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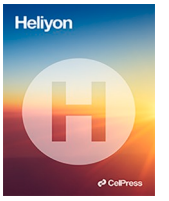

Research article

Influence of dose and exposition time in the effectiveness of *N*-Acetyl-L-cysteine treatment in A549 human epithelial cells

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ABSTRACT

N-Acetyl-L-cysteine (NAC) acts as a precursor of the tripeptide glutathione (GSH), one of the principal cell mechanisms for reactive oxygen species (ROS) detoxification. Chronic obstructive pulmonary disease (COPD) is associated with enhanced inflammatory response and oxidative stress and NAC has been used to suppress various pathogenic processes in this disease. Studies show that the effects of NAC are dose-dependent, and it appears that the efficient doses *in vitro* are usually higher than the achieved *in vivo* plasma concentrations. However, to date, the inconsistencies between the *in vitro* NAC antioxidant and anti-inflammatory *in vitro* effects, by reproducing the *in vivo* NAC plasma concentrations as well as high NAC concentrations. To do so, A549 were transfected with polyinosinic-polycytidylic acid (Poly (I:C)) and treated with NAC at different treatment periods. Oxidative stress, release of proinflammatory mediators and NFkB activation were analyzed. Results suggest that NAC at low doses in chronic administration has sustained antioxidant and anti-inflammatory effects, while acute treatment with high dose NAC exerts a strong antioxidant and anti-inflammatory response.

1. Introduction

N-Acetyl-L-cysteine (NAC) was discovered as a mucolytic agent in the early 1960s [1], and since then, it has been extensively used. NAC is derived from the amino acid L-cysteine, an amino acid that serves as a precursor to the tripeptide glutathione (GSH). GSH plays a vital role in detoxifying reactive oxygen species (ROS) in cells [2], and it has been observed that oral intake of NAC leads to an increase in GSH plasma levels [3].

NAC's mechanism of action is based primarily on its antioxidant properties. It acts as a direct scavenger of free radicals such as OH^-, H_2O_2 and O^{2-}. NAC is also a source of sulfhydryl (SH) groups, which helps in the restoration of the redox status [4]. It also modulates several signaling pathways such as p38, ERK1/2, SAPK/JNK, c-Jun and c-Fos [5] and exerts anti-inflammatory effects via inhibition of nuclear factor-kB (NFkB) [6]. Additional studies suggest that *N*-Acetyl-L-cysteine (NAC) could potentially be utilized for

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the prevention or treatment of viral illnesses [2].

Chronic obstructive pulmonary disease (COPD) is a chronic and progressive disease associated with enhanced inflammatory response in the airways in response to noxious particles or gases. The principal characteristics of COPD are airflow limitation, mucus hypersecretion, oxidative stress and airway inflammation [7]. NAC has been used to suppress various pathogenic processes in COPD: In the HIACE trial, NAC treatment 600 mg bid improved small airways function in patients with stable COPD [8]. In a larger study, PANTHEON, patients with moderate-to-severe COPD received NAC 1200 mg/day for 1 year and reported a reduction in acute exacerbations [9,10].

Other studies have shown that NAC exerts an antioxidant dose-dependent effect [11,12]. Therefore, it was suggested that NAC administered at 600 mg may elicit mainly antioxidant activity, while higher doses are necessary to produce anti-inflammatory effects [12]. The hypothesis in that case is that high-dose NAC, or prolonged use of low doses, may maintain the redox-dependent cell signaling and transcription, which can regulate pro-inflammatory genes like NFkB [13]. In fact, patients with chronic bronchitis who do not have airway obstruction may benefit from a daily 600 mg dose of NAC. In contrast, in patients with airway obstruction, a dose of at least 1200 mg is recommended to prevent exacerbations. Prolonged treatment has also been reported to be beneficial in exacerbation prevention [14,15].

Several concerns have emerged due to the discrepancy between *in vitro* and *in vivo* findings concerning the antioxidant and anti-inflammatory impact of NAC to treat COPD. After 600 mg and 1200 mg oral NAC administration, plasma concentrations reach values of 16 μM and 35 μM respectively [16,17], but in the *in vitro* setting, higher doses (>1 mM) are needed to achieve its anti-inflammatory effects [2,4]. Further, Sadowska et al. suggested that high doses would be necessary to achieve acute antioxidant and anti-inflammatory effects [4].

Therefore, the aim of this study was to investigate and corroborate the beneficial effects of both low and high doses of NAC *in vitro,* as well as to confirm the *in vivo* hypothesis that results of NAC administration depends on the concentration and duration of treatment. To do so, as respiratory viruses increase the severity of airway inflammation and the frequency of exacerbations in COPD [18], A549 cells were challenged with polyinosinic-polycytidylic acid (Poly (I:C)), a synthetic viral dsRNA analog and a TLR3 ligand, to mimic viral infection [19]. NAC concentrations tested reproduce the plasma levels reached after oral NAC 600 and 1200 mg, as well as 2 commonly *in vitro* used concentrations (in the range of mM). The results obtained will help understand the *in vivo* NAC antioxidant and anti-inflammatory effects.

