



Pivotal role of c-Fos in nitric oxide synthase 2 expression in airway epithelial cells

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ABSTRACT

The regulation of nitric oxide synthase 2 (NOS2) in airway epithelial cells plays a key role in the innate host response to a wide variety of microbial agents and also participates in the generation of pathologic airway inflammation. Among the important signalling cascades that direct NOS2 gene expression are nuclear factor κ B (NF κ B) and interferon- γ (IFN γ)/signal transducer and activator of transcription 1 (STAT-1). Previous studies suggest activator protein-1 (AP-1), in particular c-Fos component of AP-1, influences NOS2 expression. We investigated the effect of c-Fos modulation using RNA interference siRNA on NOS2 gene expression. A549 cells stably transfected with a plasmid overexpressing a c-Fos siRNA construct (FOSi) resulted in a decrease of NOS2 protein inducibility by IFN γ . In contrast, classical IFN γ inducible signal transduction pathways interferon regulated factor-1 (IRF-1) and pSTAT-1 were activated at a similar magnitude in FOSi and control cells. DNA-protein binding assays showed that c-Fos binding was present in wild type cells, but reduced in FOSi clones. FOSi clones had activation of NF κ B detectable by DNA-protein binding assays, which may have contributed to a decrease of NOS2 expression. Overall, these studies indicate that c-Fos is a requisite and specific component for inducible NOS2 expression.

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Introduction

The airway epithelium is continuously exposed to a wide variety of airborne infectious agents and particulate matter. The ability to maintain epithelial homeostasis and integrity depends largely on innate host regulation of defense mechanisms by the epithelial cells in the airway [1]. Nitric oxide (NO) production by the airway epithelial cells is a critical component of the host defense armamentarium of the airway [2–4]. NO has proinflammatory and immunomodulatory effects and is synthesized in airway epithelial cells by the inducible nitric oxide synthase (NOS2) [5,6]. NO and reactive nitrogen species (RNS) have been implicated in a wide variety of homeostatic and pathophysiological processes, such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) [1,7–9]. Investigations have identified some of the mediators and signaling pathways that direct NOS2 expression.

In general, expression of NOS2 is regulated at the transcriptional level; the 5' flanking region of the gene has binding sites for cytokine-responsive elements and redox-sensitive transcription factors such as activator protein-1 (AP-1), nuclear factor κ B (NF κ B) and IFN γ -activated sites (GAS) [3,10–12]. IFN γ alone is sufficient for induction of the NOS2 gene in primary human airway epithelial cells, but DNA sites other than GAS are required for IFN γ activation of the NOS2 promoter [10,13]. Previous study suggests that c-Fos interacts with signal transducer and activator of transcription-1 (STAT-1) in binding to the GAS element close to an AP-1 site located at 4.9 kb upstream of the transcription start site of NOS2 and is required for maximal promoter induction [14]. In this context, we hypothesized that c-Fos is a pivotal checkpoint in the IFN/STAT1 signalling pathway to NOS2 expression in airway epithelium. To test this, stable c-Fos knockdown airway epithelial cells created using RNA interference against c-Fos were evaluated for NOS2 expression and regulation of NO synthesis.

Experimental procedures

Construction of pSUPER plasmid expressing c-Fos siRNA

To knock-down expression of c-Fos, several 19-nucleotides (-nt) targeting sequences of c-Fos mRNA (GenBank Accession No.

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NM005252) were designed according to the manufacturer's instructions (OligoEngine, Inc., Seattle, WA) [15]. They included c-Fos177RNAi (target sequence CTCATTCCCACGGTCACT-nt 177–195), c-Fos498RNAi (target sequence GCGGAGACAGACCAACTAG-nt 498–516), c-Fos795RNAi (target sequence GACCGAGCCCTTGATGAC-nt 795–813), and a scrambled version of c-Fos177RNAi (GTCTCAACCCTCGTATCT). A BLAST analysis was performed to ensure that there was no significant sequence homology with other human genes. All 64 oligonucleotides, containing the unique 19-nt target in both sense and antisense orientation, 9-nt spacer sequence (hairpin), BglIII enzyme site at 5' end and HindIII enzyme site at 3' end (Fig. 1A), were synthesized by Integrated DNA Technology, Inc. (IDT, Coralville, IA). Forward and reverse 64-nts were annealed and cloned into downstream of H1 promoter of pSUPER.neo vector system. All constructs were verified by sequencing.

