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The endogenous reactive oxygen species promote NF-κB activation by targeting on activation of NF-κB-inducing kinase in oral squamous carcinoma cells

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Abstract
Reactive oxygen species (ROS) could stimulate or inhibit NF-κB pathways. However, most results have been obtained on the basis of the exogenous ROS and the molecular target of ROS in NF-κB signalling pathways has remained unclear. Here, the oral squamous carcinoma (OSC) cells, with a mild difference in the endogenous ROS level, were used to investigate how slight fluctuation of the endogenous ROS regulates NF-κB activation. This study demonstrates that NF-κB-inducing kinase (NIK) is a critical target of the endogenous ROS in NF-κB pathways. The results indicate that ROS may function as a physiological signalling modulator on NF-κB signalling cascades through its ability to facilitate the activity of NIK and subsequent NF-κB transactivation. In addition, the data are useful to explain why the altered intracellular microenvironment related to redox state may influence biological behaviours of cancer cells.

Keywords: *Endogenous ROS, NIK, NF-κB*

Introduction

The intracellular redox condition plays a crucial role in the regulation of normal physiologic signalling pathways not only in cell survival but also in major cellular activities such as growth, proliferation, differentiation, apoptosis, cell adhesion and gene expression in many cells [1–4]. All aerobic organisms have evolved complex redox-regulating systems to produce a certain amount of reactive oxygen species (ROS) and simultaneously afford protection from injury and disease caused by excessive production of ROS. Changes in the intracellular redox state following environmental stresses are mediated by the ROS production and ROS scavenging [5]. The generation of ROS or the fluctuation of the cellular redox state leads to the stimulation of various signalling systems, alteration of gene transcription patterns in response to a number of environmental stresses as well as multiple effects on cellular activities [6,7]. A moderate increase of ROS can elicit activation of signalling pathways and/or transcription factors that result in physiological responses without discernable deteriorative effects, whereas higher levels of ROS can cause harm to the cellular macromolecules, including DNA, RNA, proteins and lipids, which ultimately leads to cell death and tissue damage [8]. This

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implies that the relative level of ROS is critical for its effects. ROS has been identified as second messengers in cells and play a role in response to a number of inflammatory signalling mediators, including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), lipopolysaccharide (LPS), interferon γ (IFNγ) and post-translation modification of signalling molecules [9]. ROS is required for the downstream signalling effects.

Among many transcription factors involved in the intracellular signalling pathway, nuclear factor-kappa B (NF-κB) is one of the most important ones known to be exquisitely sensitive to cellular oxidative status. Tumour necrosis factor-alpha (TNF-α) elicits a wide range of biological effects by activating NF-κB. NF-κB-inducing kinase (NIK), inhibitor of kappa B kinase alpha and beta (IKKα and IKKβ) are kinases that locate upstream of an inhibitory protein, IκB, and act in a cascade to mediate TNF-α-initiated NF-κB pathways [10]. Recent studies have revealed that ROS, as second messengers, can stimulate or inhibit NF-κB signalling pathways elicited by TNF-α and interleukin-1 [11]. The relationship between ROS and NF-κB pathways must be quite complicated. However, the previous studies were performed largely based on direct application of the exogenous ROS to culture cells. For example, these exogenous ROS include relatively high, non-physiological concentrations of hydrogen peroxide (H_2O_2) [12] or ROS-producing agents, such as naphthoquinone [13]. Exogenous administration of H_2O_2, often at concentrations significantly higher than those within the cells under physiological conditions, is a procedure not so accurate to mimic what normally occurs in the cell. Thus, whether endogenously produced low levels of ROS are indeed capable of determining appropriate changes in signal transduction of the cell has remained unclear. Moreover, despite the fact that ROS has been linked to NF-κB activation by certain cytokines, the molecular mechanisms remain poorly defined and controversial [14]. Furthermore, among the upstream molecules NIK, IKKα and IKKβ, which is the target molecule of ROS in TNF-α-initiated NF-κB signalling pathway has remained unclear to date.

In the present study, we use the oral squamous carcinoma (OSC) cell lines with a mild difference in endogenous ROS levels to study the mechanism(s) by which the subtle fluctuation of the endogenous ROS regulates TNF-α-stimulated NF-κB activation and the possible target. Our results demonstrated that NIK is a critical molecule of ROS regulation in NF-κB signalling pathways. A slight elevation of the endogenous ROS promotes TNF-α-stimulated NF-κB activation through facilitating the activation of NIK and subsequent phosphorylation of IKKα and IKKβ as well as significant degradations of kappa B protein α (IκBα), leading to the activation of NF-κB.

