Nitric oxide regulates actin reorganization through cGMP and Ca\(^{2+}\)/calmodulin in RAW 264.7 cells

XinChen Ke, Masaharu Terashima, Yuko Nariai, Yukie Nakashima, Toumei Nabika, Yoshinori Tanigawa *

Department of Biochemistry, Shimane Medical University, Izumo 693-8501, Japan

Received 23 November 2000; received in revised form 21 March 2001; accepted 26 March 2001

Abstract

Nitric oxide (NO) has been reported to be involved in the regulation of pseudopodia formation, phagocytosis and adhesion in macrophages through the reorganization of actin. In the present study, we directly separated the globular (G) and filamentous (F) actin from quiescent or NO-stimulated macrophage-like cell line RAW 264.7 cells in order to investigate the dynamic redistribution of actin pools. We also focused on the regulatory mechanisms of actin assembly, induced by NO and its possible subsequent signaling pathway. We showed that predominant G-actin coexisted with Triton X-100-insoluble filamentous (TIF) and Triton X-100-soluble filamentous actin in resting RAW 264.7 cells. The exogenous NO produced by (+)-(E)-2-[(E)-hydroxyimino]-6-methoxy-4-methyl-5-nitro-3-hexenamide (NOR1), the endogenous NO induced by lipopolysaccharide (LPS) plus interferon-\(\gamma\) (IFN\(\gamma\)), and dibutyryl-cGMP increased the contents of TIF-actin in dose- and time-dependent manners and altered its morphology. The increase in the TIF-actin contents induced by NOR1 or LPS plus IFN\(\gamma\) was efficiently blocked by the radical scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide and the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one or the arginine analogue N\(^\text{G}\)-monomethyl-L-arginine acetate, respectively. Preincubation with the calmodulin antagonist W-7 almost completely blocked the NO-induced TIF-actin increase and morphological change. On the other hand, preincubation with C3 transferase, an inhibitor of Rho protein, efficiently prevented the change in cell morphology, but had no effect on the TIF-actin increase. We postulate that cGMP and subsequent Ca\(^{2+}\)/calmodulin may be key regulators of actin reorganization in NO-stimulated RAW 264.7 cells. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Nitric oxide; Actin; Macrophage; (+)-(E)-2-[(E)-hydroxyimino]-6-methoxy-4-methyl-5-nitro-3-hexenamide; Guanosine 3′:5′-cyclic monophosphate

Abbreviations: NO, nitric oxide; G-actin, globular actin; F-actin, filamentous actin; iNOS, inducible nitric oxide synthase; NOR1, (+)-(E)-2-[(E)-hydroxyimino]-6-methoxy-4-methyl-5-nitro-3-hexenamide; LPS, lipopolysaccharide; IFN, interferon; N\(^\text{G}\)MMA, N\(^\text{G}\)-monomethyl-L-arginine acetate; DBcGMP, 2′-O-dibutyrylguanosine 3′:5′-cyclic monophosphate; SNAP, S-nitroso-N-acetyl-\(\alpha\)-penicillamine; DBcAMP, 2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate; carboxy-PTIO, 2′-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

* Corresponding author. Fax: +81-853-20-2125; E-mail: yosinori@shimane-med.ac.jp

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PII: S0167-4889(01)00090-8
1. Introduction

Macrophages are members of a host-wide cell system that participates in the regulation of cell proliferation, homeostasis, inflammation, and defense against both microbial and neoplastic diseases. Several specific functional processes of macrophages require rapid membrane morphologic modification. For example, granular antigens are first bound and phagocytized into macrophages through FcγR and C3bR on the macrophage membrane surface and degraded to small soluble molecules of polypeptides inside the phagolysosome; then the molecules bind to major histocompatibility complex II (MHC-II) on the membrane surface and are transferred or presented to T cells by dendritic cells [1,2].

