Biophysical Chemistry 211 (2016) 1–8

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Enhanced dynamic instability of microtubules in a ROS free inert environment

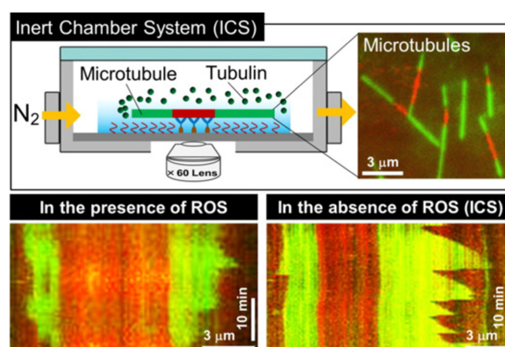
Md. Sirajul Islam [a], Arif Md. Rashedul Kabir [b], Daisuke Inoue [b], Kazuki Sada [a,b], Akira Kakugo [a,b,*]

[a] Graduate School of Chemical Sciences and Engineering, Hokkaido University, Sapporo 060-0810, Japan
[b] Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

HIGHLIGHTS

- Dynamic instability of microtubules (MTs) was studied in the presence and absence of reactive oxygen species (ROS).
- ROS significantly altered the dynamic instability of MTs.
- MTs showed enhanced dynamic instability in ROS free environment.
- ROS free environment ensured prolonged observation of dynamic instability of MTs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 October 2015
Received in revised form 19 November 2015
Accepted 22 November 2015
Available online 17 December 2015

Keywords:
Microtubule
Tubulin
Dynamic instability
Total internal reflection fluorescence microscopy
Reactive oxygen species
Oxidative stress

ABSTRACT

Reactive oxygen species (ROS), one of the regulators in various biological processes, have recently been suspected to modulate microtubule (MT) dynamics in cells. However due to complicated cellular environment and unavailability of any *in vitro* investigation, no detail is understood yet. Here, by performing simple *in vitro* investigations, we have unveiled the effect of ROS on MT dynamics. By studying dynamic instability of MTs in a ROS free environment and comparing with that in the presence of ROS, we disclosed that MTs showed enhanced dynamics in the ROS free environment. All the parameters that define dynamic instability of MTs *e.g.*, growth and shrinkage rates, rescue and catastrophe frequencies were significantly affected by the presence of ROS. This work clearly reveals the role of ROS in modulating MT dynamics *in vitro*, and would be a great help in understanding the role of ROS in regulation of MT dynamics in cells.

© 2015 Published by Elsevier B.V.

1. Introduction

Microtubules (MTs) are filamentous biopolymers formed by the polymerization of α,β-tubulin dimers and play significant roles in a wide range of cellular activities [1]. MTs are known to exhibit an intrinsic non-equilibrium behavior named 'dynamic instability', which has been reported to be essentially important in cell division, cell morphogenesis and directed cell motility, *etc.* [2,3]. In the dynamic instability the two ends of the MTs, one of which is termed as plus end and the other one as minus end, abruptly switch between phases of growth and shrinkage [4]. In cells, the dynamic behavior of MTs including their dynamic instability is strongly regulated by a number of factors including interaction with MT associated proteins (MAPs) [5,6] and other MT interacting proteins [7–9] and is influenced by different anti-mitotic

* Corresponding author at: Graduate School of Chemical Sciences and Engineering, Hokkaido University, Sapporo 060-0810, Japan.
E-mail address: kakugo@sci.hokudai.ac.jp (A. Kakugo).

http://dx.doi.org/10.1016/j.bpc.2015.11.003
0301-4622/© 2015 Published by Elsevier B.V.