Materials and methods

Expression vectors

The constructs for wild-type NIK-WT, IKKα-WT and IKKβ-WT as well as their dominant-negative mutants NIK-DN, IKKα-DN and IKKβ-DN, respectively, all epitopically tagged with Flag, were generously provided by Dr H. B. Shu (National Jewish Medical and Research Center, University of Colorado Health Sciences Center, Denver, Colorado, USA). The construct for the $5\times$NF-κB-deriven luciferase reporter gene was kindly provided by Dr R. D. Ye (University of Illinois at Chicago, USA).

Cell and cell culture

The oral squamous carcinoma (OSC) cell line stable expression of manganese superoxide dismutase (Mn-SOD) antisense in the OSC cells, i.e. OSC-AS-SOD cells, and its parental cell line OSC-4, as established previously [15,16], were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Githerburg, MD) supplemented with 10% foetal bovine serum (FBS), 10 mm glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 in air.

Detection of intracellular ROS level

2,7-Dichlorodihydrofluorescein (DCFH) diacetate form 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) can be applied in cell studies due to its ability to diffuse through the cellular membrane, being then enzymatically hydrolysed by intracellular esterases to DCFH. DCFH is widely used for the quantitative determination of ROS. DCFH is converted to dichlorofluorescein (DCF) after reaction with ROS, especially hydrogen peroxide and peroxidase. Because DCFH is non-fluorescent while DCF is fluorescent, the determination of fluorescent intensity of DCFH samples reflects the amounts of ROS that reacted with DCFH [17]. In the present study, DCFH-DA (Sigma-Aldrich Inc.) at 10 μm was coincubated with cells for 20 min. After washing once with ice-cold PBS, cells were harvested and kept on ice for an immediate detection by flow cytometry. The average intensity of DCF stands for intracellular ROS levels.

Luciferase reporter assay for NF-κB activity

Cells were cotransfected with pNF-κB-Luc and pRenilla together with the expression constructs encoding NIK and NIK-DN, IKKα and IKKα-DN, IKKβ and IKKβ-DN as well as mock DNA, respectively, using the Lipofectamine 2000 reagent

(Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were either left untreated or stimulated with 40 ng/ml of TNF-α (Promega, Madison, WI) for 6 h. Cells lysates were assayed for the expression of luciferase using the Dual luciferase assay kit (Promega, Madison, WI). Chemiluminescence representing the expression of luciferase was measured in a Junior LB9505 luminometer (Berthold, Germany). Relative luciferase activity (RLA) was obtained by normalizing the luciferase activity with the Renilla activity. The relative extent of NF-κB activation was represented by the fold increase of RLA.

Immunoblot analysis

For measurement of the protein levels of SOD, cells were left untreated. For evaluating IκBα degradation under the basal condition or over-expression of NIK and NIK-DN and for determining phosphorylation levels of IKKα and IKKβ after over-expression of NIK and NIK-DN, OSC cells and OSC-AS-SOD cells were stimulated with TNF-α (40 ng/ml) for the indicated times prior to harvest.

Cells were collected and lysed in 100 μl of sample solution (625 mm Tris, pH 6.8, 10% SDS, 25% glycerol, 100 mm DTT and 0.015% bromphenol blue) and underwent sonication followed by heat denaturation. Protein concentrations were determined by Lowry's method. Western blotting was performed using standard protocols. In brief, equal amounts of crude proteins (50 μg) from each treatment were separated on a denaturing 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Hybond C, Amersham, Piscataway, NJ). The membranes were incubated with rabbit antibodies against human SOD-2, IKKα, IKKβ, IκBα or phospho-IKKα/IKKβ (176/177) (Cell Signaling, Beverly, MA) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (secondary antibody, Promega) for a proper time before detection by an enhanced chemiluminescence (ECL) system (Amersham).

Immunoprecipitation and in vitro *kinase assays*

In order to see the difference in activity of NIK between two cell types, *in vitro* kinase assay was performed by using immunoprecipitated NIK from two cell types. Cells were cotransfected with constructors for Flag-NIK-WT plus Flag-IKKα-DN or Flag-IKKβ-DN. The dominant-negative IKKs were used to block kinase cascade at IKKs. After 48 h, the cells were stimulated for 10 min with TNF-α (40 ng/ml). Then, the cells were collected and solubilized at 4°C for 30 min in 500 μl of Nonidet P-40 lysis buffer containing 50 mm HEPES (pH 7.6), 100 mm NaCl, 10% glycerol, 1 mm EDTA, 20 mm β-glycerophosphate, 20 mm p-nitrophenylphosphate, 1 mm sodium orthovanadate, 1 mm sodium fluoride, 1 mm sodium metabisulphite, 1 mm benzamidine hydrochloride and complete protease inhibitors (Boehringer Mannheim). Cell lysates were cleared by centrifugation at 1500 × g for 10 min at 4°C and the sodium chloride concentration of the cell lysates was increased to 250 mm. The protein concentration was determined by Lowry's method. One milligram of cellular proteins was immunoprecipitated by incubating with 1 μg of anti-Flag monoclonal antibody (Sigma-Aldrich Inc.) for 60 min at 4°C followed by the addition of 25 μl of 50% (w/v) protein-A beads (BD Pharmingen, San Diego, CA, USA) at 4°C for 3 h. Immunoprecipitated beads were washed three times extensively with lysis buffer containing 250 mm sodium chloride and then kinase buffer containing 20 mm Tris- HCl (pH 7.6), 10 mm $MgCl_2$, 0.5 mm DTT, 100 mm ATP. In addition, the dominant-negative IKKs were used as substrates of NIK.