Cell culture and stable transfection

A549 cells (ATCC, Manassas, VA), an epithelial cell line derived from human lung adenocarcinoma, were cultured in MEM (Invitro-

gen, Carlsbad, CA) with 10% heat-inactivated FCS, 1% penicillin/streptomycin and 1% L-glutamine. IFN- γ was a gift from Genentech (South San Francisco, CA) and used at 10^4 U/ml. Recombinant human IL-1 β and TNF α were purchased from Biosource (Camarillo, CA) and used at 0.5 ng/ml and 10 ng/ml respectively. A549 cells were transfected with the plasmid constructs for 8 h at 30% confluence using Lipofectamine Reagent (Invitrogen) according to the manufacturer's instructions. They were split at day 1 and diluted in fresh medium. From day 2 to day 14, cells were washed with PBS and incubated with selection medium containing 600 μ g/ml G418 sulfate (Mediatech, Inc., Herndon, VA). After two weeks in selective medium, approximately 50 clones of each plasmid construct were picked and expanded separately in culture plates containing fresh medium with G418. Stable transfection clones expressing c-Fos siRNA were analyzed by Western blot and immunofluorescence to select clones with knockdown effect on c-Fos expression. All sequences were tested for c-Fos protein expression and the c-Fos177RNAi sequence (Fig. 1A) was finally kept for further analysis. We then synthesized a scrambled sequence of c-Fos siRNA (named c-Fos177scrRNAi) containing the same oligonu-

