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Inhibitor of Nuclear Factor κB Kinase Deficiency Enhances Oxidative Stress and Prolongs c-Jun NH_2-Terminal Kinase Activation Induced by Arsenic

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ABSTRACT

Stress signals activate both inhibitor of nuclear factor-κB kinase (IKKβ) and c-Jun NH_2-terminal kinase (JNK). It was shown recently that IKK-dependent nuclear factor κB activation results in attenuation of tumor necrosis factor α-induced JNK activation. How that negative cross-talk between nuclear factor κB and JNK occurs is not well-understood. By using wild-type and Ikkβ gene knockout ($Ikk\beta^{-/-}$) mouse embryo fibroblasts, we found that IKKβ deficiency results in prolongation of arsenic-induced JNK activation, which was not due to the decreased expression of GADD45β or X-linked Inhibitor of Apoptosis (XIAP), as suggested previously for $RelA^{-/-}$ cells treated with tumor necrosis factor α. This enhanced JNK activation was largely associated with an oxidative stress response as indicated by elevated expression of heme oxygenase-1 and the accumulation of H_2O_2 in $Ikk\beta^{-/-}$ cells. Expression profiling experiments revealed an increased expression of p450 family CYP1B1 mRNA in $Ikk\beta^{-/-}$ cells compared with wild-type cells. Inhibition of CYP1B1 reduced both oxidative stress and arsenic-stimulated JNK activation. Thus, increased CYP1B1 expression is central to and seems to be responsible for sensitizing $Ikk\beta^{-/-}$ cells to stress-induced JNK activation.

INTRODUCTION

Transcription factor NFκB[3] plays central roles in the cellular response to a variety of stress signals by regulating numerous genes (1–3). Signals from various stimuli induce activation of upstream kinases, mainly the IKK complex that contains two catalytic kinase subunits, IKKα and IKKβ, and a structural and regulatory component named NEMO/IKKγ. Activated IKK phosphorylates inhibitor of NFκB α and inhibitor of NFκB β, leading to their ubiquitination and degradation, and subsequent NFκB activation and nuclear translocation.

The JNK acts as an integration point for multiple intracellular biochemical signals governing a wide variety of cellular processes such as proliferation, differentiation, apoptosis, migration, transcriptional regulation, and development (4). JNK targets specific transcription factors and, thus, mediates immediate-early gene expression in response to various stress signals including UV radiation, oxidative stress, protein malfolding, osmotical shock, and inflammatory mediators. These transcription factors include activator protein 1, ATF-2, Elk-1, and p53. A number of extracellular or intracellular signaling molecules, such as cytokines or mitogen-activated protein kinase kinase kinases, that activate IKK-NFκB signaling pathways can also contribute to the activation of JNK (5). Thus, cross-talk between NFκB and JNK occurs under many circumstances. We have shown previously that inhibition of IKKβ and NFκB activities via expression of catalytically inactive IKKβ in epithelial cells enhances stress-induced JNK activation caused by As^{3+} treatment (6). In the present study, we additionally demonstrated that the enhanced JNK activation in As^{3+}-treated IKKβ-deficient cells is partially due to oxidative stress resulting from the increased expression of cyp1B1, a p450 gene family member.

MATERIALS AND METHODS

Cell Culture and Western Blotting. MEFs were cultured as described previously (7). Whole cell extracts were prepared using SDS-PAGE sample buffer and subjected to SDS-PAGE in 8 or 10% gels. The resolved proteins were transferred to a nitrocellulose membrane. Western blotting was performed using antibodies as indicated in figures or figure legends.

H_2O_2 Assay by DCFDA Staining and Confocal Microscopy. MEFs cultured on glass coverslips in six-well tissue culture plates were stained with 5 μM CM-H_2-DCFDA (Molecular Probes, Inc., Eugene, OR) in dark for 30 min. After extensive washing with PBS buffer and fixation with 10% formaldehyde for 10 min at room temperature in dark, the coverslips were mounted on slides. The H_2O_2 generation was detected as a result of the oxidation of DCFDA (excitation, 488 nm; emission, 515–540 nm) by confocal microscopy. A single rapid scan was performed for each slide with identical parameters of contrast and brightness. The cell images were randomly selected from the digital interference contrast channel for each sample.

