

Factors influencing RhoA protein distribution in the nucleus

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Abstract. The aim of the present study was to observe the influence of various factors on the nuclear distribution of the RhoA protein in the SGC-7901 human gastric cancer cell line. Immunofluorescence microscopy was used to detect the localization of the RhoA protein, and Western blotting was used to determine the quantity of RhoA in the nucleus, cytosol and membrane. The results showed that H₂O₂-mediated damage and a lipopolysaccharide (LPS)-mediated inflammatory reaction caused the translocation of RhoA from the cytosol toward the nucleus. A P38 mitogen-activated protein kinase (MAPK) inhibitor effectively hindered the LPS-triggered translocation of RhoA into the nucleus at the initial stage. Furthermore, the microtubule-targeted anticancer drug Taxol triggered the translocation of RhoA from the nucleus toward the cytosol and membrane, and Lysophosphatidic acid (LPA) enhanced this translocation. A protein modification inhibitor and a nucleus export inhibitor had no obvious effect on RhoA distribution in the nucleus. The results revealed that the distribution of RhoA protein in the nucleus was influenced by factors related to cell activities but was not affected by the modification of the protein. The translocation of RhoA into the nucleus was not dependent on the active nuclear import system.

Introduction

RhoA, with a molecular mass of 21 kDa, is the most extensively studied member of the Rho GTPase family which belongs to the Ras superfamily of small G proteins. It has been reported to regulate many biological activities including the formation of stress fibers, gene transcription, membrane transport and focal adhesions and tumor progression (1-4). RhoA cycles between a GDP-bound inactive state and a GTP-bound active state (5). These two forms can be converted by GDP/GTP exchange or GTPase reactions. The former and latter reactions are regu-

lated by GDP/GTP-exchange protein and GTPase-activating protein, respectively (6-8).

Recent research data have shown that various small G-proteins have cytoplasmic-nuclear dual distribution, and the nuclear translocation of small G protein is usually accompanied by the activation of the protein (9-12). For example, nuclear localized Ras protein exists in a GTP-bound form and this localization is possibly associated with malignancies of the cell (11). For a long time, an abundance of study data (13-15) have indicated that the RhoA protein is mainly distributed in the cytoplasm and on the membrane, but particularly in cytoplasm. However, our previous study (16) indicated that the distribution of RhoA in SGC-7901 cells was not only in the membrane and the cytoplasm but also in the nucleus. Recently, we substantiated the nuclear localization of RhoA in different cancer tissues and cell lines (17). Regarding the effect of the activation of RhoA on its localization and translocation in cells, our previous study (16) revealed that when the activity of RhoA increased, the distribution of RhoA on the membrane and in the nucleus also increased, which indicates that the nuclear distribution of RhoA has a potential role in regulating cellular activity.

To further study the mechanism of the nuclear distribution of RhoA and the function of the RhoA protein in the nucleus, this study was designed to observe the effect of several treatments including protein modification, cellular damage, inflammation and Taxol treatment on the nuclear distribution of RhoA.

Materials and methods

Cell lines. Human gastric cancer cell line SGC-7901 was obtained from the Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China).

Reagents. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). Newborn calf serum (NBCS) was from Minhai Bio-Engineering C (Lanzhou, China). Mouse monoclonal antibody against RhoA was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against glyceraldehyde phosphate dehydrogenase (GAPDH) was from Kangcheng (China). The horseradish peroxidase (HRP)-conjugated secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Simvastatin, 5-aza-2'-deoxycytidine (5-Aza-Cdr), dexamethasone, lipopolysaccharide (LPS), SB203580 and leptomycin B (LMB) were from Sigma (St. Louis, MO, USA). Electrochemiluminescence (ECL) reagents were from

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Abbreviations: LPA, Lysophosphatidic acid; LMB, leptomycin B; LPS, lipopolysaccharide; LDH, Lactate dehydrogenase.

Key words: RhoA, nucleus, damage, inflammation, Taxol

Amersham Biosciences (Buckinghamshire, UK). Taxol was from Calbiochem. The reagent for determining Lactate dehydrogenase (LDH) was from the Jiancheng Biology Engineering Institute (Nanjing, China).

