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X-linked inhibitor of apoptosis protein increases mitochondrial antioxidants through NF-κB activation

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Abstract

X chromosome-linked inhibitor of apoptosis protein is an endogenous inhibitor of caspases and is an important regulator of cell death. XIAP can also influence cell signaling, but downstream proteins affected are largely unknown. We show here using neuronal PC6.3 cells that XIAP increases the levels of antioxidants, particularly superoxide dismutase-2 that is localized to mitochondria. Studies using reporter constructs and NF-κB Rel-A deficient mouse embryonic fibroblasts showed that NF-κB signaling is required for the induction of Sod2 by XIAP. XIAP also reduced oxidative stress in the PC6.3 cells as shown by decreased production of reactive oxygen species. These findings disclose a novel role for XIAP in control of oxidative stress and mitochondrial antioxidants that may contribute to cell protection after various injuries.

Keywords: XIAP; NF-κB; Sod2; Mitochondria; Oxidative stress

Mitochondria play a crucial role in the control of cell death and apoptosis in most cells including neurons [1–3]. Different external and cell internal factors may trigger the release of pro-apoptotic proteins from the mitochondria, leading to activation of caspase-dependent and -independent cell death [1–3]. Caspases are controlled by the action of various anti-apoptotic proteins, including the family of inhibitor of apoptosis proteins (IAPs) [4,5]. X chromosome-linked IAP (XIAP) is the most versatile among the IAPs and has the highest capacity to inhibit caspase-9, -3, and -7 [4,5]. Recent studies have shown that mutant XIAP, unable to inhibit caspase-3 and caspase-9, can still attenuate cell death possibly by affecting cell signaling [5]. Particularly it has been shown that XIAP can influence transforming growth factor-β1 (TGF-β1) and bone morphogenic protein (BMP) signaling [6,7]. However, the downstream events and proteins influenced by XIAP in cell signaling are only beginning to be understood. In this respect it has been shown that XIAP can activate the transcription factor NF-κB in endothelial cells [8]. NF-κB is usually latent in the cytosol bound to a complex with the inhibitory IκB protein and is activated by translocation to the nucleus to induce transcription of different genes [9,10]. NF-κB is an important signaling system that is involved in different cell processes among others in the immune response, in cell death regulation and cell proliferation [9,10].

In the present work, we studied whether XIAP affects NF-κB signaling in neuronal PC6.3 cells and which downstream protein targets in these cells are influenced by XIAP overexpression. We focused here on various antioxidants that are able to protect against reactive oxygen species

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(ROS) and free radical-induced cell injuries [11–13]. The strict control of ROS production by antioxidants is crucial for normal metabolism and cell viability [11,12]. Elevated ROS and oxidative stress of reactive oxygen cause cell damage and accompany many diseases particularly in the nervous system [14,15]. We observed that particularly manganese superoxide dismutase-2 (Sod2) that resides in the mitochondrial matrix [13] is increased by XIAP in the PC6.3 cells concomitant with the activation of NF-κB. Data obtained using NF-κB Rel-A gene deleted fibroblasts showed that NF-κB signaling is required for the induction of Sod2 by XIAP.

Materials and methods

Cell culture and viability. PC6.3 cells were cultured in RPMI 1640 medium, containing 2 mM L-glutamine, 10% horse and 5% fetal calf serum [16]. Approximately 25×10^3 cells in collagen-coated 96-well Costar plates were transfected using 0.5 μl TransFectin™ Lipid Reagent (Bio-Rad) and 0.2 μg human XIAP-EGFP or 0.02 μg EGFP (Clontech) expression vectors. Cells were then stimulated for 24 h in 1% serum with 100 μM xanthine and 100 mU/ml xanthine oxidase (both Sigma) to induce oxidative stress [17]. Cell viability was determined using the MTT assay as described previously [18,19]. 2.5 mM acetylsalicylic acid was employed to block activation of NF-κB [20]. Control Swiss 3T3 fibroblasts and immortalized fibroblast cell lines from NF-κB Rel-A (also known as the NF-κB p65 protein) deficient mice were cultured in Dulbeccós modified Eagle medium supplemented with 10% bovine calf serum as described before [21]. Cells were transfected with 0.6 μg XIAP-EGFP or 0.06 μg EGFP plasmids and analyzed as described above.

