FEBS Letters 453 (1999) 124–128 FEBS 22162

N-Acetylcysteine protects epithelial cells against the oxidative imbalance due to *Clostridium difficile* toxins

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Received 8 May 1999

Abstract Toxins A and B from the anaerobic bacterium *Clostridium difficile* are the causative agents of the antibiotic-associated pseudomembraneous colitis. At the subcellular level, they inhibit the Rho family GTPases, thus causing alterations of the actin cytoskeleton. The cytoskeletal integrity is also controlled by the redox state of cells. Therefore, we have evaluated whether an oxidative imbalance could be involved in the toxin-induced cytopathic effects. Our results indicate that both toxins induce oxidative stress with a significant depletion of protein SH-groups. These responses and the cytoskeleton-dependent cell retraction and rounding are significantly counteracted by *N*-acetylcysteine but not by α-tocopherol. Our study provides the first evidence that the thiol supplier *N*-acetylcysteine impairs the cellular intoxication by acting on the cytoskeleton integrity. This also suggests a possible beneficial role for this drug during therapeutic intervention.

Key words: Oxidative alteration; *N*-Acetylcysteine; SH-group; Cytoskeleton; *Clostridium difficile*; Toxin

1. Introduction

The anaerobic bacterium *Clostridium difficile* has been identified as the major causative agent of antibiotic-associated pseudomembraneous colitis [1]. It produces at least two protein toxins, the enterotoxin A (CdA) and the cytotoxin B (CdB), both belonging to the group of intracellularly acting bacterial proteins which have to be internalized via receptor-mediated endocytosis to exert their cytotoxicity [2]. They are monoglucosyltransferases which catalyze the incorporation of glucose into the small GTP-binding proteins of the Rho family [3,4]. Since Rho proteins are involved in the regulation of actin assembly, such a modification renders Rho inactive, thus provoking retraction and rounding of intoxicated cells [3,4]. This can possibly contribute to the pathogenesis of the *C. difficile*-associated colitis, since only toxin-producing strains are etiologically linked to the disease.

Massive changes in the organization of the actin cytoskeleton such as those described above have also been reported to be induced by an oxidative alteration of cytoskeletal thiol groups [5,6]. Following an oxidative injury, changes in the plasma membrane, i.e. surface blebbing, as well as in cytosolic and nuclear components may occur as a consequence of cytoskeletal alterations [6,7]. Among the biological mechanisms which counteract an oxidative injury of the cytoskeleton, a pivotal role is played by the intracellular pool of soluble thiols, most of which are represented by the reduced glutathione (GSH).

In this paper, we report evidence that in epithelial cells both CdA and CdB provoke a significant depletion in the GSH levels which can be prevented by *N*-acetylcysteine (NAC) but not by α-tocopherol (the most important tocopherol of vitamin E) or its analogue trolox. Accordingly, NAC is also effective in counteracting the rounding up and the actin cytoskeleton breakdown which characterize the toxins' activity. These findings suggest a critical role for GSH levels in the development and the progression of *C. difficile* toxins-induced cytopathology.

2. Materials and methods

2.1. Cells

Human epithelial HEp-2 cells and small intestine normal crypt cells (IEC-6) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, UK), 1% non-essential amino acids, 5 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml). Cells were subcultured in 25 cm^2 Falcon plastic flasks at a density of 5×10^4 cells/ml and placed in a 37°C incubator containing a 95% air 5% CO_2 atmosphere. For IEC-6 cell cultures, insulin was added at 10 μg/ml to the culture medium. For morphological studies, cells were grown on glass coverslips on separate wells and then processed for immunofluorescence and scanning electron microscopy analyses as described below.

2.2. Chemicals and treatments

CdA was purified as previously described [8] and CdB was a generous gift from Christoph von Eichel-Streiber (Johannes Gutenberg-Universitat, Mainz, Germany). 24 h after seeding, HEp-2 cells were exposed to 3 μg/ml of CdA or 3 ng/ml of CdB for different times as previously described [8]. Only data obtained after 18 h of exposure to both toxins are described in Section 3 since, under these experimental conditions, all cells in the monolayer were rounded. After each treatment, the cell viability was evaluated by a trypan blue dye exclusion test. Anti-oxidant, NAC, [9] was purchased from Zambon Group (Milan, Italy) and α-tocopherol [10] from Aldrich (Milwaukee, WI, USA). In addition, trolox (Aldrich), a water soluble analogue of α-tocopherol, was also used [11]. In order to assay the anti-oxidant activity, NAC and trolox were directly dissolved in the culture medium and added to HEp-2 cells at 10 mM for 2 h before toxins exposure. By contrast, α-tocopherol was dissolved in DMSO and incubated at 100 μM for 24 h before challenging cell cultures with toxins. In this case, cells treated with an equal volume of DMSO alone were used as controls.