Cell culture. The cells were cultured in DMEM supplemented with 10% NBCS and maintained at 37°C in a humidified atmosphere of 5% CO₂. The medium was replaced every 2 days, and the cells were subcultured at confluence.

Immunofluorescence microscopy. The cells grown on coverslips were fixed with freshly prepared paraformaldehyde (40 g/l in PBS) for 30 min. After being penetrated with 30 ml/l Triton X-100 and blocked with 30 g/l bovine serum albumin (BSA), the cells were incubated with the primary antibody at 4°C overnight (o/n) and then with fluorescein isothiocyanate (FITC) or a tetra-rhodamine isothiocyanate (TRITC)-conjugated second antibody for 1 h at room temperature (RT), with three washes after each incubation. The distribution of the target protein of the cells was analyzed by fluorescence microscopy.

Preparation of the nuclear protein. According to a previously described method (18), 1-3 million cells were washed with 10 ml of Tris-buffered saline (TBS) and pelleted by centrifugation at 1500 x g for 5 min. The pellet was resuspended in 1 ml of TBS, transferred into an Eppendorf tube and pelleted again by spinning for 15 sec in a microcentrifuge. The cell pellet was resuspended in 400 µl of cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF) by gentle pipetting. The cells were allowed to swell on ice for 15 min, after which 25 µl of NP-40 (0.5%) was added and the tube was thoroughly mixed for 10 sec. The homogenate was centrifuged for 30 sec and the supernatant containing cytoplasm and RNA was saved for future use if needed. The nuclear pellet was resuspended in 50 µl of ice-cold buffer C (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM each of DTT, EDTA, EGTA and PMSF). The tube was mixed thoroughly and placed on a rotatory shaker for 15 min. The nuclear extract was centrifuged at 11,000 x g for 5 min (microcentrifuge). The supernatant containing the proteins from the nuclear extract was removed carefully to a fresh tube. The protein was measured in the nuclear extract, which was then aliquoted in several tubes and stored at -80°C.

Western blotting. Sample proteins were run on 10% SDS-polyacrylamide gels, and were blotted onto polyvinylidene difluoride (PVDF) membranes. The PVDF membrane was blocked with 3% (w/v) BSA in TBS-T for 1 h at RT. Membranes were incubated with the primary antibody at 4°C o/n, and with the secondary antibody for 1 h at RT, with three washes after each incubation. Electrochemiluminescence reagents were used to show the positive bands on the membrane. The bands on film were analyzed with GeneSnap/Gene Tool software from Syngene (Cambridge, UK).

Measure of LDH activity. The total LDH activity was measured by colorimetry.

Statistical analysis. Data are expressed as the mean ± SE. Statistical analysis was performed using SPSS 13.0 edition

program for ANOVA with the Scheffé multiple comparison test. A probability value of <0.05 was considered statistically significant.

Results

Effect of RhoA modification on its distribution within the nucleus. Research data has indicated that modification of small G proteins has an important effect on their distribution in the nucleus. In order to elucidate whether the modification of the RhoA protein has an effect on its nuclear distribution, inhibition of the modification of prenylation and methylation was achieved by applying the prenyltransferase inhibitor, simvastatin, and the methyltransferase inhibitor, 5-Aza-Cdr, respectively. The effect of the inhibition was observed by immunofluorescence microscopy. The results showed that neither the inhibition of prenylation nor the inhibition of methylation had any significant effect on the distribution of RhoA within the nucleus in the human gastric cancer cell line SGC-7901 (Fig. 1), which suggested that the cytoplasm-nucleus transfer of RhoA was not affected by modifications to the RhoA protein.

Distribution of RhoA within the nucleus in damaged cells. To observe the distribution of RhoA upon cellular damage, human gastric cancer cell line SGC-7901 was treated with H₂O₂. Damage to the cells was confirmed by detecting LDH activity in the culture medium (Fig. 2A). Immunofluorescence microscopy showed that the amount of RhoA increased in the damaged cells, and more RhoA was translocated to the nucleus (Fig. 2B).