2. Material and methods

2.1. Cell culture and treatment

Adenocarcinomic human bronchoalveolar basal epithelial cells (A549) were used to model ATII behavior [20]. A549 cells were purchased from ATCC (Cat.CCL-18 American Type Culture Collection; US) and cultured in high glucose DMEM (Cat. L0102, Biowest, US) supplemented with 10% FBS. *N*-Acetyl-L-cysteine (Cat.A7250) was purchased from Sigma-Aldrich. A workflow of the treatment design is included in Fig. 1G: In all the experiments performed, cells were seeded and incubated with increasing NAC concentrations of 16 μM, 35 μM, 1.6 mM and 5 mM. NAC concentrations 16 μM and 35 μM correspond to 600 mg and 1200 mg oral NAC administration [16,17], and 1.6 mM and 5 mM are amongst the commonly used *in vitro* concentrations [2,4]. The cells were incubated with NAC at different treatment periods: 24 h (1 day-T1), 72 h (3 days-T3) and 144 h (6 days-T6). After NAC treatment, cells were stimulated with 50 μg/ml of transfected polyinosinic: polycytidylic acid (poly(I: C)) (Cat.P1530. Sigma-Aldrich, US) during 24 h. Transfection was performed by using jetPRIME® transfection reagent (Cat. 114-15. Polyplus, China) according to the manufacturer's protocol and incubated for 24 h.

2.2. Cytokine determination by enzyme linked immunosorbent assay (ELISA)

A549 cells were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different time points: 24 h (T1), 72 h (T3) and 144 h (T6) and stimulated with transfected Poly I:C at 50 μg/ml, during 24 h. After completion of the experiment, the culture medium was collected and analyzed using commercially available Quantikine® ELISA kits (Cat. DY206; Cat. D8000C, R&D Systems, Spain) for the measurement of IL-8 and IL-6 cytokine levels, following the manufacturer's protocol. The obtained results were expressed as fold change in comparison to the control group.

2.3. CM-H_2DCFDA fluorescence measurement of reactive oxygen species

To quantify ROS levels, A549 were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different time points: 24 h (T1), 72 h (T3) and 144 h (T6) and stimulated with transfected Poly I:C at 50 μg/ml, during 24 h. *Tert*-Butyl hydroperoxide (Cat.458139, Sigma-Aldrich, US) at 0.77 mM was used as a positive control. At the end of the experiment, cells were harvested, rinsed with PBS and suspended in 10 μm 2′, 7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Cat. C6827, Invitrogen, UK) at 37 °C for 30 min. After incubation, cells were washed with PBS. Mean fluorescence intensity of the whole living cell population was measured by flow cytometry (FACSVerse; BD Biosciences, Madrid, Spain). BD FACSuite software was used to analyze a minimum of 10,000 cells per sample. The obtained results were expressed as CM-H_2DCFDA fluorescence intensity fold as fold change in comparison to the control group.

