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N-Acetylcysteine suppresses TNF-induced NF-κB activation through inhibition of IκB kinases

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Abstract Here, we used a reductant, *N*-acetyl-L-cysteine (NAC), to investigate the redox-sensitive step(s) in the signalling pathway from the tumor necrosis factor (TNF) receptor to nuclear factor κB (NF-κB). We found that NAC suppressed NF-κB activation triggered by TNF or by overexpression of either the TNF receptor-associated death domain protein, TNF receptor-associated factor 2, NF-κB-inducing kinase (NIK), or IκB kinases (IKKα and IKKβ). NAC also suppressed the TNF-induced activation of IKKα and IKKβ, phosphorylation and degradation of IκB, and nuclear translocation of NF-κB. Furthermore, NAC suppressed the activation of IKKα and IKKβ triggered by the overexpression of NIK. These results indicate that IKKα and IKKβ are subject to redox regulation in the cells, and that NAC inhibits NF-κB activation through the suppression of these kinases.

Key words: *N*-Acetyl-L-cysteine; Tumor necrosis factor; Nuclear factor κB; IκB kinase; Redox

1. Introduction

Cells produce reactive oxygen radicals (ROS), such as O_2^-, H_2O_2, and $OH^•$, through metabolic pathways, including that of the electron transport reaction in mitochondria. Several environmental stimuli, such as UV light and γ-ray irradiation, also generate ROS in cells. Cells possess several antioxidant systems, including cellular reductants, such as glutathione (GSH), and cellular antioxidant enzymes, such as catalase, which act against oxidative radical stress. Regulation of the cellular redox state is crucial for cell survival, and disturbance of this by increased levels of ROS induces cell death by necrosis and/or apoptosis. Recent studies have revealed that regulation of the cellular redox state plays an important role not only in cell survival, but also in cellular signalling systems linked to cell growth and cell differentiation [1–4].

Tumor necrosis factor (TNF) elicits a wide range of biological effects by induction of several genes via activation of a heterodimeric transcription factor, NF-κB, which is composed of two proteins, p65 (RelA) and p50 (NF-κB1) [5–7]. In the unstimulated state, NF-κB is bound to an inhibitory protein, IκB, which regulates the DNA binding and intracellular localization of NF-κB [5–7]. Upon binding of TNF to its receptors on the cell membrane, the signal is transduced through the TNF receptor-associated death domain protein (TRADD) [8], TNF-receptor associated factor 2 (TRAF2) [9], and NF-κB-inducing kinase (NIK) [10], and then activates IκB kinases (IKKα and IKKβ) [11–15]. The activated IKKα and IKKβ phosphorylate serine residues in the N-terminal region of IκB, which, in turn, induces the degradation of IκB by proteasomes [11–15]. This sequential reaction finally results in the release of NF-κB from IκB, allowing the activated NF-κB to enter the nucleus and promote gene transcription. As well as TNF, several extracellular stimuli, including oxidative radical stress, activate NF-κB [16–19]. Furthermore, it has been reported that antioxidants, such as *N*-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), suppress the TNF-induced NF-κB activation in the cells, indicating that the activation of NF-κB is subject to redox regulation [18–21].

NAC is a unique compound which acts as a reductant both by its own reducing power and by stimulating the synthesis of the major cellular reductant GSH [22]. It has been shown that this compound has a wide range of biological effects on cells. NAC inhibits apoptosis induced by oxidative radical stress, deprivation of growth factors or a variety of cytotoxic drugs [23–26]. NAC also stimulates mitogen-activated protein kinase (MAPK) through an upstream signalling molecule, Ras [26,27], which has been revealed to be involved in its anti-apoptotic effects [26]. Conversely, NAC uncouples the signal transduction from Ras to the MAPK cascade in rat pheochromocytoma PC12 cells when the cells are stimulated by nerve growth factor [28]. Although it is still unclear how NAC elicits these biological effects, its ability to modulate a variety of cellular functions indicates that intracellular signalling systems are subjected to redox regulation. Regarding the NF-κB signalling pathway, NAC suppresses the activation of NF-κB induced by TNF, and several extracellular stimuli, including oxidative radical stress and lipopolysaccharide [18,29]. NF-κB itself is a redox-sensitive transcription factor whose DNA binding activity is suppressed by oxidation, and which is activated by reduction in vitro [30–32]. Thus, the ability of NAC to suppress NF-κB in vivo indicates that the upstream signalling pathway to NF-κB, at least, is subject to redox regulation. However, it remains unclear which step(s) of the signal transduction pathway is suppressed by NAC. Identification of the NAC-sensitive step(s) will provide a pivotal clue to the molecular mechanism of redox regulation in cellular signalling systems. Here, we investigated the effects of NAC on the

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Abbreviations: BSO, L-buthionine-(*S*,*R*)-sulfoximine; DTNB, 5-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GSH, glutathione; IKK, IκB kinase; NAA, *N*-acetylalanine; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; TRADD, TNF receptor-associated death domain protein; TRAF2, TNF receptor-associated factor 2

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TNF-induced activation of NF-κB, and found that IKKα and IKKβ were the primary targets of NAC.

