

N-acetyl-L-cysteine inhibits antigen-mediated Syk, but not Lyn tyrosine kinase activation in mast cells

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Abstract High affinity IgE receptors ($\alpha\beta\gamma_2$) mediate the activation of the non-receptor tyrosine kinases Lyn and Syk. Here we show that the antioxidant drug *N*-acetyl-L-cysteine (NAC) inhibits antigen-mediated Syk activation whereas Lyn activation and phosphorylation of β and γ is maintained. Furthermore, NAC inhibits antigen-mediated calcium mobilization and exocytosis in a dose-dependent manner, but does not inhibit ionomycin-induced exocytosis. These data support a model in which the activation of Lyn is responsible for receptor phosphorylation and precedes the activation of Syk. The inhibition of Syk activation by NAC may be relevant to B and T cell antigen receptors, which are also linked to Syk/ZAP70 tyrosine kinases.

Key words: *N*-acetyl-L-cysteine; Fc ϵ RI receptor; tyrosine kinase

1. Introduction

Antigen/IgE complexes induce exocytosis in mast cells by clustering high affinity IgE receptors (Fc ϵ RI). Engagement of the tetrameric ($\alpha\beta\gamma_2$) Fc ϵ RI receptors mediates the activation of the non-receptor tyrosine kinases Lyn and Syk [1–6]. The activation of these kinases leads to the phosphorylation of various substrates including the β and γ receptor subunits [7–10], the phospholipase C- γ [10–12], and the GTP/GDP exchange factor vav [13]. These phosphorylation events are followed by the hydrolysis of phosphoinositides and the generation of inositol 1,4,5 triphosphate (IP₃), the later responsible for the release of calcium from the endoplasmic reticulum [14–18]. A functional role for Syk in cell surface signaling was initially recognized by the observation that a chimeric protein containing Syk induces protein phosphorylation of several substrates including PLC- γ 1, and is sufficient for the generation of a Ca²⁺ signal [19]. We have recently proposed a model of Fc ϵ RI activation in which Lyn is responsible for the phosphorylation of the β and γ receptor subunits [4]. These phosphorylation events are followed by an association of Syk with phosphorylated γ thereby promoting the activation of Syk [4]. Other studies have

also suggested that Fc ϵ RI γ associates with Syk and are consistent with our model [5, 6].

Oxidants and antioxidants are being increasingly used in studies of cell function. These include studies on growth regulation [20], apoptosis [21] and HIV-replication [22]. It is now well established that oxidizing conditions induce tyrosine kinase activation and protein phosphorylation [23–26]. However, the effect of antioxidants on tyrosine kinases has not been studied. In this report, we have investigated whether *N*-acetyl-L-cysteine (NAC), an antioxidant drug which can enter cells readily, could modulate cellular activation. We analyzed the effects of NAC on antigen-mediated protein phosphorylations, tyrosine kinase activation, calcium mobilization and exocytosis.

2. Materials and methods

2.1. Reagents and antibodies

Media and sera were purchased from Biofluids, Rockville, MD. Dinitrophenyl[30]human serum albumin (DNP-HSA, 30 molecules of DNP per molecule of HSA) was from Sigma (St. Louis, MO). *N*-acetyl-L-cysteine was obtained from Aldrich (Milwaukee, WI). Fura-2 was from Molecular Probes Inc. (Eugene, OR). Radiochemicals were purchased either from Amersham ([³²P]orthophosphate, Cat. No. PBS.13A) or DuPont-NEN ([³H]serotonin, Cat. No. NEC-225 and [γ -³²P]ATP, Cat. No. NEG 002H). The anti-phosphotyrosine (4G10) and anti-PLC- γ 1 antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY), the anti-Lyn antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit anti-Syk antibody (996), the anti-Fc ϵ RI β subunit antibody (JRK), and the DNP-specific monoclonal mouse IgE (Hi-DNP ϵ 26.82) were described previously [4,27,28].