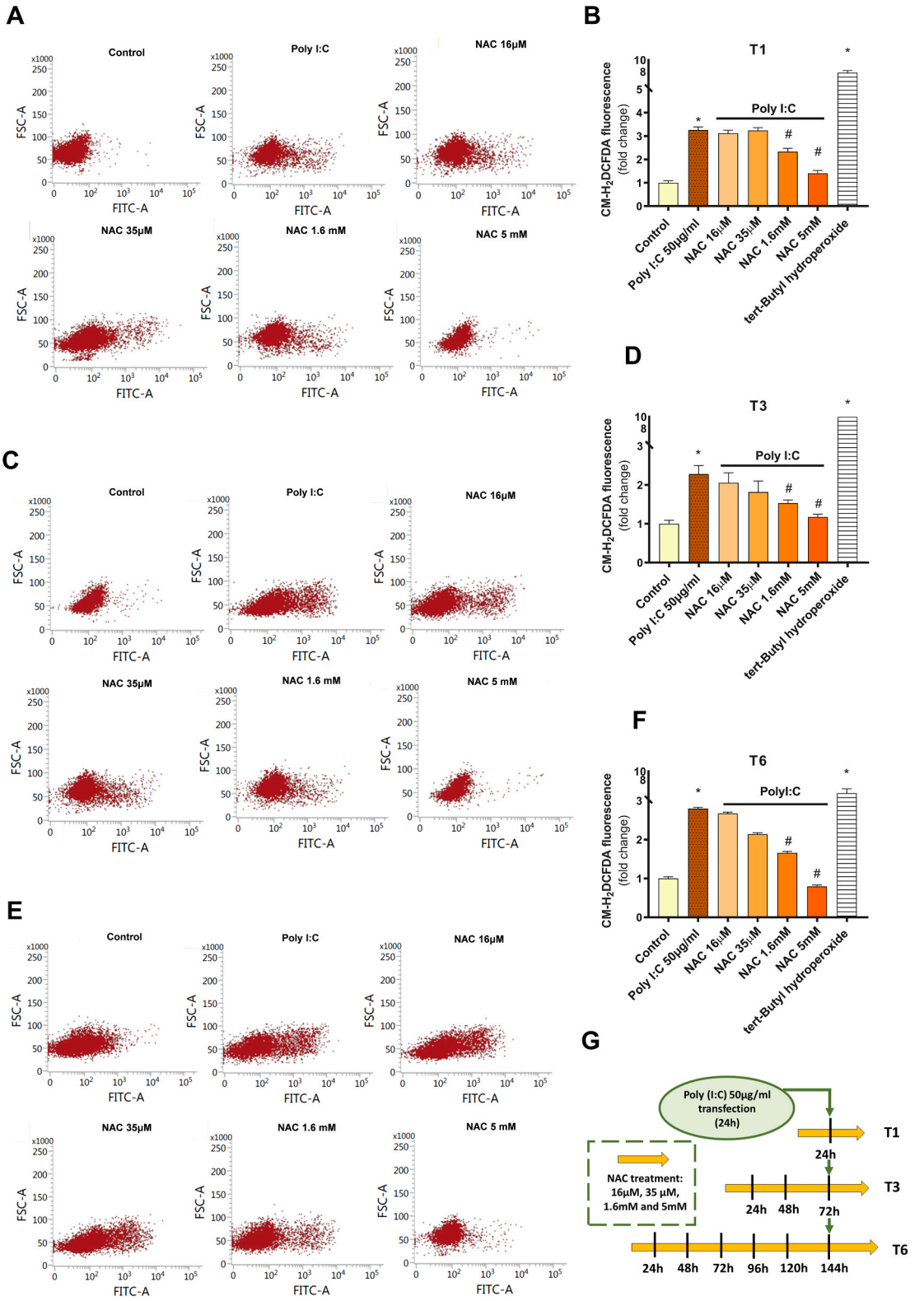

Fig. 1. NAC reduces intracellular ROS release in A549 cells. A549 cells were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different timepoints and stimulated with transfected poly I:C at 50 μg/ml. Reactive oxygen species (ROS) were measured by flow cytometric analysis using the CM-H_2DCFDA probe. (A, C, E) Representative plots for each time point are displayed: 24 h (T1), 72 h (T3) and 144 h (T6) respectively. (B, D, F) Quantification of reactive oxygen species (ROS) levels measured by the CM-H_2DCFDA assay. Mean fluorescence of the whole living cell population

was measured. (G) Workflow of the treatment. Data are expressed as CM-H_2DCFDA fluorescence intensity fold increase relative to control. Results are expressed as mean ± standard error of the mean (SEM) of three independent experiments (n = 3). Statistical analysis was performed by using Kruskal-Wallis test and Dunn's post hoc test for multiple comparisons. *$p < 0.05$ vs. control; #$p < 0.05$ vs. Poly I:C. NAC: *N*-Acetyl-L-cysteine. Poly I:C: polyinosinic-polycytidylic.

2.4. 5-CM-FDA fluorescence measurement of thiols

To quantify total intracellular thiol levels, A549 cells were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different time points: 24 h (T1), 72 h (T3) and 144 h (T6) and stimulated with transfected Poly I:C at 50 μg/ml, during 24 h. 5 mM diethyl maleate (DEM) (Cat.W505005, Sigma-Aldrich, US) was used as a positive control. At the end of the experiment, cells were harvested, rinsed with PBS and suspended in 12,5 μM 5-chloromethylfluorescein diacetate (5-CM-FDA) (Cat. C7025, Invitrogen, UK) for 15 min. Then, cells were washed, and mean fluorescence intensity of the whole living cell population was measured by flow cytometry (FACSVerse; BD Biosciences, Madrid, Spain). BD FACSuite software was used to analyze a minimum of 10,000 cells per sample. The obtained results were expressed as 5-CM-FDA fluorescence intensity fold change relative to control.

2.5. Real-time RT-PCR experiments

A549 cells were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different time points: 24 h (T1), 72 h (T3) and 144 h (T6) and stimulated with transfected Poly I:C at 50 μg/ml, during 24 h. After incubation, total RNA was extracted using the KingFisher Duo Prime automated extractor (Thermo Fisher Scientific, U.S) by using the MagMAX™ 96 Total RNA Isolation Kit (Cat. AM1830, ThermoFisher Scientific, U.S) following the manufacturer's instructions. Reverse transcription was performed in 300 ng of total RNA with the Takara PrimeScript RT Reagent kit (Cat. TKRR037A, Takarabio, Japan). The cDNA obtained from the reverse transcription reaction was subjected to amplification using primers and probes designed by Applied Biosystems for Heme oxygenase 1 (HO1) (Cat. Hs01110250_m, ThermoFisher Scientific, U.S) and NFκB (Cat. Hs00765730_m1, ThermoFisher Scientific, U.S) in a QuantStudio™ 5 Real-Time PCR System, using universal master mix (Cat. 4304437, ThermoFisher Scientific, U.S.). β-actin (Cat. Hs01060665_g1, ThermoFisher Scientific, U.S) was used as an endogenous control. The mean value of the replicates for each sample was calculated and expressed as the cycle threshold (Ct). Gene expression level was calculated as the difference (ΔCt) between the Ct value of the target gene and the Ct value of β-actin. Fold changes in the target gene mRNA levels were designated $2^{-\Delta\Delta Ct.}$

2.6. Western blotting analysis

A549 cells were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different time points: 24 h (T1), 72 h (T3) and 144 h (T6) and stimulated with transfected Poly I:C at 50 μg/ml, during 24 h. After incubation, cells were harvested in cold cytoplasmic protein extraction buffer containing 10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% NP40 and 0.5 mM PMSF. After centrifugation at 12000*g* 10 min, the pellet was washed 3 times, resuspended in nuclear protein extraction buffer (containing 20 mM HEPES pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), and incubated for 30 min. The extracts were then centrifuged and the supernatants with the nuclear extracts were recovered. Nuclear extracts were quantified using the BCA Protein Assay Kit (Cat. 23227, ThermoFisher Scientific, U.S.). 20 μg of denatured proteins were loaded into Mini-PROTEAN® polyacrylamide gels TGX ™ (Cat.4561094, Bio-Rad, UK), by application of 150 V for 1 h. Proteins were transferred to a nitrocellulose membrane Trans-Blot® Turbo ™ Transfer Pack, using the Trans-Blot® Turbo ™ Transfer System (Cat. 1704156, Bio-Rad Laboratories, UK). Then, membranes were incubated with 5% bovine serum albumin (BSA) (Cat. A7906 Sigma-Aldrich, US) for 2 h and labeled overnight at 4 °C, with NF-κB p65 antibody (Cat.ab32536, Abcam, UK). Signal visualization of proteins was carried out by incubating the membranes with chemiluminescence reagents (Cat. GERPN2232, ECL Plus, GE Healthcare, UK). Densitometry of films was performed using the Image J 1.42q software. Results of target protein expression are expressed as the percentage of the densitometry of the endogenous control lamin B1 (ab133741, Abcam, Cambridge, UK).