2. Materials and methods

2.1. Materials

[^{125}I]TNF was obtained from Amersham Pharmacia. L-Buthionine-(*S*,*R*)-sulfoximine (BSO) was obtained from Sigma (St. Louis, MO, USA). 5-Dithiobis(2-nitro-benzoic acid) (DTNB) was obtained from Nacalai Tesque (Kyoto, Japan). NAC was obtained from Merck (Darmstadt, Germany). Recombinant human TNF, anti-p50 and anti-p65 antibodies were obtained from Upstate Biotechnology (New York, USA). Anti-TRADD, anti-TRAF2, anti-NIK, anti-IκBα and anti-HA antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Flag antibody affinity resin was obtained from Sigma (St. Louis, MO, USA). The cDNA of IκBα was amplified using the polymerase chain reaction (PCR) with a primer set: 5′-ATGTTCCAGGCGGCCGAGCG-3′ and 5′-CTCATAACGTCAGACGCTGG-3′. The DNA fragment corresponding to amino acids 1–55 of IκBα was ligated with pGEX4T2 (Amersham Pharmacia), and then the bacterially expressed GST fusion IκBα(1–55) proteins were purified using GSH-Sepharose (Amersham Pharmacia). Expression plasmids of TRADD (pRK-Myc-TRADD), TRAF2 (pRK-Flag-TRAF2), NIK (pRK-Flag-NIK), IKKα (pRK-Flag-IKKα), and IKKβ (pRK-Flag-IKKβ) were a kind gift from Dr. David Goeddel (Tularik). An expression plasmid of p65 (pcDNA-p65) was a kind gift from Dr. Makoto Nakanishi (Nagoya City University) Expression plasmids of hemagglutinin (HA)-tagged IKKα (pRK-HA-IKKα) and IKKβ (pRK-HA-IKKβ) were generated by ligation of plasmids encoding the HA epitope (MYPYDVPDYA) to the DNA fragments of IKKα and IKKβ, respectively. A luciferase reporter plasmid of NF-κB, pIG3luc, encodes the triple NF-κB binding sites (AGCTTCAGAGGGGACTTTCCGAGAGG) upstream of the luciferase gene in Picca gene enhancer vector (Toyo B-Net Co., Japan).

2.2. Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 μg/ml kanamycin at 37°C in 5% CO_2 in air.

2.3. Luciferase gene reporter assay

HeLa cells (2×10^5 cells per 35 mm dish) were transfected with pIG3luc using Lipofectin (Gibco BRL). After 24 h, the luciferase activity was determined using the Luciferase Assay System (Promega) and normalized by the protein content in each sample.

2.4. GSH assay

The cells were collected and suspended in ice-cold phosphate-buffered saline (PBS). An equal volume of 10% trichloroacetic acid (TCA) was then added to the cell suspension and the mixture was centrifuged ($20\,000\times g$ for 10 min) at 4°C. The supernatant was extracted with six volumes of diethylether six times to remove the TCA. The resulting extract was incubated with 200 μM NADPH and GSH reductase (1 U/ml) for 10 min at 37°C, and then the GSH level was measured by 1 mM DTNB. The reaction was monitored by the absorbance change at 415 nm.

2.5. Kinase assays for IKKα and IKKβ

HeLa cells (2×10^5 cells per 35 mm dish) were transfected with plasmids encoding Flag-tagged IKKα or HA-tagged IKKβ by Lipofectin. Then the cells were solubilized in a buffer containing 20 mM Tris–HCl, pH 7.4, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 mM NaF, 1 mM APMSF, 50 U/ml aprotinin, 20 μg/ml pepstatin, 20 μg/ml leupeptin, 1 mM dithiothreitol (DTT), and 1% Triton X-100, and then centrifuged at $15\,000\times g$ for 20 min. The expressed Flag-IKKα and HA-IKKβ were recovered from the lysates by immunoprecipitation using anti-Flag antibody affinity resin or anti-HA polyclonal antibody, respectively. Then the immune complexes were incubated with 10 μl reaction buffer containing 20 mM HEPES–NaOH, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 100 μM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM DTT, 100 μM ATP, 0.05 μCi [γ-^{32}P]ATP, 5 μg GST–IκBα(1–55) at 30°C for 20 min. After SDS–PAGE, the phosphorylation of GST-IκBα was estimated by Imaging plate (Fuji Film).

