Involvement of RhoA/Rho kinase signaling
in pulmonary hypertension of the fawn-hooded rat

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Running Head: RhoA/Rho kinase in fawn-hooded rat

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ABSTRACT

The fawn-hooded rat (FHR) develops severe pulmonary hypertension (PH) when raised for the first 3-4 weeks of life in the mild hypoxia of Denver’s altitude (5,280 ft). The PH is associated with sustained pulmonary vasoconstriction and pulmonary artery remodeling. Furthermore, lung alveolarization and vascularization are reduced in the Denver FHR. We have recently shown that RhoA/Rho kinase signaling is involved in both vasoconstriction and vascular remodeling in animal models of hypoxic PH. In this study, we investigated the role of RhoA/Rho kinase signaling in the PH of Denver FHR. In -toxin permeabilized pulmonary arteries from Denver FHR, the contractile sensitivity to Ca2+ was increased compared to those from sea level FHR. RhoA activity and Rho kinase I protein expression in pulmonary arteries of Denver FHR (10-week old) were higher than in those of sea level FHR. Acute inhalation of the Rho kinase inhibitor fasudil selectively reduced the elevated pulmonary arterial pressure in Denver FHR in vivo. Chronic fasudil treatment (30 mg/kg/day, from birth to 10-week old) markedly reduced the development of PH and improved lung alveolarization and vascularization in Denver FHR. These results suggest that Rho kinase-mediated sustained vasoconstriction, through increased Ca2+ sensitivity, plays an important role in the established PH, and that RhoA/Rho kinase signaling contributes significantly to the development of PH and lung dysplasia in mild hypoxia-exposed FHR.

KEYWORDS: Ca2+ sensitivity, fasudil, vascular remodeling, lung dysplasia
INTRODUCTION

The fawn-hooded rat (FHR) is a genetic strain that develops severe pulmonary hypertension (PH) when raised for the first 3 to 4 weeks of life in the mild hypoxia of Denver’s altitude (5,280 feet, P_{O_2} ~120 mmHg) but not in normoxia (sea level, P_{O_2} ~150 mmHg) (15,20,25). The PH is associated with marked pulmonary artery (PA) medial thickening, vascular rarefaction, and sustained pulmonary vasoconstriction (15,25,32). Previous studies suggest several factors are involved in the development of severe PH in FHR, including increased endothelin-1 (ET-1) production (20,30) and serotonin blood levels due to platelet storage-pool deficiency (31), reduction in lung endothelial nitric oxide synthase (eNOS) expression (16,32), and decreased pulmonary vascular density associated with reduced alveolarization (15,16,32), all of which have been implicated in the pathogenesis of human PH.

Animal and human studies of systemic vascular diseases indicate that the small GTPase RhoA and one of its target proteins, Rho kinase (ROCK), contribute importantly not only to sustained vasoconstriction by inhibiting myosin light chain phosphatase and thereby increasing the Ca^{2+} sensitivity of vascular smooth muscle cell contraction, but also to vascular remodeling by regulating various cell functions, including cell migration and proliferation and gene expression (9,28). Accumulating evidence suggests that this signaling pathway is also involved in experimental pulmonary vascular disorders. For instance, our group and others have recently shown that RhoA/ROCK signaling is involved in sustained vasoconstriction and vascular remodeling in rodent models of hypoxic PH (4,6,10,21,23). Jernigan and colleagues (12) have shown that RhoA activity and ROCKII expression are increased in chronically hypoxic rat PA, and that inhibition
of this pathway, rather than a decrease in cytosolic Ca\(^{2+}\), mediates nitric oxide (NO)-induced dilation of these hypertensive vessels. Similarly, Weigand et al. (33) have found that ROCK activity contributes substantially to ET-1-induced Ca\(^{2+}\) sensitization of hypoxia-induced hypertensive intrapulmonary arteries. Abe and colleagues (1) have reported that RhoA/ROCK signaling also plays a major role in the pathogenesis of monocrotaline-induced PH in rats. Although we have previously observed that acute administration of a ROCK inhibitor elicits pulmonary vasodilation in Denver FHR (21), the role of the RhoA/ROCK pathway in development of the severe PH in this model is uncertain. Therefore the purpose of this study was to test whether RhoA/ROCK signaling contributes to the development of PH in Denver FHR. We examined Ca\(^{2+}\) sensitivity, RhoA activity, and ROCK protein expression in FHR normotensive and hypertensive PA, and the effects of acute and chronic ROCK inhibition on pulmonary hypertension in Denver FHR. Parts of this study have been previously presented in conference abstract (22) and proceedings (17) forms.