2.2. Cell culture and serotonin release experiments

The RBL-2H3 cell line culture and serotonin release assays were performed as described previously [12]. The assay buffer contained: 25 mM Na₂PIPES, pH 7.1, 100 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 1 mM CaCl₂, 5 mM D-glucose, and 0.1% BSA.

2.3. Calcium flux measurements

RBL-2H3 cells were loaded at 5×10^6 cells/ml for 45 min at 37°C with the acetoxymethyl ester of fura-2 (2 μ M). The buffer was identical to the one used for serotonin release except for the addition of 2.5 mM probenecid. Cells were analyzed in a cuvette on a Deltascan 4000 spectrofluorimeter (Photon Technology International Inc., South Brunswick, NJ) with excitation set at 340 nm and 380 nm and emission at 500 nm. Calcium concentrations were calculated using the published value of 2.24×10^{-7} for the K_d of fura-2 at 37°C [29].

2.4. Phosphorylation on intact cells, immunoprecipitation and immunoblotting

In vivo phosphorylations and immunoprecipitations were performed as previously described [4,31]. The lysis buffer (pH 8.0) contained 200 mM boric acid, 150 mM NaCl, 0.5 or 1% Triton X-100, 1% BSA, phosphatase inhibitors (1 mM sodium orthovanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, and 5 mM EDTA), and the following protease inhibitors: 10 μ g/ml aprotinin, 4 μ g/ml leupeptin,

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Abbreviations: DNP-HSA, dinitrophenyl-[30]-human serum albumin; Fc ϵ RI, high-affinity IgE receptor; NAC, *N*-acetyl-L-cysteine; PLC, phospholipase C.

10 $\mu\text{g/ml}$ pepstatin, 1 μM phenylmethylsulfonyl fluoride (PMSF). Immunoblots were performed as previously reported [4,12].

2.5. In vitro kinase assay

RBL-2H3 cells (5×10^7 cells per sample) were lysed in the buffer described above. The kinase reaction was performed as described [30]. The kinase buffer contained: 30 mM HEPES, 5 mM MgCl_2 , 5 mM MnCl_2 , 1 μM ATP, 12.5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and 100 μM sodium orthovanadate. The immunoprecipitates were separated by SDS-PAGE and the gel analyzed by autoradiography. Radioactivity in the region of the gel corresponding to the kinases were quantitated using a radioanalytic imaging system (AMBIS Systems, San Diego, CA). The proteins were then transferred to Immobilon filters and assessed by specific anti-Syk or anti-Lyn immunoblots. Amount of precipitated kinases was quantitated with a computing densitometer (Molecular Dynamics System, Sunnyvale, CA). The activity of the kinase was estimated before and after cell activation by normalizing the kinase activity for the amount of precipitated kinase, and the ratio triggered/non triggered of kinase activity was determined.

3. Results

3.1. Inhibition of antigen-mediated protein phosphorylation by NAC in RBL-2H3 cells

We first tested the effect of NAC on the viability of RBL-2H3 cells under our experimental conditions. During an incubation time of 30 min, NAC (10 mM) did not affect cell viability as assessed by trypan blue exclusion (data not shown). We then analyzed the effect of NAC on the phosphorylation of substrates induced by antigen. RBL-2H3 cells were saturated with anti-DNP IgE, labeled in vivo with $[\text{}^{32}\text{P}]\text{phosphoric acid}$ and incubated or not with NAC (10 mM) for 2 min. Cells were then stimulated (or not) with the multivalent antigen DNP-HSA (100 ng/ml, 1 min). After cell lysis, the phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies (anti-PY), separated by SDS-PAGE and autoradiographed (Fig. 1, panel a). The proteins from the same gel were then transferred to Immobilon filters and analyzed on anti-Fc ϵ RI β or anti-Syk immunoblots (Fig. 1, panel b).