2.6. Western blot analysis

Cell lysates were fractionated by SDS–PAGE, and then transferred to ECL membrane (Amersham Pharmacia). Western blot analysis was performed using a Western Blotting Detection System (Amersham Pharmacia) according to the manufacturer's instructions.

2.7. Immunofluorescent cell staining

HeLa cells grown on coverslips were washed with 0.1 M potassium phosphate buffer (pH 7.4) and fixed with 4% formaldehyde in 0.1 M potassium phosphate buffer (pH 7.4) for 1 h at room temperature. The cells were permeabilized with 0.1% Triton X-100 in PBS for 1 h,

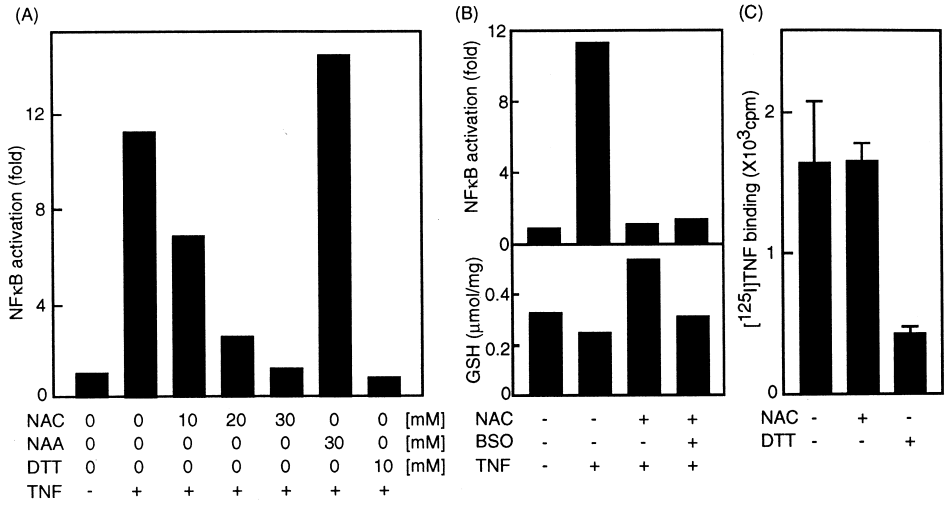

Fig. 1. The inhibitory effects of NAC on NF-κB activation. A: Inhibitory effects of reductants on TNF-induced NF-κB activation. HeLa cells were transfected with pIG3luc plasmids. After 24 h, the cells were treated with NAC, NAA, or DTT for 1 h, and then were stimulated with TNF (20 ng/ml) for 4 h. The activity of NF-κB was estimated by the luciferase assay. B: The inhibitory effects of NAC were independent of GSH. The cells were treated with NAC (30 mM) in the presence or absence of 100 μM BSO for 1 h, and then were stimulated with TNF (20 ng/ml) for 4 h. The activity of NF-κB was estimated by the luciferase assay, and the cellular GSH levels were estimated using DTNB. C: NAC did not inhibit the binding of TNF to the receptor. HeLa cells (8×10^5 cells) were incubated with 0.1 μCi [^{125}I]TNF for 5 h on ice in the presence or absence of 30 mM NAC or 10 mM DTT. The binding of [^{125}I]TNF to the cells was estimated using a γ-counter. The results are presented as mean ± S.D. from three independent experiments.