Effect of Taxol on RhoA distribution within the nucleus. Taxol is an antitumor drug targeting the microtubule structure of tumor cells. To observe the effect of microtubule structure dynamics on the distribution of RhoA, human gastric cancer cell line SGC-7901 was treated with Taxol, and Taxol combined with Lysophosphatidic acid (LPA). Immunofluorescence microscopy showed that Taxol triggered the translocation of RhoA from the nucleus towards the cytosol and membrane, and LPA enhanced this translocation (Fig. 3). These results suggest that the distribution of RhoA is related to the microtubule structure in cells.

Effect of LPS on the distribution of RhoA within the nucleus. To observe the inflammatory effect on the distribution of RhoA, human gastric cancer cell line SGC-7901 was treated with LPS to mimic an inflammatory effect. Results of the immunofluorescence staining (Fig. 4A) and Western blotting (Fig. 4B and C) showed that, in LPS-treated cells, the nuclear RhoA increased significantly, the translocation of RhoA from the cytosol toward the nucleus was obvious, and some cells even showed that without RhoA distribution in the cytosol, the nucleus moved largely towards one side of the cell, resembling a 'shining ring'.

Effect of P38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580) on the translocation of RhoA within the nucleus triggered by LPS. To further confirm the inflammatory effect on RhoA distribution, human gastric cancer cell line SGC-7901 was treated with a P38 MAPK inhibitor

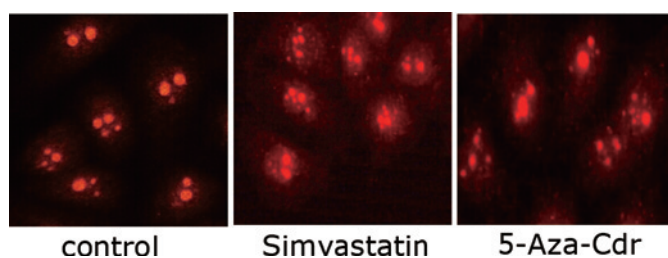


Figure 1. Effect of RhoA modification on its distribution within the nucleus. Human gastric cancer cell line SGC-7901 was treated with prenyltransferase inhibitor simvastatin (0.125 μ l/ml) and methyltransferase inhibitor 5-Aza-Cdr (0.25 μ l/ml), respectively, for 24 h, and the cells were then immunofluorescently stained with a specific mAb against RhoA. The results revealed that the inhibition of neither prenylation nor methylation had any significant effect on the distribution of RhoA within the nucleus (x400).

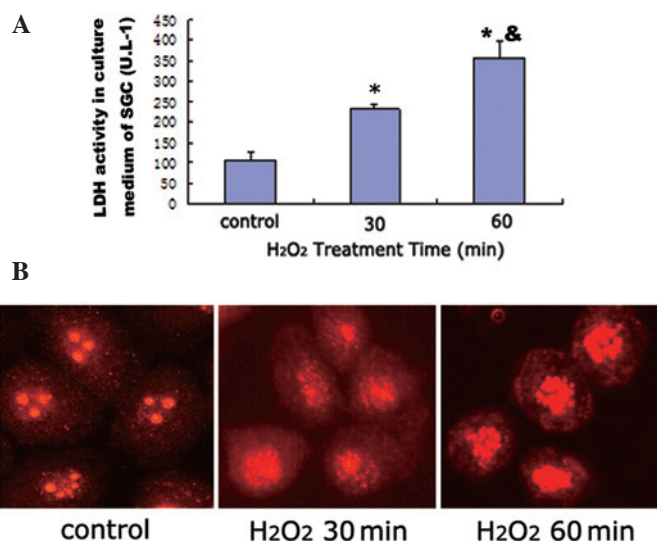


Figure 2. Distribution of RhoA within the nucleus in damaged cells. Human gastric cancer cell line SGC-7901 was treated with 1 M H₂O₂, and the LDH activity in the culture medium was measured at different time points (0, 30 and 60 min). (A) LDH activity was analyzed by SPSS 13.0 Tool software. Each bar represents the mean \pm SE obtained from five independent experiments. Bar 1, expression of LDH activity of the control group; SGC-7901 cells were not treated with H₂O₂; bar 2, expression of LDH activity of SGC-7901 cells treated with H₂O₂ for 30 min; bar 3, expression of LDH activity of SGC-7901 cells treated with H₂O₂ for 60 min. *P<0.05, compared with bar 1; [&]P<0.05, compared with bar 2. (B) Results of the immunofluorescent staining with a specific mAb against RhoA, indicating that the total amount of RhoA and nuclear RhoA increased significantly as cells underwent damage at 30 and 60 min after H₂O₂ treatment (x400).