As already reported [8–13], Fc ϵ RI crosslinking by antigen induced phosphorylation of various substrates (Fig. 1a, lanes 1–2). Specific immunoblots (Fig. 1b) revealed that the 32 kDa and the 72 kDa molecules were, respectively, the Fc ϵ RI β chain and the tyrosine kinase Syk. Similarly, the 10 kDa molecule was identified as the Fc ϵ RI γ chain (data not shown). Note that the β chain appears as a doublet on the immunoblot as reported previously [7,9], while only the top band corresponds to phosphorylated β .

In NAC treated cells, the antigen-mediated phosphorylation of several substrates, including the 72 kDa molecule, was abolished whereas the phosphorylation of other substrates including the 10 kDa, 32 kDa, and 180 kDa molecules was maintained (Fig. 1a, lanes 3–4). Immunoblots (Fig. 1b) revealed that Fc ϵ RI β chain (the doublet around 32 kDa), and Fc ϵ RI γ chain (data not shown) but not Syk kinase (the 72 kDa molecule) are among the remaining phosphorylated substrates following receptor clustering in NAC treated cells. Phosphorylation of a non identified 180 kDa molecule was also unaffected by NAC. Thus NAC inhibits antigen-mediated Syk phosphorylation, but not β and γ phosphorylation.

3.2. Effect of NAC on antigen-mediated tyrosine kinase activation in RBL-2H3 cells

We then determined by in vitro kinase assays whether the

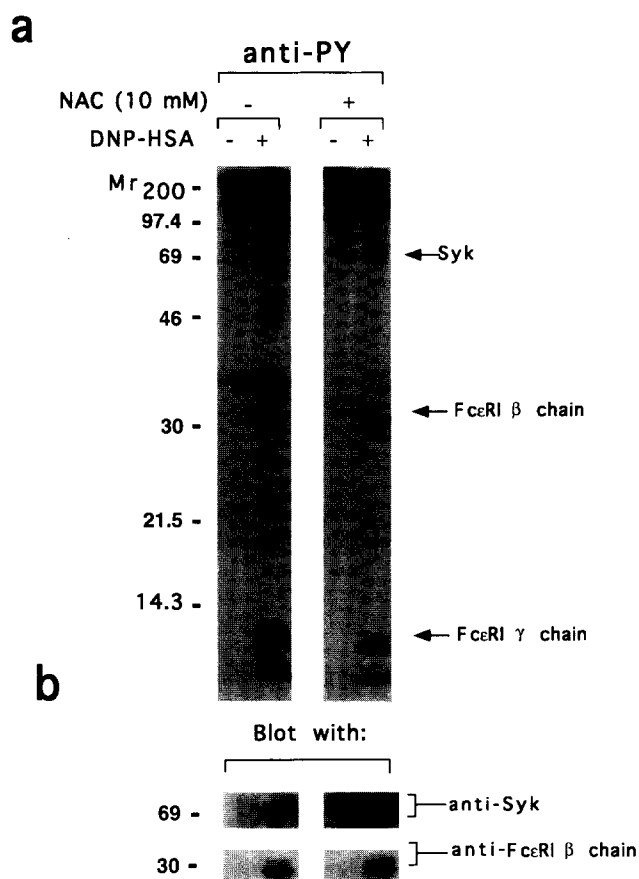


Fig. 1. Effect of NAC on antigen-induced protein tyrosine phosphorylations. RBL-2H3 cells (5×10^7 cells per sample), were saturated with anti-DNP IgE and in vivo labelled with $[\text{}^{32}\text{P}]\text{phosphoric acid}$ at 37°C as described in section 2. Cells were incubated (or not) with 10 mM of NAC (for 2 min) and triggered (or not) with antigen (DNP-HSA, 100 ng/ml) for 1 min in the continuous presence of NAC. After lysis of the cells, proteins were immunoprecipitated, as indicated, and separated on 13% SDS-PAGE under reducing conditions. (a) Autoradiograph of proteins immunoprecipitated with anti-phosphotyrosine (anti-PY) antibodies. (b) The same immunoprecipitates were analyzed by immunoblotting with either anti-Fc ϵ RI β chain or anti-Syk antibodies, as indicated.

