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The NFκB-mediated control of RS and JNK signaling in vitamin A-treated cells: Duration of JNK–AP-1 pathway activation may determine cell death or proliferation

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ABSTRACT

Nuclear factor kappa B (NFκB) has emerged as a crucial regulator of cell survival, playing important functions in cellular resistance to oxidants and chemotherapeutic agents. Recent studies showed that NFκB mediates cell survival through suppression of the accumulation of reactive species (RS) and a control of sustained activation of the Jun-N-terminal kinase (JNK) cascade. This work was undertaken in order to evaluate the role of NFκB in modulating the pro-oxidant effects of supplementation with vitamin A (retinol, ROH) in Sertoli cells, a major ROH physiological target. In this work, we reported that ROH treatment increases mitochondrial RS formation leading to a redox-dependent activation of NFκB. NFκB activation played a pivotal role in counteract RS accumulation in ROH-treated cells, since NFκB inhibition with DNA decoy oligonucleotides or pharmacological inhibitors (BAY-117082) potentiated ROH-induced RS accumulation and oxidative damage. In the presence of NFκB inhibition, ROH-induced oxidative stress promoted a prolonged activation of the JNK–activator protein 1 (AP-1) pathway and induced significant decreases in cell viability. Inhibition of JNK–AP-1 with decoy oligonucleotides to AP-1 or JNK inhibitor SP600125 prevented the decreases in cell viability. Antioxidants blocked the persistent JNK–AP-1 activation, cell oxidative damage, and the decreases in cell viability induced by NFκB inhibition. Finally, our data point superoxide dismutase (SOD)2 as a potential antioxidant factor involved in NFκB protective effects against ROH-induced oxidative stress. Taken together, data presented here show that NFκB mediates cellular resistance to the pro-oxidant effects of vitamin A by inhibiting RS accumulation and the persistent and redox-dependent activation of JNK–AP-1 cascade.

1. Introduction

Reactive species (RS) are implicated on the modulation of both proliferation and apoptosis events depending on stimuli and on cell type [1]. High and persistent RS production is frequently related to cell death by inducing extensive oxidative damage to cellular components, whereas low oxidant levels are associated with proliferative events [1]. In this way, several studies have suggested a dualistic effect of mitogen-activated protein kinases (MAPKs) and transcription

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factors as nuclear factor kappa B (NFκB) and activator protein 1 (AP-1) in oxidative stress environments, since they may induce both proliferation or death depending on persistence of their activation, and this transient or persistent activation is directly related to duration of oxidative insult [2–4]. In pro-oxidant environments, rapid and transient activation of MAPKs as Jun-N-terminal kinase (JNK)1/2 and extra cellular signal-regulated kinase (ERK)1/2 are frequently reported mediating proliferative events [1,4,5], whereas a prolonged activation may promote cell death [2–4]. Thus, cells need to orchestrate the balance between its pro-oxidant and anti-oxidant factors in order to counteract RS production and oxidative damage, avoiding the persistent activation of some redox-sensitive pathways, and finally promoting resistance/adaptation to pro-oxidant environments [5–7]. In this context, activation and/or expression of antioxidant enzymes as superoxide dismutases (SOD1/2), catalase and glutathione peroxidases, and the synthesis of non-enzymatic antioxidants as glutathione play important roles in keeping the intracellular redox balance thus preventing the persistence of oxidative stress and the extensive cell damage [6,7]. The ability of transcription factors as NFκB in modulating anti-apoptotic and antioxidant genes as SOD2 (mitochondrial SOD), ferritin heavy chain, glutathione S-transferase, inhibitor of apoptosis proteins (IAPs), and caspase 8 homologue FLICE-inhibitory protein (c-FLIP) [6,8–10], which ultimately lead to cellular resistance to oxidative stress and death inductors agents, have addressed NFκB as a important factor involved on cellular resistance to oxidants and chemotherapeutic drugs [8–10].

Our previous studies have demonstrated the pro-oxidant effects of vitamin A supplementation in rats and in cultured cells [11–15]. In Sertoli cells, a major physiological target of ROH in mammalian, treatment with ROH induces an interesting effect; incubation of Sertoli cells with ROH increases RS production and stimulates proliferation through a transient and redox-dependent activation of the JNK1/2 pathway [15]. Antioxidant treatment blocks ROH-induced RS production, JNK1/2 activation, and proliferation, suggesting a mechanism mediated by oxidants. The investigation of potential sites of RS indicates that ROH leads to impairment on electron transfer system causing a significant increase in mitochondrial superoxide production [15]. Thus, it is comprehensible that attenuation of the intracellular RS accumulation, significantly in mitochondria, was involved in the control of RS levels and in triggering Sertoli cells to a redox-mediated proliferation and not to a cell death pathway following ROH treatment in our experimental model.

