, Japan) [9,22]. Because capsaicin activates cholinergic reflexes [5], atropine (1 mol/kg) was administrated 15 min before drug challenge in all the animals. Ten minutes after vehicle, L-NAME (10 mg/kg), aminoguanidine (10 mg/kg), L-arginine (150 mg/kg), L-732138 (1 μmol/kg), or SR-48968 (1 μmol/kg) pretreatment ($n = 6$ each), the rats were received intravenous injection of capsaicin (0–100 nmol/kg).

For electromyogram recordings, epoxy-coated stainless steel wire (50 μm; M.T. Giken Co., Ltd, Tokyo, Japan) was placed into the inner layer of airway smooth muscle under the dissecting microscope (Nikon, Japan). The EMG electrodes were embedded into the smooth muscle ∼1–2 mm. EMG signals were connected to an EMG amplifier (EMG 100C, Biopac Systems) and recorded on the recording system [8].

For evaluation of plasma extravasation, 10 min after drug pretreatment, the rats were received intravenous injection of India ink (1 mg/kg, over 5 s, Chroma-Gesellschaft, Kongen, Germany) ($n = 4$) [24] or Evans blue dye (50 mg/kg, $n = 5$) [4] followed by capsaicin stimulation (100 nmol/kg). Five minutes after capsaicin, the animal was exsanguinated by infusion of 50 ml of 0.9% (w/v) saline, at 37 °C, into the left cardiac ventricle. The trachea of India-ink was analyzed with a microscope (Leica DMRD) and of Evans blue was determined with a spectrophotometer (Beckman Coulter DU 6408, USA) at the absorbance maximum of 620 nm wavelength after extraction in a known volume of formamide at 60 °C for 24 h. Plasma extravasation was expressed as the content of Evans blue dye in μg/g of tissue.

All the signals collected are stored in the IBM computer (ThinkPad R40) and analyzed with software (AcqKnowledge 3.7.3 Biopac System). The rate of rise of EMG activity was calculated by dividing peak integrated EMG activity by the time to peak, and expressed the result as a percentage of the maximal activity.

Values are mean ± standard error of mean. Differences in parameters among groups were analyzed with analysis of variance. Post hoc analyses were performed by means of the Newman–Keuls test. For all tests, differences were considered significant if $P < 0.05$.

In our experiments, the baseline level of arterial blood pressure was 102–109 mmHg. Atropine treatment did not significantly increase the arterial blood pressure in all the animals. Intravenous capsaicin (from 25 to 100 nmol/kg) evoked hypotension, apnea, or bradypnea (decreased respiratory frequency) in a dose-dependent manner (Fig. 1). The cardiovas-