Gene Profiling and RT-PCR. Total RNA was isolated from MEFs and subjected to gene profiling using Affymetrix mouse GeneChip following the manufacturer's instructions. To verify the gene profiling data, some of the differentially expressed genes in WT and $Ikk\beta^{-/-}$ MEFs were analyzed by RT-PCR.

RESULTS AND DISCUSSION

To understand the mechanisms by which IKKβ inhibition potentiates JNK activation, we used MEFs derived from WT and Ikkβ gene knockout ($Ikk\beta^{-/-}$) mice (7). Expression of IKK subunits in these cells was determined by immunoblotting after treatment with As^{3+}, a classic stress inducer (8, 9), for various time periods. A relatively higher concentration of As^{3+}, 50 μM, rather than the lower concentrations relevant to the chronic environmental exposure, was used in this study for the purpose of short experimentation. As expected, whereas equal levels of IKKα were noted between WT and $Ikk\beta^{-/-}$ cells, expression of IKKβ was limited to WT cells (Fig. 1). Both types of cells exhibited activation of JNK in response to As^{3+}, as described previously (10). However, JNK activation detected by immunoblotting with an antibody against Thr183/Tyr185-phosphorylated JNK protein occurred with different kinetics in the two cell types. In WT cells, JNK activation was elevated rapidly by As^{3+}, peaking ~4 h after stimulation, and declined by 24 h. In $Ikk\beta^{-/-}$ cells, however, JNK activation was sustained and peaked at 24 h after stimulation (Fig. 1*A*). Similarly, an enhanced activation of p38 and Erk, two additional members of mitogen-activated protein kinase family, was noted in $Ikk\beta^{-/-}$ cells. However, the enhancement of p38 and Erk in $Ikk\beta^{-/-}$ cells was much weaker than that of JNK. Fig. 1*B* shows a normalized value of four detailed time course experiments for JNK

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[3] The abbreviations used are: NFκB, nuclear factor κB; IKK, inhibitor of nuclear factor κB kinase; JNK, c-Jun NH_2-terminal kinase; As^{3+}, arsenic; MEF, mouse embryo fibroblast; DCFDA, 2′,7′-dichlorofluorescein diacetate; RT-PCR, reverse transcription-PCR; WT, wild-type; MEKK, mitogen-activated protein kinase kinase kinase; MKP, mitogen-activated protein kinase phosphatase; HO-1, heme oxygenase-1; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; AhR, aryl hydrocarbon receptor; FBS, fetal bovine serum.