agents [10,11]. Understanding the intracellular regulation of MT dynamics is of utmost importance for perceiving the cellular processes. In recent years, *in vitro* studies using MTs assembled from purified tubulins have been promising in disclosing various features of isolated MTs, which were helpful for understanding the intrinsic properties of MTs in cells [12–14]. Likewise *in vitro* studies have also played an indispensable role in clarifying the correlations between regulation of MT dynamics and its relation to the cellular functions, which was difficult to understand directly from the *in vivo* studies due to complicated cellular environment [15,16]. Recently, accumulating evidences have just started to shed light on the role of another factor *i.e.*, reactive oxygen species (ROS), an essential regulator in various biological processes, in suppressing the MT dynamics inside cell [17–20]. However, no *in vitro* study has been carried out so far that could reveal the role of ROS in altering the dynamic behavior of MTs and clarify it quantitatively, which is critically important for understanding the perturbation of MT dynamics by ROS in cells.

In this work, by demonstrating simple *in vitro* investigations, we have revealed the role of ROS in altering the dynamics of MTs. We have studied the dynamic instability of MT *in vitro* using the recently developed inert chamber system (ICS) that keeps the dissolved oxygen (DO) concentration less than 0.1 ppm in the system and maintains a ROS free environment during investigation [21–24]. In parallel, we studied the dynamic instability of MTs *in vitro* by employing the conventional scavenger system *i.e.*, a mixture of glucose, glucose oxidase and catalase. Although a scavenger system can initially reduce the DO concentration in the experimental system, the DO cannot be completely removed out rather rises gradually with time (˜0.1–0.15 ppm) [21]. The increased DO concentration in the conventional scavenger system is responsible for generating ROS under a total internal reflection fluorescence (TIRF) microscopy (a widely used tool for studying MT dynamics), thereby was considered a means for studying the effect of ROS on the MT dynamics [21,25–27]. Finally comparison of growth and shrinkage rates, frequency of rescue and catastrophe of MTs obtained in the ROS free environment and in the presence of ROS *i.e.*, in the conventional scavenger system confirmed the role of ROS in modulating the MT dynamics *in vitro*, where a significant level of enhancement of MT dynamics was observed in the ROS free environment. The growth and shrinkage rates of MTs were found much higher in the ROS free environment than that obtained in the presence of ROS. Moreover, the frequencies of rescue and catastrophe of MTs were also significantly different in the ROS free environment compared to that observed in the presence of ROS. This work is the first report that clearly unveils the role of ROS in altering the MT dynamics *in vitro*, and hence would be a big aid in accounting for the regulation of MT dynamics by ROS in cells. At the same time, our results emphasize the importance of the employment of a ROS free environment in the *in vitro* study of dynamic instability of MTs, which now appears a prerequisite for obtaining the intrinsic dynamic behavior of MTs.

2. Materials and methods

2.1. Tubulin preparation and labeling

Tubulin was purified from porcine brain using a high-concentration PIPES buffer (1 M PIPES, 20 mM EGTA, and 10 mM $MgCl_2$; pH adjusted to 6.8 using KOH). The high-concentration PIPES buffer and BRB80 buffer (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$; pH adjusted to 6.8 using KOH) were prepared using PIPES from Sigma [28]. The tubulin purified following the protocol of two cycles of polymerization and depolymerization is MAP-free [28].

Rhodamine-labeled tubulin was prepared using tetramethylrhodamine succinimidyl ester (TAMRA-SE; Invitrogen) according to the standard techniques [29]. Rhodamine-tubulin was obtained by chemical cross-linking and the labeling ratio was 1.4. This ratio was determined by measuring the concentration of protein and tetramethylrhodamine. The concentration of protein was measured using Bradford method where bovine serum albumin (BSA) was used as a standard. The concentration of tetramethylrhodamine was calculated spectrophotometrically by measuring the absorbance at 555 nm using a NanoDrop 2000c spectrophotometer (Thermo Scientific). Alexa 488-labeled tubulin was prepared using Alexa Fluor 488 succinimidyl ester (Alexa Fluor 488-SE®; Invitrogen) following the standard technique [29]. The labeling ratio of Alexa 488-modified tubulin was 1.0. This ratio was determined by measuring the absorbance of the protein and Alexa 488 at 280 nm and 495 nm respectively using a NanoDrop 2000c spectrophotometer (Thermo Scientific).