In vitro kinase assays were performed by mixing immunoprecipitated NIK and the substrate IKKα-DN or IKKβ-DN with 10 μCi of [γ-^{32}P] ATP in 15 μl kinase buffer. The mixture was incubated at 30°C for 30 min. Samples were then heated at 95°C for 5 min before separation on 10% SDS-PAGE. After SDS-PAGE, the levels of phosphorylation of IKKα or IKKβ were detected by a FLA-5000 Science imaging system (Fujifilm, Japan). The amounts of the total NIK, IKKα-DN and IKKβ-DN in immunoprecipitated proteins were determined by immunoblot using anti-Flag antibody.

Results

The NF-κB activation differs between OSC and OSC-AS-SOD cells that have mild difference on ROS level

This study was based on the OSC cells model with a mild difference in ROS level. The higher inherent ROS was constituted by stable expression of Mn-SOD antisense in OSC-AS-SOD cells compared to that in parent OSC cells. We first confirmed the differences of Mn-SOD expression and intracellular ROS levels in both cell lines by immunoblot and flow cytometry, respectively. Indeed, as shown in Figure 1A and B, the higher inherent ROS was constituted by stable expression of Mn-SOD antisense in the OSC cells, i.e. OSC-AS-SOD cells, as established previously [15,16]. Our results showed that a slighter elevation of the endogenous ROS promoted NF-κB transactivation (Figure 1C). Accordingly, the degradation rate of IκBα was faster in the OSC-AS-SOD cells than in the OSC cells (Figure 1D). These data indicate that Mn-SOD antisense expression in OSC cells is capable of promoting ROS generation and rendering influence on NF-κB signal pathway. NF-κB activation was