**METHODS**

All chemicals were purchased from Sigma unless specifically stated.

*Animals* All experimental procedures were approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center (Denver, CO). Animals were obtained from a breeding colony of FHR that was previously established at our institution (25). Experiments were performed with two groups of FHR (140-250 g); the mildly hypoxic, pulmonary hypertensive group was kept at Denver’s altitude (Denver
FHR), and the normoxic, pulmonary normotensive group was raised from birth to 10 weeks in a hyperbaric chamber in which the pressure was increased to simulate sea level PO2 (sea level FHR) (16). The chamber was continuously flushed with room air to prevent accumulation of CO2, NH3, and H2O. Hyperbaric exposure was 24 h/day, except when the chamber was opened for 10 – 15 min every 2 days to remove or clean cages and replenish food and water. All rats were exposed to a 12:12-h light-dark cycle and allowed free access to standard rat food and water. At the end of the experimental protocols, all rats were euthanized by an overdose of pentobarbital (150 mg/kg, ip), and tissues were collected as described below. Wet weights of right ventricle (RV) and left ventricular wall plus septum (LV + S) were measured to assess RV hypertrophy in all rats.

\[\text{\underline{$\phi$}-toxin permeabilized pulmonary artery rings}\]  The left and right first branches of the extralobar PA rings were prepared from sea level and Denver FHR as described previously (2). Endothelium intact rings were placed on steel wires attached to force transducers and suspended in baths containing 10 ml of physiological salt solution (Earle’s balanced salt solution), and gassed with 21 % O2, 5 % CO2, and 74 % N2. Resting passive force was adjusted to an optimal tension of 0.75 (sea level FHR) or 1.5 g (Denver FHR) (determined by maximal response to 80 mM KCl). After 60-min equilibration, rings were incubated in relaxing solution for 15 min, gassed with 21 % O2 and 79 % N2, and then permeabilized as previously described (13,27) with minor modifications. Briefly, a 10 \[\mu\text{l}\]-droplet of pCa 6.3 solution containing 750 units of staphylococcus aureus \[\phi\]-toxin and 10 \[\mu\text{M}\] A23187, to deplete the sarcoplasmic reticulum of Ca2+, was placed onto each ring segment. After tension development reached a plateau, the rings were equilibrated in relaxing solution and then exposed to activating
solution. All experiments were done at room temperature. Relaxing solution consisted of (in mM) PIPES 30, sodium creatine phosphate 10, Na₂ATP 5.16, magnesium chloride 7.31, potassium methane sulfonate 74.1, K₂EGTA 1: pH is adjusted to 7.1 with KOH. In the [-toxin or activating solution, 10 mM EGTA was used, and a specified amount of CaCl₂, calculated using the Maxcheletor program (WINMAX Cv 2.00 by C Patton, http://www.Stanford.edu/~cpatton/maxc.html), was added to give the desired concentration of free Ca²⁺ ions (pCa).

Tissue preparation for western blot analyses PA segments (from 1st to 5th branch) were isolated from sea level and Denver FHR lungs for western blot analyses. Freshly isolated PA segments were equilibrated for 1 h in physiological salt solution at 37 °C to allow the tissue to recover from the mechanical effects of isolation, before they were snap frozen and kept at - 80°C for subsequent measurements.

RhoA translocation Previous reports have shown that the active form of RhoA is translocated from cytosol to membrane (5). To assess RhoA translocation (activation), each arterial sample was homogenized in lysis buffer (10 mM HEPES, 2 mM EDTA, 1 mM MgCl₂) containing protease inhibitors (Pierce). Homogenate was centrifuged at 40,000 g for 30 min and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in lysis buffer and centrifuged again (40,000 g for 15 min) to generate the membrane fraction. Equal amounts of protein (15 µg) were loaded in wells. RhoA protein in membrane and cytosolic fractions was determined by standard western blot analysis using a specific mouse monoclonal anti-RhoA antibody (1 : 250 dilution, Santa Cruz Biotechnology) and a peroxidase-labeled anti mouse IgG antibody (Vector).
Relative density of membrane to cytosolic RhoA was determined using NIH image software.