Reporter assay for NF-κB. PC6.3 cells were transfected with 1 μg human XIAP-myc or with 1 μg pcDNA3.1 plasmids together with 1 μg NF-κB Luciferase Reporter and 0.1 μg *Renilla* luciferase pRL-TK plasmids. Cells were harvested after 24 or 48 h using buffer containing 25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol and 1% Triton X-100 (Stratagene). *Renilla* and firefly luciferase activities were measured using dual luciferase substrate (Promega) and a luminometer (TD-20/20, Luminometer Turner Designs) [22]. Results are shown as fold increase in luciferase normalized to the *Renilla* activity.

Analyses of ROS production. PC6.3 cells were cultured on 6 cm Nunc dishes and transfected with 8 μg XIAP-EGFP and 1.6 μg EGFP. Two days later cells were stimulated for 1 h with 100 μM xanthine and 100 mU/ml xanthine oxidase in serum-free RPMI medium. 10 μM dihydroethidium (DHE; Molecular Probes) was added for the last 15 min and the cells were examined by fluorescent activated cell sorter Aria (FACS) (BD Biosciences). The number of DHE positive cells was measured by gating GFP expressing cells using excitation at 488 nm and emission at 502 for GFP and 595 nm for DHE, and expressed as relative to controls.

Immunocytochemistry. Cells were fixed for 20 min using 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 5 min, and washed in PBS [19,23]. After incubation with 5% BSA-PBS, anti-NF-κB p65 antibody (1:100, Santa Cruz sc-8008) was added overnight at 4 °C, followed by 1 h with a Cy3-conjugated secondary antibody (1:200, Jackson ImmunoResearch Laboratories). Nuclei were stained using 4 μg/ml Hoechst 33258 (Sigma), and cells mounted with Gel Mount™ Medium (Sigma G0918). Negative controls were incubated without primary antibodies.

Immunoblotting. Cells were lyzed in a buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% natriumdeoxycholate, 150 mM NaCl, 1 mM EDTA, and protease inhibitors (Roche). 20 mM NaF and 0.2 mM Na_3VO_4 were added to inhibit phosphatases [19,23]. Following protein assay (BioRad) 20–40 μg total protein were separated using SDS–PAGE, transferred to nitrocellulose membranes (Hybond-C Extra, Amersham, UK), blocked for 1 h in TBS and 5% skim milk, and incubated overnight at 4 °C with primary antibodies against Bcl-xL (1:2000, BD Biosciences), catalase (1:2000, LabFrontier), IκB-α (1:200, Santa Cruz, CA), P-IκB-α (1:100, Santa Cruz), NF-κB (p-65) (1:500, Santa Cruz), Sod1 (1:1000, Calbiochem), Sod2 (1:15,000, LabFrontier), Thioredoxin 2 (Trx2, 1:1000, LabFrontier), XIAP (1:5000, BD Biosciences), and β-actin (1:1000, Sigma) that was used as control. Appropriate peroxidase-conjugated antibodies (1:2500, Jackson Laboratories) were added for 1 h and detection was performed using SuperSignal West Pico Substrate (Pierce). Quantification was performed using GelDoc (BioRad).

RT-PCR. Total RNA was extracted from control and XIAP transfected cells and cDNA was synthesized using dTT primers according to the manufacturers manual (ThermoScript RT-PCR system). PCR was carried out for 30 cycles at 95, 60, and 72 °C each for 30 s and using DNA polymerase, buffer (Biotools, Madrid, Spain) and dNTP Mix (Finnzymes) [23]. Triplicate cDNA samples were analyzed using the following primers: Sod1, upstream, 5-CAA GCG GTG AAC CAG TTG TG-3, downstream, 5-TGA GGT CCT GCA GTG GTA C-3′; Sod2, upstream, 5-GCC TGC ACT GAA GTT CAA TG-3, downstream, 5-ATC TGT AAG CGA CCT TGC TC-3; and β-actin upstream, 5′-CAC ACT GTG CCC ATC TAT GA-3, downstream, 5-CCA TCT CTT GCT CGA AGT CT-3. To detect human XIAP, we used human specific primers, upstream, 5′-CCC AAA TTC AAC AAA TCT-3, downstream, 5-GAC TTG ACT CAT CTT GCA T-3.