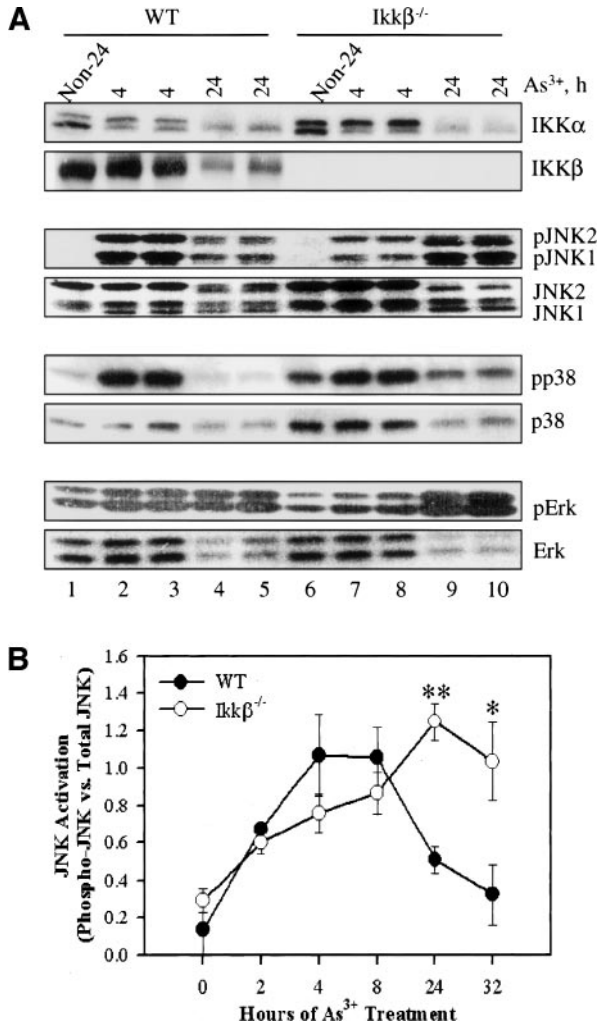

Fig. 1. Prolonged JNK activation in Ikk$\beta^{-/-}$ cells. *A*, WT and Ikk$\beta^{-/-}$ cells were cultured in six-well tissue culture plates in medium containing 5% FBS for 2 days followed by culture in medium containing 0.1% FBS for 1 day. Cells were then treated with 50 μM As^{3+} for different time periods as indicated. Total cellular protein was extracted and subjected to immunoblot analysis using antibodies against IKKα, IKKβ, phospho- or non-phospho-JNK, phospho- or non-phospho-p38, and phospho- or non-phospho-Erk as indicated. *Lanes 1* and *6* indicate that cells cultured for 24 h without As^{3+} treatment. *B*, a more detailed time course study for As^{3+}-induced JNK activation in both WT cells and Ikk$\beta^{-/-}$ cells. JNK activation was normalized by determining the density ratios of phospho-JNK bands *versus* non-phospho-JNK bands using TotalLab software. * indicated significant difference of JNK activation induced by As^{3+} between WT and Ikk$\beta^{-/-}$ cells at the same time point (**, $P \leq 0.005$; * $P \leq 0.05$).

activation. In WT cells, the decline of JNK activation by As^{3+} started at 8 h. The activation of JNK, however, was still near maximum at 32 h of As^{3+} treatment in Ikk$\beta^{-/-}$ cells (also see Supplementary Fig. 1).

The prolonged activation of JNK in Ikk$\beta^{-/-}$ cells may indicate enhanced activity of upstream kinases that activate JNK. MEKK1 is a M_r 196,000 mammalian serine/threonine kinase that may be responsible for stress-induced JNK activation. After stress signals, MEKK1 is cleaved by proteases into an active COOH-terminal M_r 91,000 kinase fragment (11). To determine the status of proteolytic activation of MEKK1, an immunoblot was performed using an antibody that recognizes both a full-length and an active COOH-terminal kinase fragment of MEKK1. As depicted in Fig. 2*A*, Ikk$\beta^{-/-}$ cells showed basal cleavage of MEKK1 that was not seen in WT cells. In addition, Ikk$\beta^{-/-}$ cells exhibit higher basal levels of activated caspase-3, a cysteine protease that cleaves MEKK1 (Ref. 11; Fig. 2*B*), as evidenced by the appearance of the activated M_r 12,000 caspase-3 fragment. However, there was no correlation between late-phase JNK activation by As^{3+} and MEKK1 cleavage or caspase-3 activation, which were most apparent in Ikk$\beta^{-/-}$ cells in the absence of As^{3+} (compare Fig. 1 with Fig. 2, *A* and *B*).

It should be noted that previous studies have shown that JNK activation by As^{3+} is largely dependent on inhibition of MKPs, which normally dephosphorylate and inhibit the activity of JNK (10). To investigate whether potentiated JNK activation in Ikk$\beta^{-/-}$ cells is due to deficiency or reduced expression of MKPs, the protein levels of several MKPs in both WT cells and Ikk$\beta^{-/-}$ cells were determined. As indicated in Fig. 2*C*, immunoblot analysis revealed an increase rather than a decrease in MKP1 and MKP2 expression or induction in Ikk$\beta^{-/-}$ cells (Fig. 2*C*). PAC1, another dual-specific MKP, was expressed equally in WT and Ikk$\beta^{-/-}$ cells, whereas the expression of MKP3 could be detected only in Ikk$\beta^{-/-}$ cells. Both cells did not express a relatively specific JNK phosphatase M3/6 as indicated by RT-PCR analysis (data not shown). Therefore, it is less likely that decreased expression of MKPs is responsible for the prolonged JNK activation by As^{3+} in Ikk$\beta^{-/-}$ cells.

There are a number of other factors that contribute to the activation of JNK signaling, among which oxidative stress is most prominent (12, 13). Furthermore, the MKPs responsible for JNK inhibition are cysteine-dependent enzymes, of which the activity require the presence of reduced cysteine and are, therefore, sensitive to oxidative stress (10). To ascertain whether the enhanced JNK activation by As^{3+} in Ikk$\beta^{-/-}$ cells was the result of redox imbalance, we first