Actin is one of the most important constituents of proteins found in all eukaryotes. It has a molecular mass close to 43 000 Da due to the high degree of conservation of amino acid sequence and number. Besides muscle contraction, actin is involved in many processes such as phagocytosis, secretion, cell migration, and the maintenance of cell shape [3]. Structurally and functionally distinct polymers coexist with actin monomers in cells [4]. The plasma membrane of eukaryotic cells is supported by a network of actin filaments. The actin cytoskeleton is believed to provide both the protrusive and contractile forces required for cell motility, via a combination of actin polymerization, depolymerization, actin filament cross-linking, and the interaction of actin-binding proteins with actin filaments [5]. Because cell movement is a dynamic process, actin at the leading edge of the cell must be continuously depolymerizing and recycling the actin subunits for further rounds of polymerization. In this way, an individual actin molecule is constantly moving from and to different actin pools that alter with the shift of dynamic level when the milieu changes.

Nitric oxide (NO), a radical gas, has been established as a diffusible universal messenger mediating a wide array of physiological and pathological activities in neural, vascular, and immune systems [6]. NO exerts several protective functions: causing vasodilation and thereby improving tissue perfusion, inhibiting platelet aggregation and thus acting as an anti-thrombotic agent [7], inhibiting leukocyte adhesion to endothelial cells and recruitment of inflammatory cells [8,9], and inhibiting smooth muscle cell proliferation and thereby preserving tissue and organ architecture [10]. Besides mediating normal functions, NO has been implicated in pathophysiologic states as diverse as septic shock, stroke, and neurodegenerative diseases [6,11,12]. NO synthesis is catalyzed by the NO synthases (NOSs), and NOS isoforms are classified into constitutive enzymes (eNOS and nNOS) or an inducible enzyme (iNOS) that is typically induced in response to inflammatory or proinflammatory mediators [6,13]. Soluble guanylate cyclase (sGC) catalyzes the conversion of guanosine 5’-triphosphate (GTP) to cyclic guanosine 3’:5’-monophosphate (cGMP), and the cyclase is accepted to be a conclusive receptor for NO. The activation of sGC and the subsequent accumulation of cGMP transmit NO signals to the downstream elements of the signaling cascade: cGMP-dependent protein kinase, cGMP-gated cation channels, and cGMP-regulated phosphodiesterase [14].

NO is known to be involved in dynamic morphological and mobility changes in several cells. For instance, NO alters cell shape and motility in aortic smooth muscle cells via protein tyrosine phosphatase 1B activation [10], or induces dose-dependent Ca2+ transients and causes temporal morphological hyperpolarization in human neutrophils [15]. NO and cGMP regulate endothelial permeability and filamentous (F) actin distribution in hydrogen peroxide-treated endothelial cells [16]. It has been reported that NO may be involved in pseudopodia formation, phagocytic activity, and adhesion in macrophages through the modification of actin [17]. However, the influence of NO on the actin pools and its downstream pathway remains obscure. Thus, we investigated the distribution of actin pools by exogenous and endogenous NO in murine macrophage cell line RAW 264.7 cells. We also focused on the regulatory mechanisms of actin assembly induced by NO and its possible signal pathway. In order to confirm that alteration of actin contents is induced by NO, we chose a NO donor, (+)-(E)-2-[(E)-hydroxylimino]-6-methoxy-4-methyl-5-nitro-3-hexenamide (NOR1), that automatically produces pure NO. We show here that a predominant globular (G) actin pool coexists with two F-actin pools in resting RAW 264.7 cells. Exogenous and endogenous NO increased Triton X-100-insoluble actin contents and altered cell...
morbidity. Furthermore, we suggest that cGMP and Ca\(^{2+}\)/calmodulin may be key regulators of NO-induced actin reorganization in RAW 264.7 cells.