Since NFκB is an important factor in resistance to oxidative stress [6,8–10], this work was undertaken in order to evaluate the involvement of this transcription factor in modulating ROH pro-oxidant effects in cultured Sertoli cells. The results show that a redox-dependent activation of NFκB lead to increases in manganese SOD (SOD2) activity and reduction of RS accumulation in ROH treatment. Inhibition of NFκB increased ROH-induced RS accumulation at later time points of incubation (6–24 h), and it induced extensive oxidative damage and persistent activation of JNK1/2–AP-1 cascade, which promoted significant decreases in cell viability. Data suggest that NFκB modulates the levels of RS, the duration of the JNK1/2-AP1 pathway activation, and the cell fates during a vitamin A-induced oxidative stress.

2. Methods and materials

2.1. Materials

All-*trans* retinol alcohol (retinol, ROH), 2′,7′-dichlorohydrofluorescein diacetate (DCFH-DA), 3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide (MTT), *N*-acetyl-L-cysteine (NAC), (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile) (BAY-117082), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), rotenone, Nonidet P-40, dithiothreitol, 2-thiobarbituric acid (TBA), anti-phospho-JNK1/2 (Thr 183/ Tyr 185), anti-JNK2, anti-β-actin, anti-β-tubulin antibodies, and culture analytical grade reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Anti-SOD2, anti-p65, and anti-lamin B antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). SP600125 was from Promega Corporation (Madison, WI, USA), anti-c-Fos antibody was from Calbiochem (San Diego, CA, USA), and electrophoresis/immunoblotting reagents were from Bio-Rad Laboratories (Hercules, CA, USA). DNA oligonucleotides were synthesized by The Midland Certified Reagent Company, Inc. (Midland, TX, USA).

2.2. Sertoli cells cultures

Sertoli cells were isolated as previously described [15]. Briefly, testes of 15-day-old rats were removed, decapsulated, and digested enzymatically with trypsin for 30 min at 37 °C, and centrifuged at 750 × *g* for 5 min. The pellet was mixed with soybean trypsin inhibitor, then centrifuged and incubated with collagenase, hyaluronidase and deoxyribonuclease for 30 min at 37 °C. After centrifugation (10 min at 40 × *g*), the pellet was taken to isolate Sertoli cells. Cells were plated in multi-well plates (2.1×10^5 cells/cm^2, 80% confluence) in Medium 199, pH 7.4, 1% FBS, and maintained in a humidified atmosphere at 34 °C for 24 h to attach. The medium was then changed to serum-free medium and cells were taken for assays after 48 h of culture. ROH and inhibitors were dissolved in dimethylsulphoxide (DMSO), and solvent controls were performed for each condition.

2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot

Proteins (20 μg) were separated by SDS-PAGE on 10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide gels and electrotransferred onto nitrocellulose membranes. Membranes were then incubated in Tris-buffered saline Tween-20 [TBS-T; 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 0.05% (v/v) Tween 20] containing 1% (w/v) non-fat milk powder for 1 h at room temperature. Subsequently, the membranes were incubated for 12 h with the appropriate primary antibody. After washing in TBS-T, blots were incubated with horseradish peroxidase-linked anti-immunoglobulin G (IgG) antibodies for 1.5 h at room temperature. Chemiluminescent bands were detected, and densitometric analysis was performed by Image-J® software.

2.4. Nuclear and cytoplasmic extracts preparation

To prepare nuclear extracts, 2.7×10^6 cells were collected by centrifugation 750 × *g* for 5 min, and resuspended in 300 μl of hypotonic buffer consisting of 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 μg/ml pepstatin A, 1 μg/ml leupeptin and incubated in ice for 15 min. Later, 12 μl of 10% Nonidet P-40 was added and the swollen cells were disrupted by vortexing (15 seg). Nuclei were isolated by centrifugation 14,000 × *g* for 30 s, and pellet (intact nuclei) and supernatant (cytoplasmic) fractions were separated. Nuclear fraction was resuspended in 70 μl of high salt buffer [10 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml pepstatin A, 1 μg/ml leupeptin], and incubated for 40 min in ice bath releasing soluble proteins from the nuclei. After extraction, the nuclei debris (insoluble fraction) was removed by centrifugation 14,000 × *g* for 10 min, and supernatant containing soluble nuclear proteins was stored at −80 °C until experiments.

2.5. Electro-mobility shift assay (EMSA)

To determine NFκB and AP-1 DNA-binding activity, biotin 3'end-labeled AP-1 and NFκB oligonucleotide consensus sequence were carried out by EMSA. Briefly, oligonucleotide consensus sequences were labeled with biotin-ddUTP in accordance with manufacturer instructions (LightShift Chemiluminescent EMSA kit, Pierce, Rockford, IL, USA). In binding reactions, 20 μl of reaction mixture comprising 15 mM HEPES (pH 7.9), 1 mM dithiothreitol, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM $MgCl_2$, 2.5% glycerol and 50 ng/μl poly (dI·dC), 5 μg of nuclear extracts, and 30 fmol of biotin-3'end-labeled DNA probes were incubated for 30 min in ice bath. Nucleo-protein complexes were loaded onto the pre-electrophoresis 5.5 % non-denaturing polyacrylamide gels in 0.5× Tris–boric acid–EDTA buffer (TBE) and run at 120 V. The electrophoresed binding reactions were electrotransferred (100 mA for 3 h) in 0.5× TBE to a nylon membrane positively charged in ice-cold bath. The biotin-ddUTP 3'end-labeled DNA probe was cross-linked with ultraviolet-C (UVC) exposure for 15 min and detected using streptavidin–horseradish peroxidase conjugated. The membranes were exposed to X-ray film for 1–5 min to obtain the adequate signal.