2.3. Fluorescence microscopy

Following treatments, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline solution (PBS) with 2% bovine serum albumin (BSA), for 10 min at room temperature. After washing in the same buffer, cells were permeabilized with 0.5% Triton X-100 (Sigma

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PII: S0014-5793(99)00706-1

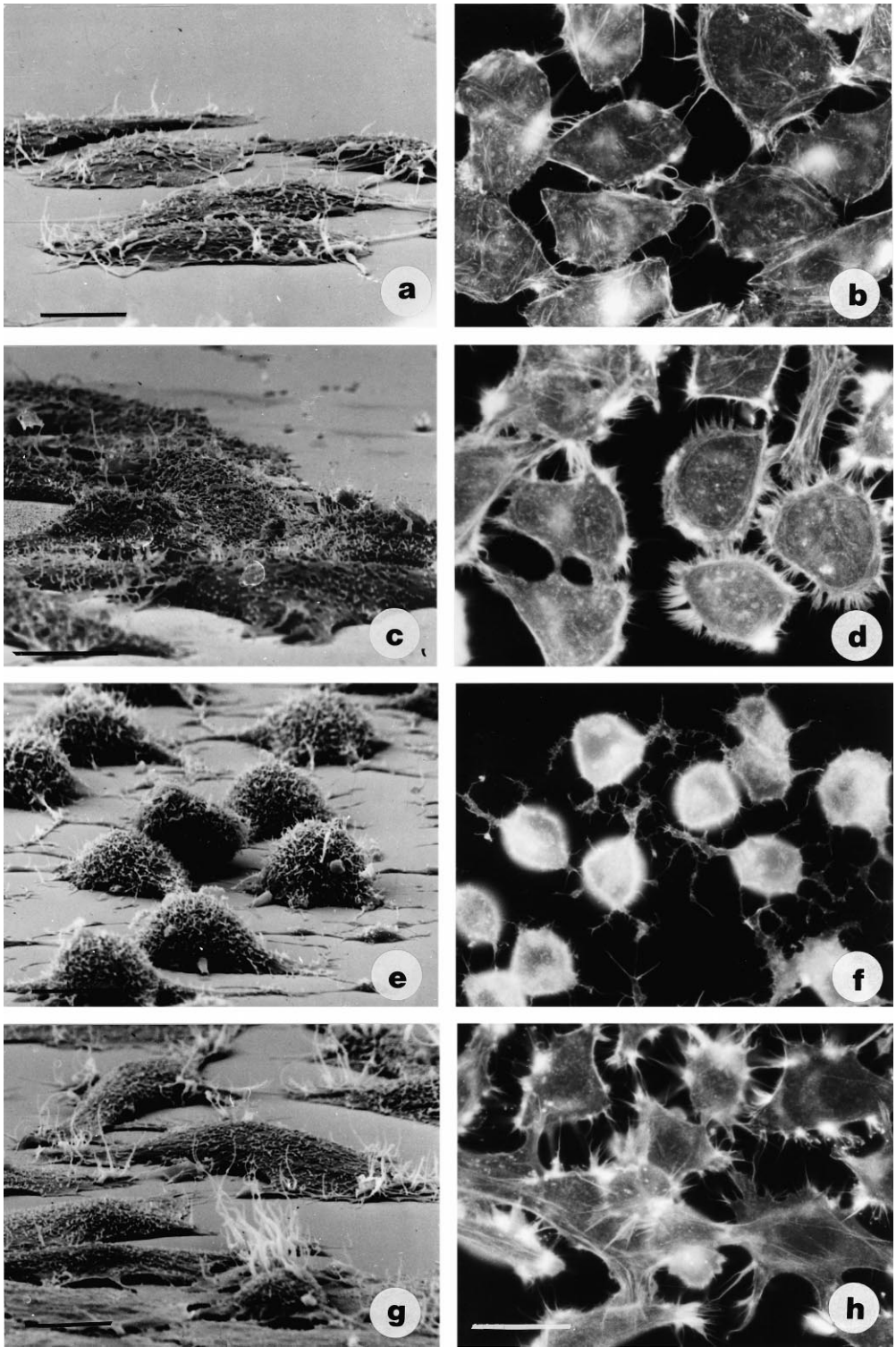

Fig. 1. NAC prevented the CdB-induced cytoskeletal changes in epithelial cells. Scanning electron micrograph (on the left) and fluorescence micrograph (on the right) of HEp-2 cells. Control cells (a, b), cells treated with NAC (c, d), cells exposed to CdB (e, f), cells pre-treated with NAC before exposure to CdB (g, h). Bars represent 10 μm.