2. Materials and methods

2.1. Reagents

Mouse anti-actin monoclonal antibody was purchased from Chemicon International, and anti-mouse horseradish peroxidase (HRP)-conjugated IgG was from Medical and Biological Laboratories. NOR1, \(\text{S-nitroso-N-acetyl-DL-penicillamine (SNAP)}\), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO), \(\text{C3 ADP-ribosyltransferase, } \text{N}^\text{G}-\text{monomethyl-L-arginine, acetate (N\text{G}-\text{MMA}) were from Wako Pure Chemical Industries (Japan). Dibutrylguanosine 3':5'-cyclic monophosphate (DBcGMP) and calmodulin antagonist W-7 were from Seikagaku (Japan). Dipotassium \(\text{N}^\text{4,2P-O-Dibutyrylcytidine 3':5'-cyclic monophosphate (DBcAMP) and 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) were bought from Sigma. Lipopolysaccharide (LPS) (Escherichia coli, 0127:B8 Westphal type) was obtained from Difco Laboratories (Detroit, MI, USA). Recombinant mouse interferon-\(\gamma\) (IFN\(\gamma\)) was purchased from Genzyme (Cambridge, MA, USA). N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (NBD-phallacidin) was from Molecular Probes (USA).

2.2. Cell culture

The mouse macrophage-like cell line RAW 264.7 was obtained from the Riken Cell Bank (Japan) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum at 37\(^\circ\)C in a humidified atmosphere of 5% CO\(_2\) in air. The cells (1 \times 10^6 cells/well) were seeded into six-well cell culture dishes (Nunc). Cellular viability during all experiments was determined by cell number and trypan blue dye exclusion test (cell survival rate: 95%).

2.3. Preparation of the actin pools

Actin pools were separated using the method described by Watts [4]. RAW 264.7 cells treated with or without NOR1 were scraped off, washed twice with PBS, and lysed for 15 min at room temperature in 0.5 ml lysis buffer containing 1% Triton X-100, 10 mM imidazole (pH 7.2), 40 mM KCl, 10 mM EGTA and 0.2 mM PMSF. Triton X-100-insoluble filamentous actin (TIF-actin) was maintained in the precipitate after a 15 min centrifugation at 16000 \(\times\) g. The supernatant was further separated by ultracentrifugation at 105 000 \(\times\) g for 1 h. Triton X-100-soluble filamentous actin (TSF-actin) was then located in the supernatant. Each resultant F-actin preparation was resolved into 50 \(\mu\)l of lysis buffer containing 2% SDS. The protein concentration of samples was determined by the method of Bradford using bovine serum albumin as a standard [18].

2.4. Western blot analysis

Actin samples separated using 12.5% SDS-PAGE were electrotransferred to nitrocellulose membranes. The membranes were then incubated with 0.5% ovalbumin in PBS containing 0.05% Tween 20 (PBS-T) for 1 h, washed, and exposed to mouse anti-actin monoclonal antibody for 1 h. After being washed with PBS-T four times, the membranes were incubated for 1 h with HRP-conjugated anti-mouse IgG. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech.). Laser densitometric analysis was carried out using a LKB 2222-020 Ultroscan XL laser densitometer (Bromma, Sweden).

2.5. Determination of NO\(^{-}_2\) production and cGMP accumulation in RAW 264.7 cells

RAW 264.7 cells were incubated with LPS (1.0 \(\mu\)g/ml) and IFN\(\gamma\) (100 U/ml) in the presence or absence of 0.1–0.4 mM N\text{G}-MMA for 18 h. Cell-free supernatant was collected, and the nitrite concentration was measured and used to provide indirect estimates of NO production as described previously [19]. Briefly, aliquots of the conditioned medium (100 \(\mu\)l) were incubated with 10 \(\mu\)l of 0.005% 2,3-diaminonaphthalene for 15 min at room temperature. The reaction was terminated by adding 5 \(\mu\)l of 2.8 M NaOH. Each sample was diluted with distilled water.
to 4 ml, and the fluorescence intensity was measured with excitation and emission settings of 365 nm and 450 nm, respectively, using a fluorescence spectrophotometer (Hitachi 850 type).

cGMP accumulated in the NOR1-stimulated RAW cells was measured using an enzyme immunoassay (EIA) kit (Amersham Pharmacia Biotech.) under the manufacturer’s protocol. cGMP content after treatment with DBcGMP or DbcAMP failed to be determined accurately in this study, probably because of their cross-reactivity to the antiserum of the EIA system.