2.6. Determination of intracellular RS production [real-time dichlorofluorescein (DCF) assay]

Intracellular RS production was detected using DCFH-DA [16]. This reagent enters the cells and reacts predominantly with highly oxidizing species of RS such as hydroxyl radicals (•OH), hydroperoxides, and peroxynitrite, thus producing the fluorophore DCF. Briefly, cells were seeded in 96-well plates and 25 μM DCFH-DA dissolved in medium containing 1% fetal bovine serum (FBS) was added to the cell culture 30 min before ROH/RA incubation to allow cellular incorporation. Then, the medium was discarded and cells were treated in complete medium. The DCFH oxidation was monitored with 5 min interval at 37 °C in a 96-well plate fluorescence reader with an emission wavelength set at 535 nm and an excitation wavelength set at 485 nm.

2.7. Decoy targeting to NFκB and AP-1

To decoy experiments, double-stranded oligonucleotide decoy (dsODN) to NFκB and AP-1were prepared by annealing of sense and antisense oligonucleotides in vitro in 1× annealing buffer (20 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, and 50 mM NaCl). The mixture was heated at 95 °C for 2 min and allowed to cool to room temperature slowly over 6 h [17]. These oligonucleotides are specifically designed to bind NFκB and AP-1 factors and are used to dampen the content of these factors within the cell. Cells were transfected with dsODN–liposome complexes containing 2 μg/ml dsDNA and 8.3 μl/ml lipofectamine for 6 h at 37 °C, 5% CO_2 before exposing them to the different treatments. The sequence oligonucleotide decoy to NFκB and AP-1 used was (1) NFκB consensus sequence: 5'-CGACACCCCTCGGGAATTCCCCCACTGGGCC-3', 3'-GCTGTGGGGAGCCCTTAAGGGGGTGACCCGG-5' and (2) AP-1 consensus sequence: 5'-TGACACACATTAGTCACATATTAAT-3', 3'-ACTGTGTGTAATCAGTGTATAATTA-5'. A non-related oligonucleotide sequence Oct2A (5'-AGCTTAGGGCTCGTTGACGTCTCCAAG-3') was used as control. In preliminary experiments, we confirmed the ability of NFκB or AP-1 decoy ODNs to block, respectively, NF(B or AP-1DNA binding activities. Oct2A did not alter NFκB or AP-1 pathways.

2.8. MTT assay

Cell viability was estimated by the quantification of the MTT reduction to a blue formazan product by cellular dehydrogenases [15]. At the end of treatments, the medium was discarded and a new medium containing 0.5 mg/ml MTT was added. The cells were incubated for additional 30 min at 37 °C. After the medium was removed, cells were washed three times with phosphate buffered saline, and DMSO was added for 10 min. Formazan salt formation was determined at 560 nm. Data were expressed as percentage of formazan formation in untreated cells.

2.9. Thiobarbituric acid reactive species (TBARS)

As an index of lipid peroxidation, we used the formation of TBARS during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described [18]. Briefly, 300 μl cell extracts were mixed with 600 μl of 10% trichloroacetic acid (TCA) and 0.5 ml of 0.67% TBA, and then heated in a boiling water bath for 25 min. TBARS were determined in spectrophotometer at 532 nm. Results are expressed as TBARS/mg protein.

2.10. SOD2 activity

To measure manganese SOD2 activity, NaCN was added to the reaction mix at a final concentration of 2 mM and incubated for 30 min. High concentrations of cyanide (1–2 mM) were reported to inhibit SOD1 up to 97–99% at pH 10.00 [19]. SOD2 activity was assessed by quantifying the inhibition of the superoxide-dependent adrenaline auto-oxidation at pH 10.2 in