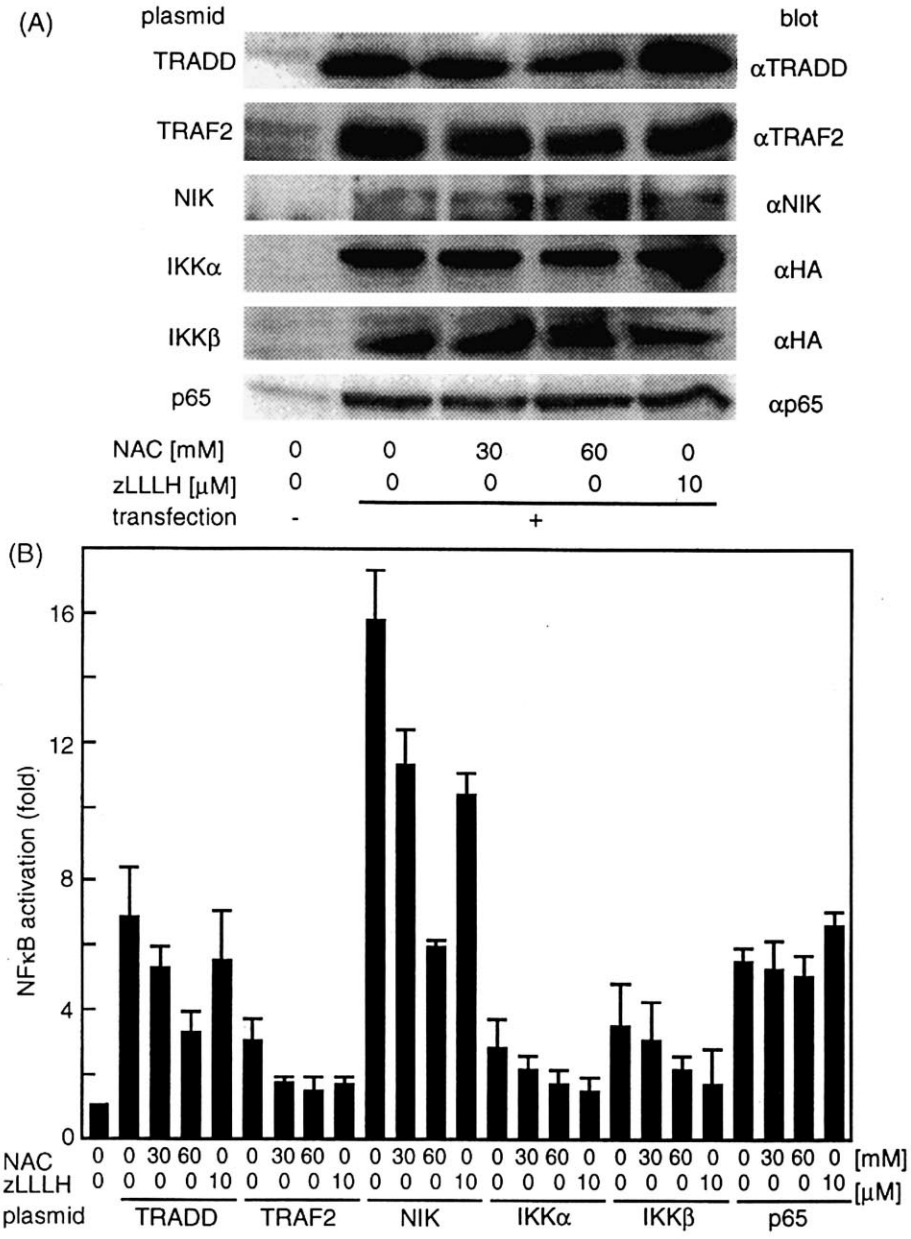

Fig. 2. The inhibitory effects of NAC on NF-κB activation induced by overexpression of either TRADD, TRAF2, NIK, IKKα, or IKKβ. HeLa cells were transfected with pIG3luc plasmids together with expression plasmids encoding TRADD (pRK-Myc-TRADD), TRAF2 (pRK-Flag-TRAF2), NIK (pRK-Flag-NIK), IKKα (pRK-HA-IKKα), IKKβ (pRK-HA-IKKβ), p65 (pcDNA-p65) or the control vectors. A caspase inhibitor, z-VAD-fmk (10 μM), was added to the cell culture transfected with TRADD to prevent apoptosis. After 24 h, the cells were treated with various concentrations of NAC or 10 μM zLLLH for 6 h. A: The expression levels of transfected TRADD, TRAF2, NIK, IKKα, IKKβ and p65 proteins in the cells treated with various concentrations of NAC or zLLLH were estimated by Western blotting using anti-TRADD, anti-TRAF2, anti-NIK, anti-HA, or anti-p65 antibodies. B: NF-κB activity was estimated by the luciferase assay. The results are presented as mean ± S.D. from three independent experiments.

incubated with anti-p65 or anti-p50 antibody at room temperature for 1 h, and then stained with fluorescein isothiocyanate-conjugated anti-IgG antibody (Jackson Immunoresearch Laboratories) for 1 h at room temperature.

3. Results

3.1. DTT and NAC suppress TNF-induced NF-κB activation

The luciferase gene reporter assay revealed that TNF-induced NF-κB activation in HeLa cells was suppressed by NAC in a dose-dependent manner. Another reductant, DTT, also suppressed NF-κB activation, while *N*-acetylalanine (NAA) failed to inhibit it (Fig. 1A), indicating that a thiol residue on NAC is required for the suppression of NF-κB. It is known that NAC stimulates the metabolic production of the major cellular reductant GSH in cells [22]. Indeed, cellular GSH levels were increased when cells were treated with 30 mM NAC for 5 h (Fig. 1B). To elucidate whether the increase in GSH was involved in the inhibitory effects of NAC, BSO, a specific inhibitor of GSH synthesis, was added to abolish the increase in GSH. Treatment of the cells with BSO completely inhibited the increase in cellular GSH (Fig.