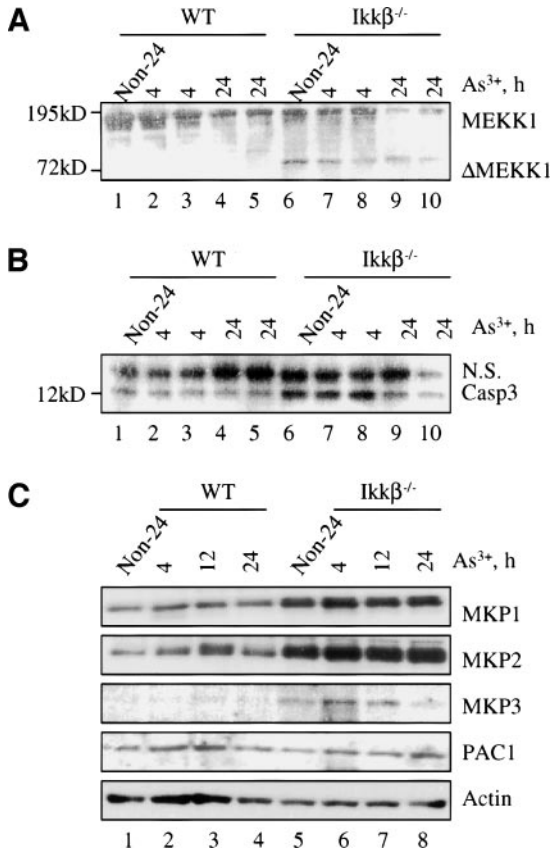

Fig. 2. Spontaneous cleavage of MEKK1 and enhanced caspase-3 activation in Ikk$\beta^{-/-}$ cells. Cells were cultured as described in Fig. 1. Total cellular protein was used for immunoblot using antibodies against MEKK1 (*A*) and caspase-3 (*B*), respectively. Results are representation of three experiments. *Lanes 1* and *6* in each figure indicate that cells cultured for 24 h without As^{3+} treatment. *C*, Expression of MKPs in WT and Ikk$\beta^{-/-}$ cells. Total cellular proteins were prepared from WT or Ikk$\beta^{-/-}$ cells cultured in the absence or presence of 50 μM As^{3+}. Immunoblotting was performed by sequential hybridization of the same membrane with antibodies against MKP1, MKP2, MKP3, PAC1, and Actin.