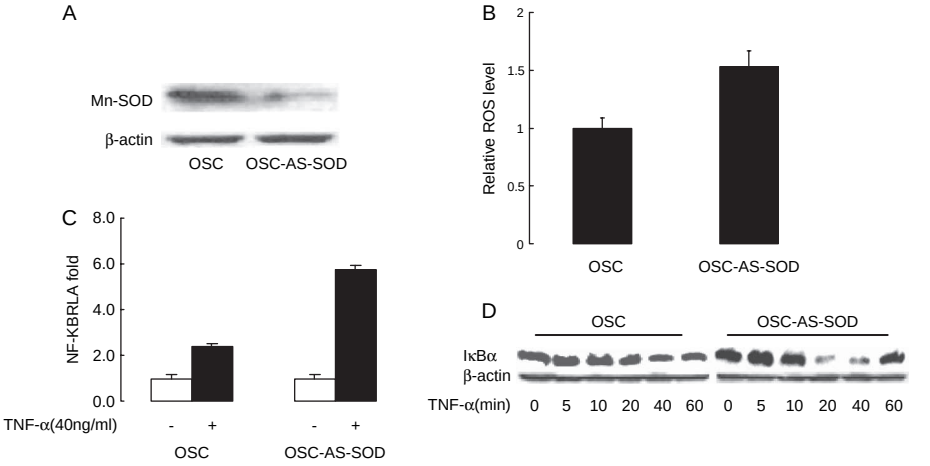

Figure 1. The difference of Mn-SOD expression, endogenous ROS level, and NF-κB activation between OSC and OSC-AS-SOD cells. (A) Stable expression of Mn-SOD antisense was confirmed by immunoblot using an anti-SOD-2 antibody in OSC and OSC-AS-SOD cells. Cell lysates were extracted and equal amounts (50 μg) of protein were subjected to immunoblot analysis using an anti-SOD-2 antibody. β-actin was shown as a loading control. SOD expression was strongly decreased in OSC-AS-SOD cells compared to in OSC cells. (B) The endogenous ROS level was detected by flow cytometry in OSC and OSC-AS-SOD cells. Two cell types were loaded with DCFH-DA for 20 min after TNF-α (40 ng/ml) stimulation for 6 h. The cells were then subjected to flow cytometric analysis. The endogenous ROS level in OSC-AS-SOD cells was slightly higher than that in OSC cells. The examination was done in triplicate and each bar indicates the mean $\pm$SD. (C) Luciferase assays for NF-κB activation in OSC and OSC-AS-SOD cells. Two cell types were transiently cotransfected with pNF-κB-Luc (50 ng/ml) and pRenilla (10 ng/ml). After 24 h, cells were cultured with or without TNF-α (40 ng/ml) for 6 h prior to cell lysis. Luciferase activity was determined to quantify NF-κB transcription activity. Relative luciferase activity (RLA) for NF-κB activation was increased in OSC-AS-SOD cells compared with OSC cells. The samples were duplicated and the data were presented as mean $\pm$SD from three independent experiments. (D) The IκBα degradation in OSC and OSC-AS-SOD cells were detected by immunoblot. Two cell types were cultured with or without TNF-α (40 ng/ml) at the indicated times. TNF-α-stimulated IκBα degradation rate was rapider in OSC-AS-SOD cells than that in OSC cells. Upper panel: Blot; Lower panel: Densitometry analysis. Three independent experiments were performed.

higher in the cells that possessed higher ROS level and the difference occurred at the step upstream of IκBα degradation. We hence believe that these data reflect a relatively physiological situation regarding the magnitude of ROS fluctuation.

The difference of NF-κB activation in two cell types is augmented by over-expression of wild-type NIK and abolished by over-expression of dominant-negative mutant of NIK

NIK and IKKα/IKKβ are key kinases and act in a cascade to activate the NF-κB pathway. To examine if activation or inactivation of NF-κB is affected by ROS through these components, NIK, IKKα, IKKβ and their dominant-negative mutants were over-expressed in OSC-AS-SOD cells or OSC cells. NF-κB-specific luciferase reporter assay showed that the difference in NF-κB activation between these two cell types was enhanced by over-expression of NIK and abolished by over-expression of its dominant-negative mutant NIK-DN, with or without TNF-α stimulation (Figure 2A). Over-expression of either IKKα or IKKβ also promoted NF-κB activation. However, unlike NIK-DN, the difference in NF-κB activation between these two cell types was not completely abolished by over-expression of either IKKα-DN or IKKβ-DN (Figure 2A).

To confirm that the abrogation of NF-κB activation was specifically caused by expression of the mutant NIK, a series of concentration of plasmid DNA carrying NIK or NIK-DN, IKKα or IKKα-DN, and IKKβ or IKKβ-DN were cotransfected in OSC-AS-SOD cells or OSC cells. The results showed that NF-κB activation was promoted in a does-dependent manner by the wild-type NIK with or without TNF-α stimulation (Figure 2B). However, the difference in TNF-α-induced NF-κB activation was really abrogated in these two cell types following over-expression of the mutant NIK in all concentrations examined. Although IKKα and IKKβ are both involved in TNF-α-mediated activation of NF-κB, the difference in NF-κB activation between these two cell types was not abolished by over-expression of IKKα-DN or IKKβ-DN at the same concentrations (data not shown).

Meanwhile, to evaluate whether a slight elevation of endogenous ROS level influences NF-κB activation through changing IκBα degradation under the condition of over-expression of NIK or NIK-DN, immunoblot for IκBα quantity was performed. As shown in Figure 2C, IκBα was degraded more rapidly in response to TNF-α in OSC-AS-SOD cells than that in OSC cells after over-expression of the wild-type NIK, but the difference in IκBα degradation rate between these two cell types almost