Chemical, St. Louis, MO, USA) in PBS for 10 min at room temperature. For F-actin detection, cells were stained with fluorescein-phalloidin (Sigma, working dilution 1:500) at 37°C for 30 min. Finally, after washing, coverslips were mounted with glycerol-PBS (2:1) and analyzed with a Nikon Microphot fluorescence microscope.

2.4. Scanning electron microscopy

Control and treated cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min. Following post-fixation in 1% OsO_4 for 30 min, cells were dehydrated through graded ethanols, critical point-dried in CO_2 and gold-coated

by sputtering. The samples were examined with a Cambridge 360 scanning electron microscope.

2.5. Determination of oxidized glutathione (GSSG) and GSH

To determine the total intracellular glutathione content, the enzymatic recycling assay with glutathione reductase (type IV, Sigma) and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Sigma) was used, essentially according to Anderson [12]. At the end of experiments, cells were harvested, centrifuged for 5 min at 900 rpm, washed twice with PBS, resuspended in the same buffer and homogenized by sonication for 30 s in a Soniprep 150. Cell lysates were diluted with 1 volume of 10% (w/v) ice-cold 5-sulfosalicylic acid (Sigma) and centrifuged in a microfuge (6000 rpm) at 4°C for 10 min to remove the protein precipitate. The clear supernatants were stored at 4°C until assayed. Total glutathione was estimated by monitoring the formation of chromophoric product 2-nitro-5-thiobenzoic acid at 412 nm in the presence of glutathione reductase and NADPH. For the measurement of GSSG, the acidified homogenates were submitted to derivatization with undiluted 2-vinylpyridine (Aldrich) in the presence of triethanolamine (Sigma) for 1 h at room temperature. The amounts of both total glutathione and GSSG in the sample were determined from a standard curve obtained by plotting a known amount of GSH and GSSG (both from Sigma), incubated in the respective experimental conditions, versus the rate of the change in absorbance at 412 nm. The amount of GSH present in the samples was calculated as the difference between total glutathione and GSSG levels. Data, expressed as nmol of GSH or GSSG per mg of protein, were calculated on the basis of a GSH calibration curve.

2.6. Determination of protein sulfhydryl groups and the protein content

Protein sulfhydryl groups were measured as described by Di Monte and coworkers [13]. Cell pellets previously precipitated with ice-cold 5-sulfosalicylic acid were washed twice with 5% (w/v) of the same solution and were finally resuspended in 0.5 M Tris-HCl, pH 7.6. DTNB (100 μM final concentration) was then added and, after 20 min, the absorbance was measured at 420 nm. Data were expressed as nmol of SH per mg protein, calculated on the basis of a GSH calibration curve.

The protein concentration was measured by the commercially available Coomassie brilliant blue dye-binding assay (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. BSA was used as a standard.

2.7. Statistical analysis

Each experiment was repeated at least three times and data were presented as the mean ± S.D. Statistical significance among the groups was determined using the unpaired two-tailed Student's t test. A P value < 0.05 was taken to reflect a significant difference.

3. Results

3.1. NAC prevents cytoskeletal changes induced by C. difficile toxins in epithelial cells

Since the two toxins induced identical responses in epithelial cells, only data obtained with CdB are shown here. When observed by scanning electron microscopy, both control and NAC-treated cells formed monolayers of spread cells adhering well to the substrate (Figs. 1a and c, respectively). Exposure to 3 ng of CdB per ml for 18 h caused retraction and rounding up in the whole cell population (Fig. 1e), phenomena clearly prevented by pre-exposure to NAC (Fig. 1g). Fluorescence micrographs of cells stained for F-actin detection showed the dramatic breakdown of the actin network provoked by the toxin (Fig. 1f) which could be counteracted by NAC pre-exposure (Fig. 1h). In accordance to our previous reports [7], NAC alone maintained the stress fiber organization (Fig. 1d) typical for control cells (Fig. 1b). By contrast, pre-treatment with trolox or α-tocopherol could not prevent the toxin-induced morphological changes (data not shown).