2.6. NBD-phallacidin staining and flow cytometric analysis

RAW 264.7 cells incubated with 0.5 mM NOR1 and/or 1 mM carboxy-PTIO or LPS (1.0 μg/ml) plus IFNγ (100 U/ml) were washed twice and suspended in 0.9 ml of HBSS. The cells were fixed, permeabilized, and stained by addition of 0.1 ml of 37% phosphate-buffered formalin containing 3.3 μM NBD-phallacidin and 200 μg/ml lysophosphatidylcholine for 10 min at 37°C [20], then washed three times, and resuspended in a buffer containing 140 mM KCl, 1 mM MgCl₂, 1 mM ATP, 10 mM glucose, 10 mM HEPES, 1 mM EGTA, and 0.2 mM CaCl₂. The cells were filtered through a 50 μm nylon mesh filter to remove large aggregates. NBD-phallacidin-stained cells (10,000 cells per sample) were analyzed by flow cytometry using FACStar (Becton Dickson Immunocytometry Systems). The shift in the population distribution of the fluorescence was taken as an index of actin polymerization.

2.7. Confocal laser scanning microscopy (CLSM)

Cells seeded on micro slide glasses in six-well plates (Nunc) treated with 0.5 mM NOR1, 1 mM DBCGMP, or LPS (1.0 μg/ml) plus IFNγ (100 U/ml) were fixed, permeabilized, and stained by addition of 0.1 ml of 37% phosphate-buffered formalin containing 3.3 μM NBD-phallacidin and 200 μg/ml lysophosphatidylcholine for 20 min at 37°C. The slides were then taken out carefully, washed and dried in the dark, and the stained cells were observed by CLSM as described previously [21]. Photographs were taken on Kodak Plus-X Pan 125 film.

2.8. Statistical analysis

Data are presented as the means ± S.E.M. for the indicated number of independent experiments. Statistical significance was evaluated by Student’s t-test and P values of less than 0.05 were considered significant.

3. Results

3.1. Distribution of actin pools in resting RAW 264.7 cells

We determined the distribution of distinct actin pools in resting RAW 264.7 cells in the first series of experiments. Due to the different combinations that exist, we separated F-actin into two types according to the method of Watts [4]. One is TIF-actin, the massive part of which participates in the constitution of the cytoskeleton, and the other is TSF-actin, short cytoplasmic filaments capped with gelsolin. The third actin pool is monomeric G-actin. To quantify the proteins in the cell lysates, Western blot and laser densitometric analysis were carried out. As shown in Fig. 1, RAW 264.7 cells contain defined pools of actin. The total cellular actin contents of cells is noted to be 866.1 ± 41.06 ng/10⁶ cells. The apportionment of actin was: TIF-actin, 164.5 ± 9.15 ng/10⁶ cells; TSF-actin, 18.8 ± 8.14 ng/10⁶ cells; and G-actin, 682.8 ± 41.99 ng/10⁶ cells (n = 4).

![Western blot analysis of actin pools in resting RAW 264.7 cells.](image)

Table 1. Western blot analysis of actin pools in resting RAW 264.7 cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TIF-actin</th>
<th>G-actin</th>
<th>TSF-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer</td>
<td>-</td>
<td>19.0</td>
<td>78.8</td>
</tr>
</tbody>
</table>

Fig. 1. Western blot analysis of actin pools in resting RAW 264.7 cells. Actin samples were prepared as described in Section 2, and then subjected to SDS-PAGE and ECL Western blot analysis. Lanes: 1, sample buffer only; 2, TIF-actin; 3, G-actin; 4, TSF-actin.
There is a predominant G-actin pool in the quiescent cells, giving potentiating formation of large amounts of F-actin rapidly when the cells undergo specific situations. Liu and Sundqvist reported that NO produced by hydrogen peroxide or phorbol myristate acetate altered permeability and redistributed F-actin, producing a rise in F-actin contents in bovine aortic endothelial cells [16,22]. Thus, we investigated the effect of NO on the F-actin contents in a macrophage cell line.