2.7. Statistical analyses

Results were statistically analyzed with GraphPad Prism 8. The data from cellular *in vitro* experiments were presented as mean ± standard error of the mean (SEM) of n experiments. Multiple comparisons analysis of variance (ANOVA) with Dunn's post hoc test was used for statistical analysis. p-values lower than 0.05 were considered statistically significant. A detailed Table with exact p values can be found in the supplemental material named as "supplemental-statistical table".

3. Results

3.1. NAC treatment prevents oxidative stress in vitro

Previously to the assessment of oxidative stress and inflammation, the effects of NAC treatment on cell viability were examined at the different experimental conditions and concentrations used in the study. Results of the cytotoxicity test showed viability rates higher than 90% in all the conditions (data not shown). The protective effects of NAC against the poly I:C-induced oxidative stress

response was analyzed in A549 cells by measuring intracellular ROS and thiols production and by measuring the antioxidant HO1 gene expression levels. As shown in Fig. 1A–F, treatment with NAC, prevented the ROS release at higher concentrations, NAC 1,6 mM and 5 mM at all time points. Further, the 35 μM dose became effective when incubated for the longest period T6 (Fig. 1F).

The intracellular thiol levels were decreased upon stimulation with poly I:C. NAC showed a tendency to partially revert such response at the highest dose of 5 mM at T1 (Fig. 2A and B). Treatment for a longer period, at T3 and T6 significantly prevented the thiol content decrease, even at the lower NAC doses (Fig. 2C–F). Regarding HO1 expression, HO1 was upregulated after incubation with Poly I:C and NAC prevented dose-dependently such increase (Fig. 3A–C). Nonetheless, while mM concentrations were always effective in preventing HO1 upregulation, the decrease in HO1 gene expression was significant at the lowest doses when NAC was incubated for a longer period at T6 (Fig. 3C).

3.2. NAC treatment prevents the increase of pro-inflammatory mediators in vitro

The protective effects of NAC against the poly I:C-induced cytokine release was analyzed in A549 cells. Incubation of A549 cells with Poly I:C induced an increase in IL-6 and IL-8 release. At T1, the increase of both cytokines was prevented significantly by incubation with NAC 5 mM (Fig. 4A and D A, D). At T3 and T6, the reduction of IL-6 was significant for all NAC concentrations (Fig. 4B and C). While the decrease of IL-8 at T3 was only significant for the 5 mM concentration (Fig. 4E), the reduction of IL-8 was significantly at all doses after incubation at T6 (Fig. 4F).

To analyze if the transcription factor NFκB might be implicated in the NAC anti-inflammatory effects in A549 cells, gene expression of NFκB and nuclear protein expression of NFκB p65 were measured. Poly I:C induced upregulation in the expression of NFκB. This induction was not significantly prevented by any NAC concentration at T1 (Fig. 5A), but after NAC incubation for T3 and T6 (Fig. 5B and C), NFκB expression was reduced dose-dependently by NAC, being significant at the mM concentrations at T3 and T6. As for the nuclear expression of NFκB p65, NAC 5 mM significantly prevented translocation of NFκB p65 to the nucleus at T1, and the same effect was achieved by NAC 35 μM at T6 (Fig. 5D and E).

4. Discussion

N-Acetyl-L-cysteine (NAC) has been in clinical use for more than 40 years [21]. Acute exacerbation is the principal cause of lung function worsening in COPD patients. Administration of high-dose NAC has been suggested for the prevention and decrease of the rate of exacerbations in COPD, and chronic administration requires lower doses for its effectiveness [22]. However, studies show that low NAC doses exert a better anti-oxidant than anti-inflammatory activity [11,12].

Regarding the *in vitro* results, there are some disagreements about the effective doses of NAC because the efficient doses are usually higher than the plasma concentrations achieved *in vivo* [2,4]. These inconsistencies may be due by the possibility that *in vivo*, a prolonged NAC treatment maintains the redox-dependent cell signaling and transcription [13]. However, to date, the mechanisms that underly these events have yet to be elucidated. Therefore, we suggested that similar to what happens *in vivo*, low and high NAC doses can exert anti-oxidant and anti-inflammatory activities, but its effectiveness is influenced by the duration of treatment.

To test this hypothesis, this study compares in A549 cells, different NAC concentrations; the achieved plasma levels (16 μM and 35 μM), which correspond to 600 mg and 1200 mg oral NAC administration [16,17], and two commonly used *in vitro* concentrations (1,6 mM and 5 mM). The treatment duration times were 24 h (T1), 72 h (T3) and 144 h (T6). Deterioration in COPD is usually induced by respiratory viruses. Therefore, in this study, A549 cells were stimulated for 24 h with transfected polyinosinic-polycytidylic acid (Poly (I:C)), to mimic viral infection, one of the common triggers for COPD exacerbations [19,23]. Poly (I:C) has been shown to promote the production of pro-inflammatory cytokines, in mice and in a variety of airway epithelial cell lines, including BEAS-2B, 16HBE14o- and A549 [24,25].