tyrosine kinases Lyn and Syk, i.e. two kinases known to interact with Fc ϵ RI [4–6], were affected by treatment of intact cells with NAC. RBL-2H3 cells were saturated with anti-DNP IgE, treated (or not) with 10 mM NAC for 2 min and then stimulated (or not) with 1 $\mu\text{g/ml}$ DNP-HSA for 30 s. The cells were lysed and Syk or Lyn kinases were immunoprecipitated with specific antibodies. The immunoprecipitates were subjected to in vitro kinase reactions with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and analyzed by SDS-PAGE and autoradiography. The amount of precipitated kinases in each condition was assessed by specific anti-Syk or anti-Lyn immunoblots. As already reported [1–6], Fc ϵ RI crosslinking by antigen induces Syk (Fig. 2a, lanes 1–2) and Lyn activation (Fig. 2b, lanes 1–2). NAC inhibited antigen-mediated Syk activation (Fig. 2a, lanes 3–4) whereas Lyn activation is maintained (Fig. 2b, lanes 3–4). Quantitative analysis performed by using a radioanalytic imaging system and a computing densitometer revealed that the triggered/non triggered ratio of Lyn activity (see section 2) was equivalent in non treated cells and in NAC treated cells. By contrast, the triggered/non

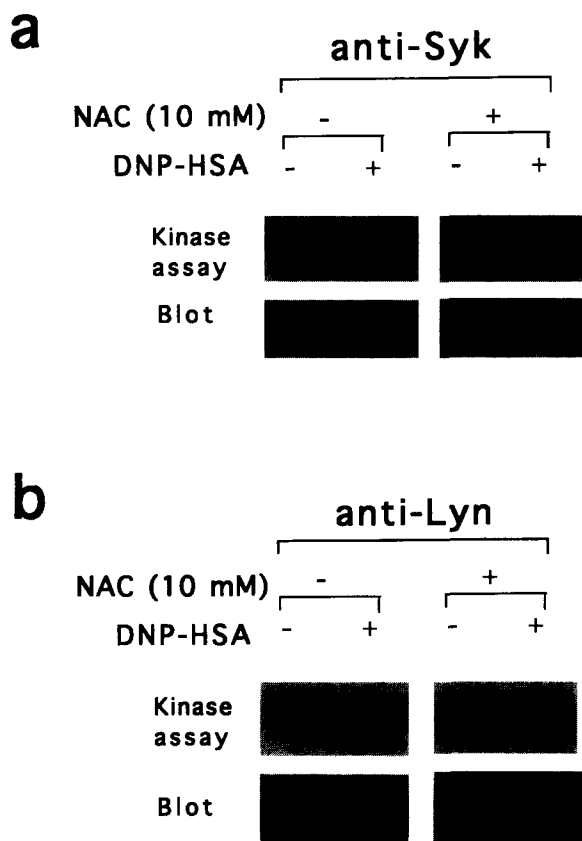


Fig. 2. In vitro kinase assays: effect of NAC on antigen-induced Lyn, and Syk activation. RBL-2H3 cells saturated with anti-DNP IgE were incubated (or not) with NAC (10 mM) and triggered (or not) with DNP-HSA (1 μ g/ml for 30 s). Immunoprecipitates were subjected to in vitro kinase assays as described in Material and Methods, and in vitro phosphorylated proteins were separated by SDS-PAGE and the gel exposed for autoradiography. (a) In vitro kinase assay after anti-Syk immunoprecipitates (top). The same immunoprecipitates were also analyzed by immunoblotting with anti-Syk antibodies (bottom). (b) In vitro kinase assay after anti-Lyn immunoprecipitates (top). Immunoblot with anti-Lyn antibodies (bottom).

triggered ratio of Syk activity was 2.6 times higher in non treated cells than it was in NAC treated cells. Thus NAC inhibits antigen-mediated Syk activation, whereas Lyn activation is maintained at the same level.