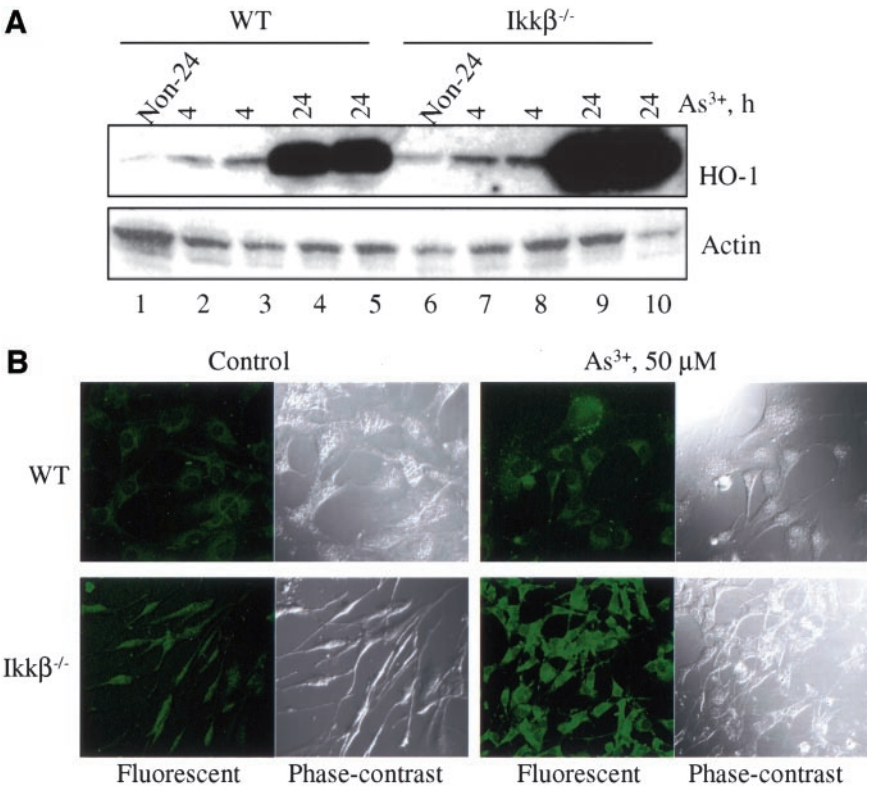

Fig. 3. IKKβ deficiency causes oxidative stress. *A*, WT and Ikk$\beta^{-/-}$ cells treated with or without 50 μM As^{3+} for 0, 4, or 24 h. The cell lysates were used for an immunoblot of HO-1 (*top panel*) or β-actin (*bottom panel*). Results are representative of three experiments. *Lanes 1* and *6* indicate that cells cultured for 24 h without As^{3+} treatment. *B*, both WT and Ikk$\beta^{-/-}$ cells were cultured in six-well tissue culture plates (10^5/well) containing glass slides in 5% FBS for 2 days and then in 0.1% FBS for 1 day. The cells were additionally cultured in 0.1% FBS containing 50 μM As^{3+} for 12 h. H_2O_2 accumulation was determined by incubation of the cells with DCFDA for 30 min followed by confocal microscopic analysis.

measured the expression of HO-1, a sensitive and reliable indicator of oxidative stress (14). To visualize the basal expression of HO-1 in both WT and Ikk$\beta^{-/-}$ cells, we overexposed the immunoblot membrane. Whereas HO-1 was nearly undetectable under basal conditions in WT cells, a notable HO-1 band was present in nonstimulated Ikk$\beta^{-/-}$ cells (Fig. 3*A*, *top panel*). As^{3+} treatment caused a much stronger induction of HO-1 in Ikk$\beta^{-/-}$ cells than that in WT cells (Fig. 3*A*). To investigate whether oxidative stress was responsible for the increased basal and As^{3+}-induced HO-1 expression in Ikk$\beta^{-/-}$ cells, we examined H_2O_2 accumulation in both WT and Ikk$\beta^{-/-}$ MEFs using DCFDA, of which the fluorescence is proportional to the level of intracellular H_2O_2. Confocal microscopy analysis indicated that only a marginal increase of H_2O_2 could be detected in the WT cells upon treatment with 50 μM of As^{3+} for 12 h (Fig. 3*B*). In contrast, significant levels of H_2O_2 were present in Ikk$\beta^{-/-}$ cells even before treatment with As^{3+} (Fig. 3*B*). Treatment with As^{3+} resulted in an additional increase its H_2O_2 production in these cells, possibly because of partial depletion of intracellular reduced glutathione pool as suggested (15), although the change of reduced glutathione did not reach statistical significance (Supplementary Fig. 2). To exclude the possible nonspecific detection of H_2O_2 by DCFDA, the increased H_2O_2 generation in Ikk$\beta^{-/-}$ cells was additionally confirmed biochemically using OXIS Bioxytech H_2O_2 detection kit (data not shown).

ROS are generated from molecular oxygen either by mitochondrial electron transport chains or by cytosolic metabolic reactions. Spontaneous or enhanced oxidative stress may reflect either overproduction of ROS or accumulation of ROS resulting from dysfunction of endogenous antioxidant enzymes. A number of enzymes with peroxidase activity, such as cytochromes p450 family members, cyclooxygenases or lipoxygenases, are responsible for generation of ROS in the cytosol (16, 17). Whereas expression profiling experiments by DNA microarray analysis revealed no decrease in mRNA expression of several major antioxidant enzymes in Ikk$\beta^{-/-}$ cells, a 2.8-fold increase in the mRNA level of p450 protein CYP1B1 was noted (Table 1). To verify the DNA microarray data, we performed RT-PCR analyses using total RNA derived from both WT and Ikk$\beta^{-/-}$ cells (Fig. 4*A*). A roughly equal expression of GADD45β, GADD45γ, and GAPDH mRNAs was observed between WT and Ikk$\beta^{-/-}$ cells. In agreement with the DNA microarray results, a substantial increase in CYP1B1 mRNA and a marginal increase of SOD1 mRNA were detected in Ikk$\beta^{-/-}$ cells. In addition, an appreciable decrease of metallothionein II, a major metal binding protein that may act as a scavenger of hydroxyl radicals (18, 19), was observed in Ikk$\beta^{-/-}$ cells.