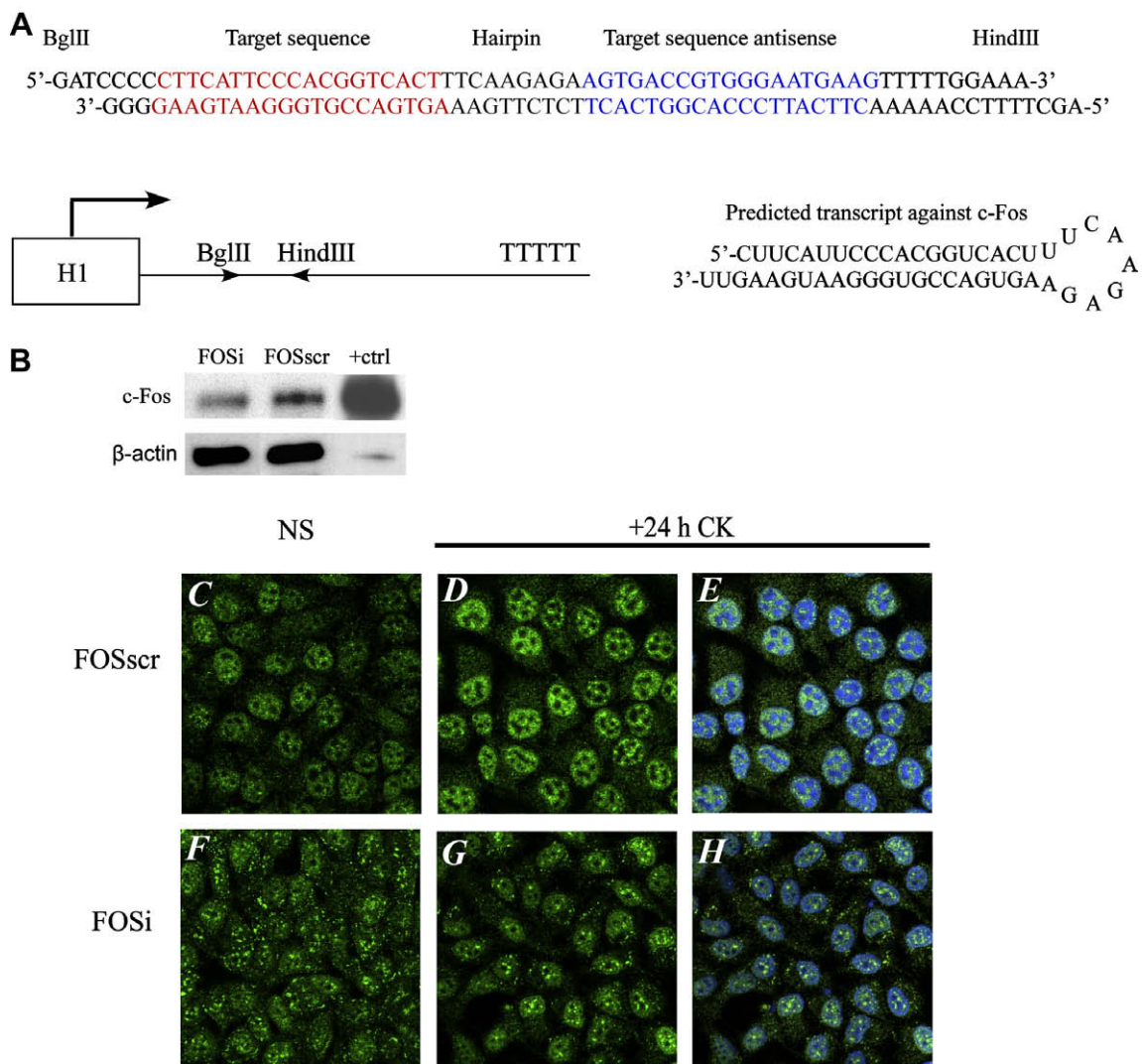


Fig. 1. (A) Structure of the 64-mer DNA chemically synthesized oligonucleotide containing the unique 19-nt sequence to c-Fos mRNA (red: sense, blue: antisense) and the predicted transcript against c-Fos generated from c-Fos 177RNAi/pSUPER.neo plasmid. (B) Western blot of immunoprecipitated c-Fos. Expression shows a decrease of c-Fos in A549 cells stably transfected by c-Fos 177RNAi/pSUPER.neo plasmid (FOSi) as compared to scrambled clone FOSscr. 293T cell line transfected with pRSV-c-Fos overexpression construct was used as positive control for c-Fos (+ ctrl). Results are representative of three different gels. (C–H) Cytokine (CK including IL1- β , TNF α and IFN γ) stimulation of FOSi cells (G and H) have less nuclear localization of c-Fos as compared to FOSscr (D and E). c-Fos antigen detection by immunofluorescence staining is in green (C, D, F, and G) and nuclei identified by DAPI positive staining in blue (E and H). Results are representative of three or more separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

cleotide content as c-Fos177RNAi but in a random order to use as control. Clones transfected by c-Fos177RNAi/pSUPER.neo vector and c-Fos177scrRNAi/pSUPER.neo vector were further named FOSi clones and FOSscr clones respectively.

Western blot analysis

Whole cell protein extracts from A549 cells were prepared as previously described [14,16]. Proteins (70 µg/lane) were separated by electrophoresis on 15% SDS-PAGE HCl gel and transferred to nitrocellulose membrane or onto Immuno-Blot™ PVDF Membrane (0.2 µm, Bio-Rad Labs, Hercules, CA). Primary antibodies (Ab) used included anti-c-Fos, anti-IRF-1, anti-STAT-1, anti-pSTAT-1, anti-MHC-I, anti-p50, anti-p65 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-NOS2 polyclonal Ab NO53 (Merck, Rahway, NJ) and mouse anti-β-actin monoclonal Ab AC-15 (Sigma-Aldrich, St Louis, MO). The secondary Ab was conjugated with horseradish peroxidase (GE Healthcare, Piscataway, NJ). The detection of signal was performed with enhanced chemiluminescent system ECL (GE Healthcare).

Immunoprecipitation

A549 cells were lysed with nonreducing lysis buffer as previously described [14]. The whole cell lysis was incubated with anti-c-Fos antibody overnight (Cell Signaling, Danvers, MA), followed by Protein G-Sepharose (Amersham Laboratories, Arlington Heights, IL) to extract the immunoblobulins. The captured beads were washed and boiled in denaturing, nonreducing buffer then analyzed by Western blot as described above, using a second anti-c-Fos antibody (Santa Cruz). To ensure equal protein loading, the whole cell lysates were further analyzed by western analyses for the presence of β-actin.

Immunofluorescence

Cells were grown on cover slides in 6-well plates. They were incubated in medium with 1% FCS and then stimulated with cytokine mixture (CK), containing 10⁴ U/ml IFN-γ, 0.5 ng/ml IL-1 β and 10 ng/ml TNFα for 24 h. Cells were then washed with PBS and fixed for 30 min with ice-cold acetone/methanol (1:1). After fixation, cells were blocked with 2% fetal bovine serum (FBS) for 30 min, incubated with rabbit anti-c-Fos Ab (Santa Cruz Biotechnology) in PBS/2% FBS for 30 min at room temperature and then incubated with Alexa Fluor 488 (green) conjugated anti-rabbit IgG Ab A21441 (Invitrogen) in PBS/2% FBS for 1 h at room temperature. The slides were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI, Vector Labs, Burlingame, CA), sealed and analyzed by confocal laser-scanning microscopy (TCS-40; Leica Microsystems, Cambridge, UK).