3.3. Effect of NAC on antigen-mediated calcium mobilization

We next investigated whether NAC could inhibit the calcium mobilization induced by antigen. RBL-2H3 cells were first saturated with anti-DNP IgE, loaded with the calcium-sensitive dye fura-2, treated or not (for 2 min) with increasing concentrations of NAC, transferred into the cuvette of a spectrofluorometer and triggered with 100 ng/ml of DNP-HSA. As expected, calcium mobilization was seen after addition of antigen in non treated cells. However, NAC inhibited the antigen-mediated calcium mobilization in a dose-dependent manner (Fig. 3).

3.4. Effect of NAC on antigen-induced and ionomycin-induced exocytosis in RBL-2H3 cells

We then investigated the effects of NAC on the release of serotonin induced by antigen or by the calcium ionophore ionomycin. RBL-2H3 cells were loaded with [3 H]serotonin, satu-

rated with anti-DNP IgE and incubated with increasing concentration of NAC (for 2 min) before being triggered (or not) with 100 ng/ml of DNP-HSA or with ionomycin (20 μ M). After 30 min, the amount of serotonin released in the supernatant was quantitated. Treatment of RBL-2H3 cells with NAC did not induce serotonin release (Fig. 4, filled bars). However NAC treatment inhibited the antigen-induced exocytosis in a dose-dependent manner (Fig. 4, grey bars). On the contrary, ionomycin-induced exocytosis was maintained (Fig. 4, dashed bars) except at high NAC concentration (15 mM). Thus NAC inhibits antigen-mediated exocytosis whereas ionomycin-induced exocytosis is maintained.

4. Discussion

It is well established that oxidants such as hydrogen peroxide or peroxidase are capable of activating tyrosine kinases possibly because of the inhibition of phosphatases [23–26]. However, the effect of antioxidants on the activity of tyrosine kinases has not been studied so far. In this report, we demonstrate that NAC inhibits the activation of Syk and the phosphorylation of various substrates induced after engagement of Fc ϵ RI with antigen (Figs. 1 and 2). However, the activation of Lyn is maintained (Fig. 2). Therefore it appears that NAC discriminates between the two tyrosine kinases Lyn and Syk. The activation of Syk is thought to be mediated by the binding of its two Src-homology 2 (SH2) domains to the two phosphotyrosines of the ARAM (antigen recognition activation motif) motif of the Fc ϵ RI γ chain [4–6]. We have recently proposed that the phosphorylation of γ and of β is due to Lyn [4]. The data presented here support this model of activation. NAC inhibits the Syk-related but not the Lyn-related events such as receptor phosphorylation and seems to uncouple the activation of Syk