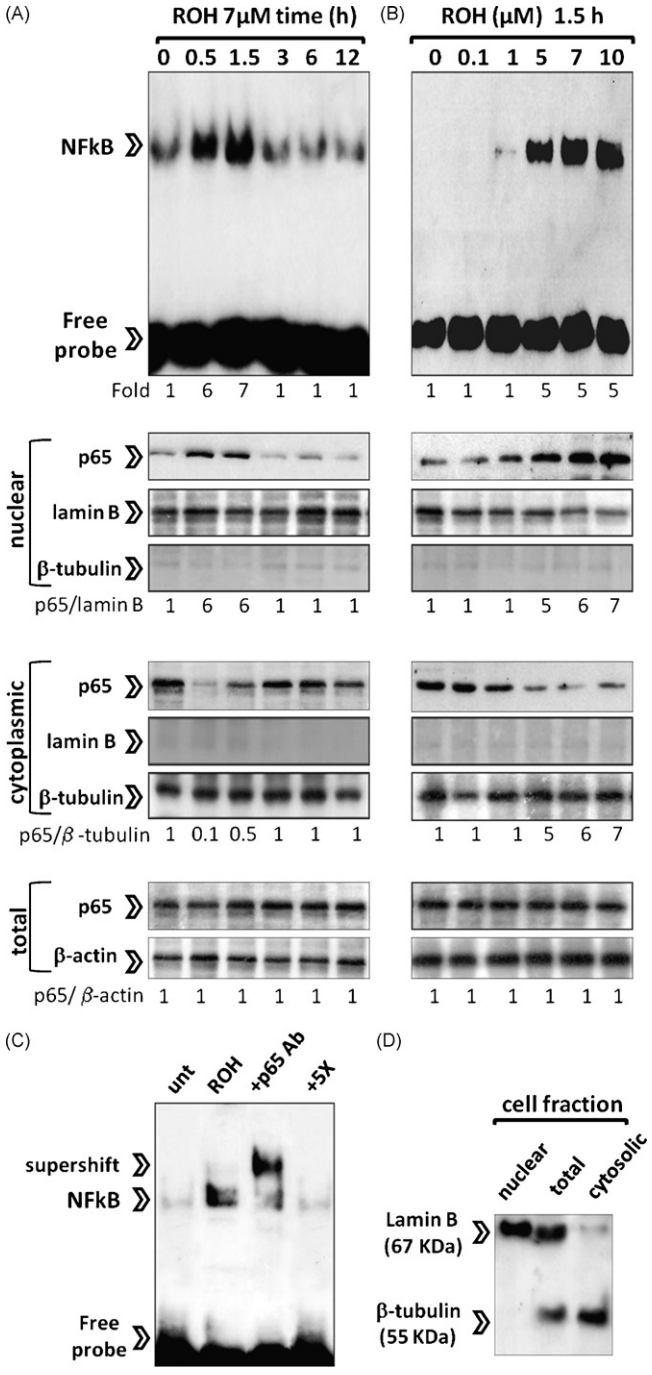

Fig. 1 – NFκB activation in ROH-treated Sertoli cells. Cells were treated with 7 μM ROH for different times and NFκB DNA-binding activity or p65 immunocontent in cell subfractions was determined. (A) Time-course effect of 7 μM ROH in NFκB DNA-binding activity and p65 protein translocation. (B) Effect of different ROH concentrations (μM) on NFκB binding and p65 translocation. Cells were treated for 1.5 h, and cell extracts were isolated for EMSA or immunoblotting. (C) Supershift and competition assays for NFκB. In binding reactions, nuclear extracts of 7 μM ROH-treated cells were pre-incubated with 0.01 μg anti-p65 NFκB antibody (+p65 Ab lane) or 5× excess of an unlabeled NFκB oligonucleotide (+5× lane) for 15 min prior labeled oligonucleotide addition. Later, EMSA were performed. (D) For control of the purity of nuclear and cytoplasmic fractions, proteins were separated by SDS/PAGE and blots were incubated with a solution containing anti-lamin B (1:1000) and anti-β-tubulin (1:1000) antibodies. Legends: unt, untreated; ROH, retinol; +5×, nuclear extracts of ROH-treated cells plus five-fold excess of unlabeled NFκB oligonucleotides; +p65 Ab, ROH-treated extracts +p65 Ab, supershift. Representative of four independent experiments ($n = 4$).

a 0.1 M glycine–NaOH buffer in spectrophotometer at 480 nm, as previously described [20]. The results are expressed as U SOD2/mg protein.

2.11. Cell proliferation assay

[Methyl-^{3}H] thymidine incorporation was assessed as indicative of the DNA synthesis and proliferation rate in Sertoli cells [15]. At 24 h prior ROH treatment, cells were pre-warmed with 0.5 μCi/ml [methyl-^{3}H] thymidine (248 GBq/mmol; Amersham, UK). After the medium was removed and cells were treated. After treatments, 1 μCi/ml of [methyl-^{3}H] thymidine diluted in medium was added for additional 18 h. After DNA precipitation, scintillant liquid was added, and incorporated radionucleotide was measured using a Packard Tri-Carb Model 3320 scintillation counter.

2.12. Protein quantification

Protein contents of each sample were measured by Lowry method [21].