Statistics. Statistical comparisons were performed using Student's *t*-test when comparing two groups, or one-way ANOVA followed by a Bonferroni post hoc test comparing three or more groups.

Results

NF-κB is known to activate transcription of various genes involved in inflammation and in cell death regulation [9,10]. To study whether XIAP can activate NF-κB in neuronal cells, we transfected PC6.3 cells with the plasmid encoding full-length human XIAP. Data showed that overexpression of XIAP in the PC6.3 cells led to phosphorylation of the inhibitory molecule IκB (lower band, Fig. 1A), indicative of activation of the NF-κB pathway. As a control, the cells were stimulated by xanthine and xanthine oxidase that induces oxidative stress and is known to activate NF-κB (Fig. 1A). The level of the p65, NF-κB Rel-A protein was not changed by XIAP, but the protein was found in the nucleus in keeping with the activation of the NF-κB pathway (Fig. 1B). Stimulation of PC6.3 cells expressing a reporter construct for NF-κB confirmed that XIAP activates NF-κB in these cells, as shown by a 11-fold increase in NF-κB promoter activity after 48 h (Fig. 1C).

We then studied whether the NF-κB signaling activated by XIAP may alter expression of antioxidant genes important in the control of oxidative stress. RT-PCR showed that particularly the expression of the mitochondrial antioxidant Sod2 is increased by overexpression of XIAP (Fig. 2A). Immunblotting confirmed that the levels of Sod2 protein was significantly (by $70 \pm 10\%$, $n = 3$) increased in the PC6.3 cells overexpressing XIAP, with no significant change observed in Sod1 or catalase (Fig. 2B). In addition, the anti-apoptotic Bcl-xL protein that associates with mitochondria was not influenced by XIAP (Fig. 2B). The increase in Sod2 by XIAP was larger in cells showing higher expression of XIAP after transfection (Fig. 2C).

To study whether the induction of Sod2 is linked to the activation of NF-κB, we employed acetylsalicylic acid