It is well known that p450 family members catalyze a large spectrum of oxidation of endobiotics and xenobiotic chemicals, resulting ROS generation (20). To determine whether increased CYP1B1 expression in Ikk$\beta^{-/-}$ cells might be responsible for the observed changes in H_2O_2 production and JNK activation, we pretreated the cells with NAC and p450 inhibitors. NAC, as has been shown previously (10), inhibited the induction of HO-1 and JNK activation in Ikk$\beta^{-/-}$ cells treated with As^{3+} for 16 h (Fig. 4, *B* and *C*, *Lanes 3* and *4*). SKF525A, an inhibitor for CYP1A1, 2A, and 4A (21, 22), showed no effect on either HO-1 induction or JNK activation (Fig. 4, *B* and *C*, *Lanes 5* and *6*). In contrast, the CYP1B1 inhibitor, α-naphthoflavone,

Table 1 *Relative levels of gene expression in Ikk$\beta^{-/-}$ MEF versus WT MEF*

Genes	Ikk$\beta^{-/-}$ *vs.* WT
GADD45α	2.7
GADD45β	1.3
GADD45γ	1.5
SOD1	1.2
GST8.7	2.7
Thioredoxin	2.0
CYP 1B1	2.8
Lysyl oxidase	−2.4
Metallothionein I	−3.2
Metallothionein II	−8.2