2.13. Statistical analysis

Data are expressed as means ± SD and were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. ROH induces redox-dependent NFκB activation

Cells were incubated for different times (h) with 7 μM ROH, and NFκB-binding activity was evaluated by EMSA. Fig. 1A shows that ROH induced a transient increase in NFκB DNA-binding activity. NFκB activation occurred as early as 0.5 h after ROH incubation, continued for up to 1.5 h, and decreased at 3 h. The increase in NFκB activity was accompanied by a time-related translocation of the NFκB subunit p65 from cytoplasm to nucleus of 7 μM ROH-treated cells (Fig. 1A). Total p65 protein immunocontent was unaltered by 7 μM ROH treatment. Thus, a 1.5 h incubation period was used for subsequent experiments. We also tested the effect of different ROH concentrations on NFκB-binding activity and p65 translocation (Fig. 1B). Data show that NFκB activity and p65 translocation from cytoplasm to nuclear compartment were stimulated from 5 μM ROH; total p65 immunocontent remained unaltered (Fig. 2B). To give assay specificity, supershift analyses were performed by pre-incubating nuclear extracts from ROH-treated cells with

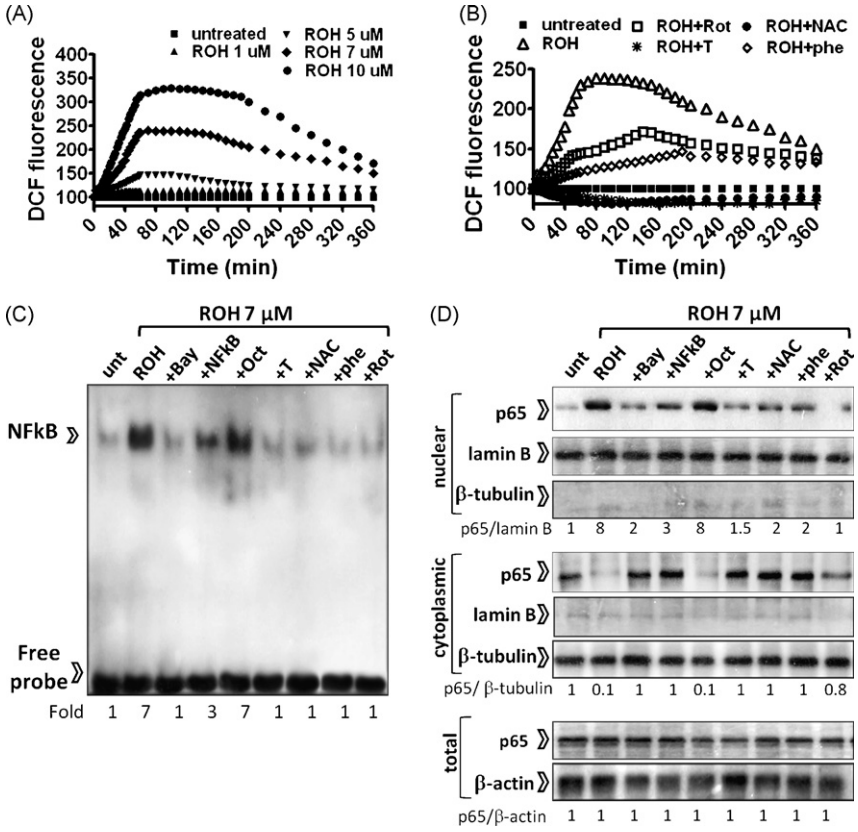

Fig. 2 – ROH induces redox-dependent NFκB activation. (A) Representative experiment showing the time-course effect of different ROH concentrations on RS production in Sertoli cells. (B) Mitochondrial electron transfer inhibitor (rotenone) and RS scavengers inhibit RS-dependent DCFH oxidation. Cells were pre-incubated for 30 min with 5 μM rotenone, 100 μM phenantroline (iron chelator), N-acetylcysteine (NAC 1 mM), or 50 μM Trolox (general antioxidant) prior 7 μM ROH addition, and DCF formation was monitored. (C and D) Effect of different antioxidants and classical NFκB inhibitors on (C) NFκB-binding activity and (D) p65 immunocontent in subcellular fractions. For experiments presented in (C) and (D), cells were pre-treated with antioxidants and NFκB inhibitors for 30 min prior 7 μM ROH addition, and cellular extracts were isolated after 1.5 h of incubation with ROH. Legends: ROH, retinol (7 μM); Rot, rotenone; NAC, N-acetylcysteine; T, Trolox; Bay, BAY-117082 (10 μM); +NFκB, NFκB decoy; +Oct, Oct2A decoy; phe, phenantroline. Representative of three independent experiments (*n* = 3).

purified anti-p65 NFκB antibody. The presence of supershift following p65 antibody incubation (+p65 Ab lane), and the absence of shift in competition assays with five-fold excess of unlabeled NFκB oligonucleotides (+5× lane) confirmed that shifted band visualized on EMSA are indeed due to the binding of NFκB proteins (Fig. 1C). The purity of cytoplasmic and nuclear lysates was confirmed by the absence of β-tubulin immunoreactivity in the soluble nuclear proteins isolated for EMSA, and the absence of lamin-B in cytoplasmic extracts. In whole lysates, immunoreactivity for both β-tubulin and lamin-B was observed (Fig. 1D). β-Actin was used as loading control to total cell lysates.