**ROCKI and II expression** To examine total expression of ROCKI and II, isolated arteries were equilibrated and then homogenized in buffer containing 0.25 M sucrose, 25 mM imidazole, 1 mM EDTA, and 1% NP-40. Homogenate was centrifuged at 10,000 g for 10 min. Equal amounts of protein (30 μg) were loaded in wells. ROCK I and II protein levels were detected by western blotting using specific anti-ROCK I and II antibodies (1: 250, 1: 1000 dilution, respectively, BD Transduction) and a peroxidase-labeled anti mouse IgG antibody. Relative density of blots was determined using NIH image software.

**Catheterized rats** Rats were anesthetized with intramuscular ketamine (100 mg/kg) and xylazine (15 mg/kg) for placement of catheters in the right jugular vein and pulmonary and right carotid arteries (20). Anesthetized rats were placed in a ventilated plastic box, and mean pulmonary (MPAP) and systemic arterial pressures (MSAP) were measured with pressure transducers. Cardiac output (CO) was determined by a standard dye-dilution method. Total pulmonary resistance was calculated by dividing MPAP by CO.

**Acute inhalation of fasudil** We tested concentration response effects of inhaled ROCK inhibitor, fasudil (Asahi Kasei Corporation) (9, 21), in catheterized Denver FHR under anesthesia. Aerosol exposure was done as previously described (21). Briefly, after baseline hemodynamic measurement, rats were exposed to aerosolized fasudil (30 and 100 mM) or vehicle (saline) generated by jet nebulizer in a small Plexiglas chamber for 5
minutes. Hemodynamic measurements were repeated 15 minutes after each 5-minute exposure and successive doses were administrated at 20-minute intervals.

**Chronic fasudil treatment**  Fasudil (30 mg/kg/day) was given to Denver FHR from immediately after birth to 10-wk old (from birth to 4-wk old through mother’s milk and thereafter by drinking water). After chronic treatment, hemodynamic measurements were made under anesthesia. We chose this dose based on previous pharmacokinetic studies (11,26) and a recent study examining its chronic effects on monocrotaline-induced PH (1). Although the exact concentration of fasudil the pups received from their mother [which received fasudil (30 mg/kg/day) by drinking water] was not determined, it has been preliminarily found that fasudil passes to mother’s milk at a concentration similar to that of blood when administered intravenously (personal communication with Dr. S-I Sato, Asahi Kasei Corp.).

**Measurements of PA medial wall thickness, arterial density, and alveolar size**

Histological changes of PA were quantified by morphometry as previously described (7). After the hemodynamic measurement, the PA was cannulated and perfused with heparinized physiological salt solution (37 °C, 20 cm H₂O pressure) to remove residual blood, and then injected with a barium-gelatin mixture (60 °C) at 74 mmHg for 3 min. The trachea was cannulated, and the lung was distended with 10 % formalin at 10 cm H₂O pressure. A block of tissue was taken from the midportion of the left lung parallel to the hilum. Paraffin sections of 5 μm thick were cut (using a standard microtome) and mounted onto Superfrost Plus Slides (Fisher Scientific Co.). Sections were stained with hematoxylin-eosin and assessed microscopically for degree of arterial wall thickness. In each section, at least 30 consecutive barium-filled arteries (50 – 100
m external diameter) were analyzed at 400. Medial wall thickness was expressed as the summation of the two points of medial thickness/external diameter 100 (%).

For the measurement of arterial density, barium filled PA were counted per high-power field (100 magnification) (15). Five fields were counted per lung.

Intra-alveolar distance was measured as the mean linear intercept (MLI) (19), which reflects alveolar size. Images of each section were captured using the 20 objective as a high resolution PICT image by a ProgRes 3008 digital camera (JenOptik; 1,928 1,450 pixel resolution) and were analyzed with the Stereology Toolbox (Davis) software. MLI was determined by dividing the total length of 42 lines drawn across the lung section by the number of interprets encountered as determined by the investigator. Twenty randomly selected fields in each section were utilized to calculate the MLI.