3.2. Effect of NOR1 on the distribution of actin pools in RAW 264.7 cells

We used NOR1 and SNAP as involuntary NO donors to investigate the effect of NO on the distribution of each actin pool in RAW 264.7 cells. As shown in Table 1, we measured the NO$_3$ production of NOR1 by the fluorescent method using 2,3-diaminonaphthalene and confirmed that NO$_3$ production correlated with the increasing concentrations of these compounds in the culture medium as described elsewhere [23,24].

When the RAW 264.7 cells were incubated with 0.5 mM NOR1 for 1, 2, and 4 h, the cellular actin pools were evidently redistributed (Fig. 2A). TIF-actin increased significantly after 1 h of stimulation and reached a maximum at 4 h. A similar dose-dependent increase was also seen (Fig. 2B). On the contrary, monomeric G-actin decreased with increasing TIF-actin contents in both time- and dose-dependent manners, although the change was not significant (Fig. 2A,B). The increased percentage of TIF-actin may come from the predominant G-actin pool, whose population was sufficient to support the increase of TIF-actin contents. In addition, we used another pure NO donor, SNAP, a stable S-nitrosothiol [24]. After 2 h incubation, 0.5 mM SNAP induced a significant increase of TIF-actin contents up to 180.1 ± 5.774% (P < 0.008, compared with the value without SNAP).

Because the TSF-actin pool was trifling and remained unchanged after NOR1 treatment (data not shown), we will simply define TIF-actin contents as the F-actin contents hereafter.

3.3. Effect of LPS plus IFN$\gamma$ on the distribution of actin pools

We considered whether the endogenously produced NO also regulates actin distribution, as was seen in the case of the exogenous NO donor, NOR1. To prove this, we treated the RAW cells with LPS (1.0 $\mu$g/ml) plus IFN$\gamma$ (100 U/ml) in the presence or absence of NGMMA, an iNOS inhibitor [25], and measured the concentration of NO$_3$ as an estimate of the NO production and distribution of actin pools in the cells. LPS plus IFN$\gamma$ induced a marked increase in the NO$_3$ concentration in the culture medium that was significantly inhibited by NGMMA dose-dependently (Fig. 3A). As shown in Fig. 3B, LPS and IFN$\gamma$ induced a higher contents of F-actin compared to that of the control. The increase in F-actin contents was also significantly inhibited by NGMMA dose-dependently, although no significant change in the G-actin contents was observed (Fig. 3B). Thus, a positive correlation between NO concentrations and F-actin contents induced by LPS.

Table 1

<table>
<thead>
<tr>
<th>NO$_3$ production (μM)</th>
<th>cGMP (f mol/mg protein)</th>
<th>relative TIF-actin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.40 ± 0.020</td>
<td>185.00 ± 4.041</td>
</tr>
<tr>
<td>0.25 mM NOR1</td>
<td>111.25 ± 2.529*</td>
<td>367.67 ± 8.090*</td>
</tr>
<tr>
<td>0.5 mM NOR1</td>
<td>169.00 ± 3.719*</td>
<td>441.00 ± 18.193*</td>
</tr>
<tr>
<td>10 μM ODQ</td>
<td>0.40 ± 0.020</td>
<td>165.67 ± 8.686</td>
</tr>
<tr>
<td>10 μM ODQ+0.5 mM NOR1</td>
<td>167.50 ± 3.227*</td>
<td>191.67 ± 11.780</td>
</tr>
<tr>
<td>1.0 mM DBcGMP</td>
<td>0.40 ± 0.020</td>
<td>–</td>
</tr>
<tr>
<td>1.0 mM DBcAMP</td>
<td>0.40 ± 0.020</td>
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The cells were treated with the indicated reagent for 2 h. The cell-free supernatant and cell lysate were subjected to determination of the amounts of NO$_3$, the intracellular amounts of cGMP (n = 10) and relative TIF-actin contents (n = 3), respectively, as described in Section 2. The data shown are means ± S.E.M. *P < 0.005, compared with control.
3.4. Flow cytometry of the change in relative F-actin contents