2.2. Preparation of seed MTs

Rhodamine-labeled seed MTs were obtained by polymerizing 70.0 μM tubulin mix (rhodamine-tubulin: non-labeled tubulin = 1:9 in molar ratio) at 37 °C for 30 min using a polymerization buffer containing 2 mM of the guanosine-5′-triphosphate (GTP) analogue, guanosine-5′-[(α,β)-methyleno]triphosphate (GMPCPP, Jena Biosciences, Jena, Germany) and 4 mM $MgCl_2$. The solution containing the seed MTs was diluted with BRB80 buffer.

2.3. Experimental assay for the observation of dynamic instability of MT

Flow cell with approximate dimensions of $2 \times 9 \times 0.15$ mm^3 ($W \times L \times H$) was prepared by placing a cover glass (9×18 mm^2, Matsunami) on a glass slide (40×50 mm^2, Matsunami) where double-sided tape was used as a spacer. Plasma treatment was applied on the surface of glass slide before preparing flow cell to increase its hydrophilicity. First, the flow cell was filled with 5 μL of 0.5 mg mL^{-1} protein A (Biovision Inc., USA) and incubated 5 min, followed by a wash with 15 μL BRB80 buffer. Then 5 μL of 73 mg mL^{-1} anti-tubulin antibody (Sigma) was passed through the flow cell and incubated for 5 min. After washing with 15 μL BRB80 buffer, the flow cell was incubated for another 5 min with 1% pluronic F127 in BRB80 and finally the GMPCPP seed MTs in BRB80 were applied and allowed to bind to the anti-tubulin antibodies for 10 min. Finally, to allow the seed MTs for showing dynamic instability in a scavenger system, a polymerizing mixture (3 mM GTP, 4 mM $MgCl_2$, 5% DMSO, 4.5 mg mL^{-1} D-glucose, 50 U mL^{-1} glucose oxidase, 50 U mL^{-1} catalase) containing 15.0 μM Alexa 488-labeled tubulin mix (Alexa-tubulin: non-labeled tubulin = 4:1 in molar ratio) was applied into the flow cell. Then after ~40 min, observation was performed under TIRF microscopy. For the experiments performed inside ICS, the preparation of flow cell was the same as described above, except that in the final step a polymerization mixture excluding the scavenger system (3 mM GTP, 4 mM $MgCl_2$, 5% DMSO) containing 15.0 μM Alexa 488-labeled tubulin mix (Alexa-tubulin: non-labeled tubulin = 4:1 in molar ratio) was applied into the flow cell. Then the flow cell was placed inside the inert chamber and microscopic observation was performed after ~40 min of passing humid nitrogen gas through the chamber to remove out the oxygen present in the experimental system.

2.4. Determination of the hydrogen peroxide (H_2O_2) concentration

Hydrogen peroxide (H_2O_2) concentration in the scavenger system was measured using an Amplex red assay kit (Invitrogen). In this system, glucose reacts with DO in a glucose oxidase catalyzed reaction to form H_2O_2. Catalase first reacts with the H_2O_2 to decompose it and next Amplex red reagent (AR) (10-acetyl-3,7-dihydroxyphenoxazine) reacts with any unreacted H_2O_2 to form fluorescent resorufin in a horseradish peroxidase (HRP)-coupled reaction [30,31]. Determination of H_2O_2 produced in scavenger system was accomplished from a calibration curve of different H_2O_2 standard solutions. In each H_2O_2 standard solution, 100 μM AR and 0.2 U/mL HRP reacted with H_2O_2 and absorbance was measured at 560 nm using a NanoDrop 2000c spectrophotometer

Download English Version:

https://daneshyari.com/en/article/5370764

Download Persian Version:

https://daneshyari.com/article/5370764

Daneshyari.com