(SB203580) 1 h before LPS treatment. Immunofluorescence microscopy showed that after 3 h, the P38 MAPK inhibitor SB203580 effectively hindered the LPS-triggered translocation of RhoA into the nucleus, which was represented by increased distribution of RhoA within the cytosol, resembling 'dispersive sand'; while after a prolonged treatment of LPS, significant translocation of RhoA from the cytosol into the nucleus was observed (Fig. 5).

Effect of nucleus-export inhibitor on the distribution of RhoA within the nucleus. To study the molecular mechanism of RhoA transportation to the nucleus, human gastric cancer cell line SGC-7901 was treated with the nucleus export inhibitor, lepto-

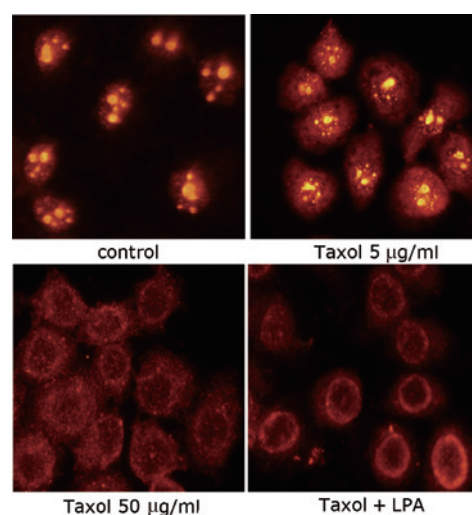


Figure 3. Effect of Taxol on RhoA distribution within the nucleus. Human gastric cancer cell line SGC-7901 was treated with 5 or 50 μ g/ml Taxol or 5 μ g/ml Taxol combined with 1 μ M LPA, respectively, for 6 h. Results of the immunofluorescent staining with a specific mAb against RhoA showed that Taxol triggered the translocation of RhoA from the nucleus toward the cytosol and the membrane, and that LPA enhanced this translocation (x400).

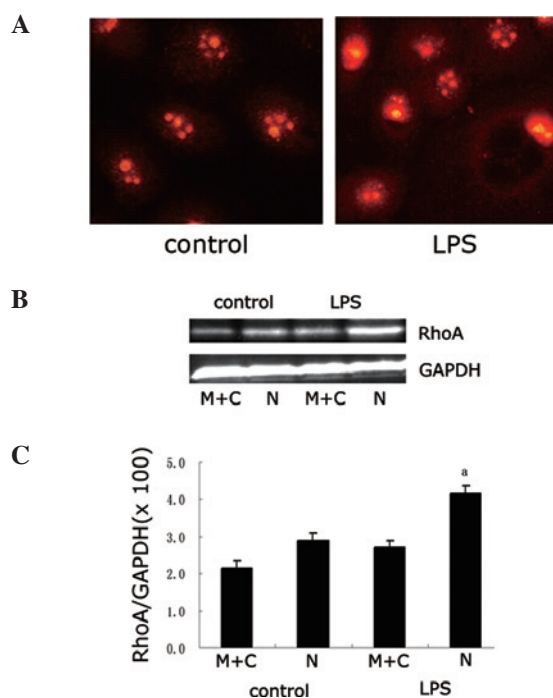
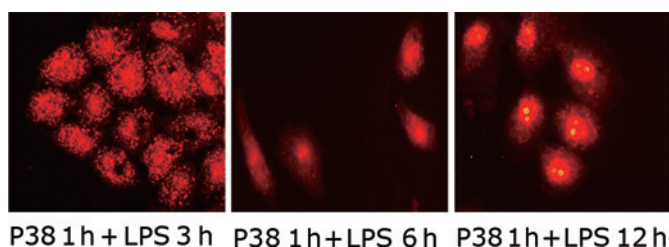
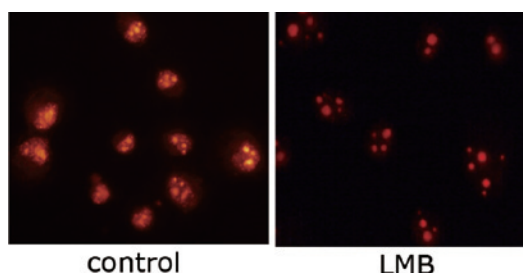