We first examined the antioxidant effects in the different experimental conditions. Intracellular ROS generation was measured with CM-H_2DCFDA fluorescent probe which reacts with several ROS including hydrogen peroxide, hydroxyl radicals and peroxynitrite [26]. NAC prevented the ROS increase induced by poly I:C transfection at the higher doses of 1.6 mM and 5 mM. Of note, the 35 μM dose became effective against ROS induction at the longer incubation period T6. In line with these results, Calzetta et al., reported that NAC at low concentrations (16 μM), did not modulate the levels of nitric oxide after LPS challenge in isolated airways from COPD patients [27] while NAC 1 mM could reduce hydrogen peroxide in epithelial cells [28]. Similarly, *in vivo*, long-term administration, during 9 and 12 months, of 600 mg NAC proved to decrease H_2O_2 exhalation in COPD subjects [29] while Benedetto et al. showed that H_2O_2 was reduced by NAC at a dosage of 600 mg bid for 2 months [30]. These differences show the importance of both dose and treatment duration of NAC.

As GSH levels are reduced in COPD patients during exacerbations [31] and chronic treatment with NAC has been useful to prevent COPD exacerbations [14], we next measured the cellular content of nonprotein thiols with the 5-CM-FDA probe. 5-CM-FDA fluorescence correlates with the cellular content of glutathione [32,33]. Poly I:C transfection decreased the thiol levels and NAC 5 mM significantly prevented such a decrease at T1 incubation. Interestingly, treatment with NAC for longer periods at T3 and T6, increased its effectiveness and even the smallest concentration (16 μM) augmented the thiol levels significantly. It has been shown that medium-to-high concentrations are effective in preventing the reduced GSH response, in an *ex vivo* model of COPD exacerbation [11], and in neutrophils, the GSH content increased only after 10 mM NAC [34]. Further, NAC administered *in vivo* has been also reported to require a longer time to achieve sustained effects on the cellular thiols [35]. Moreover, NAC exerted an earlier response in the case of thiols, which could explain its higher and faster effect as a GSH precursor. Our suggestion is that these increased levels of thiols induced by NAC might be the drivers of the reduction of ROS.