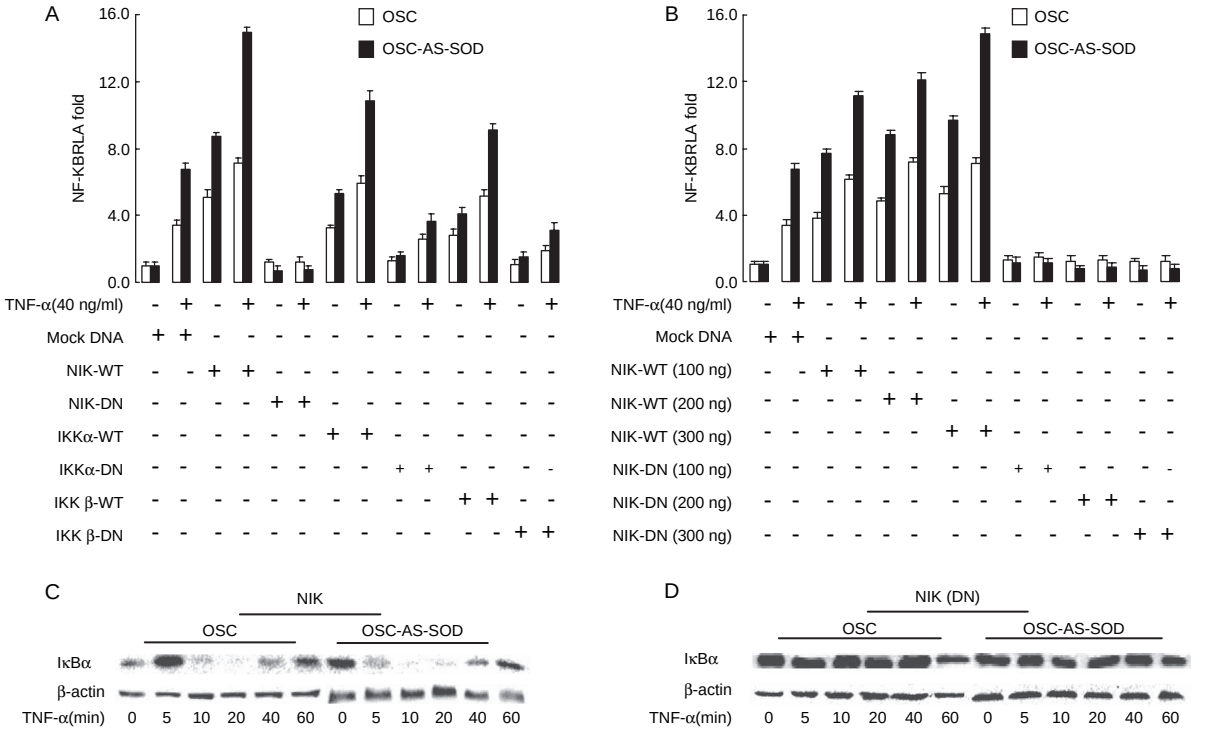

Figure 2. Effects of over-expression of NIK, IKKα, IKKβ and their dominant-negative mutants NIK-DN, IKKα-DN, IKKβ-DN on NF-κB transactivation following TNF-α treatment between OSC and OSC-AS-SOD cells. (A) Two cell types were cotransfected with pNF-κB-Luc (50 ng/ml) together with expression plasmids encoding NIK and NIK-DN, IKKα and IKKα-DN, IKKβ and IKKβ-DN, as indicated. For each transfection, 300 ng/transfection of every expressing plasmid was used. After 24 h, cells were incubated with or without TNF-α (40 ng/ml) for 6 h prior to cell lysis. Luciferase activity was determined to quantify NF-κB transcription activity. The difference in NF-κB activation between two cell types was enhanced by over-expression of NIK, IKKα and IKKβ, and NF-κB activation in OSC-AS-SOD cells was higher than that in OSC cells. NF-κB activation was abrogated by over-expression of NIK-DN, but not abolished by over-expression of IKKα-DN or IKKβ-DN. (B) Two cell types were transiently transfected with expression plasmids encoding NIK or NIK-DN at the indicated concentrations, together with pNF-κB-Luc (50 ng/ml). After 24 h, cells were incubated with or without TNF-α (40 ng/ml) for 6 h prior to cell lysis. Luciferase activity was measured to quantify NF-κB transcription activity. NF-κB activation was dose-dependent on the concentrations of NIK and increased along with the increasing concentrations of NIK with TNF-α stimulation in these two cell types. The difference in NF-κB activation between two cell types was abrogated by over-expression of NIK-DN at all concentrations tested. Conditions for transfection and treatment were indicated below each graph. Results depict the mean relative luciferase activity (RLA). (C and D) Immunoblot analysis of the degradation of IκBα in OSC and OSC-AS-SOD cells. Cells were transiently transfected with NIK-WT or NIK-DN, respectively. The cells were treated with TNF-α (40 ng/ml) for the indicated times prior to cell lysis. Total cell lysates were prepared. β-actin was shown as a loading control. IκBα degradation pattern showed that IκBα in OSC-AS-SOD cells in response to TNF-α was more rapidly degraded than that in OSC cells after over-expression of NIK-WT (C), but this difference between two cell types almost disappeared after over-expression of NIK-DN (D). Upper panel: Blot; Lower panel: Densitometry analysis. A representative experiment is shown here from three independent ones.

disappeared after over-expression of the mutant NIK (Figure 2D). Taken together, these data suggest that NIK is required for the role that ROS played in the IκBα degradation and NF-κB transactivation and that it might be a critical target of the endogenous ROS.