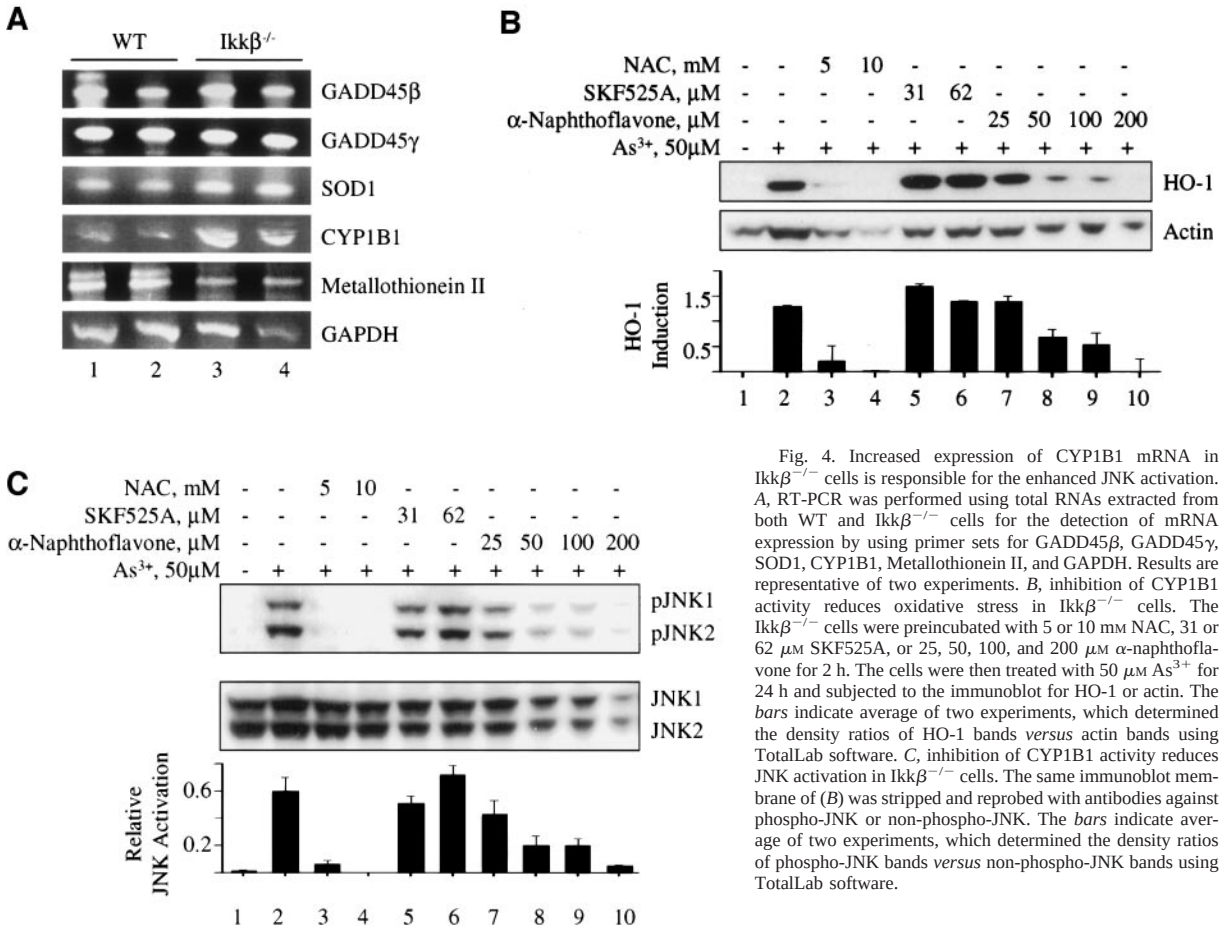

Fig. 4. Increased expression of CYP1B1 mRNA in Ikk$\beta^{-/-}$ cells is responsible for the enhanced JNK activation. *A*, RT-PCR was performed using total RNAs extracted from both WT and Ikk$\beta^{-/-}$ cells for the detection of mRNA expression by using primer sets for GADD45β, GADD45γ, SOD1, CYP1B1, Metallothionein II, and GAPDH. Results are representative of two experiments. *B*, inhibition of CYP1B1 activity reduces oxidative stress in Ikk$\beta^{-/-}$ cells. The Ikk$\beta^{-/-}$ cells were preincubated with 5 or 10 mM NAC, 31 or 62 μM SKF525A, or 25, 50, 100, and 200 μM α-naphthoflavone for 2 h. The cells were then treated with 50 μM As^{3+} for 24 h and subjected to the immunoblot for HO-1 or actin. The *bars* indicate average of two experiments, which determined the density ratios of HO-1 bands *versus* actin bands using TotalLab software. *C*, inhibition of CYP1B1 activity reduces JNK activation in Ikk$\beta^{-/-}$ cells. The same immunoblot membrane of (*B*) was stripped and reprobed with antibodies against phospho-JNK or non-phospho-JNK. The *bars* indicate average of two experiments, which determined the density ratios of phospho-JNK bands *versus* non-phospho-JNK bands using TotalLab software.

inhibited HO-1 induction and JNK activation in a dose-dependent manner in Ikk$\beta^{-/-}$ cells treated with 50 μM As^{3+} (Fig. 4, *B* and *C*, *Lanes 7–10*). In WT cells, although both NAC and α-naphthoflavone were able to inhibit JNK activation induced by As^{3+} treatment for 4 h, neither NAC nor α-naphthoflavone showed a significant effect on JNK activation in WT cells treated with As^{3+} for 16 h, possibly because of the fact that As^{3+}-induced JNK activation at this time point had already declined to an undetectable level (Supplementary Fig. 3).

There are several possibilities that explain the increased expression of CYP1B1, and the resulting oxidative stress and prolonged JNK activation in Ikk$\beta^{-/-}$ cells. Deficiency in IKKβ reduced the basal activity of NFκB (data not shown), a transcription factor that antagonizes the AhR (23, 24), a nuclear protein required for the transcription of *CYP1B1* gene that contains a number of AhR binding sites (XRE; Ref. 25). In addition, impairment of NFκB signaling possibly decreases expression of the AhR repressor gene, leading to the enhancement of AhR function and increased expression of CYP1B1.

Increased JNK activation in RelA$^{-/-}$ MEF treated with tumor necrosis factor α has been attributed previously to the decreased expression of GADD45β and XIAP (26, 27). Intriguingly, both GADD45β and XIAP have also been shown to be capable of activating JNK through affecting upstream kinase (28, 29). In the present study, no substantial difference of basal GADD45β mRNA was observed in WT cells and Ikk$\beta^{-/-}$ cells (Table 1; Fig. 4*A*). In addition, no difference in the levels of XIAP expression between WT and Ikk$\beta^{-/-}$ MEF was detected (data not shown). Therefore, it is unlikely that decreased GADD45β or XIAP expression contributed to an enhanced As^{3+}-induced JNK activation in Ikk$\beta^{-/-}$ cells. Instead, we believe that oxidative stress partially resulted from the increased expression of CYP1B1 in Ikk$\beta^{-/-}$ cells is responsible for the sustained As^{3+}-induced JNK activation. Although oxidative stress itself is not very potent in basal JNK activation, it is synergistic for stress-induced JNK activation, possibly through inactivation of MKPs or endogenous JNK signaling inhibitors, such as glutathione *S*-transferase π and thioredoxin (30, 31).

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Note Added in Proof

During the reviewing period of this manuscript, Sakon *et al*. reported, in the August 1st issue of EMBO J (22:5530), that increased ROS accumulation is responsible for the prolonged TNFa-induced JNK activation in rela−/− cells.
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