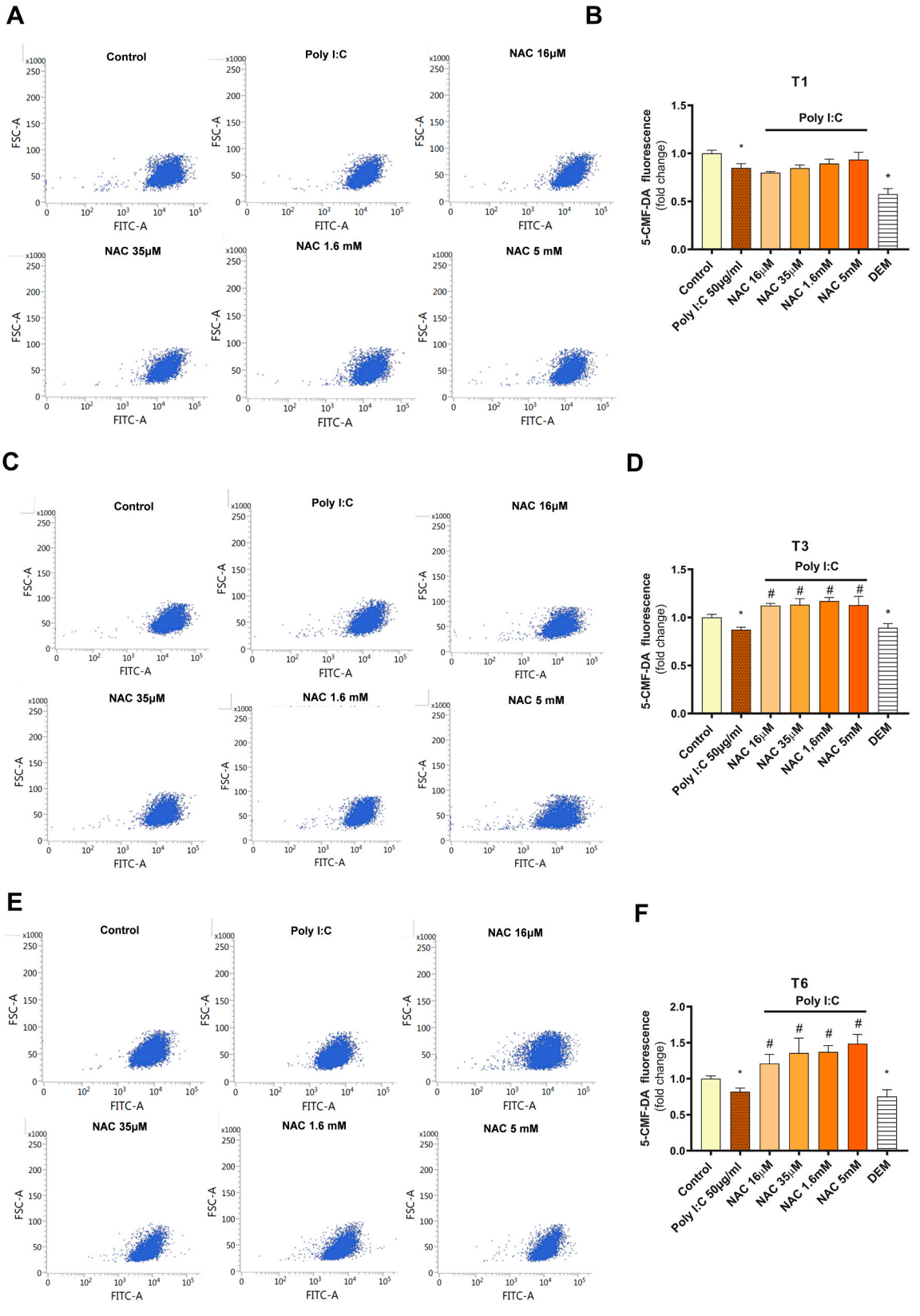

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Fig. 2. NAC increases total intracellular thiol levels in A549 cells. A549 cells were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different timepoints and stimulated with transfected Poly I:C at 50 μg/ml. Total intracellular thiol levels were measured by flow cytometric analysis using the 5-CM-FDA probe. (A, C, E) Representative plots for each time point are displayed: 24 h (T1), 72 h (T3) and 144 h (T6) respectively. (B, D, F). Quantification of thiols measured by the 5-CM-FDA assay. Mean fluorescence of the whole living cell population was measured. Data are expressed as thiols 5-CM-FDA fluorescence intensity as fold change. Results are expressed as mean ± standard error of the mean (SEM) of three independent experiments (n = 3). Statistical analysis was performed using Kruskal-Wallis test and Dunn's post hoc test for multiple comparisons * $p < 0.05$ vs. control. #$p < 0.05$ vs. Poly I:C. NAC: *N*-Acetyl-L-cysteine. POLY I:C: polyinosinic-polycytidylic. DEM: diethyl maleate.

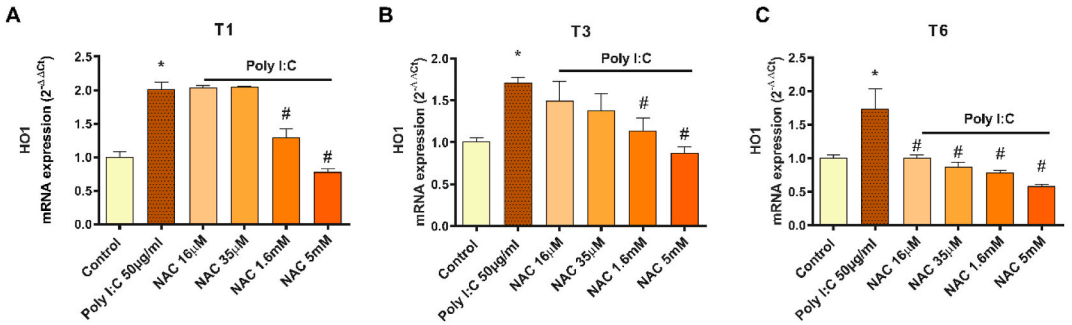

Fig. 3. NAC prevents upregulation of the antioxidant gene heme oxygenase 1 (HO1) in A549 cells. A549 cells were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different timepoints and stimulated with transfected Poly I:C at 50 μg/ml. HO1 mRNA levels were measured by real-time PCR at (A) 24 h (T1), (B) 72 h (T3) and (C) 144 h (T6). Data are expressed as $2^{-\Delta\Delta Ct}$. Results are expressed as mean ± standard error of the mean (SEM) of three independent experiments (n = 3). Statistical analysis was performed by using Kruskal-Wallis test and Dunn's post hoc test for multiple comparisons * $p < 0.05$ vs. control. #$p < 0.05$ vs. Poly I:C. NAC: *N*-Acetyl-L-cysteine. POLY I:C: polyinosinic-polycytidylic.

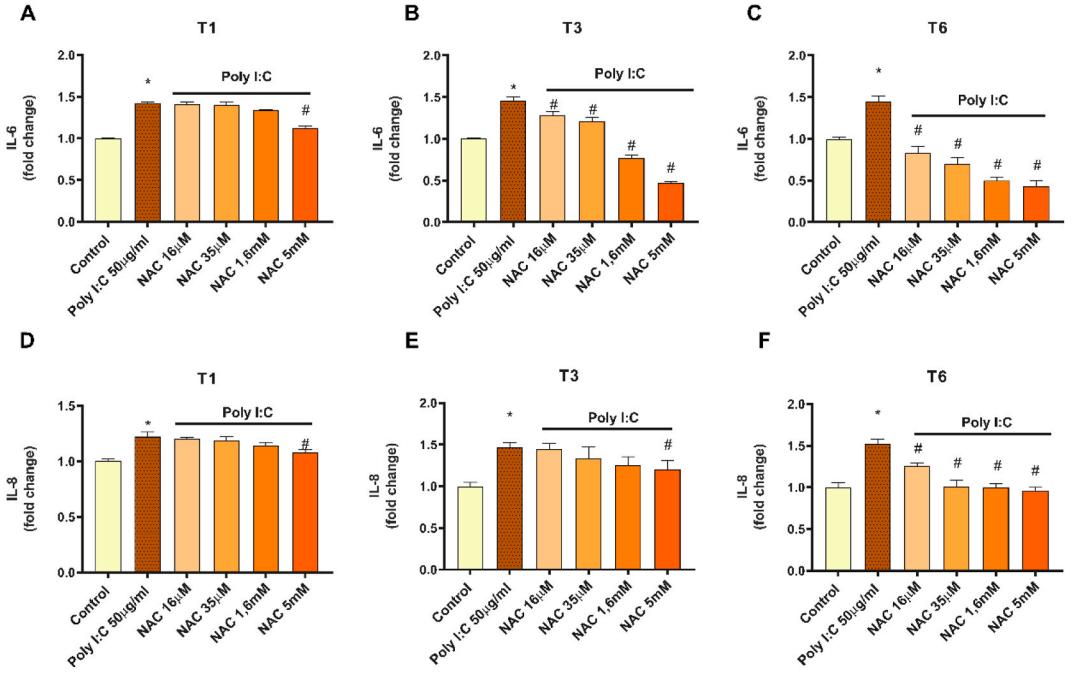

Fig. 4. NAC reduces IL-6 and IL-8 release in A549 cells. A549 cells were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different time points 24 h (T1), 72 h (T3) and 144 h (T6) and stimulated with transfected Poly I:C at 50 μg/ml (A, B, C) IL-6, and (D, E, F) IL-8 levels were measured by ELISA. Results are expressed as mean ± standard error of the mean (SEM) of three independent experiments (n = 3). Statistical analysis was performed by using Kruskal-Wallis test and Dunn's post hoc test for multiple comparisons. *$p < 0.05$ vs. control. #$p < 0.05$ vs. Poly I:C. NAC: *N*-Acetyl-L-cysteine. POLY I:C: polyinosinic-polycytidylic.