3.2. NAC prevents the oxidative imbalance induced by C. difficile toxins in epithelial cells

It is known that oxidative stress may be involved in several pathways leading to cellular retraction and rounding associated to the cytoskeletal rearrangement. Also in this case, we show only the results obtained with CdB (totally overlapping those obtained with CdA). We observed that exposure to CdB

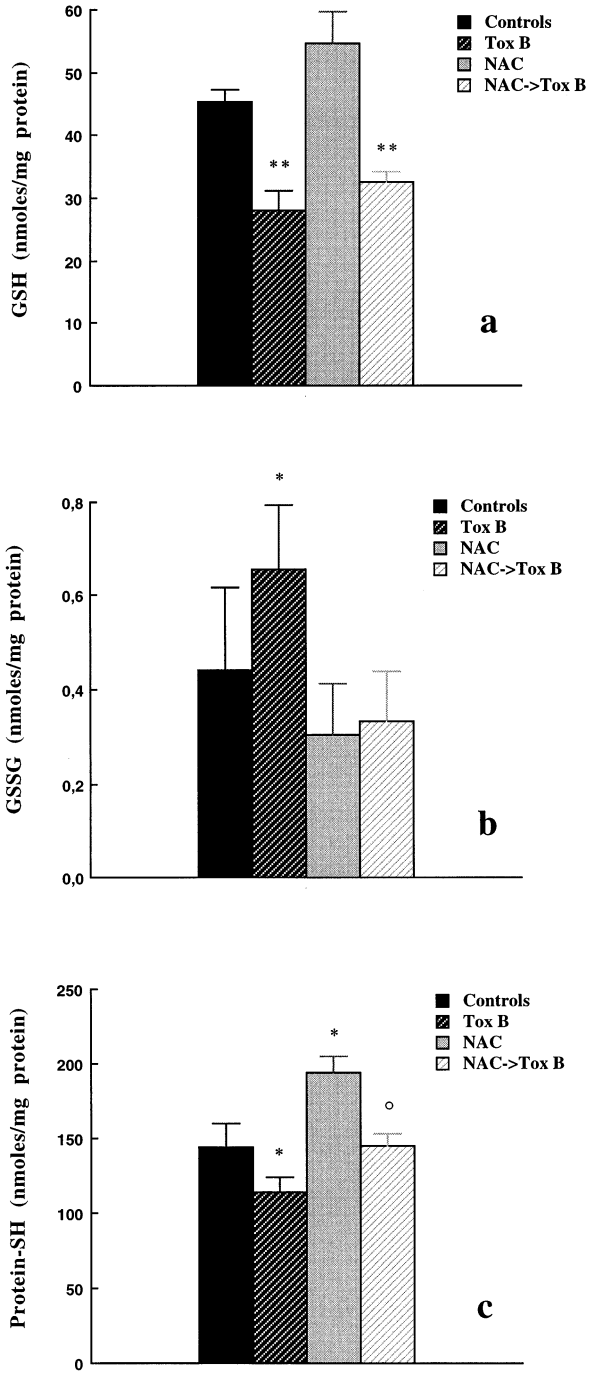

Fig. 2. NAC prevented the CdB-induced oxidative imbalance in epithelial cells. Evaluation of the redox state of HEp-2 cells after NAC and CdB treatments. Three different parameters have been considered in the present investigation: GSH (a), GSSG (b), protein thiol (SH) groups (c). $*P < 0.05$ and $**P < 0.001$ versus untreated cells: $°P < 0.05$ versus CdB-treated cells.