Electrophoretic mobility shift assay

Whole cell extract was prepared as previously described [14,17,18]. Oligonucleotides used in EMSA included the NOS2 AP-1u site (5'-GCCAGCTTGAGTCACACTCCA-3' and the NOS2 GAS site (5'-CGGGCGTTTCCAGTAAAATC-3') synthesized by Operon (Alameda, CA) and then end-labeled with [³²P]ATP by polynucleotide kinase [14,17,18]. NFκB site (5'-AGTTGAGGGGACTTCCAGGC-3') was from Santa Cruz Biotechnology (Santa Cruz, CA). Underlined sequences represent the consensus elements for AP-1, GAS and NFκB. To specifically identify AP-1, GAS binding-factor, and NFκB proteins in binding complexes, rabbit anti-c-Fos, p50 or p65 polyclonal Ab (Santa Cruz Biotechnology), rabbit anti-STAT-1 polyclonal Ab [17,19], or nonimmune rabbit IgG (Biosdesign, Saco, ME) was added to the binding reaction mix.

Nitrite and nitrate in the supernatant

To evaluate NO synthesis, total nitrite and nitrate (NOx) concentrations in the culture supernatants were measured using chemiluminescence as previously described [20]. Briefly, total NOx was converted to NO by a saturated solution of VC1₃ in 1 M HCl, and NO generated was detected by the Sievers NOA 280i (GE Analytical Instruments, Boulder, CO). Sample NOx concentration was determined by adding 20 µl of cell supernatant to the reaction vessel. All samples were measured in triplicate and the NOx concentrations were determined by interpolation using authentic standards of nitrate.

Statistical analysis

Statistical comparisons were performed using JMP® statistical software, version 7.0 (SAS Institute, Cary, NC). For NOx measurements, data were expressed as fold increase from baseline levels, and comparison between groups was analyzed using the one way ANOVA. All pairwise were analyzed with the Tukey test. Relationships were considered statistically significant when *P* < 0.05.

Results

c-Fos protein expression is decreased in A549 cells stably transfected with c-Fos siRNA plasmid vector

Whole cell extracts were evaluated at baseline for c-Fos expression by western blot on every clone stably transfected with pSUPER.neo plasmid containing c-Fos177RNAi (Fig. 1A), c-Fos498RNAi or c-Fos795RNAi sequences. For further analysis, we selected clones stably transfected with pSUPER.neo plasmid containing c-Fos177RNAi (named FOSi) which displayed approximately 40% decrease of c-Fos expression (FOSscr 0.52 ± 0.28; FOSi

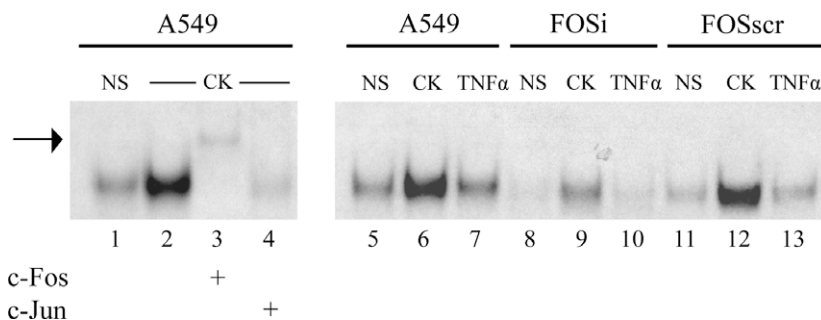


Fig. 2. Decreased NOS2 AP-1 binding in FOSi. EMSA of the NOS2 AP-1-binding activity in extracts from A549, FOSi, and FOSscr cells after exposure to CK or TNFα for 3 h using the radiolabeled NOS2 AP-1u oligonucleotide. c-Fos and c-Jun antibodies were added to identify binding proteins (lanes 3 and 4). The arrow indicates supershift of the binding complex by anti-c-Fos Ab (lane 3). The autoradiograph is representative of two separate experiments.