The available data *in vitro* claims that NAC is antioxidant at high concentrations but our results prove that low doses are also effective. Further, we analyzed Heme oxygenase 1 (HO1) gene expression. HO1 is a protein with pleiotropic effects, that can detect and respond to cellular stress, and contributes in regulating antioxidant responses [36]. Several studies have demonstrated a correlation between the induction of HO1 activity and the elevation in total antioxidant capacity [37]. In line with ROS, and thiols results, NAC at

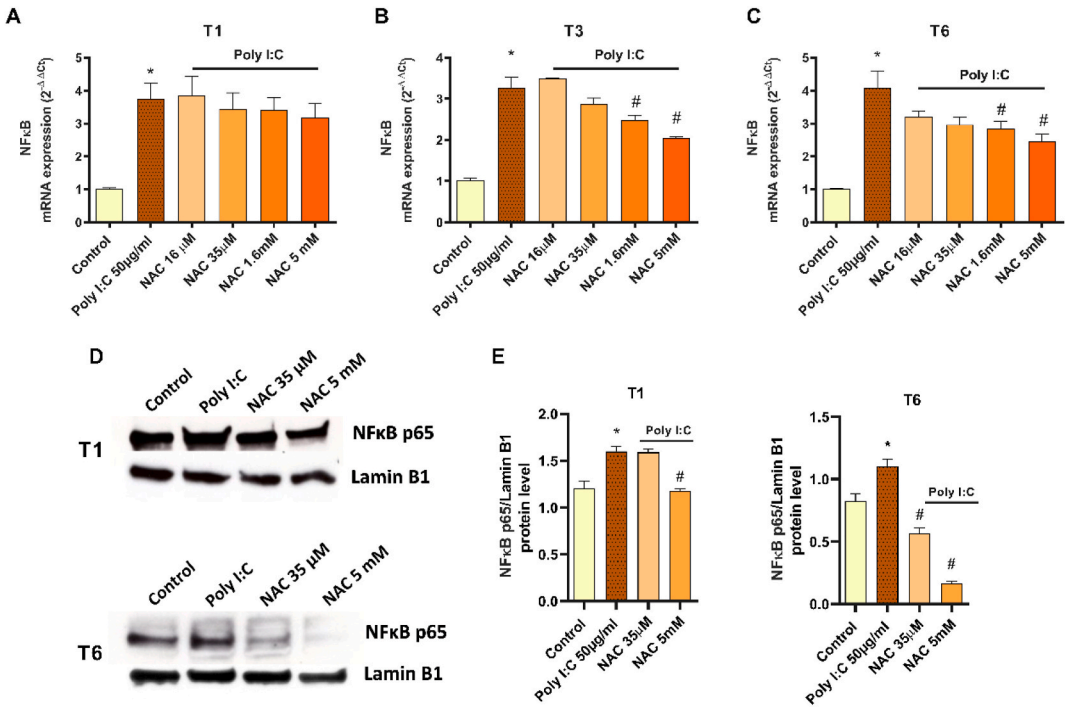

Fig. 5. NAC prevents activation of NFκB transcription factor in A549 cells. A549 cells were treated with NAC 16 μM, 35 μM, 1.6 mM and 5 mM at different time points: and stimulated with transfected Poly I:C at 50 μg/ml (A–C) NFκB mRNA levels were measured by real-time PCR at 24 h (T1), 72 h (T3) and 144 h (T6). Data are expressed as $2^{-\Delta\Delta Ct}$. (D, E) NFκB p65 subunit protein levels were analyzed by Western blotting. Quantification was performed by densitometry and normalized to Lamin B1. Uncropped versions of the blots can be found in the supplemental material named "Uncropped images of the original WB". Results are expressed as mean ± standard error of the mean (SEM) of three independent experiments (n = 3). Statistical analysis was performed by using Kruskal-Wallis test and Dunn's post hoc test for multiple comparisons * $p < 0.05$ vs. control. #$p < 0.05$ vs. Poly I:C. NAC: *N*-Acetyl-L-cysteine. POLY I:C: polyinosinic-polycytidylic.

mM concentrations protected from de Poly I.C-induced HO1 gene upregulation, at T1 and T3 incubation periods. However, the smallest μM concentrations were protective when the incubation period was increased (T6). The same response has been reported at high mM concentrations in alveolar type II cells stimulated with cigarette smoke extract (CSE) [38], and in respiratory syncytial virus -infected primary human bronchial epithelial cells [39]. High concentrations of NAC have also prevented HO1 induction in osteoblasts. Nonetheless, to our knowledge, this is the first study that demonstrates *in vitro* that longer incubation periods with low dose NAC, can exert the same antioxidant effects as higher NAC doses.