for 18 h provoked a significant decrease ($P < 0.001$) in the intracellular GSH with respect to untreated cells (Fig. 2a), with a concomitant 1.5-fold increase of GSSG basal levels ($P < 0.05$, Fig. 2b). By contrast, when the cells were pre-treated with 10 mM NAC for 2 h before CdB exposure, the depletion of GSH did not appear to be efficiently hindered. By contrast, Nac exposure induced a significant reduction of GSSG intracellular levels with respect to CdB-treated cells. The ability of NAC to maintain the oxidative balance within the cells was more evident evaluating the GSSG/GSH ratio, considered to be a more precise marker of the redox potential of the cell. In fact, while CdB-treated cells showed a severe increase of this ratio (0.026) with respect to controls (0.009, $P < 0.001$), NAC pre-treatment was able to maintain this parameter unaffected (0.0087). Interestingly, the drop of the GSH level due to CdB was not a cell-specific phenomenon. In fact, in IEC-6 cells, which are normal crypt cells derived from the small intestine, CdB induced, besides an alteration of the microfilament system [18], a drop of the GSH level (0.027 nmol GSH/μg of proteins versus 0.013 nmol GSH/μg of proteins in CdB-treated cells), which was counteracted by pre-exposure to NAC (0.035 nmol GSH/μg of proteins in NAC-treated cells versus 0.065 nmol GSH/μg of proteins in NAC-treated cells subsequently exposed to CdB).

The effects of CdB exposure on the GSH homeostasis prompted us to investigate the influence of treatment on protein-bound sulfhydryl groups. As shown in Fig. 2c, there was a significant ($P < 0.05$) disappearance in protein thiols after addition of 3 ng/ml of CdB to the incubation medium. By contrast, NAC pre-treatment allows the cells to maintain their protein thiol group profile ($P < 0.05$ versus CdB-treated cells) in the reduced state, the values observed being very similar to those of controls. Hydroxyl radical scavenger agents, such as α-tocopherol and trolox, failed in hindering such toxin-induced effects, i.e. cell retraction and rounding (data not shown).

4. Discussion

Protein toxins from *C. difficile* are important etiological agents involved in the pathogenesis of pseudomembraneous colitis [1]. Pathogenic strains are always toxigenic and, although CdA is the recognized enterotoxin causing fluid secretion in vivo, several lines of evidence indicate that CdB as well plays a role in the disease [2]. In this paper, we have reported that both toxins A and B can induce oxidative stress in epithelial cells, notably at doses lower than those utilized to prove monoglucosylation of Rho [3,4]. Moreover, the cellular 'intoxication' can be partially counteracted by those anti-oxidant compounds which act as thiol suppliers, such as NAC. This drug is actually able to counteract the protein thiol group depletion due to the toxins' activity, maintaining the SH-values similar to that of control cells. By contrast, radical scavenger agents such as α-tocopherol and trolox failed in preventing the oxidative imbalance provoked by the toxins. This points to the requirement of SH-group supply (furnished by NAC) to effect a significant protection.

Probably, since the oxidative state of actin is critical for its organization [14,15], the reduced state of actin, due to the anti-oxidant activity of NAC, may 'buffer' those effects strictly dependent on cytoskeletal thiol group oxidation. This drug could inhibit the actin filament side by side cross-linking which is typical of cell injury processes leading to retraction, rounding and blebbing [13,16]. Thus, while an anti-oxidant drug with a thiol supplier activity, such as NAC, is capable of preventing (or counteracting) the toxin-induced oxidative stress, a 'simple' hydroxyl radical scavenger, such as α-tocopherol or its analog trolox, whose effects are mainly ascribable to a lipid peroxidation protection exerted at the plasma membrane level, does not.

The drop of GSH following the challenge with both toxins has been observed also in IEC-6 cells, normal epithelial cells derived from rat crypt small intestine, where the two toxins have been described to act as apoptotic inducers [17,18]. Very little, however, is known about toxins provoking a redox imbalance. Toxins produced by cyanobacteria, which contaminate water and cause serious health problems, induce an oxidative damage which is probably involved in cyanobacteria extract-induced hepatotoxicity [19]. Data obtained in animals also point to the connection between a certain enterotoxin activity and the redox state of cells. Cholera toxin, for example, induces fluid secretion and decreases the level of mucosal GSH [20], although prior modulation of the thiol status of the mucosa was ineffective on toxin-induced fluid secretion. This is in accordance with our findings showing that also CdB, which is involved in the disease but does not cause fluid secretion, is capable of altering the oxidative state of the cells.

In conclusion, taken together, all the above reported results clearly indicate that *C. difficile* toxins cause oxidative imbalance in epithelial cells which can be counteracted by anti-oxidant compounds acting as thiol suppliers, such as NAC. A role for NAC as an anti-dote for poisoning has already been reported [21]. Hence, the observation that certain bacterial toxins acting as enteropathogenic virulence factors provoke oxidative stress in cells is a new point of view which may provide some clues to the pharmacological control of their intoxication-associated cytopathology.

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