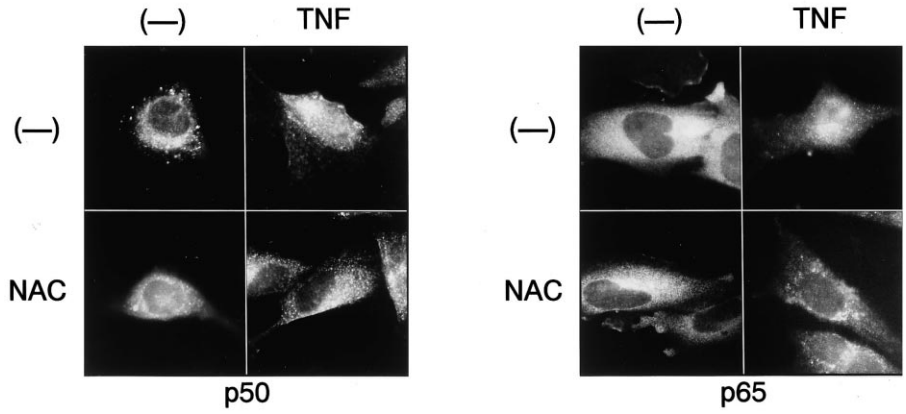

Fig. 3. NAC inhibits nuclear translocation of NF-κB. The cells were incubated in the presence or absence of 30 mM NAC for 1 h, and then stimulated with 20 ng/ml TNF. After 30 min TNF stimulation, the cells were fixed and stained with anti-p65 or anti-p50 antibodies, and then analyzed by immunofluorescence microscopy.

1B). In these BSO-treated cells, NAC effectively suppressed TNF-induced NF-κB activation, indicating that NAC inhibits NF-κB activation irrespective of GSH production.

TNF itself has a disulfide bond in the molecule [33], and the TNF receptor also contains several disulfide bonds in the extracellular domain [34]. A plausible mechanism for reductant-mediated suppression of TNF-induced NF-κB activation is that the reductant cleaves these disulfide bonds, resulting in the inhibition of TNF binding to the receptor. This turned out to be the case in DTT-induced suppression of NF-κB activation as revealed by inhibition of [^{125}I]TNF binding to the cells (Fig. 1C). However, the [^{125}I]TNF binding was not affected at all by 30 mM NAC (Fig. 1C), indicating that NAC and DTT suppressed NF-κB activation in a different manner; DTT inhibits the binding of TNF to its receptor, whereas the inhibitory effect(s) of NAC is on the downstream signalling pathway.

3.2. *NAC suppresses TRADD-, TRAF2-, NIK-, IKKα-, and IKKβ-induced activation of NF-κB*

TNF induces the activation of NF-κB through cellular signalling pathways involving TRADD, TRAF2, NIK, IKKα, and IKKβ. To elucidate the NAC-sensitive step(s) from the TNF receptor to NF-κB activation, we analyzed the effect of NAC on the activation of NF-κB in cells overexpressing either TRADD, TRAF2, NIK, IKKα, IKKβ, or p65. Transfection of the plasmids encoding these genes led to significant expression of these proteins, and incubation with NAC or the proteasome inhibitor zLLLH did not change the amount of these proteins in the cells (Fig. 2A). Overexpression of these signalling molecules led to significant induction (3–16-fold) of NF-κB reporter gene expression (Fig. 2B). The induction of reporter genes by TRADD, TRAF2, NIK, IKKα, or IKKβ decreased partially when the cells were treated with NAC for 6 h in a dose-dependent manner. When compared with the inhibitory effect of NAC on TNF-induced NF-κB activation (Fig. 1A), it was apparent that NAC led to much less suppression of NF-κB activation in cells overexpressing these signaling molecules. This was not surprising since NAC was added after the transfection of these genes followed by an incubation period required for expression of the genes, during which NF-κB was fully activated. Rather, the partial inhibition of TRADD-, TRAF2-, NIK-, IKKα-, and IKKβ-induced expression of the reporter gene reflects a significant suppression of NF-κB by NAC. Consistently, the proteasome inhibitor zLLLH, which attenuates the degradation of IκB (see Fig. 4), also revealed a partial inhibition in the reporter gene assay (Fig. 2). In contrast, NAC failed to inhibit the induction of reporter gene expression in cells overexpressing p65 (Fig. 2B), indicating that NAC did not inhibit either the nuclear translocation or the DNA binding of NF-κB. Taken together, these results indicate that NAC inhibited NF-κB activation through suppression of a step(s) between these signalling molecules and NF-κB.