Airway inflammation can lead to exacerbations and worsening of COPD [40], therefore, we examined the anti-inflammatory effect of NAC by measuring IL-6 and IL-8 cytokines. IL-8 serves as a marker of severity of airway inflammation [41] and both IL-6 and IL-8 are elevated in patients with exacerbations and may predict the frequency of future episodes [42]. Our results showed that both IL-6 and IL-8 increased after poly I:C transfection. NAC prevented IL-6 increase only when was administered at 5 mM at T1 incubation time, but, increasing the incubation periods to T3 and T6 improved its effectiveness at all concentrations tested. The same effect was observed for IL-8, but the lower concentrations prevented IL-8 increase only during T6 incubation. The anti-inflammatory effects of NAC have been questioned because it seems that higher doses are needed *in vitro* to achieve anti-inflammatory activity [4]. *In vivo*, it has been shown that IL-8 levels decreased significantly in high dose NAC group after 10 days of treatment in patients with COPD exacerbations [12] and NAC administered at high concentrations inhibited the IL-8 release induced by lipopolysaccharide (LPS) in an *ex vivo* model of COPD exacerbation [11]. Other *in vitro* studies show that NAC concentrations in the mM range are needed to reduce diesel-induced IL-8 release in primary human bronchial epithelial cells from a COPD cohort [43], fMLP-induced IL-8 in neutrophils [34], viral-induced IL-8 in A549 cells [18] and CSE-induced IL-8 In epithelial airway cells (Calu-3) [44]. These results are in line with our findings at the T1 incubation time, where NAC was only effective at the highest concentration of 5 mM. However, we confirmed that NAC al low doses is also able to exert anti-inflammatory activity, when incubated for a prolonged time (T6). Regarding IL-6, NAC at 4 mM has been shown to prevent BaP-induced upregulation of IL-6 in lung epithelial cells [45], viral-induced IL-6 release in A549 cells [18] and CSE-induced IL-6 In epithelial airway cells (Calu-3) [44], similarly to our results at T1. What is more, unlike IL-8, the micromolar NAC doses were also effective in reducing IL-6 at T3. The longest incubation period (T6) allowed the smallest NAC concentration to be anti-inflammatory. This event has been observed by other authors, for example, in an *ex vivo* COPD exacerbation model, NAC 1 μM, prevented IL-6 elevation. This means that the positive impact of NAC in IL-6 regulation might be attributed by its antioxidant properties, as well as other anti-inflammatory mechanisms against IL-6 [11,27].

It is also interesting to mention that while the smallest concentration 16 μM at T6 was able to increase thiol levels and reduce the

inflammatory markers, this was not seen in the case of ROS assessment. The probe used to detect ROS reacts with hydrogen peroxide, hydroxyl radicals and peroxynitrite. It is possible that the antioxidant effects of NAC are faster in inducing the increase of the thiol levels than the decrease of ROS, due to the direct effect of NAC as a precursor of reduced glutathione (GSH). However, based on the very interesting relationship described among dose and time of incubation, we speculate that 16 μM at longer periods than 144 h (T6) would have produced an effect on ROS.

Next, we aimed to explore if the anti-inflammatory effect of low-dose NAC for long-term treatment was mediated by NFκB activation. As expected, the concentration of NAC 35 μM incubated at T6 was effective in reverting the upregulation of NFκB gene expression and the elevation NFκB p65 subunit in the nuclear extracts. This shows that, independently of the concentration, NAC has an influence on NFκB that varies with the treatment duration. It is well known that *in vitro* high mM NAC concentration attenuates NFκB activation in CSE-induced Calu-3 cells [44], BaP-induced NFκB activation in A549 cells [45] and silica-induced NFκB activation in bronchial epithelial cells [46]. Nonetheless, our results suggest for the first time that the NAC *in vitro* suppressive effects on NFκB activation can also be achieved at low doses when incubated for longer a period. It is notable that the data available in the *in vitro* settling suggest that mM concentrations are necessary for the anti-inflammatory effects of NAC. However, this is the first report that confirms the hypothesis that *in vitro*, low NAC doses for longer incubation periods, can also exert anti-inflammatory activity through NFκB modulation.

These results present novel evidence in A549 cells by confirming the hypothesis raised by Sadowska et al. [4] that the effects of NAC are concentration dependent not only *in vivo* but also in the *in vitro* settling. The effects of NAC might be due to the sustained antioxidant effect, along with its influence on intracellular events like modulation of NFκB activation. However, the mechanisms by which NAC regulates transcription factors, are yet to be discovered.

Of note, there are a few limitations to be mentioned, the study was designed with A549, a cell line that has increased glutathione levels [47,48] due to the requirements of the experimental method. This method requires long-term incubation periods, starting with low plating densities, and A549 cells proved to have a good growth curve and viability rates for the performance of the experiments. It is important to mention that it would have been more relevant for the extrapolation of the clinical results if the experiments were performed on epithelial primary cells. However, primary cells are not suitable for this type of experimental design as plating at low densities induces cell death over the days. Furthermore, the low transfection efficiency rates in primary cells increase the difficulty of performing this type of study.

Bearing in mind these limitations, our results have to be extrapolated to the clinical observation with caution. The findings presented in this work should be confirmed in a different lung epithelial cell line, and ideally in primary cells. Nonetheless, these results suggest and support the idea that chronic administration of low NAC doses is a good strategy to maintain the redox state and thus prevent possible exacerbations, while in patients with an already stablished airway obstruction, an acute treatment with high doses would be beneficial for COPD patients. Overall, these findings add new data to the existing body of experimental and clinical evidence of the beneficial activity profile of NAC in chronic inflammatory lung diseases such as COPD.

Author contribution statement

Paula Montero Magalló: Performed the experiments; Wrote the paper.
Inés Roger; Cristina Estornut: Performed the experiments.
Javier Milara: Conceived and designed the experiments; Analyzed and interpreted the data.
Julio Cortijo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data associated with this study has been deposited at Mendeley data under the accession number DOI: 10.17632/hdt99r9ny6.1.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15613.

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