Next, we evaluated whether ROH-induced NFκB activation involved alterations in intracellular redox state. ROH induced a rapid (within 15 min) and dose-dependent (5–10 μM) increase in RS production as assessed by DCF assay (Fig. 2A). Pre-incubation of cells with RS scavengers as NAC (1 mM), Trolox (50 μM), phenantroline (50 μM) attenuated 7 μM ROH-induced RS production (Fig. 2B), NFκB-binding activity (Fig. 2C) and p65 nuclear translocation (Fig. 2D). We also tested the effect of the mitochondrial electron transport inhibitor rotenone (5 μM), which we previously showed to inhibit RS production and MAPKs activation in ROH treatment [11]. Rotenone pre-treatment not only blocked RS formation (Fig. 2B), but also prevented NFκB activation and p65 translocation into nucleus (Fig. 2C and D).

Finally, we use decoy ODNs to NFκB, and the IκB kinase (IKK) inhibitor BAY-117082 (10 μM) in order to specifically inhibit NFκB. DNA decoy and BAY-117082 inhibited 7 μM ROH-induced NFκB-binding activity (Fig. 2C) and p65 translocation from cytoplasm to nucleus (Fig. 2D). Decoy with a non-related sequence Oct2A did not alter the pattern of ROH induced NFκB.

3.2. *NFκB inhibition induces persistent RS production, potentiates oxidative damage, and promotes decreases cell viability in ROH-treated cells*

Incubation of Sertoli cells with 7 μM ROH increased RS formation as showed in Fig. 2A. The increase in RS started at early time points (10 min), reached a plateau between 1 and

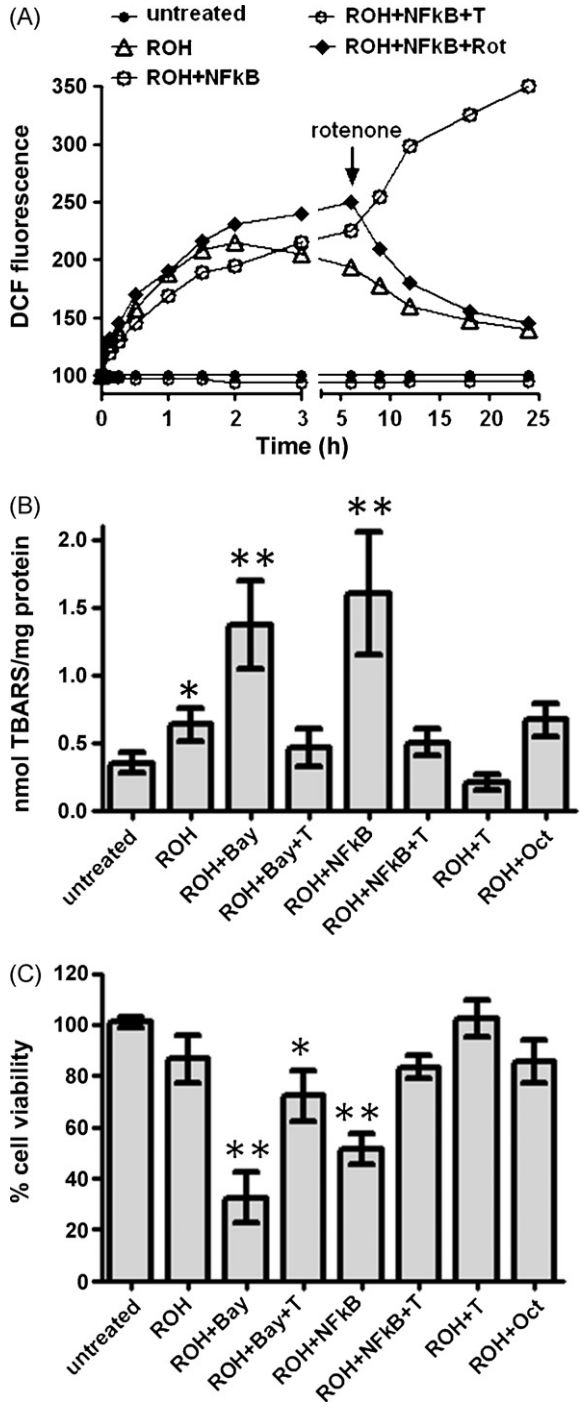

Fig. 3 – NFκB inhibition potentiates ROH-induced oxidative stress leading to decreases in cell viability. (A) Representative experiment showing the time-course effect of NFκB inhibition on 7 μM ROH-induced RS formation in Sertoli cells. Cells were pre-incubated with NFκB inhibitors prior ROH addition and DCF fluorescence was monitored at different times. (B) NFκB inhibition potentiates ROH-induced lipoperoxidation. Damage to lipids was assessed by TBARS assay at 24 h after 7 μM ROH addition. (C) NFκB inhibition induces decreases in cell viability following 7 μM ROH treatment. Legends: ROH, retinol (7 μM); T, Trolox; Bay, BAY-117082 (10 μM); NFκB, NFκB decoy; Oct, Oct2A decoy. *Different from untreated cells, **different from untreated and from ROH-treated cells. Representative of three experiments ($n = 3$).