Statistical Analysis Values are means ± SE. Comparisons between groups were made with Student’s t-test, analysis of variance (ANOVA) with Fisher’s post-hoc test for multiple comparisons. Differences were considered significant at P<0.05.

RESULTS

RV hypertrophy The presence of PH in the Denver FHR was reflected in RV/LV + S, which averaged 0.66 ± 0.01 (n = 31) vs. 0.29 ± 0.01 (n = 17) in sea level FHR (P < 0.001).

Ca²⁺ sensitivity of PA in FHR In -toxin permeabilized PA, the Ca²⁺ concentration-contraction curve of Denver FHR was slightly but significantly left-shifted (pCa range from 6.0 to 6.5) compared to that of sea level FHR (Figure 1).
**RhoA translocation in PA** We measured RhoA protein expression in cytosol and membrane fractions separately to assess RhoA activation. Expression of membrane RhoA was significantly higher in Denver FHR hypertensive PA than in sea level FHR normotensive PA (Figure 2A and B). Membrane to cytosolic ratio of RhoA expression by densitometry was also higher in Denver FHR than in sea level FHR (Figure 2C).

**ROCKI and II expression in PA** ROCKI protein expression in PA was greater in Denver FHR than in sea level FHR (Figure 3A). ROCKII expression was not significantly increased (P = 0.15) in PA of Denver FHR compared to that of sea level FHR (Figure 3B).

**Acute hemodynamic effects of inhaled fasudil in Denver FHR** In Denver FHR with PH, acute inhaled fasudil caused a marked reduction in MPAP (from 70 ± 5 to 58 ± 4 and 54 ± 9 mmHg by 30 and 100 mM, respectively) with no effects on MSAP or cardiac output (Figure 4). Calculated total pulmonary resistance (TPR) was reduced significantly by inhaled fasudil (from 4,232 ± 552 to 2,923 ± 389 mmHg/l/min by 100 mM).

**Chronic effects of fasudil in Denver FHR** Denver FHR had much higher MPAP than sea level FHR (49 ± 6 vs. 26 ± 1 mmHg, P<0.05) (Figure 5A). Chronic in vivo fasudil treatment markedly reduced the development of PH in Denver FHR (MPAP; 49 ± 6 vs. 33 ± 2 mmHg, P<0.05) (Figure 5A) with no effect on MSAP (Figure 5B). Similarly, RV/LV+S in Denver FHR was much higher than that in sea level FHR, and this RV hypertrophy was markedly attenuated by chronic fasudil treatment (RV/LV+S ratio; 0.65 ± 0.03 vs. 0.43 ± 0.02 %, P<0.05) (Figure 5C).
Consistent with previous studies (15,19,25), Denver FHR showed increased PA medial wall thickness (Fig 6A and 6C) and alveolar size (reflected by increased MLI) (Fig. 6B and 6D) and decreased small barium-filled PA vessel count (Fig. 6B and 6E) compared to sea level FHR. These abnormalities were also significantly improved by the chronic fasudil treatment (Figure 6A-E).

**DISCUSSION**

The major findings of this study were that: in pulmonary hypertensive Denver FHR: 1) Ca\(^{2+}\) sensitivity, membrane/cytosol ratio of RhoA (an indirect measure of Rho A activation), and ROCKI expression of PA were greater than those in sea level FHR; 2) acute inhalation of the Rho kinase inhibitor fasudil caused a dose-dependent selective pulmonary vasodilation; and 3) chronic fasudil treatment from the time of birth until 10 wks of age reduced the development of PH and improved alveolarization and vascularization.