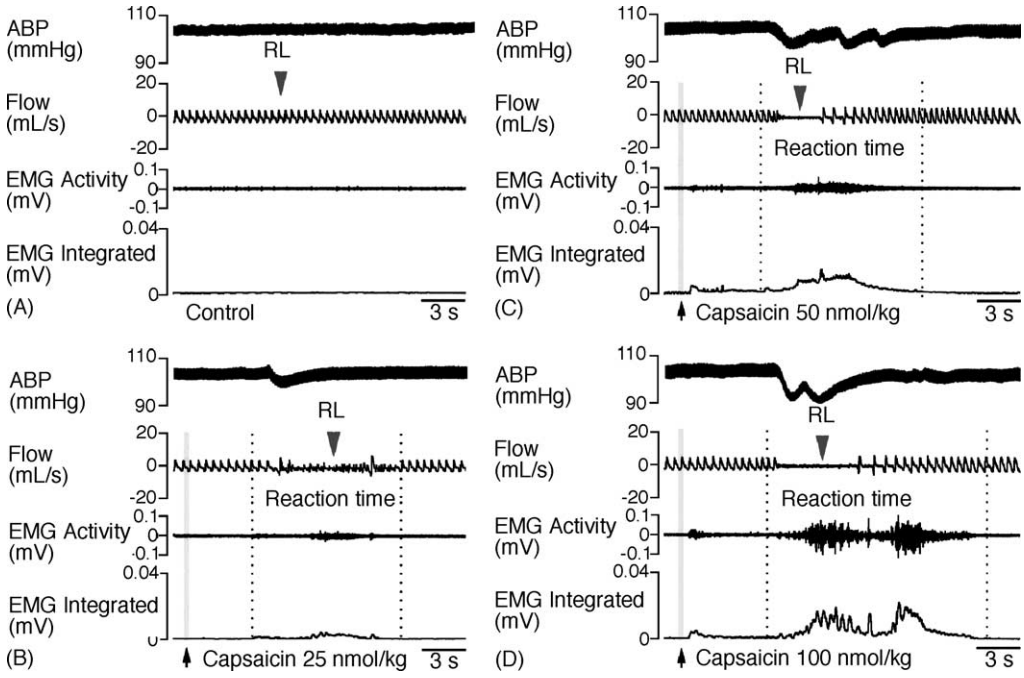

Fig. 1. Experimental recordings of responses to intravenous capsaicin in anesthetized rats. (A) Control responses; (B–D) responses to intravenous capsaicin. Capsaicin evokes hypotension, bradypnea (a decrease in respiratory frequency) and augmented electromyographic (EMG) activity in a dose-dependent manner. Downward arrow (▼) indicates time of total pulmonary resistance (R_L) measurement.

cular and breathing pattern data (Fig. 2) confirmed that lung C-fiber nerve endings are indeed stimulated by capsaicin.

After capsaicin stimulation (0, 50, and 100 nmol/kg), the increased R_L was 0.35 ± 0.03 cmH_2O/mL/s, 1.5 ± 0.2 cmH_2O/mL/s, and 4.7 ± 0.6 cmH_2O/mL/s, whereas the enhanced airway smooth muscle EMG activity was 3 ± 0.3 μV, 52 ± 13 μV, and 103 ± 15 μV (Fig. 2). India ink stain can be retained by basement membrane while leakage and can easily be identified as a severity index of neurogenic inflammation [24]. Capsaicin (100 nmol/kg) significantly increased the area densities of India ink-labeled blood vessels in the trachea (Fig. 3B) when compared to the vehicle control treated trachea (Fig. 3A). The quantitative data of plasma extravasation obtained from Evans blue stain was also increased by capsaicin stimulation (Fig. 3E). These lung mechanics and pathohistologic data indicate that capsaicin induces neurogenic airway responses, characterized by bronchoconstriction and plasma exudation.

L-732138, and SR-48968 pretreatment significantly reduced 100 nmol/kg of capsaicin-induced increases in R_L by 21%, and 36% and in smooth muscle EMG activity by 19%, and 33%, respectively. L-732138, and SR-48968 pretreatment also reduced 100 nmol/kg of capsaicin-induced plasma extravasation by 44%, and 20% (Fig. 3E). These data confirmed that the observed neurogenic airway responses are due to the effects of tachykinins.

The baseline level of plasma NO level was 90–120 counts/10 s. NK receptor antagonists did not affect the baseline NO level. The increased NO amount (1201 ± 150 counts/10 s) by capsaicin stimulation was partly inhibited by L-732138 (588 ± 70 counts/10 s) and SR-48968 (325 ± 50 counts/10 s in NO amounts) pretreatment (Fig. 2), indicating that tachykinins increase the production of endogenous NO during the development of neurogenic airway responses.

L-NAME pretreatment increased the baseline (245%) and capsaicin-enhanced R_L (28%) and EMG activity (22%), whereas aminoguanidine pretreatment exaggerated capsaicin-induced increases in R_L (30%) and in EMG activity (19%). L-NAME and aminoguanidine exerted a similar inhibition (66% versus 63%) on capsaicin-induced plasma extravasation (Fig. 3E). These results suggest that NO generated from iNOS exert their distinct effects on the airway smooth muscle and airway microvasculature. L-Arginine as an exogenous NO source inhibited capsaicin-induced EMG activity (55%) and R_L (53%), but potentiated capsaicin induced plasma extravasation (24%). These data indicate that enhanced production of NO by exogenous source would exert the same effects as that of NO generated from iNOS.

Capsaicin administration has been demonstrated to produce the so-called cardiopulmonary chemoreflex, including apnea, bradycardia, and hypotension [10]. In our study capsaicin also increased plasma NO level, R_L, airway smooth muscle EMG activity, and plasma extravasation in anesthetized and atropinized rats. Bronchoconstriction, vasodilation, plasma extravasation, and increased plasma NO level induced by capsaicin were mediated by NK_1 and NK_2 receptors, because these responses were partially blocked by pretreatment with the selective antagonist of NK_1-receptor L-732138 [5,13] and NK_2-receptors SR-48968 [5,20]. The