To assess how an NO-induced increase in F-actin contents was expressed in RAW 264.7 cells, the cells were treated with both NOR1 and LPS plus IFNγ caused a right shift of the cell population compared to that of control (Fig. 4A: control, B: NOR1, and D: LPS plus IFNγ). The right shift caused by NOR1 was recovered by adding carboxy-PTIO (Fig. 4C). These results indicated that NO derived from either an exogenous donor or an endogenous source increased the relative F-actin contents in the cells.

3.5. NO induces F-actin reorganization and morphological modification

Because F-actin is an important component of the cellular structure, to see whether the increasing F-actin contents affect the cell shape, the morphology of the cells treated with NO was observed by CLSM.

Fig. 2. Effect of NOR1 on relative actin contents in RAW 264.7 cells. The cells were incubated with 0.5 mM NOR1 for the indicated time (A), or for 2 h with increasing concentrations of NOR1 (B). Actin samples were isolated and subjected to SDS-PAGE and ECL Western blot analysis as described in Section 2. The developed films were analyzed by laser densitometry. The data shown are the means ± S.E.M. of four independent experiments. *P < 0.005, **P < 0.008, compared with the value without NOR1.

Fig. 3. Effects of LPS plus IFNγ and N6MMA on nitrite (NO₂⁻) production and relative actin contents in RAW 264.7 cells. Cells were incubated with LPS (1.0 μg/ml) plus IFNγ (100 U/ml) in the presence or absence of N6MMA (0.1 mM, 0.2 mM, 0.4 mM) for 18 h. The NO₂⁻ secreted into the medium (A) and relative actin contents of the cells (B) were determined as described in Section 2. The data shown are the means ± S.E.M. of three independent experiments. *P < 0.002, **P < 0.008, compared with the value of LPS+IFNγ.
While resting cells exhibited regular round shapes (Fig. 5A), NOR1-induced reorganization in actin distribution of the cells led to a striking difference in cell morphology. Many cells were polarized and had an extended oval-shaped configuration (Fig. 5B). In addition to this morphological change, NOR1 induced cell polarization indicating cell migration [27]. Typically, one sharp side of the cells displayed long actin-rich pseudopodia, characteristic of the leading edge of a motile cell, whereas the opposite edge was rounded or displayed retraction fibers, typical of the trailing edge of a motile cell. The effect of NOR1 was completely blocked by carboxy-PTIO: the cells remained round and unpolarized, and no pseudopodium was seen (Fig. 5C).

3.6. Influence of DBcGMP and DBcAMP on F-actin contents and morphologic change

The only conclusively proven target for NO is guanylate cyclase, which catalyzes the conversion of GTP to cGMP [14]. To test whether NOR1 influences the RAW cells through cGMP, the cells were treated with DBcGMP. As shown in Fig. 6, the increase in F-actin contents was seen in both dose- (Fig. 6A) and time-dependent (Fig. 6B) ways, as was shown in the NOR1 treatment (see Fig. 2). Neither 4 or 8 mM DBcGMP nor prolonged incubation over 4 h changed the F-actin contents any further (data not shown). The cells were clearly observed polarizing, with long pseudopodia as leading edges and with round retraction fibers as trailing edges (Fig. 5D). These results showed that not only NO but also cGMP contributed equally to the actin reorganization.