The increased ROS facilitate the activation of NIK and subsequent phosphorylation of IKKα and IKKβ

Next, we tested if NIK-controlled IKKα and IKKβ activation in response to TNF-α stimulation is different between OSC-AS-SOD cells and OSC cells. The ability of NIK to phosphorylate both IKKα and IKKβ *in vivo* was examined by immunoblot. The results showed that the phosphorylation of both IKKα and IKKβ was advanced approximately 10 min following TNF-α treatment in the OSC-AS-SOD cells comparative to OSC cells after over-expression of the wild-type NIK (Figure 3A), but the extent of IKKβ phosphorylation was weaker than that of IKKα. In contrast, the extents for inhibition of IKKs phosphorylation by NIK-DN appeared similar in these two cell types (Figure 3B). Phosphorylations of both IKKα and IKKβ were delayed similarly in both cell types by over-expression of the mutant NIK (Figure 3B). The total cellular protein levels of both IKKα and IKKβ were also examined, showing no changes occurred following TNF-α stimulation (Figure 3A and B).

Moreover, *in vitro* kinase assay was used to further confirm the ability of NIK to phosphorylate both IKKα and IKKβ in response to TNF-α stimulation. The results showed that immunoprecipitated

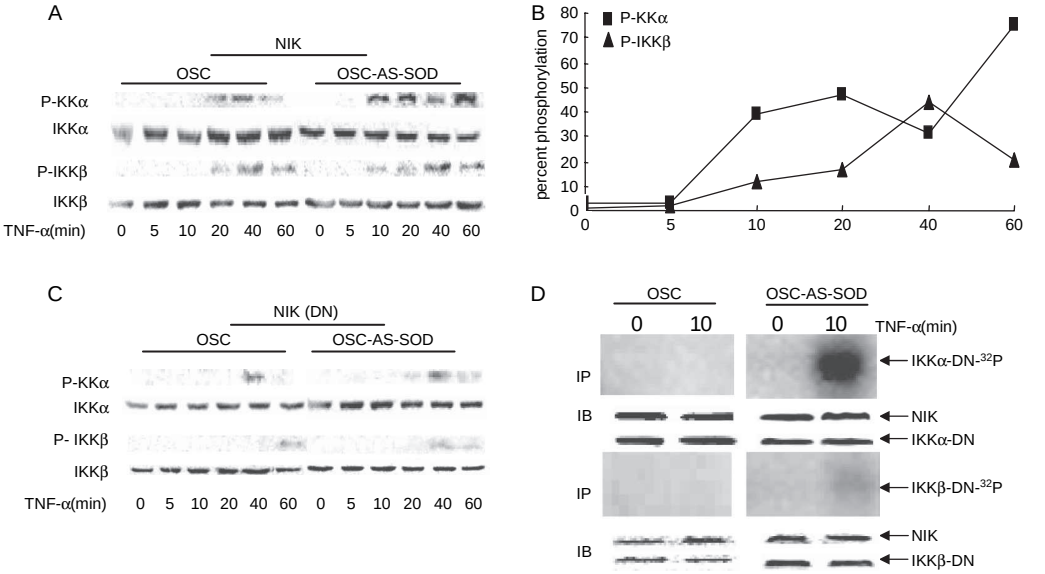

Figure 3. The ability of the endogenous ROS to activate NIK kinase activity on IKKα and IKKβ phosphorylations was evaluated in both *in vivo* and *in vitro* experiments. (A and B) Effects of over-expression of NIK-WT and NIK-DN on the phosphorylation of IKKα and IKKβ. Two cell types were transiently transfected with NIK-WT and NIK-DN independently. Forty-eight hours post-transfection, cells were stimulated with TNF-α (40 ng/ml) at the indicated times. Equal amounts (50 μg) of protein from cell lysates were subjected to immunoblot analysis using an anti-phospho-IKKα/IKKβ (176/177) antibody. The blots were stripped and reprobed with antibodies against total IKKα or IKKβ. Phosphorylation of both IKKα and IKKβ was advanced in OSA-AS-SOD cells than that in OSA cells by over-expression of NIK-WT (A), but it was delayed in these two cell types by over-expression of NIK-DN (B). Upper panel: Blot; Lower panel: Densitometry analysis. Three independent experiments were performed. (C and D) *In intro* kinase assays. Two cell types were cotransfected with constructors for Flag-NIK-WT plus Flag-IKKα-DN (C) or Flag-IKKβ-DN (D), respectively. Forty-eight hours post-transfection, the cells were stimulated for 10 min with TNF-α (40 ng/ml) prior to cell lysis and subsequently immunoprecipitated with anti-Flag antibody. Immunoprecipitated NIK and the substrate IKKα-DN or IKKβ-DN were then incubated with 10 μCi of [γ-^{32}P] ATP in 15 μl kinase buffer, respectively. The amounts of NIK, IKKα and IKKβ used in the reactions were determined by immunoblot with anti-Flag antibody (bottom). Immunoprecipitated Flag-NIK from OSC-AS-SOD cells significantly phosphorylated IKKα and IKKβ after 10 min of TNF-α treatment, but NIK derived from OSC cells had little effect on the phosphorylation. The extent of phosphorlation by NIK was stronger in IKKα than in IKKβ.