3.3. *NAC inhibits phosphorylation and degradation of IκB, and nuclear translocation of NF-κB*

Translocation of NF-κB to the nucleus is a crucial step in NF-κB activation. Immunofluorescence analyses using either anti-p65 or anti-p50 antibody have revealed that TNF-induced localization of NF-κB in the nucleus was completely suppressed in NAC-treated cells (Fig. 3). NAC-mediated suppression of nuclear translocation was also confirmed by Western blotting analysis of nuclear extracts (data not shown). The inhibition of nuclear translocation suggested that NAC suppressed upstream signalling systems, such as phosphorylation

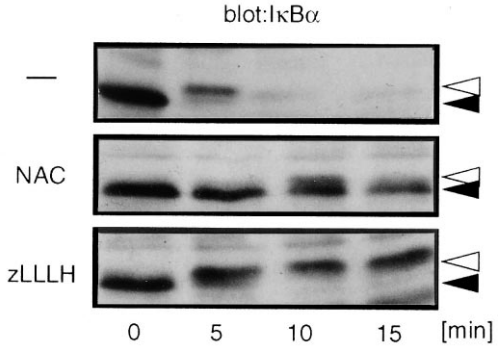

Fig. 4. NAC inhibits phosphorylation and degradation of IκB. The cells were incubated in the presence or absence of 30 mM NAC or 10 μM zLLLH for 1 h, and then stimulated with TNF. At the indicated times, cellular proteins were recovered and analyzed by Western blotting using anti-IκB antibody. The black arrowheads indicate IκB, and the white arrowheads indicate the phosphorylated form of IκB.