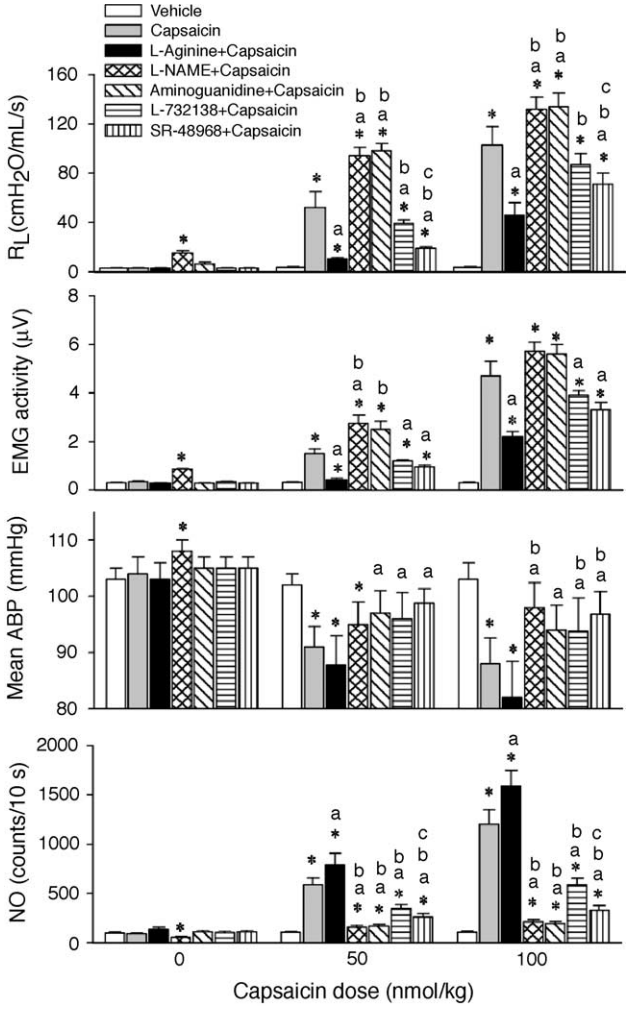

Fig. 2. Mean response of L-arginine, L-NAME, aminoguanidine, and tachykinin antagonists on capsaicin-induced total pulmonary resistance (R_L), airway smooth muscle EMG activity, mean arterial blood pressure (ABP), and plasma NO counts. Capsaicin enhances R_L, EMG activity, hypotension, and plasma NO counts in a dose-dependent manner. L-NAME or aminoguanidine exacerbated capsaicin-evoked R_L and EMG activity, whereas L-arginine, L-732138, or SR-48968 reduced these responses. The degree of capsaicin-induced hypotension was enhanced by L-arginine, but inhibited by L-NAME, aminoguanidine, L-732138 or SR-48968. $^{*}P < 0.05$ vs. respective baseline control without capsaicin treatment. $^{a}P < 0.05$ vs. capsaicin alone, $^{b}P < 0.05$ vs. L-arginine treatment, $^{c}P<0.05$ vs. L-732138. For all tests, significant difference (*, a–c) between the groups was performed by analysis of variance and post hoc contrasts by means of the Newman–Keuls test.