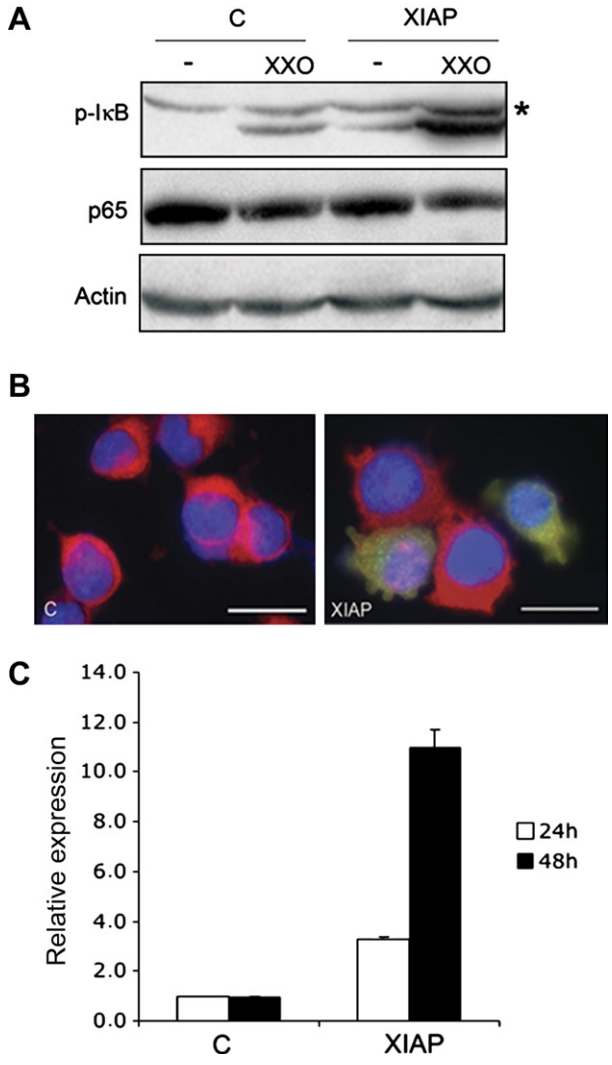

Fig. 1. XIAP activates NF-κB signaling in neuronal PC6.3 cells. PC6.3 cells were transfected using control EGFP and XIAP-EGFP (XIAP) expression plasmid for 24 h. Half of the cells were treated with 100 μM xanthine and 100 mU/ml xanthine oxidase (XXO). (A) Immunoblotting was performed as described in Materials and methods using specific antibodies against phospho-IκB (p-IκB) and p65, NF-κB Rel-A, β-actin was used as control. Note increases in p-IκB by XIAP and XXO (lower band). The antibody recognized also an upper nonspecific band in all samples shown by the star. (B) Control, or XIAP-EGFP expressing cells (green fluorescence) were immunostained with antibodies against the NF-κB-p65 protein (red fluorescence). Nuclei are stained with Hoescht blue. Arrow, note the presence of p65 in the nucleus (yellow color) after XIAP. (C) Cells were transfected with NF-κB-Luciferase (LUC) reporter plasmid together with control pcDNA3.1 (C) or XIAP-myc (XIPA) expression plasmid as described in Materials and methods. Cotransfection with *Renilla* expression vector was used as control to assay transfection efficiency. Results are fold induction of LUC activity after 24 and 48 h of transfection. Values are means ± SEM, $n = 3$. $p < 0.005$ for XIAP vs. C at 24 and 48 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that is a blocker of NF-κB [20]. This drug inhibited the XIAP-induced increase in Sod2, suggesting an involvement of NF-κB in its regulation by XIAP (Fig. 2D). To study this in more detail, we analyzed mouse embryonic fibroblasts derived from Rel-A deficient mice with an inhibition of NF-κB signaling [21]. XIAP-induced Sod2 expression in control cells with intact NF-κB signaling, but not in fibroblasts lacking Rel-A (Fig. 3A). In these experiments, XIAP also elevated the antioxidant, Trx2 in the controls, but not in the Rel-A deficient cells (Fig. 3B). In contrast, Sod1 was not influenced by XIAP in any of the cells (Fig. 3C).

Data obtained thus show that XIAP increases the levels of the mitochondrial antioxidants, Sod2 and Trx2 via the NF-κB pathway. We then studied whether XIAP has an effect on the production of ROS and on oxidative stress in the cell. Using neuronal PC6.3 we observed that XIAP overexpression counteracted the increase in ROS, as measured by the compound DHE (Fig. 4A and B). XIAP also decreased cell death induced by ROS in these cells (Fig. 4C).

Discussion

In this study, we reveal a novel function for the anti-apoptotic protein XIAP in the regulation of oxidative stress in cells through activation of NF-κB and regulation of the mitochondrial antioxidants. The increase in the antioxidant, Sod2 by XIAP was evident in neuronal PC6.3 cells and in cultured fibroblasts. In the latter cells, XIAP also increased Trx2 that is localized to mitochondria. Trx2 belongs to the thioredoxin family of proteins that take part in redox reactions and in cellular defense against ROS [24,25]. In contrast, we did not observe any effect of XIAP on the antioxidants Sod1 and catalase, neither on Bcl-xL that associates with mitochondria.

Previous studies have shown that XIAP can affect signaling events [6–8]. Using reporter constructs we observed that overexpression of XIAP activated NF-κB in the PC6.3 cells. In addition, the increase in Sod2 by XIAP was blocked by acetylsalicylic acid inhibiting the NF-κB pathway. To substantiate these findings, we employed mouse embryonic fibroblasts obtained from NF-κB Rel-A null mutant mice. The results showed that Sod2 and Trx2 are increased by XIAP overexpression in a NF-κB-dependent manner. Sod1 was not increased by XIAP in the mouse fibroblasts, showing a preferential effect of XIAP on the mitochondrial antioxidants.