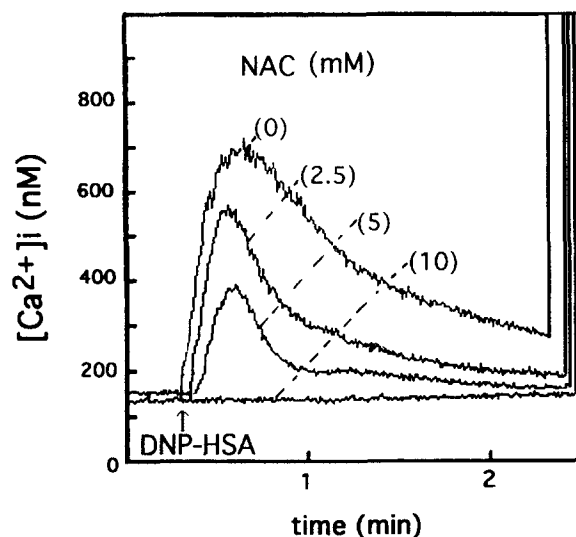


Fig. 3. NAC inhibits antigen-induced calcium mobilization. RBL-2H3 cells were saturated with anti-DNP IgE, loaded with fura-2 (2 μ M). The cells were then incubated, for 2 min with increasing concentrations of NAC, as indicated, before being challenged (arrow) with DNP-HSA (100 ng/ml). The calcium mobilization was measured on a Photon Technology International Deltascan 4000 spectrofluorimeter (cuvette system). Triton X-100 (0.1% final) and EGTA (18 mM final) were added for calibration (off scale). Calcium concentrations were calculated using the published value of 2.24×10^{-7} for the K_d of fura-2 at 37°C.

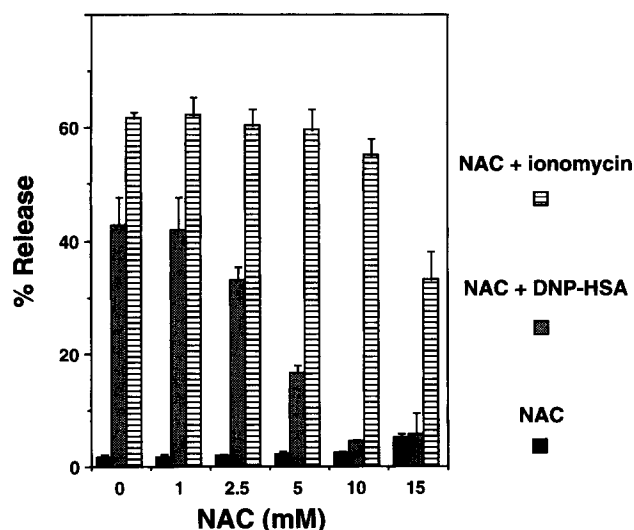


Fig. 4. Effect of NAC on antigen-mediated and on ionomycin-mediated exocytosis. RBL-2H3 cells were labeled with [3 H]serotonin, saturated with anti-DNP IgE, and incubated for 2 min with increasing concentrations of NAC. Cells were then stimulated with 100 ng/ml DNP-HSA or 20 μ M ionomycin or vehicle (as indicated), in the continuous presence of NAC. The amount of [3 H]serotonin released in the supernatant after 30 min is expressed in percent of the total [3 H]serotonin incorporated into the cells.

from Lyn activation. The mechanism of this differential inhibition of the two kinases by NAC remains to be defined. Phosphatases but also kinases contain conserved cysteines residues which are essential for their activity [31,32]. NAC, which is a thiol-containing compound, could affect the redox buffering capacity of the cytosol thereby affecting the redox status of sulfhydryl groups of various enzymes such as Syk. A difference of sensitivity to the redox environment between Lyn and Syk could explain the results.

We also show that NAC inhibits calcium mobilization and exocytosis induced by clustering Fc ϵ RI with antigen. A specific inhibition of Syk by NAC could explain our results. Nevertheless, we cannot rule out that NAC has multiple cellular targets, some of which could be involved in the degranulation process. However, the fact that ionomycin-induced exocytosis is maintained, suggests that the target involved in the inhibition of exocytosis by NAC precedes the calcium signal. This would also explain why calcium mobilization after Fc ϵ RI aggregation is inhibited. Studies with other kinases and phosphatases will be necessary to define the specificity of NAC. Looking at other systems should also be informative. The B cell antigen receptor (BCR), like Fc ϵ RI, activates also the tyrosine kinases Lyn and Syk [33]. The T cell antigen receptor (TCR) activates other kinases: the Src-family kinase Fyn and Lck and the 72 kDa ZAP-70 homologous to Syk [34]. One can speculate that, like in mast cells, NAC will inhibit Syk/ZAP-70 kinases in B and T cells without affecting the other Src-family kinases.

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