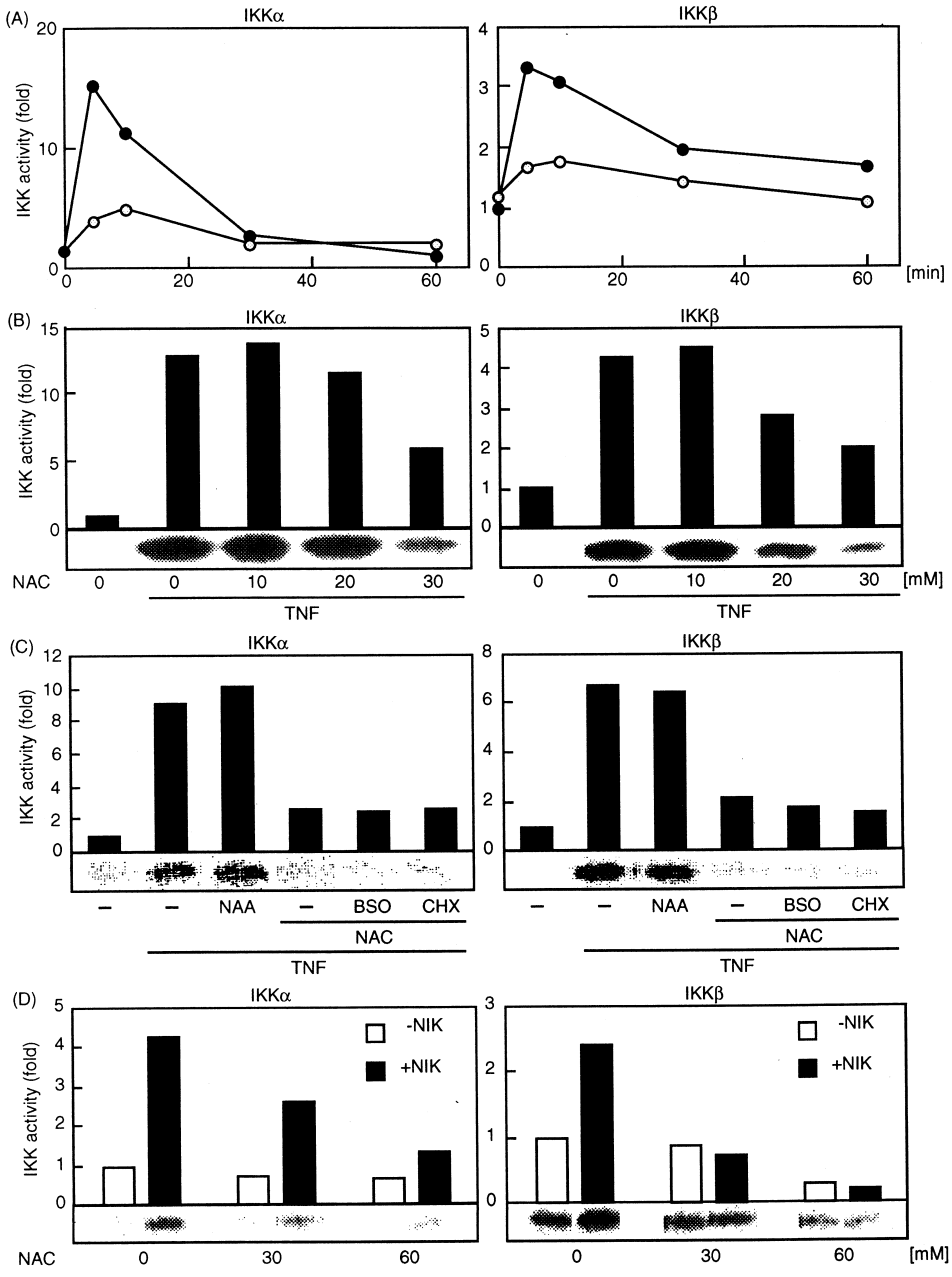

Fig. 5. Inhibitory effects of NAC on the activation of IKKα and IKKβ in the cells. A: NAC inhibits the TNF-induced activation of IKKα and IKKβ. The cells were transfected with plasmids encoding Flag-tagged IKKα (pRK-Flag-IKKα) and HA-tagged IKKβ (pRK-HA-IKKβ). After 48 h, the cells were incubated with or without 30 mM NAC for 1 h, and then were stimulated with 20 ng/ml TNF. At the indicated times, the transfected IKKα and IKKβ were recovered by immunoprecipitation, and then the kinase activity was estimated using GST–IκBα(1–55) as substrate. B: Dose-dependent inhibitory effects of NAC on IKKα and IKKβ. After 48 h of transfection, the cells were incubated with various concentrations of NAC for 1 h, and then were stimulated with 20 ng/ml TNF for 10 min. C: NAC inhibits IKKα and IKKβ independently of cellular GSH and new protein synthesis. After 48 h of transfection, the cells were incubated in the presence or absence of 30 mM NAC or NAA with or without 100 μM BSO or 1 μg/ml cycloheximide for 1 h, and then were stimulated with 20 ng/ml TNF for 10 min. D: NAC suppresses NIK-induced activation of IKKα and IKKβ. The cells were transfected with plasmids encoding HA-tagged IKKα or HA-tagged IKKβ together with expression plasmids encoding NIK (pRK-Flag-NIK) or the vector (pRK-Flag). After 24 h, the cells were incubated with various concentrations of NAC for 6 h, and then the transfected IKKα and IKKβ were recovered by immunoprecipitation. The phosphorylation of GST–IκBα(1–55) was detected by Imaging plate (figures), and then the activity of IKK was estimated (graphs).

of IκB and/or release of NF-κB from IκB. This prompted us to investigate the effects of NAC on the phosphorylation and degradation of IκB. IκB was rapidly phosphorylated within 5 min of TNF stimulation, and then completely degraded within 15 min (Fig. 4). Even though a small fraction of IκB was phosphorylated 10 min after TNF stimulation in the presence of NAC, a large fraction of IκB was neither phosphorylated nor degraded. It should be noted that the phosphorylated forms of IκB were effectively degraded within 15 min even in the presence of NAC. In contrast, although zLLLH did not inhibit the phosphorylation of IκB, it effectively suppressed the degradation of IκBα. Thus, NAC suppressed IκB

phosphorylation without any effect on the proteasome system degrading phosphorylated IκB.

3.4. NAC suppresses the activation of IKKα and IKKβ

The results obtained above suggested that NAC suppressed the upstream kinases, IKKα and IKKβ. To assess this, we next introduced plasmids encoding Flag-tagged IKKα and HA-tagged IKKβ into the cells, and the activity of these kinases was directly determined after immunoprecipitation. Treatment of the cells with TNF rapidly activated these kinases within 10 min, whereas TNF-induced activation of both IKKα and IKKβ was markedly suppressed in the cells pretreated with NAC (Fig. 5A). The dose dependence of NAC on the inhibition of these kinases was similar to that of the NAC-mediated suppression of NF-κB activation (compare Fig. 5B with Fig. 1A). Again, NAA failed to inhibit the activity of these kinases, and the NAC-mediated suppression was not abolished by either BSO or cycloheximide, indicating that NAC, as a reductant, inhibits both IKKα and IKKβ independently of cellular GSH and new protein synthesis (Fig. 5C). IKKα and IKKβ are activated by the upstream kinase, NIK, in the cells. Indeed, the activity of IKKα and IKKβ was increased when the cells were transfected with NIK expression plasmids (Fig. 5D). Noteworthy was that the NIK-induced activation of IKKα and IKKβ was markedly suppressed when the cells were cultured with NAC (Fig. 5D). Thus, it was evident that NAC suppressed the signalling from NIK to IKKα and IKKβ, which in turn resulted in the suppression of NF-κB.