0.33 ± 0.17) compared to c-Fos177scrRNAi (named FOSscr) (Fig. 1B). The phase contrast microscopy appearance of clones was comparable to A549 cells (data not shown). Cytokine induction of c-Fos nuclear trans-localization was reduced substantially in FOSi cells as compared to FOSscr or A549 wild type cells (Fig. 1C–H).

c-Fos binding to NOS2 AP-1 site is reduced in FOSi

To evaluate the NOS2 AP1 binding activity of lysates from FOSi cells stimulated with CK, EMSA was performed using duplex oligonucleotides from the AP-1 site of the human NOS2 promoter region (from –5574 to –4909 bp) (GeneBank Accession No. AF017634). Fig. 2 shows the presence of DNA binding activity at baseline in A549 and FOSscr which increases after CK stimulation but not with TNF α stimulation alone. In contrast, DNA binding was diminished in FOSi cells under all three conditions. The AP-1 binding activity present in CK stimulated FOSi is attributable to c-Fos binding as shown by the supershift in lane 3. Thus, the FOSi had reduction of c-Fos protein, nuclear translocation and DNA binding.

Loss of NOS2 expression in FOSi

We previously reported that c-Fos is involved in NOS2 expression [14]. Here, CK stimulation induced NOS2 expression in A549 and FOSscr cells, whereas FOSi cells had decreased NOS2 protein expression as shown in different clones (Fig. 3A). The c-Fos knockdown did not affect STAT-1, phospho-STAT-1 (pSTAT-1), or interferon-related factor-1 (IRF-1) (Fig. 3B). This indicates that the interferon system was not activated by the siRNAs [21].

Impairment of NO production by FOSi

The functional consequence of c-Fos silencing on NO production in response to cytokine stimulation (IL-1 β , TNF α and IFN γ) was evaluated. The cells were incubated in serum free medium overnight, with or without CK for 24 h. The supernatant was collected for the measure of end products of NO in solution, nitrate and nitrite (NOx). The basal level of NOx in A549, FOSscr and FOSi were similar. CK stimulation led to increase of NOx production by FOSscr cells but not in FOSi (Fig. 3C). A549 cells produced NOx at levels similar to those previously published [17,22].

pSTAT-1 binding to NOS2–GAS is preserved in FOSi

We previously reported a physical interaction between c-Fos and STAT-1 after IFN γ activation of cells [14]. The STAT-1/c-Fos heterodimer binds at the GAS that is in proximity to the AP-1 site, 4.9 kb upstream of the NOS2 transcriptional start site. To evaluate the impact of c-Fos knockdown on binding of pSTAT-1 to the GAS element of the NOS2 promoter, EMSA was performed using extracts from A549, FOSi, and FOSscr cells that were exposed to IFN γ for 30 min. Phospho-STAT–GAS binding in FOSi was similar to that observed in A549 and FOSscr. Supershift of the binding complex was noted with anti-STAT-1 and anti-c-Fos Ab (Fig. 3D). Thus, reduction of NOS2 expression in FOSi was not necessarily related to less binding at the GAS element, or loss of c-Fos from the binding complex.

Consequence of AP-1 impairment on NF κ B activation in FOSi

NF κ B was also evaluated in the FOSi [10,11]. A549, FOSi and FOSscr cells were exposed to TNF α for 30 min. The whole cell extract was evaluated in EMSA with the radiolabeled consensus NOS2 NF κ B sequence. Unexpectedly, NF κ B–DNA binding was strongly increased in FOSi, suggesting an effect of c-Fos knockdown

on NF κ B activation (Fig. 4A). The presence of p50 and p65 in the DNA–protein binding complex was confirmed by supershift using anti-p50 and anti-p65 Ab (Fig. 4A). Western analysis identified a slight increase of p50, but not p65 protein in FOSi cells (Fig. 4B). Since redox is a major regulator of NF κ B activation, we also evaluated the redox sensitive Bcl-2 protein expression. Bcl-2 was induced in FOSi at baseline and was similar after CK expression. These findings suggest c-Fos knockdown modified redox in the cell which was associated with abrogation of NOS2 induction (Fig. 4B).

Discussion

Targeting c-Fos using RNA interference successfully reduced c-Fos expression, retards nuclear translocation, and inhibits DNA binding. As hypothesized, the knockdown of c-Fos resulted in a decrease in NOS2 gene induction and NO synthesis in cytokine-stimulated lung epithelial cells. NOS2 expression is primarily regulated at the transcription level, and the decrease in NOS2 in the FOSi was related to less c-Fos/c-Jun heterodimer binding to the NOS2 AP-1u site. However, STAT1–GAS binding was not affected and c-Fos was detectable within the STAT1–GAS binding complex. Thus, reduction of NOS2 expression in FOSi was not related to less binding at the GAS element, or loss of c-Fos/STAT1 interaction.