Activation of NF-κB signaling is known to regulate the expression of different genes, involved among others in the control of cell death, proliferation and the immune response [9,10]. The effects of NF-κB activation vary between cell types, inducers and with the time of stimulation [26]. In neurons, NF-κB signaling is mainly protective, but the downstream gene products are largely unknown [27]. Sod2 has been shown to be a target for NF-κB in some cell types, and the Sod2 promoter contains a functional κB cis-element responding to NF-κB [13,28]. The activation mechanism by which XIAP can induce NF-κB in neurons is unknown, and may involve the protein kinase, TAK1 that was shown to be stimulated by XIAP in endothelial cells [8].

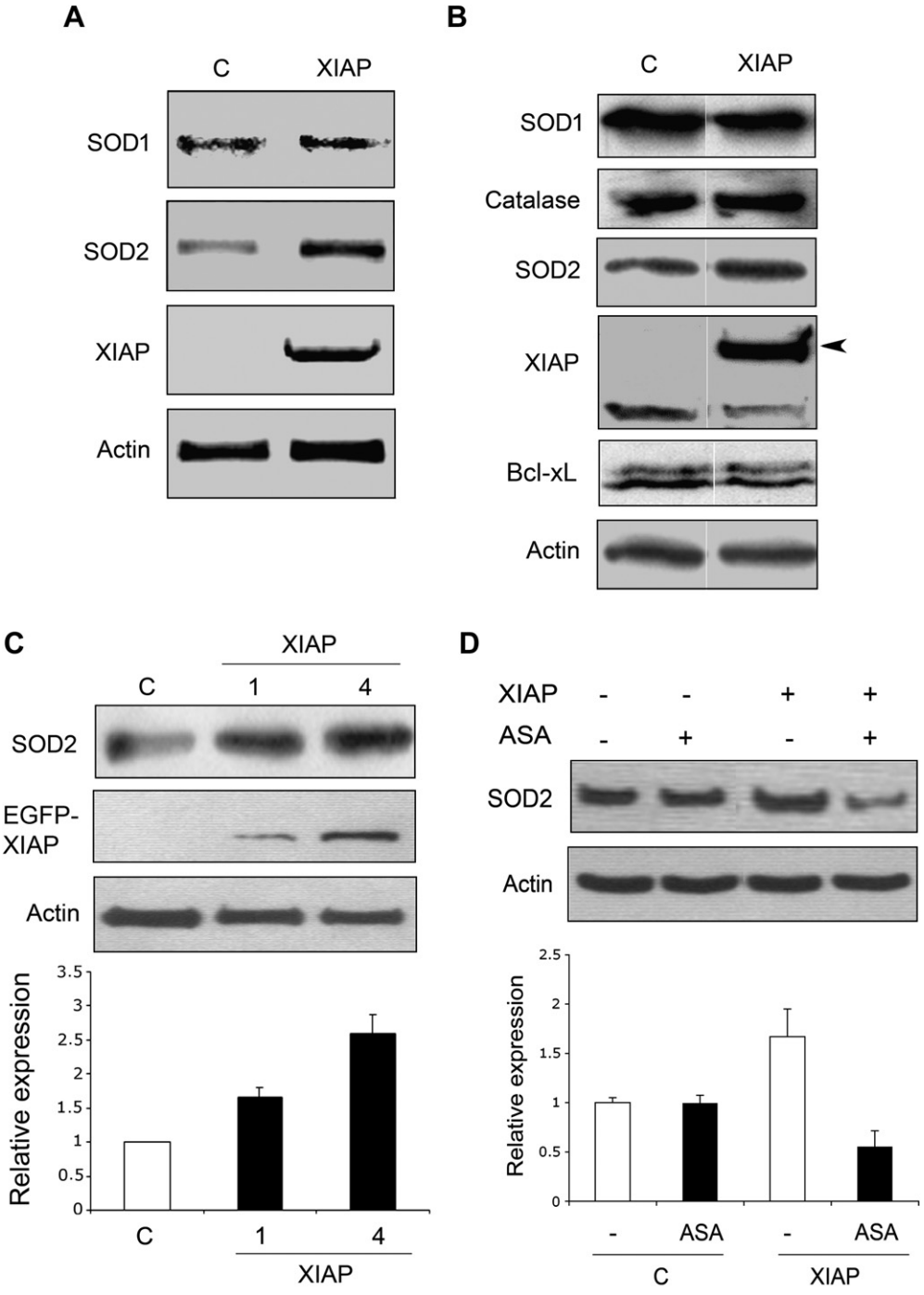

Fig. 2. XIAP increases Sod2 levels in PC6.3 cells. Cells were transfected using control, EGFP and XIAP-EGFP (XIAP) expression plasmids for 48 h and cells analyzed for RNA and protein expression using RT-PCR or immunoblotting using specific antibodies as described in Materials and methods. (A) RT-PCR was done after 48 h of transfection using specific primers as described in Materials and methods. Note increase in Sod2 expression by XIAP. Levels of human XIAP is also shown. β-Actin was used as control. C, control. A typical experiment is shown and was repeated three times. (B) Immunoblotting was performed using specific antibodies as described in Materials and methods Levels of Sod2 were increased by XIAP whereas Sod1, catalase, and Bcl-xL remained unchanged. and XIAP-EGFP fusion protein expressed is shown by the arrowhead, and endogenous XIAP is below. Immunoblot of one experiment is shown that was repeated three times. (C) Different amounts, 1 and 4 μg of XIAP-EGFP plasmid was transfected as shown in the middle panel. Quantification was performed as described in Materials and methods. Note elevated Sod2 by XIAP. Values are means ± SEM, $n = 4$. $p < 0.01$ for 1 μg XIAP vs. C and for 4 μg XIAP vs. C. (D) Cells were transfected and treated with 2.5 mM acetyl salicylic acid (ASA) as described in Materials and methods. Note a decrease in Sod2 after inhibition of NF-κB. One immunoblot is shown that was repeated 3 times. Values are means ± SEM. $p < 0.01$ for XIAP + ASA vs. XIAP.

Previously, it has been shown that XIAP itself may be regulated by NF-κB pathway pathway [29]. However, in the neuronal PC6.3 cells we did not observe an increase in endogenous rat XIAP after transfection with human