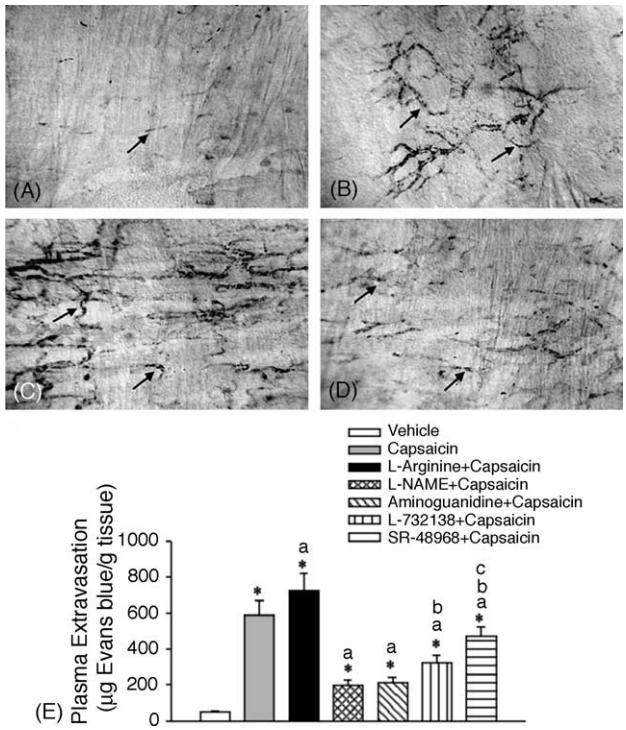

Fig. 3. Effects of L-NAME, aminoguanidine, L-arginine, NK_1 and NK_2 receptor antagonist on 100 nmol/kg capsaicin-induced plasma extravasation in the rat trachea. In the trachea, India-ink labeled plasma extravasation (indicated by arrow) is significantly induced by intravenous capsaicin (B) when compared to vehicle (A). L-Arginine pretreatment potentiated trachea plasma extravasation (C), whereas L-NAME (D) reduced the India-ink intensity of plasma extravasation. The mean response of plasma extravasation in the trachea indicated by Evans blue leakage is illustrated in E. $^{*}P < 0.05$ vs. vehicle. $^{a}P < 0.05$ vs. capsaicin treatment, $^{b}P < 0.05$ vs. L-NAME treatment, $^{c}P < 0.05$ vs. L-732138. For all tests, significant difference (*, a–c) between the groups was performed by analysis of variance and post hoc contrasts by means of the Newman–Keuls test.

present experiments also show that capsaicin via NK_1 and NK_2 receptor activation increases iNOS activity and NO synthesis, which directly ameliorates capsaicin-induced bronchoconstriction by a reduction of airway smooth muscle EMG activity, but exaggerates capsaicin-induced plasma protein extravasation possibly by an impairment of NO-dependent relaxation as well as contraction.

Capsaicin can activate a subpopulation of neuropeptide-containing C fibers and minor small-diameter A-δ fibers [2] and the activation of these two groups of fibers may play a role in airway physiology and pathophysiology. Capsaicin can cause the release of tachykinins from these sensory nerve endings [5]. The tachykinins act on NK_1 and NK_2 receptor located on the airway smooth muscles and vessels to produce airway responses. The mechanism that we have described in this report is the involvement of the NK receptor-NOS-NO pathway in the modulation of bronchoconstriction, vasodilation, and plasma extravasation induced by capsiaicin in Sprague–Dawly rats. Three pieces of evidence point to this conclusion. First, capsaicin via vanilloid type 1 receptor to activate NK_1 and NK_2 receptor and to evoke bronchoconstriction, vasodilation, plasma extravasation [6], and NO level, which can be inhibited by the selective NK_1 or NK_2 receptor antagonists. Second, the increase in smooth muscle EMG activity and R_L caused by capsaicin is inhibited in the presence of L-arginine, but is potentiated by L-NAME or aminoguanidine, an iNOS inhibitor. Third, the degree of capsaicin-induced plasma extravasation is ameliorated by L-NAME or aminoguanidine pretreatment, but is potentiated by L-arginine pretreatment. Because aminoguaindine exerts a similar effect in bronchoconstriction induction and plasma extravasation inhibition to L-NAME, our data also addressed

the possible mechanism that capsaicin via the action of iNOS causes the release of bronchodilative and vasorelaxant NO.

NO has been identified as an endothelium-derived relaxing factor in the airway smooth muscle [29] and vascular cells [17]. A deficiency of NO contributes to the increased responsiveness of isolated perfused trachea to various drugs stimulation [11]. Neurokinin A or capsaicin activates NOS in the airway smooth muscle layer and generates exhaling NO to counteract neurokinin A- [19] or capsaicin-induced bronchoconstriction (out data). NO donor, L-arginine, as a bronchodilator can reduce the tachykinins-induced bronchoconstriction. However, L-arginine can exacerbate capsaicin-induced increases in plasma extravasation possibly by the enhancement of iNOS activity, and the increment of blood flow in postcapillary venules [23]. Previous study has demonstrated that increased expression of iNOS by gene transfer in blood vessels produces impairment of NO-dependent relaxation as well as contraction [18]. The impaired contraction and relaxation may contribute to the exacerbated plasma extravasation and can be improved by aminoguanidine and L-*N*-iminoethyl lysine, inhibitors of iNOS [18]. In our study, L-NAME and aminoguanidine can functionally inhibit capsaicin-induced plasma leakage, also supporting the dysfunctional role of iNOS in vascular permeability. The differential responses between airway smooth muscle and vascular cells may be explained by the different levels of NO, different sites of NO production [14], different actions of NO [18], and different sources of NO. Our results implicate that different sources of NO have beneficial and deleterious effects and caution should be taken when chemicals relevant to the increase the production of NO or interfering endogenous NO are considered as potential therapeutic drugs.

In summary, capsaicin stimulates lung C-fiber sensory nerve endings to cause the release tachykinins, which act on NK_1 and NK_2 receptors located on the airway smooth muscles and vessels and enhance NO formation, bronchoconstriction, vasodilation, and plasma extravasation in the trachea. Our findings further indicate that the released tachykinins increase the production of NO via iNOS, and iNOS-evoked NO counteracts tachykinin-mediated bronchoconstriction, but exacerbates tachykinin-mediated plasma extravasation.

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