The expression and regulation of NOS2 in airway epithelial cells is closely related to activation of transcription factors, including the JAK/STAT-1, NF κ B and AP-1 signal transduction pathways [10,11,13,18,23,24]. In primary human airway epithelial cells, IFN γ alone induces NOS2 *in vitro* highlighting the central role of this pathway in NOS2 induction [25]. Moreover, inhibition of IFN–JAK/STAT pathway blocks NOS2 expression in A549 cells [23]. Promoter analyses of the NOS2 gene also identify that mutations of NF κ B sites result in about 85% decrease of promoter activity with cytokine stimulation [10]. While STAT1 and NF κ B have been clearly identified as important signal transduction pathways to NOS2 gene expression [18,23–25], the role of AP-1 in NOS2 expression has not been extensively studied. Mice genetically deficient in c-Fos do not have abnormality in lung growth or development, but the model was not specifically evaluated for NOS2 induction or host defense [26]. An antisense strategy against c-Fos suggested that induction of NOS2 following cytokine stimulation is dependent upon c-Fos [14]. Here, even though a level of c-Fos persists in FOSi, it appears that lowering its level is sufficient to abrogate translocation and DNA binding at AP-1 sites and reduce NOS2 induction. The fact that reduction of c-Fos is adequate to diminish NOS2 expression supports the concept that multiple transcription factor interactions are involved in the NOS2 gene regulation. On the other hand, previous study showing that c-Fos over-expression does not increase NOS2 expression suggests a permissive effect of c-Fos [14].

One potential mechanism for near complete loss of AP-1–DNA binding in the FOSi, despite the presence of c-Fos protein, is that a modulation of the intracellular redox homeostasis occurs in response to a reduction of the c-Fos protein [27]. AP-1 binding activity depends on the redox state of the c-Fos and c-Jun proteins. Reversible redox modification of cysteine residues located in the basic DNA binding domain of c-Fos and c-Jun by redox factor 1 (ref-1) or thioredoxin has been shown to be a critical event in the AP-1-mediated responses [27–29]. It is possible that the redox or phosphorylation status of the remaining c-Fos impairs its nuclear localization even in the presence of protein in the cytoplasm. FOSi cells had greater NF κ B binding complex of the NOS2–NF κ B DNA consensus oligonucleotide. To our knowledge, this is the first demonstration that knockdown of c-Fos increases NF κ B activation. AP-1 and NF κ B are both redox-sensitive transcription factors. There is an increase in Bcl-2 expression in FOSi cells, which supports an altered redox state, since Bcl-2 induction occurs in response to increased intracellular oxidative milieu [30]. *In vivo*