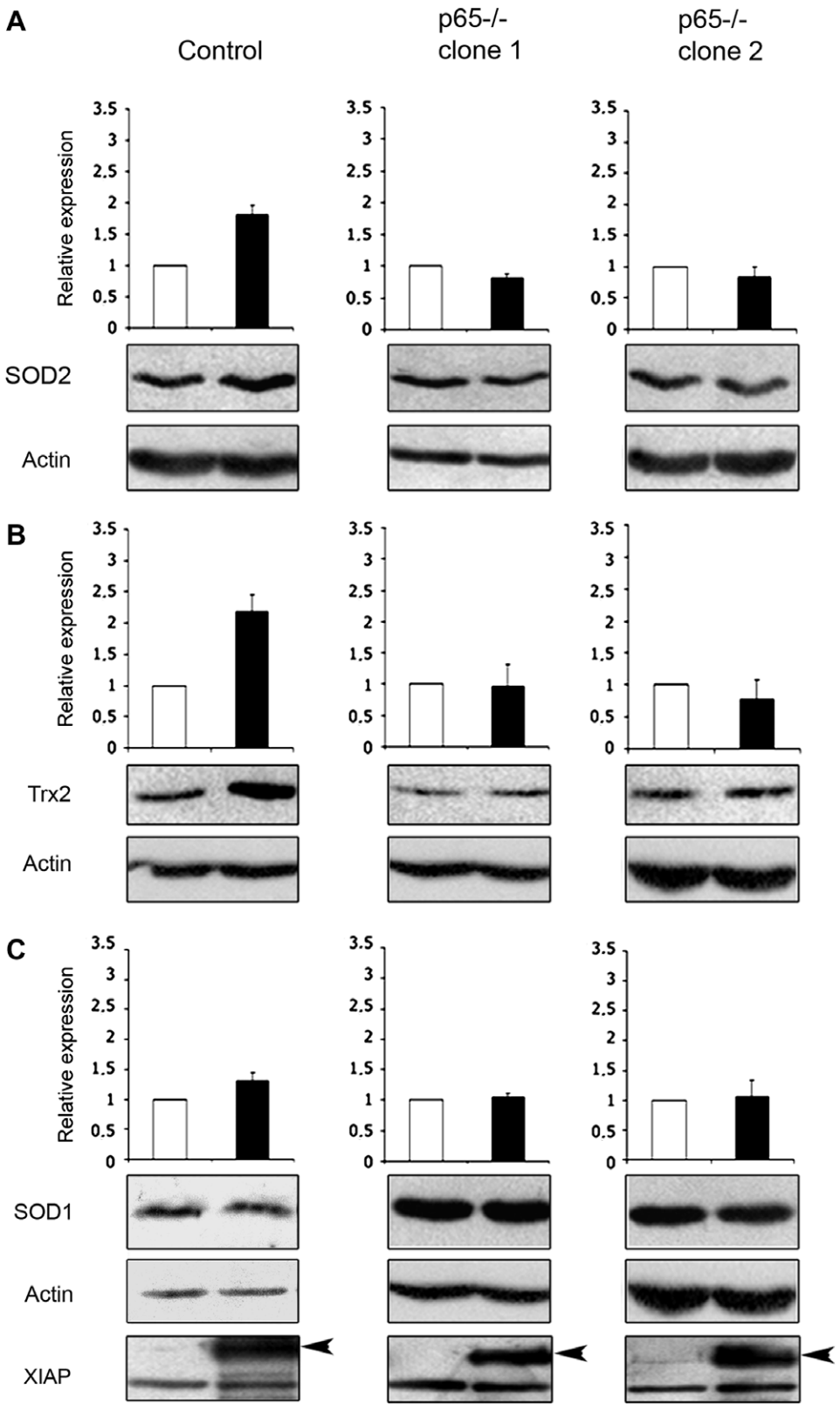

Fig. 3. Role of NF-κB in XIAP mediated increases in antioxidants. Mouse embryonic fibroblast cell lines lacking NF-κB Rel-A (p65−/−) and control fibroblasts were transfected with EGFP (white bars) or XIAP-EGFP (black bars) for 24 h and subjected to Western blotting using specific antibodies. Two different Rel-A deficient cell lines were studied in parallel. Immunoblots of one experiment is shown that was repeated three times. β-Actin was used as control. Quantification was done using GelDoc. Values are means ± SEM. Lower panel shows XIAP-EGFP fusion protein (arrowhead) and endogenous XIAP below. (A–B) Sod2 and Trx2 were increased by XIAP in control but not in p65−/− cells. $p < 0.02$ for XIAP vs. C in control fibroblasts. (C) Sod1 remained unchanged in all cell types.