Next, we tested the effect of DBcAMP on the actin distribution in RAW 264.7 cells. When the cells were treated with 1 mM DBcAMP for 2 h, the relative F-actin contents were slightly increased, though the extent of the increase was less than that of DBcGMP (Table 1 and see also Fig. 6A). It has been noted that cAMP may modulate the NO/cGMP pathway in vascular smooth muscle cells, and that there is cross-talk between the cAMP- and cGMP-mediated signal transduction systems [28]. Thus, DBcAMP may partly mimic the effect of cGMP on the F-actin content, although the effect was not significant in this study.
3.7. Effect of ODQ on cGMP accumulation and increase in F-actin contents induced by NOR1

To confirm the NOR1-induced actin reorganization could be mediated through cGMP, we measured NOR1-induced cGMP production in the cells by EIA, and further investigated the effect of the soluble guanylate cyclase inhibitor ODQ [29] on NOR1-induced increase in F-actin contents. As shown in Table 1, the accumulation of both NO$_3^-$ and cGMP is in proportion to the increasing concentration of NOR1, suggesting that NOR1 apparently induced cGMP production in RAW 264.7 cells. When 10 µM ODQ was added to the culture medium, NOR1-induced cGMP accumulation was effectively blocked, and the NOR1-induced increase in F-actin contents was significantly reduced. These results clearly show that NOR1 and the subsequently produced cGMP are involved in the regulation of actin reorganization.

3.8. Influence of W-7 on F-actin contents and morphological alteration induced by NOR1

It has been reported that NO donors such as SNAP, NOR1 and sodium nitroprusside (SNP) can increase the intracellular calcium concentration in cultured nodes of ganglion neurons or single PU5-
1.8 cells [30,31]. To investigate the signaling pathway of the cytoskeletal and the morphologic modification induced by NO in RAW cells, the cells were incubated with the calmodulin antagonist W-7 for 1 h before NOR1 was added. Fifty μM W-7 completely blocked the NOR1-induced increase in F-actin contents (Fig. 7A), and W-7 dose-dependently prevented cell morphological change induced by NOR1 (Fig. 8C,D). Furthermore, W-7 inhibited the DBcGMP-induced increase in F-actin contents (data not shown), indicating that Ca²⁺/calmodulin or calmodulin-dependent kinase might locate in the downstream pathway of NO and cGMP. These results indicate that NO, cGMP and Ca²⁺/calmodulin may be involved in the redistribution of actin.

3.9. Effect of Rho protein on F-actin contents and morphological alteration induced by NOR1

To test whether Rho proteins are involved in the increase in F-actin contents, the RAW cells were preincubated for 16 h with 0.02 μg/ml C3 transferase, which ADP-ribosylates Rho protein and disrupts its function, then NOR1 was added and incubated for a further 2 h. C3 had no effect on the increase in the
F-actin contents induced by NOR1 (Fig. 7B). On the other hand, 0.02 μg/ml C3 completely prevented NOR1-induced morphologic change: the cells remained round like the control (Fig. 8E). Rho protein is recognized as a key regulator of the actin cytoskeleton [32,33], although these results indicate that Rho protein may not be involved in NO-induced actin reorganization.

Fig. 8. Effects of the calmodulin inhibitor W-7 and C3 ADP-ribosyltransferase on morphology in RAW cells treated with NOR1. Cells seeded on cover glasses were preincubated with 25 or 50 μM W-7 for 1 h or 0.02 μg/ml C3 for 16 h, then 0.5 mM NOR1 was added for another 2 h. The cells were permeabilized, fixed, stained and subjected to CLSM as described in Section 2. (A) Control; (B) NOR1; (C) 25 μM W-7; (D) 50 μM W-7; (E) C3.
4. Discussion