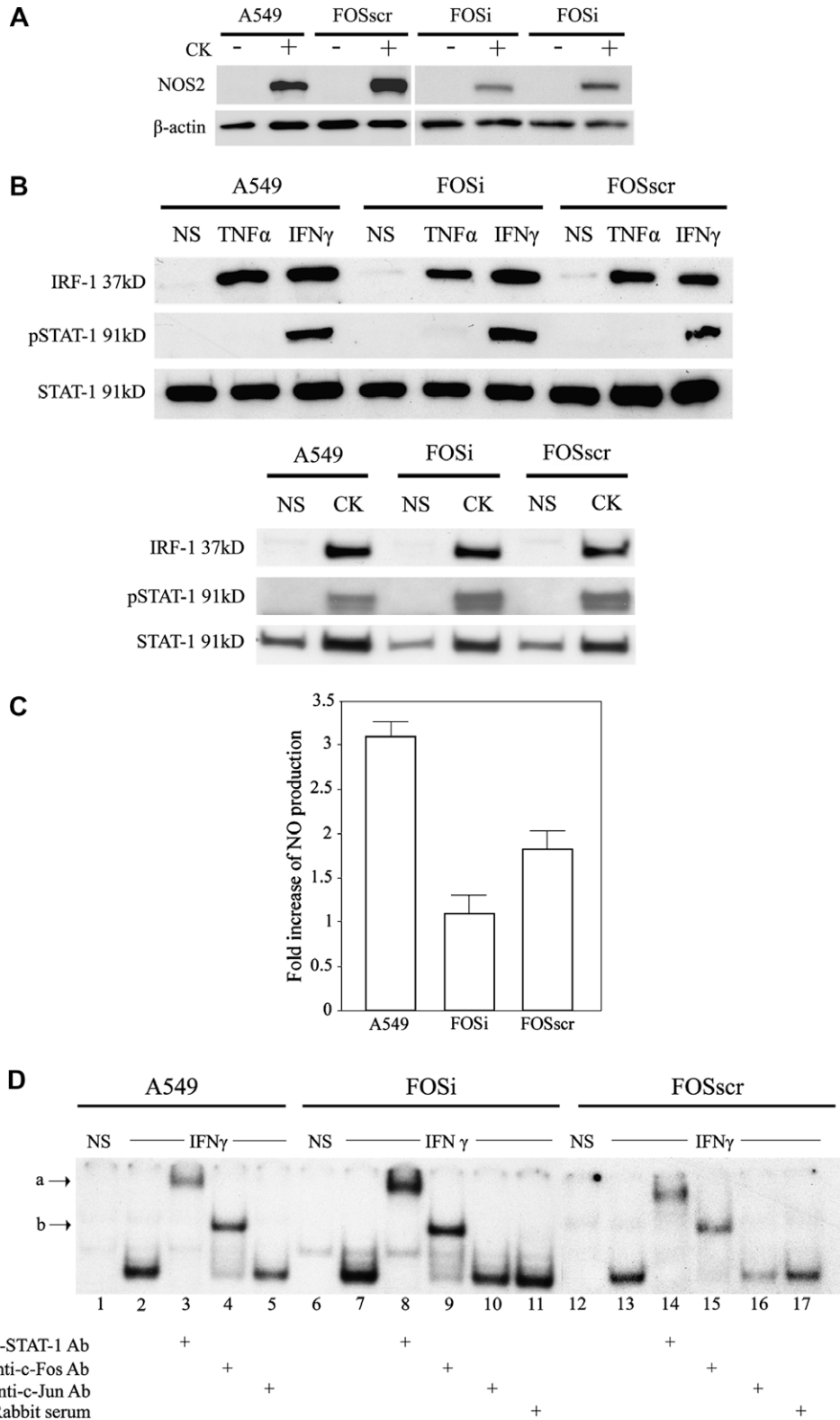


Fig. 3. (A) Reduction of NOS2 protein expression in FOSi. After 24 h exposure to CK, NOS2 is induced in A549 and FOSscr clone, but reduced in two FOSi clones. Results shown are representative of three separate experiments. (B) Western blot of IRF-1, pSTAT-1 and STAT-1 expression. After 24 h stimulation by TNFα or IFNγ or cytokine mix (CK: IL-1β, TNFα and IFNγ), levels of expression were similar among FOSi and FOSscr and A549. pSTAT-1 was induced by IFNγ or CK exposure. Results shown are representative of three separate experiments. (C) Decreased NOx production by FOSi cells after 24 h CK stimulation (IL1-β, TNFα and IFNγ). Results are presented as fold increase of NO production over baseline (means ± SD of 15 measures in three separate experiments). (D) NOS2-GAS binding activity using radiolabeled NOS2 GAS in A549, FOSi, and FOSscr cells after exposure to IFNγ for 30 min. Anti-STAT-1 (lanes 3, 8, and 14), anti-c-Fos (lanes 4, 9, and 15), anti-c-Jun antibodies (lanes 5, 10, and 16), and nonimmune rabbit IgG (lanes 11 and 17) were added to the binding reactions to identify the presence of proteins in the binding complex. The supershift of the binding complex is observed with STAT-1 (arrow a) and c-Fos (arrow b). The autoradiograph is representative of two separate experiments.