Figure 4. Effect of LPS on the distribution of RhoA within the nucleus. Human gastric cancer cell line SGC-7901 was treated with LPS (1 μ g/ml) for 12 h. (A) Results of the immunofluorescent staining with a specific mAb against RhoA revealed that the translocation of RhoA from the cytosol toward the nucleus was significant. In some cells even showing a lack of RhoA in the cytosol, the nucleus moved largely towards one side of the cell, resembling a 'shining ring' (x400). (B) Two groups of SGC-7901 cells were lysed, and the membrane and cytosol (M+C), and the nuclear (N) fractions were obtained. Expression of RhoA and GAPDH in each fraction was detected by Western blotting. (C) Western blotting results were analyzed by SPSS 13.0 Tool software, and the volume ratio of RhoA/GAPDH input was calculated and presented. Each bar represents the mean \pm SE obtained from three independent experiments. Bar 1, expression of RhoA in the M + C fraction of the control group; SGC-7901 cells were not treated with LPS; bar 2, expression of RhoA in the N fraction of the control group; bar 3, expression of RhoA in the M + C fraction of SGC-7901 cells treated with LPS for 12 h; bar 4, expression of RhoA in the N fraction of SGC-7901 cells treated with LPS for 12 h. ^aP<0.05, compared with bar 2.



P38 1h+LPS 3 h P38 1h+LPS 6 h P38 1h+LPS 12 h

Figure 5. Effect of the P38 MAPK inhibitor (SB203580) on the translocation of RhoA within the nucleus triggered by LPS. Human gastric cancer cell line SGC-7901 was cultured in serum-free medium for 24 h, and the cells were then divided into 4 groups; one group was set as the control and the other 3 groups were treated with 2 μ g/ml P38 MAPK inhibitor (SB203580) for 1 h before treatment with LPS (1 μ g/ml). The samples were collected at 3, 6 and 12 h, respectively, and immunofluorescent staining was carried out with a specific mAb against RhoA. The results revealed that after 3 h, the inhibitor SB203580 effectively hindered the LPS-triggered translocation of RhoA into the nucleus, which was represented by increased distribution of RhoA within the cytosol, resembling 'dispersive sand'; while after a more prolonged treatment of LPS, significant translocation of RhoA from the cytosol into the nucleus was observed (x400).



control LMB

Figure 6. Effect of a nucleus-export inhibitor on the distribution of RhoA within the nucleus. Human gastric cancer cell line SGC-7901 was treated with nucleus export-inhibitor leptomycin B (LMB, 20 ng/ml) for 12 h. The results of the immunofluorescent staining with a specific mAb against RhoA reflected the existence of RhoA within the nucleus, indicating no significant effect of the nucleus export-inhibitor on RhoA distribution within the nucleus (x400).

mycin B (LMB). Immunofluorescence microscopy showed that RhoA still existed within the nuclei in LMB-treated cells, indicating no significant effect of LMB on RhoA distribution within the nucleus (Fig. 6).

Discussion

Research data have shown that modification of some small G-proteins is related to the distribution of the proteins within cells. For example, members of the Ras superfamily are subject to post-translational modifications and the spectrum of modifications depends on the composition of the carboxyl terminus. In the case of Ha-Ras, the protein is subject to prenylation, proteolysis, carboxylmethylation and S-acylation/palmitoylation (19). The prenylated Ha-Ras in the cytoplasm is then transferred by the endomembrane system to the plasma membrane (20). Research data also suggest that prenylated Rab acceptor protein (PRA1) binds prenylated small G-proteins to act as an escort protein for the GTPases (21). Prenylation is followed by proteolysis of the carboxyl-terminal tripeptide and methylation of the newly generated carboxyl-terminal amino acid in the endoplasmic reticulum (ER) (19,21). Without

prenylation, the subsequent processing events do not occur, and the Ras proteins remain soluble (19,21). Statins may inhibit the prenylation of protein, disorder or destroy the cell framework, and remodel activation of many nuclear transfer genes by the signaling pathways of JNK/ SAPK and P38 MARK. Our study revealed that neither the inhibition of prenylation nor the inhibition of methylation had any significant effect on the distribution of RhoA within the nucleus. This suggests that the cytoplasmic-nuclear transfer of RhoA is not affected by modifications.