Flag-NIK from OSC-AS-SOD cells significantly phosphorylated both IKKα and IKKβ after 10 min of TNF-α treatment and the extent of phosphorylation by NIK was much weaker in IKKβ. However, NIK derived from OSC cells failed to phosphorylate both IKKα and IKKβ under the same condition (Figure 3C and 3D). These *in vivo* immunoblot and *in vitro* kinase assays indicate that a mild increase of the endogenous ROS facilitates the activation of NIK and subsequent phosphorylation of IKKα and IKKβ as well as degradation of IκBα.

Discussion

ROS are essential to cell survival, but the fluctuation of ROS level in response to intra- and extracellular changes brings complex effects of ROS to the cells. This implies that the relative level of ROS determines the effects of ROS. As it has been long known, the exposure of living cells to high intensity of oxidants or ion radiation can result in an 'oxidative stress', i.e. a series of biochemical changes capable of provoking cell injury and death. An excessive accumulation of ROS leads to alterations and damage of all type of macromolecules and eventually to slowed growth, cell cycle arrest, apoptosis and necrotic cell death [18,19]. On the other hand, a moderate increase of ROS can elicit activation of signalling pathways and/or transcription factors that result in physiological responses without discernable deteriorative effects [8]. While ROS are predominantly implicated in causing cell damage, they also play a major physiological role in several aspects of intracellular signalling. Therefore, it is now recognized that low, more 'physiological' levels of pro-oxidants can exert regulatory roles within the cell and hence the term 'oxidant-mediated regulation' has been proposed as a more accurate alternative to 'oxidative stress' [12]. The knowledge about role of ROS has been dramatically renewed owing to the studies during the past decade. However, the conclusions mentioned above are largely based on the studies using exogenous ROS directly, for instance employing relatively high, non-physiological concentrations of H_2O_2 or ROS-producing agents, such as naphthoquinone [13,20]. Exogenous administration of H_2O_2, often at concentrations significantly higher than those even reached within the cell in physiological conditions, is probably a procedure not so accurate to mimic what normally occurs in the cell, considering the low diffusion of many ROS as well as the possible compartmentalization of

ROS-generating systems. And the ROS-producing agents themselves may have effects other than interfering ROS. In the present study, we use a pair of oral squamous cell carcinoma cell lines with a mild difference in endogenous ROS levels to study the relation of ROS with NF-κB signalling pathway. The level of ROS depends not only on the initial production of ROS but also on the speed of their disappearance and transitions of different forms of ROS at the given moment. Mn-SOD is an enzyme responsible for clearance of the superoxides and transition into hydrogen peroxides. In this OSC cell model ROS level is stably 50% higher in cells where expression of Mn-SOD is knocked down than their wild-type counterpart. Mn-SOD antisense expression in OSC cells is in fact capable of promoting oxidative reaction in the intracellular microenvironment. We hence believe that the data shown in this study reflects a more physiological situation regarding to ROS fluctuation.

NF-κB is among the most crucial transcription factors shown to respond directly to a variety of ROS [21–23]. NIK, IKKα, IKKβ and IκBα are four key factors in the NF-κB pathway and act in a cascade to activate the pathway. TNF-α activates this pathway through binding to its receptor on the cell membrane. The signals are passed from the intracellular receptor associated proteins to NIK, which in turn stimulates the IKKα and IKKβ that trigger phosphorylation and degradation of IκBα [10]. The relationship of ROS and NF-κB pathway must be quite complex and multifaceted. TNF-α is the most frequently applied inducer for activation of NF-κB pathway and thus we take TNF-α-induced NF-κB activation as one example in the present study to explore the role of ROS on NF-κB pathways [6,7]. Intriguingly, it has been noticed for a long time that TNF-α promotes ROS production, probably through disturbance of mitochondrial electron transport [24]. Furthermore, TNF-α-mediated activation of NF-κB can be blocked by antioxidants, suggesting that TNF-α activity is dependent on generation of oxidant challenge. However, Hayakawa et al. [14] have provided evidence against a model proposing ROS as second messengers for TNF-α-induced NF-κB activation. They show that endogenous ROS produced through Rac/NADPH oxidase do not mediate TNF-α-induced NF-κB signalling. Hence, the process that oxidant challenge plays a role in the TNF-α signalling pathway leading to NF-κB activation has not been adequately studied [11] and the steps at which ROS affect TNF-α-induced NF-κB activation are unclear [25].