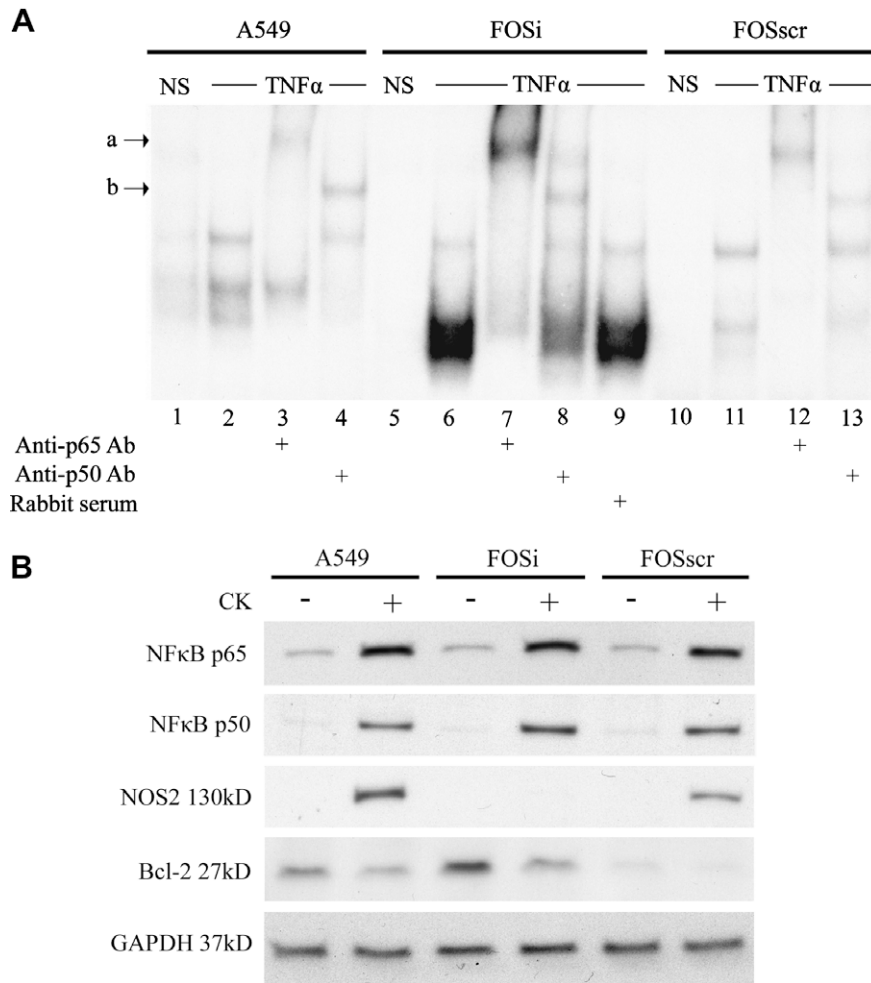


Fig. 4. (A) NOS2–NFκB binding activity in A549 and FOSi cells after exposure to TNF α for 3 h using the radiolabeled NFκB binding consensus oligonucleotide. The supershift of the binding complex was observed with p65 (lanes 3, 7, and 12) and p50 Ab (lanes 4, 8, and 13) (arrows a and b, respectively). Increased NFκB binding activity was present in the FOSi cells induced by TNF α . The autoradiograph is representative of three separate experiments. (B) Expression of p50, p65 and Bcl-2 in FOSi cells as compared to FOSscr and A549 cells. iNOS expression in the FOSi clone following CK stimulation was significantly reduced, whereas Bcl2 is increased at baseline and after CK stimulation in FOSi compared to FOSscr.

studies indicate that NFκB and Bcl-2 increase in transgenic mice with alterations in redox homeostasis [31]. The greater NFκB activation may also participate in abrogation of NOS2 induction. Binding of STAT-1 to the NOS2 promoter region that contains the critical GAS element is competitive with NFκB due to proximity of a NFκB binding site. Thus, if c-Fos knockdown is associated with activation of NFκB, accessibility of the STAT-1 to the GAS site may be limited and affect NOS2 transcription [10,23]. Similarly, even if STAT-1 binding to GAS occurs in the NOS2 promoter, the inability of the c-Fos to bind to the AP-1 site may influence the tertiary structure imposed by the multiple DNA–protein interactions and hence impede transcription. Conversely, lesser NOS2 expression may contribute to the greater NFκB–DNA binding activation since NO induces S-nitrosation of p50 and its DNA binding but with less ability to transactivate gene expression [32–34].

There are limitations to the study. We used a stable transfection RNAi technology against c-Fos to obtain long-term and higher intracellular concentrations of siRNAs, which allows the study of clones for a longer time in different conditions compared to the antisense or transient transfection approach [15]. Although this is a powerful and valid approach for studying the signal transduction pathways, the efficiency of silencing depends in part on the RNAi sequence designed and the variability of expression among clones. In fact, variability in NOS2 inhibition was seen among FOSi

clones. Despite this, clones displayed inhibition of cytokine-stimulated NOS2 expression providing reassurance that c-Fos gene silencing was the cause [35]. RNAi may activate the interferon system. Since regulation of NOS2 is modulated by IFNs, this may have confounded our experiments. However, the lack of IFN inducible genes expression in FOSi clones provides reassurance that IFN was not significantly activated [21].

The expression of NOS2 is pivotal to lung health as shown by susceptibility to infections in murine models deficient in NOS2, and in human conditions associated with loss of NOS2 expression [36,37]. For example, cystic fibrosis airways lack NOS2 expression due to impairment of STAT1 activation [36], and the decrease in STAT-1 and NOS2 is linked to an increase in viral replication and bacterial colonizations [37,38]. This study expands our understanding of the regulation of the innate host response and may offer strategies to augment host defense of the airway, or inhibit the excessive inflammatory processes in disease.

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