4. Discussion

Here we have demonstrated that NAC effectively suppresses the TNF-induced activation of NF-κB as a result of the suppression of both IKKα and IKKβ. These kinases are the key enzymes which phosphorylate the IκB molecule associated with NF-κB, which is followed by degradation of IκB by the proteasome system, leading to the activation and the nuclear translocation of NF-κB. Thus it is intriguing that the signalling pathway of TNF is subjected to redox regulation not at the receptor but at the downstream kinase level. A similar observation has been reported that another antioxidant, PDTC, also inhibits TNF-induced NF-κB activation accompanied by the suppression of IκB degradation [20]. Furthermore, PDTC suppresses carbachol-induced activation of IKKα and IKKβ in canine gastric parietal cells [35]. Combined together, it is most plausible that IKKα and IKKβ are regulated by redox.

It has been reported that NAC activates a transcription factor AP-1 through the stimulation of both c-*jun* and c-*fos* expression [16]. This raises the possibility that NAC stimulates the synthesis of certain proteins which, in turn, suppress the activation of IKKα and IKKβ. However, this was ruled out since NAC effectively suppressed the activation of these kinases even in the presence of a protein synthesis inhibitor, cycloheximide. In addition, the inhibitory effects of NAC were independent of the synthesis of GSH, suggesting that the suppression of NF-κB through suppressing IKKα and IKKβ is a result of the direct reducing power of NAC. However, this does not necessarily rule out a role for GSH in the redox regulation of NF-κB activation, since it has been reported that treatment of cells with GSH ester leads to the suppression of TNF-induced IκB phosphorylation and NF-κB activation [36].

Regarding the mechanism underlying NAC-mediated suppression of IKKα and IKKβ, a possible explanation is that NAC directly inhibits the kinase activity of IKKα and IKKβ. In this respect, however, it should be noted that NAC failed to inhibit IKKα and IKKβ activity in vitro (data not shown), indicating that NAC mediates the suppression of these kinases through an unknown cellular redox-sensitive mechanism(s). Another plausible mechanism is that TNF treatment produces ROS which act as signal mediators to activate NF-κB, and the blockade of ROS production by NAC leads to the suppression of NF-κB. Indeed, the role of ROS as signal mediators has been established in the signalling system of TNF activating c-Jun N-terminal kinase (JNK) [37,38]. However, the involvement of ROS in TNF-induced NF-κB activation is still unclear. Some reports have presented evidence that serine residues of IκB are phosphorylated by IKKα and IKKβ [11–15], while others have shown that oxidative radical stress induces the phosphorylation of tyrosine residues, not serine residues, on IκB [17,39]. Although either form of IκB phosphorylation results in NF-κB activation, it is clear that the latter reaction is not mediated by IKKα or IKKβ but by a tyrosine kinase, and hence oxidative radical stress activates NF-κB in a manner quite different from that of TNF signalling. The situation was made more confusing by the finding that NF-κB activation by ROS is cell type-specific: H_2O_2 stimulates NF-κB in some cell types, whereas it does not activate NF-κB at all in other cells [19,40,41]. Furthermore, an antioxidant suppresses TNF-induced NF-κB activation even in cells in which H_2O_2 did not stimulate NF-κB [41]. Taking these lines of evidence together with our present results, it may be pertinent to conclude that, although the role of ROS as second messengers in TNF signalling is unclear, an appropriate redox state is essential for TNF-induced NF-κB activation, and the disturbance of the cellular redox state by NAC results in the attenuation of NF-κB through the suppression of IKKα and IKKβ.

It should be noted that many other molecules involved in TNF signalling as well as IKKα and IKKβ are subject to redox regulation. For instance, NAC suppresses TRAF2-induced JNK activation by uncoupling the signalling from TRAF2 to MEKK1 [42]. Also, an apoptosis signal-regulating kinase, which is involved in TNF-induced activation of JNK and p38 MAPK, is subject to redox regulation through a cellular reductant enzyme, thioredoxin (TRX) [43]. An intriguing feature in this context is that oxidants and reductants have opposite effects upstream and downstream of the signalling pathways from TNF to NF-κB. Oxidative radical stress induces NF-κB activation through tyrosine kinase(s) as described [17,39], whilst the reductant suppresses NF-κB activation through the suppression of IKKα and IKKβ, as shown in this study. In contrast, the DNA binding activity of NF-κB itself is abolished by oxidation, whilst it is fully activated by reduction [30–32]. These results indicate that NF-κB is subject to dual redox regulation in the cells. The dual redox regulation has also been confirmed by the observation that TRX has dual and opposing roles in the regulation of NF-κB: TRX interferes with the degradation of IκB in the cytoplasm, whilst it enhances NF-κB transcriptional activity in the nucleus [44]. Thus, NF-κB activity might be regulated by extracellular stimuli, such as TNF, in concert with the dual redox regulation system. Our data suggest that IKKα and IKKβ play

pivotal roles in both the signalling from TNF to NF-κB and the redox regulation of NF-κB activation.

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