Recent studies have indicated that RhoA/ROCK signaling contributes to the pathogenesis of chronic hypoxia- (4,6,10,12,21,23,33) and monocrotaline- (1) induced PH in rodents. In contrast to other strains of rat, the FHR develops severe PH in the mild hypoxia of Denver’s altitude but not in the normoxia of sea level (15,20,25). Although the exact mechanism of PH in Denver FHR is not fully understood, several factors that are closely related to the pathogenesis of human PH are implicated in the development and maintenance of severe PH in this strain. For example, mild hypoxia-exposed FHR lungs have elevated expression and production of ET-1 compared to normal Sprague
Dawley rat or sea level FHR lungs (20,30). Serotonin may play a role, since the FHR has increased plasma serotonin levels due to an inherited platelet storage-pool deficiency (31). Although a report by Nagaoka et al. questions whether platelet storage-pool deficiency alone is sufficient to cause the PH of this strain (20), a recent study has shown that expression of lung serotonin 1B receptor and serotonin-mediated PA contraction are greater in FHR than in Sprague Dawley rats (18), suggesting an increased pulmonary vascular responsiveness to serotonin in FHR.

RhoA can be activated by various vasoconstrictors, including ET-1 and serotonin, whose receptors are coupled to G proteins, and plays a central role in the stimulation of ROCK and thereby inhibition of myosin light chain phosphatase activity and increased Ca\(^{2+}\) sensitization (24,29). We found in this study that membrane/cytosol ratio of RhoA and Ca\(^{2+}\) sensitivity of PA in Denver FHR were higher than those in sea level FHR. In addition, acute in vivo ROCK inhibition effectively reduced the sustained pulmonary vasoconstriction in Denver FHR. These results suggested that RhoA was activated in PA, possibly by increased ET-1 and serotonin levels and/or responsiveness and deficient NO production (12,32), which lead to ROCK-mediated increased Ca\(^{2+}\) sensitivity of contraction and sustained pulmonary vasoconstriction in the mildly hypoxic pulmonary hypertensive FHR. The acute reduction in MPAP by a ROCK inhibitor indicated that active vasoconstriction, in addition to the pulmonary vascular remodeling and lung dysplasia (reduced vascularity), played a significant role in the elevated pulmonary artery pressure in 10-wk old Denver FHR.

We also observed that ROCKI protein expression was greater in PA of Denver FHR than in those of sea level FHR. This is similar to the findings in chronically
hypoxia-exposed Sprague Dawley rat PA in which both RhoA activity and ROCKII expression were increased compared to normoxic PA (6,12). Although little is known about the regulation of ROCK expression, we speculate that in hypertensive FHR lungs increased stimulation of vascular smooth muscle by ET-1 and serotonin, and perhaps other unidentified inflammatory mediators, might activate nuclear factor-κB (34) which leads to upregulation of ROCK expression (8).

We also examined if chronic ROCK inhibition would attenuate the development of severe PH in Denver FHR. We found that 10-wk treatment of Denver FHR with fasudil (from birth to 10-wk old) markedly reduced the increases in MPAP, RV/LV+S, and PA medial wall thickness. These results indicated that, similar to the findings in the hypoxia- (4,6,10) and monocrotaline-induced (1) PH models, ROCK activity contributed importantly to the development of severe PH in the FHR model. The chronic effect of ROCK inhibitors on PH may be attributable to their ability to reduce sustained abnormal pulmonary vasoconstriction, as shown in this and previous studies (10,21,23) and to attenuate PA remodeling by inhibiting proliferation and inducing apoptosis of pulmonary vascular smooth muscle cells (1).

In addition to inhibition of PH, chronic fasudil treatment improved the lung dysplasia in the Denver FHR (i.e., fasudil decreased alveolar size and increased pulmonary vascular density), suggesting that ROCK activation might also play a significant role in inhibiting lung development after birth. Further experiments are necessary to elucidate whether ROCK activation inhibits postnatal lung vascularization and alveolarization directly or through modulation of some other mediators, such as eNOS (3,14).
In summary, this study demonstrated that RhoA/ROCK signaling was involved in the elevated pulmonary vascular tone, development of PH, and lung dysplasia in mild hypoxia-exposed FHR. Results of this and previous studies (1,4,6,10,21,23) indicate that RhoA/ROCK signaling plays key roles in the development of at least three different animal models of PH (i.e., Denver FHR, chronic hypoxia-, and monocrotaline-induced PH models). We thus propose that activation of this intracellular signaling pathway might be a common factor in the development of various forms of PH independent of their pathogenesis, and that RhoA/Rho kinase signaling might be a promising target for the treatment of human PH.