A number of publications have reported the effects of ROS on NF-κB activation induced by various environmental insults other than TNF-α and explored the possible targets. However, the conclusions are diverse. Using GPx-1 expression to modulate cellular H_2O_2, Li et al. [26] report that redox regulation of NF-κB activation by intercellular H_2O_2 may be specific for the unique subunit in the IKK complex [26]. The same authors recently found that H_2O_2 imparts NF-κB activation by promoting NIK and potentially inhibiting phosphatases that inactivate NIK. What's more, a very narrow range of H_2O_2 concentration (1–10 μm) facilitates NIK activation, whereas higher levels of H_2O_2 inhibit NIK activity. They have demonstrated that interleukin-1β (IL-1β)-induced NF-κB activation is partially regulated by H_2O_2-mediated activation of NIK [27]. However, the detailed steps linking the various upstream TNF-α signal transduction cascades with the multiple IκBα degradation mechanisms are not completely understood. Our present study focuses on the effect of the endogenous change of ROS on TNF-α-induced NF-κB activation and the critical molecule of ROS regulation. Herein, we have revealed that the function of NIK in regulating NF-κB activation with a slight elevation of the endogenous ROS in OSC cells. We have demonstrated that a slight elevation of the endogenous ROS promotes TNF-α-stimulated NF-κB activation through facilitating the activation of NIK and subsequent phosphorylation of IKKα and IKKβ as well as degradations of IκBα, leading to the activation of NF-κB. Thus, NIK has been placed as a central redox-regulated signalling component in the TNF-α-mediated activation of NF-κB, indicating that the increased ROS may act on NIK or its upstream parts to exert its modulation.

NIK is a member of the MEKKK family, which has Ser/Thr kinase activity. NIK can phosphorylate and thereby activate the IKKs in response to both TNF-α and IL-1β [2]. Many reports have shown that the effect of NIK on TNF-α-induced NF-κB activation is cell type-dependent. For example, NIK is sufficient to induce NF-κB activation in 293 cells, but fails to activate NF-κB in Fibroblasts and Keratinocytes [4,28]. Our results indicate that NF-κB activity in OSC cells is also NIK-dependent. These findings shed light on the molecular mechanisms by which the endogenous ROS regulate the TNF-α-induced NF-κB pathway. It is evidenced that ROS may function as physiological signalling modulators on NF-κB signalling cascades through its ability to facilitate the activity of the critical molecule NIK, which holds true at least in the contexts of our experiments and tested cell type. Our findings are helpful for interpretation of the molecular mechanisms by which the endogenous ROS regulates the TNF-α-induced NF-κB pathway. NIK seems the common point through which the IL-1β and TNF-α pathways mediate NF-κB activation [2]. We believe that the present study is the first report to demonstrate that NIK may be a critical factor of the endogenous ROS regulation of the TNF-α-stimulated NF-κB pathway.

The redox response of NF-κB appears to be complex; slight difference in redox state may result

in either activation or suppression. NF-κB is known as a proinflammatory transcription factor and is now confirmed as an antiapoptotic and prosurvival force in the most cell types [29–31]. It maintains a constitutively higher activation in a variety of the tumour cells and is subjected to further activation when cells encounter the stress or challenges such as TNF-α and chemotherapeutic agents [25,30,32]. We herein show that TNF-α-elicited NF-κB activation is more intensified in cells with slightly higher ROS, indicating that mild increase of ROS promotes NF-κB signalling. This may explicate why tumour cells that usually possess higher ROS levels maintain higher NF-κB activation.

Given that ROS generation and NF-κB activation are increased in cancer cells, and these two events are causative related, a number of approaches may be designed to interfere redox state to suppress NF-κB activation in cancer cells. Logically, antioxidants can be used to antagonize ROS-promoted NF-κB activation [33]. Paradoxically, elevating ROS to suppress NF-κB activation is becoming a novel strategy. Along with ROS increase, it first promotes NF-κB activation by triggering NIK activation, which usually leads to prosurvival transcriptional events. However, as ROS become overwhelming, the nuclear environment is shifted from reductive to oxidative, which inhibits activation of NF-κB and other transcriptional factors by preventing them from binding to DNA. As a consequence, the prosurvival transcriptional activity is abolished [12]. This may elucidate how the increasing ROS shift survival/death control. The present study is of potential clinical relevance, since NIK is at a pivotal place for ROS modulating the NF-κB pathway, the enzymatic activity of NIK can be interfered by pharmacological and molecular means and could therefore represent a suitable target for anticancer drugs and therapeutic strategies.

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