Taxol, an antimicrotubule agent, has the ability to destroy karyokinesis of cancer cells by strengthening the aggregation of canaliculus protein and inhibiting division, as well as the ability to inhibit and kill cancer cells (22-24). It has previously been shown to be very effective against ovarian and breast cancer, and recent clinical trials have shown that Taxol may also be a useful agent in the treatment of non-small cell lung cancer, head and neck cancer and various other forms of the disease (25). Taxol treatment may cause defects in mitotic spindle assembly, chromosome segregation and cell division. The ability of Taxol to inhibit spindle function is generally attributed to its suppression of microtubule dynamics. Our results showed that Taxol hindered the translocation of RhoA into the nucleus. On one hand, this suggests that the distribution of RhoA in the nucleus is related to the microtubule structure in the cells. On the other hand, due to the extreme importance of microtubules in the process of mitosis and tubulin being one of the most important targets for new anticancer drugs (26), RhoA may be a novel target for the diagnosis and therapy of tumors.

Lipopolysaccharide (LPS) is a primary factor which induces inflammatory diseases. When stimulated by bacterial LPS, many intracellular signaling pathways are activated, leading to the generation of nuclear factor NF- κ B, which in turn promotes pro-inflammatory cytokine production and release (27). Caplan *et al* (28) reported the up-regulation of MD-2, one of the bacterial LPS co-receptors, in patients with neonatal necrotizing enterocolitis. Inflammatory mediators released during acute and chronic diseases can activate multiple intracellular signals inducing cascades of the MAPK signaling transduction pathway. MAPK plays a significant role in the recruitment of leukocytes to the sites of inflammation (29). The present study showed that LPS promoted the translocation of RhoA from the cytosol toward the nucleus, and that a P38 MAPK inhibitor effectively hindered the LPS-triggered translocation of RhoA into the nucleus. This suggests that nuclear RhoA may play various roles in the inflammatory reaction. The possible mechanism still requires further elucidation.

This study also confirmed that more RhoA was distributed in the nucleus of damaged cells. Research data have indicated that when the cell encounters oxidation stress damage, expression of some proteins may increase, and the proteins may be rapidly transported into the nucleus to congregate there (30). Generally, stimuli promoting protein expression are also able to promote their intracellular movement, most of which are cytoplasm-to-nucleus translocations (31). In response to stress conditions or other stimuli, such as hypoxia, hypoxia, mechanical stress, cAMP, hypotonic media, vasoactive agents and inflammation, several cell types release ATP via either a non-lytic or a lytic mechanism from necrosis and

apoptosis, reaching high local concentrations (32,33). High ATP-mediated metabolic stimuli may induce quick cytoplasm to nucleus translocation of the protein. On the other hand, when the tissue cells in the body undergo pathogeny, they induce certain inflammatory factors or media, such as TNF- α (34). These inflammatory and damage factors are also likely to induce the translocation of RhoA into the nucleus. However, the exact mechanism which induces the nuclear distribution of RhoA is still unknown and warrants further study.

In order to enter the nucleus, proteins larger than ~60 kDa generally require a specific nuclear localization signal (NLS) (35). Protein molecules can bind with transporting protein such as importin via NLS and enter the nucleus through an ATP-dependent process (36). However, the molecular mass of RhoA is 21 kDa and there is no NLS in its protein structure. Moreover, treatment with a nucleus-export inhibitor had no effect on the nuclear distribution of RhoA. Thus, we propose that the molecular mechanism of RhoA protein transportation into the nucleus was not through an active transportation pathway, but through a diffusion pathway.

Acknowledgements

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