ACKNOWLEDGEMENTS

This study was supported by grants from the National Institute of Health (HL 14985 and HL 07171) and the American Heart Association (SDG 0335208N and SDG 0330199N). The authors thank Asahi Kasei Corporation (Tokyo, Japan) for generous supply of fasudil.
REFERENCES


FIGURE LEGENDS

Figure 1. Concentration-response curves to calcium in ⧲-toxin permeabilized pulmonary arteries from sea level (normotensive) and Denver (hypertensive) FHR. Values are means ± SE; n = 4 each. * P < 0.05 vs. Denver FHR.

Figure 2. A: RhoA protein levels of pulmonary arteries from sea level (SL) and Denver (D) FHR in cytosolic and membrane fractions. B: Densitometric analysis of
RhoA protein levels. C: Membrane to cytosol ratio of RhoA. Values are means ± SE; n = 4 each. * P < 0.05 vs. SL.

**Figure 3.** Protein levels of ROCK I (A) and II (B) in pulmonary arteries from sea level (SL)- and Denver (D)-FHR. Upper panels show immunoblots and lower panels show densitometric assessments. Values are means ± SE; n = 4 each. * P < 0.05.

**Figure 4.** Concentration response effects of inhaled fasudil on mean pulmonary arterial pressure (MPAP; upper left), mean systemic arterial pressure (MSAP; upper right), cardiac output (lower left), and total pulmonary resistance (TPR; lower right) in Denver FHR. Values are means ± SE; n = 5 for fasudil and n = 4 for vehicle. * P < 0.05 vs. BL (baseline).

**Figure 5.** Mean pulmonary arterial pressure (MPAP; A), mean systemic arterial pressure (MSAP; B), and right ventricle (RV)/left ventricle plus septum (LV + S) weight ratio (C) in sea level (SL)-, Denver (D)- and fasudil treated D-FHR (D + FAS). Values are means ± SE; n = 5 - 9. * P < 0.05.

**Figure 6.** Hematoxylin eosin stained representative barium-filled small pulmonary arteries (60–70 μm in external diameter) (A) and lung sections (B) from sea level (SL)- (left), Denver (D)- (center), and fasudil-treated Denver (D + FAS)- (right) FHR. (C); % Medial wall thickness of 50 ~ 100 μm diameter pulmonary arteries. (D); Mean linear
intercept. (E); Pulmonary arterial density. Values are means ± SE; n = 4 for SL; n = 4 for D; n = 5 for D + FAS. * P < 0.05.
Figure 1

![Graph showing relative tension (%) vs. pCa for Denver FHR and Sea level FHR.](image-url)
Figure 2

A

RhoA

Cytosol

Membrane

SL  D

SL      D             SL     D

Cytosol          Membrane

B

Arbitrary Units

SL  D  SL  D

C

Membrane/Cytosol Ratio

SL  D

*
Figure 3

A  ROCK I

B  ROCK II

Arbitrary Units

Arbitrary Units

SL  D  SL  D

*
Figure 4

- **Δ MPAP (mmHg)**
  - Vehicle: BL 30 100
  - Fasudil: BL 30 100
  - BL 30 100

- **Δ MSAP (mmHg)**
  - Vehicle: BL 30 100
  - Fasudil: BL 30 100

- **CARDIAC OUTPUT (ml/min)**
  - Vehicle: BL 100
  - Fasudil: BL 100

- **TPR (mmHg/l/min)**
  - Vehicle: BL 100
  - Fasudil: BL 100

*Significantly different from baseline.*
Figure 5

A

\[ \text{n.s} \]

\[ \ast \ast \ast \]

\[
\begin{array}{ccc}
\text{SL} & \text{D} & \text{D+ FAS} \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{MPAP (mmHg)} \\
\end{array}
\]

B

\[
\begin{array}{ccc}
\text{SL} & \text{D} & \text{D+ FAS} \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{MSAP (mmHg)} \\
\end{array}
\]

C

\[
\begin{array}{ccc}
\text{SL} & \text{D} & \text{D+ FAS} \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{RV/LV+S (%)} \\
\end{array}
\]
Figure 6

A

SL  D  D + FAS

B

C  Medial Wall Thickness (%)  D  Mean Linear Intercept (μm)  E  Vessel Counts

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*  *  *

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