Actin has long received attention as an important component of the cytoskeleton because of its dynamic involvement in cell mobility and functions in eukaryotes. In the present study, we quantified the G- and F-actin contents from NO-stimulated macrophage-like cell line RAW 264.7 cells in order to investigate the dynamic redistribution of actin contents in addition to the fluorescent microscopic analysis. We demonstrated that the predominant G-actin coexisted with TIF- and TSF-actin in quiescent RAW 264.7 cells. Both NOR1 and LPS plus IFN-γ or DBcGMP increased the contents of TIF-actin in dose- and time-dependent manners, and the cells became oval, polarized and extended long actin-rich pseudopodia. We confirmed that exogenous or endogenous NO and subsequent cGMP production could be involved in the regulation of actin reorganization by using carboxy-PTIO, N\textsuperscript{G}MMA and ODQ, respectively. Carboxy-PTIO, a scavenger of free radical NO [26], blocked NOR1-induced actin reorganization analyzed by FACS and CLSM (Fig. 3B,C; Fig. 4B,C). N\textsuperscript{G}MMA, a useful NOS inhibitor because of its chemical stability, low toxicity and apparent specificity for NOS [25], simultaneously inhibited NO\textsuperscript{3} production and actin reorganization induced by LPS plus IFN-γ (Fig. 3). Furthermore, ODQ, a specific inhibitor of NO-dependent soluble guanylate cyclase [29], blocked the NOR1-induced increase in both cGMP concentration and the change in F-actin contents (Table 1). These results clearly show that NO and cGMP are the key regulators of actin reorganization. Preincubation with the calmodulin antagonist W-7 almost completely blocked the NO- and DBcGMP-induced increase in F-actin contents and morphological change, indicating that Ca\textsuperscript{2+}/calmodulin may also be involved in the NO- and cGMP-induced signaling pathway in RAW 264.7 cells. In fact, Wang et al. reported that NO signaling mediates stimulation of Ca\textsuperscript{2+} current elicited by the withdrawal of acetylcholine in cat atrial myocytes [35]. Furthermore, in human neutrophils, W-7 had dual effects on fMLP-induced actin polymerization [36]. Taken together with the above results and our data, we suggest that NO and subsequent cGMP may act through Ca\textsuperscript{2+}/calmodulin to increase the F-actin contents, thus resulting in actin reorganization and morphological alteration.

The small GTP-binding proteins of the Rho family have a close relationship with the rearrangement of the actin network. Several studies indicate the participation of Rho GTPases in the regulation of cell shape and motility [32,33]. In the present study, a Rho inhibitor, C3 exoenzyme, efficiently prevented the NOR1-induced shape change of the cells, but had no effect on the increase in F-actin contents. These results suggest that Rho protein might function in the submembranous portion to coordinate the actin network, but it does not take part in NO-induced actin reorganization in RAW 264.7 cells.

It has been reported that NO produced by phorbol myristate acetate or hydrogen peroxide reduces the endothelial permeability and increases the dense peripheral bands of actins or alternatively increases the F-actin contents in endothelial cells [16,22]. These effects indicated that NO and cGMP may have a role in regulating F-actin distribution in the endothelial cells. On the contrary, NO induced by SNP decreased pseudopodia formation estimated by rhodamine-phalloidin staining, probably via ADP-ribosylation of actin in murine macrophages [17]. It has been noted that SNP may possess some as yet unknown action other than NO production, unlike the pure production of NO by NOR1 or SNAP,
because the nitroprusside anion is a coordinate complex of a ferrous ion with five cyanide anions and a nitrosonium ion [37,38]. Moreover, in the present study, we detected no ADP-ribosylation of actin induced by NO (X.-C. Ke and M. Terashima, unpublished observation) in addition to the reports of NO-independent ADP-ribosylation of actin [39–41]. Thus, the unknown action by SNP other than NO production might cause the different results.

Actin polymerization has accounted for the involvement of many actin-binding proteins [3,5], indicating that the NO- or subsequently produced cGMP- or Ca^{2+}/calmodulin-induced alteration in the function of the actin-binding proteins may have a rational role in actin polymerization. We are now investigating the precise mechanism of NO-induced increase in F-actin contents.

In summary, we demonstrated that predominant G-actin coexists with two F-actin pools in macrophage-like cell line RAW 264.7 cells. NO and DBcGMP increased TIF-actin contents and altered the cell morphology, and W-7 completely blocked the NO-induced changes. We postulate that cGMP and subsequent Ca^{2+}/calmodulin may be key regulators of NO-induced actin reorganization in RAW 264.7 cells.

Acknowledgements

The content of this report serves as the basis of the dissertation of X.-C. Ke for the degree of Doctor of Medical Science. This investigation was supported in part by the Izumo Rotary Club and Grants-in-Aid of Scientific Research 12670117 from the Ministry of Education, Science and Culture, Japan.

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