3 h treatment, and decreased after 6 h to levels approximately 1.4-fold higher than that observed in untreated cells (Fig. 2A). Pre-treatment of cells with NFκB decoy ODNs – which ultimately lead to NFκB inhibition as showed in Fig. 2C – or BAY-117082 induced a prolonged increase in RS formation following 7 μM ROH treatment (Fig. 3A). At 6–24 h ROH treatment, RS production was approximately 3.5-fold higher in the presence of NFκB inhibitors. In some experiments, the addition of the electron transfer chain inhibitor rotenone (5 μM) at 6 h attenuated ROH-induced RS accumulation in the presence of decoy ODNs to NFκB, suggesting an involvement of mitochondria on RS accumulation. The NFκB inhibitors BAY-117082 and decoy ODNs alone did not present effects on RS production and oxidative damage (not shown).

In agreement, NFκB inhibition with BAY-117082 or DNA decoy potentiated 7 μM ROH-induced lipoperoxidation at 24 h as assessed by TBARS assay (Fig. 3B). Pre-treatment with the RS scavenger Trolox, besides to block RS formation (Fig. 2B), blocked the increase in TBARS levels in both ROH and ROH plus NFκB inhibitor groups, confirming a redox mechanism (Fig. 3B). Data obtained from MTT assay show that 7 μM ROH alone did not decrease cell viability at 24 h treatment, but the presence of NFκB inhibition with BAY-117082 or NFκB decoy induced significant decreases in cell viability following ROH treatment (Fig. 3C). BAY-117082 (10 μM) or DNA decoy ODNs alone did not alter cell viability or lipoperoxidation (not shown). Again, pre-treatment with Trolox prevented the decrease in viability. Taken together, these data suggest a protective role of NFκB against ROH-induced oxidative stress.

3.3. *NFκB inhibition induces persistent activation of JNK1/2–AP1*

In previous studies, we reported that a transient and redox-dependent activation of JNK1/2 mediates the proliferative effects of ROH in Sertoli cells [15]. The aforementioned data showed that NFκB inhibition potentiates RS production and oxidative damage in ROH-treated cells. In addition, NFκB inhibition decreased cell viability in ROH treatment. Thus, we decided to test whether the decreases in cell viability could be related to a prolonged stimulation of JNK1/2 pathway during the persistent oxidative stress observed in presence of NFκB inhibitors as previously reported in other studies [2,22]. Treatment with 7 μM ROH alone induced a rapid and transient increase in JNK1/2 phosphorylation (Fig. 4A). JNK1/2 phosphorylation (i.e. activation) increased at 15 min, continued up to 2 h, and decreased to basal levels at 3 h up to 24 h treatment. In agreement, 7 μM ROH induced a transient increase in DNA-binding activity of transcription factor AP-1. AP-1 activation occurred as early as 1.5 h, continued up to 3 h, and decreased to basal levels at 6 h treatment (Fig. 4B).

NFκB inhibition changed the pattern of JNK1/2 and AP-1 activation from a transient to a prolonged time profile in 7 μM ROH-treated cells (Fig. 4A and B, respectively). JNK1/2 phosphorylation and AP-1-binding activity remained