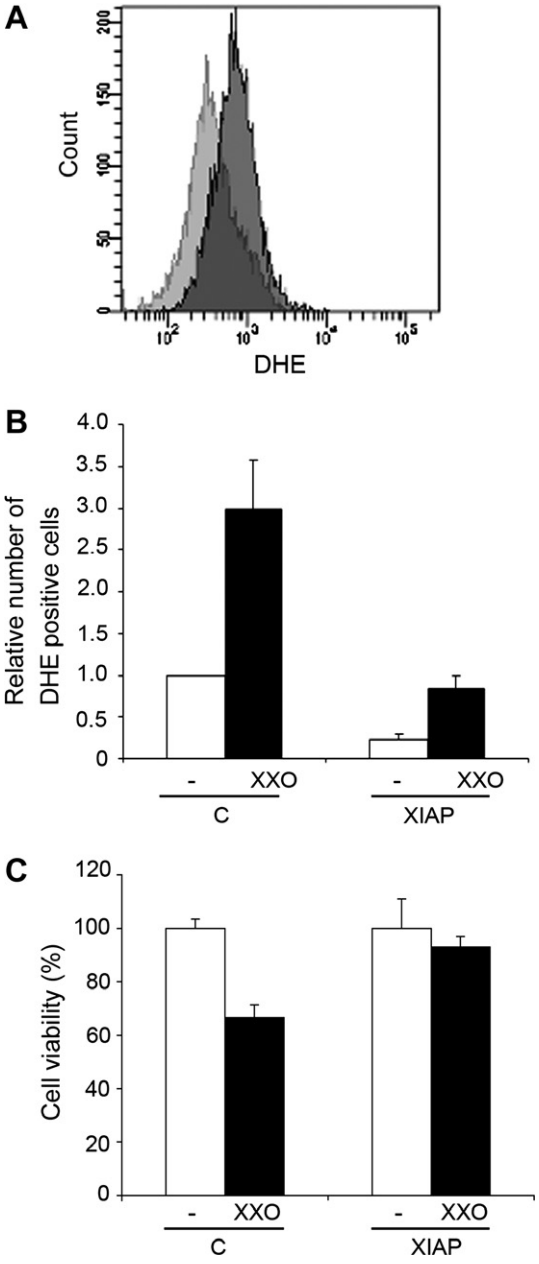

Fig. 4. XIAP decreases ROS production and cell death after oxidative stress. (A) PC6.3 cells were transfected with control EGFP (C) or XIAP-EGFP (XIAP) expression plasmids 24 h before induction of oxidative stress for 60 min with 100 μM xanthine and 100 mU/ml xanthine oxidase (XXO). ROS levels in transfected cells were determined using the dye, DHE and fluorescent activated cell sorting as described in Materials and methods. Controls, right trace; XIAP cells, left trace. Representative picture is shown. (B) Quantification of data. XXO treated cells are shown as black columns. Values are means ± SEM, $n = 4$. $p < 0.01$ for XXO vs. C, and $p < 0.05$ for XIAP + XXO vs. XIAP. $p < 0.01$ for XIAP + XXO vs. XXO. Note decreased ROS levels in XIAP expressing cells. (C) Effect of XIAP on cell viability. Transfected cells were treated for 24 h with 100 μM xanthine and 100 mU/ml xanthine oxidase (black columns). Untreated cells are set to 100%. Cell viability was determined using the MTT assay. Values are means ± SEM, $n = 6$. $p < 0.01$ for XXO vs. C. $p > 0.5$ for XIAP vs. XIAP + XXO.

XIAP (Fig. 2B) nor did the XIAP levels differ between control cells and fibroblasts lacking Rel-A protein (Fig. 3). This suggests that the NF-κB pathway does not regulate XIAP levels in these cells to any significant degree. Apart from transcription, it has been shown that XIAP is regulated by translational mechanisms that can fine-tune the cellular levels of the protein [30].

Mitochondria are important for cell death control by regulating the release of cytochrome *c* and other pro-apoptotic factors [1–3]. Mitochondria are also the major source of ROS production in the cell, and are themselves targets for oxidative stress [15,31]. Oxidative stress contributes to cell injury by impairment of mitochondrial respiration [15,31]. Increased oxidative stress is known to accompany many diseases particularly in the brain. The precise role of oxidative stress in the different disorders is largely unknown as are the signaling events contributing to their regulation.

Studying the physiological significance of the regulation of antioxidants by XIAP, we observed that XIAP decreases production of ROS and cell death induced by oxidative stress in the PC6.3 cells. This suggests that increased levels of mitochondrial antioxidants including Sod2 by XIAP are involved in the regulation of ROS and the sensitivity of these cells against oxidative stress Previous studies have shown that transgenic overexpression of Sod2 and Trx2 can decrease focal ischemic injury in adult mice [24,32].

Taken together the results reveal a function for XIAP in the control of oxidative stress and mitochondrial antioxidants via the NF-κB pathway. In view of this, modulation of XIAP remains an interesting possibility to consider in various therapies to reduce cell injuries caused by enhanced oxidative stress.

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