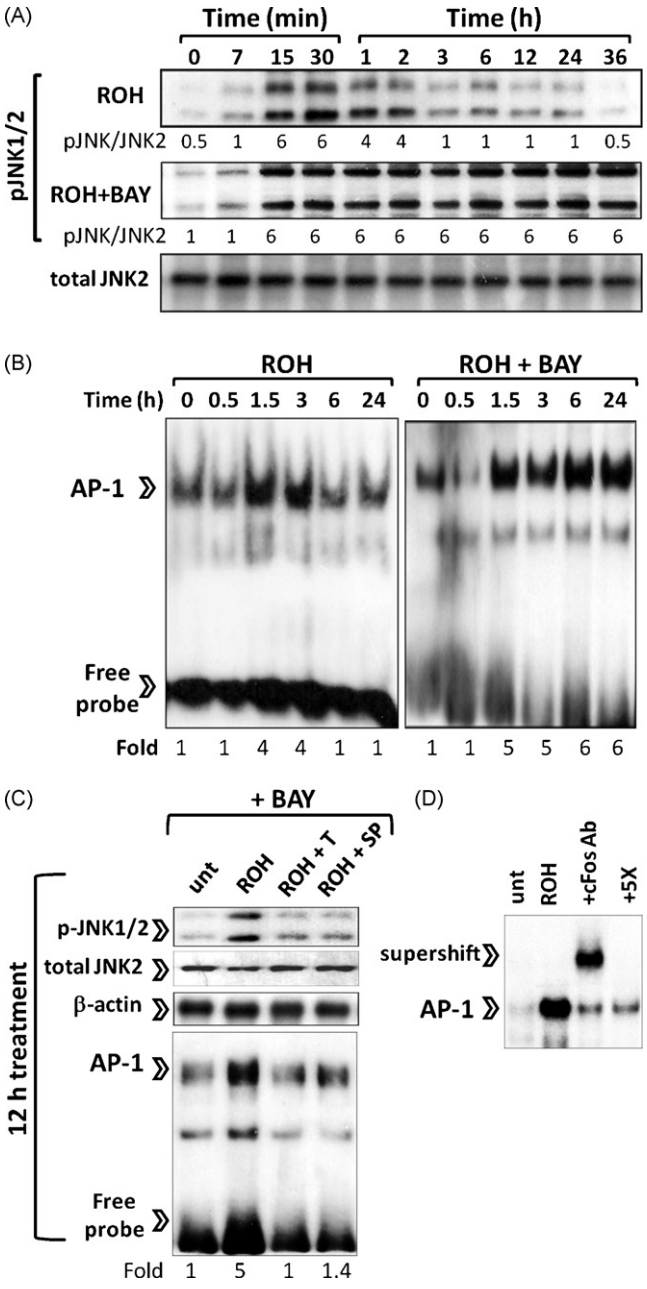

Fig. 4 – NFκB inhibition induces persistent activation of JNK–AP-1 in ROH treatment. (A) Time-course effect of 7 μM ROH on JNK1/2 phosphorylation in Sertoli cells in the presence or absence of the NFκB inhibitor BAY-117082 10 μM. (B) Time-course effect of 7 μM ROH on AP-1 DNA-binding activity in the presence of absence of NFκB inhibition. (C) Effect of 50 μM Trolox and JNK1/2 inhibitor SP600125 (10 μM) on 7 μM ROH plus 10 μM BAY-117082-induced persistent AP-1 activation. Cells were pre-treated with BAY-117082 for 30 min, after 7 μM ROH plus Trolox or SP600125 was added, and cells were incubated for additional 12 h. Nuclear extracts were isolated and EMSA were performed. (D) Supershift and competition assays for AP-1. In binding reactions, nuclear extracts of 7 μM ROH-treated cells were pre-incubated with 0.01 μg anti-cFos AP-1 subunit antibody (+cFos Ab lane) or 5× excess of an unlabeled AP-1 oligonucleotide sequence (+5× lane) for 15 min prior labeled AP-1 oligonucleotide addition. Legends: ROH, 7 μM retinol; T lanes, Trolox; SP lanes, 10 μM SP600125; BAY, 10 μM BAY-117082; +5×, five-fold excess of unlabeled AP-1 oligonucleotides; +cFos Ab, ROH + cFos antibody, supershift. Representative of three experiments (*n* = 3).

increased up to 24 h treatment with ROH in the presence of DNA decoy to NFκB (not shown). The same pattern was observed with 10 μM BAY-117082 as NFκB inhibitor (Fig. 4A and B). Pre-treatment with a pharmacological JNK1/2 inhibitor (SP600125, 10 μM) inhibited AP-1 activation as assessed at 12 h incubation, suggesting that JNK1/2 mediates AP-1 activation (Fig. 4C). The presence of the antioxidant Trolox (50 μM) prevented the prolonged activation of JNK1/2 and AP-1 suggesting that the persistent activation of this pathway is mediated by RS (Fig. 4C). To confirm the specificity of EMSA to AP-1, we performed supershift to c-Fos (a major AP-1 subunit) and competition assays. The presence of a total shift following c-Fos antibody incubation (+cFos Ab lane), and the significant reduction of shifted band in competition assays with five-fold excess of unlabeled AP-1 oligonucleotide (+5× lane) confirmed that band on EMSA was indeed due to the binding of AP-1complexes (Fig. 4D).

3.4. *Persistent JNK–AP-1 pathway activation mediates the decreases in cell viability following treatment with ROH plus NFκB inhibitors*

The evaluation of cell viability showed that cytotoxic effects of treatment with NFκB inhibitors in combination with ROH were prevented by inhibiting JNK1/2–AP1 pathway activation with SP600125 or DNA decoy ODNs to AP-1 (Fig. 5A, see ROH + NFκ‴ B + AP-1 and ROH + NFκB + SP lanes), suggesting that the persistent JNK–AP-1 pathway activation observed in ROH plus NFκB inhibitors induce decreases in cell viability. DNA decoy to AP-1 in absence of NFκB inhibition (ROH + AP-1 lane) did not alter cell viability (Fig. 5A).

Finally, in absence of NFκB inhibitors, Sertoli cells proliferate following 7 μM ROH treatment (Fig. 5B), and DNA decoy to AP-1 or JNK1/2 inhibition with SP600125 attenuated ROH-induced proliferation. Thus, these data altogether suggest that a transient activation of the JNK–AP-1 pathway induces cell proliferation, whereas its prolonged activation induces cell death, and NFκB activation plays a key role in inhibiting persistent activation of JNK–AP-1 by attenuating RS accumulation and oxidative stress in ROH-treated cells.

3.5. *ROH induces NFκB dependent increases in mitochondrial SOD*

In our previous studies and here, we showed that mitochondria act as a primary source of RS in ROH-treated Sertoli cells [15]. Thus, it is plausible that the effects of NFκB in inhibiting prolonged RS formation and oxidative damage in our model could be related to modulation of mitochondrial oxidative stress. SOD2 (mitochondrial SOD) is a major mitochondrial antioxidant enzyme regulated through NFκB [8,9,23], and authors showed that retinoids could increase SOD2 in an

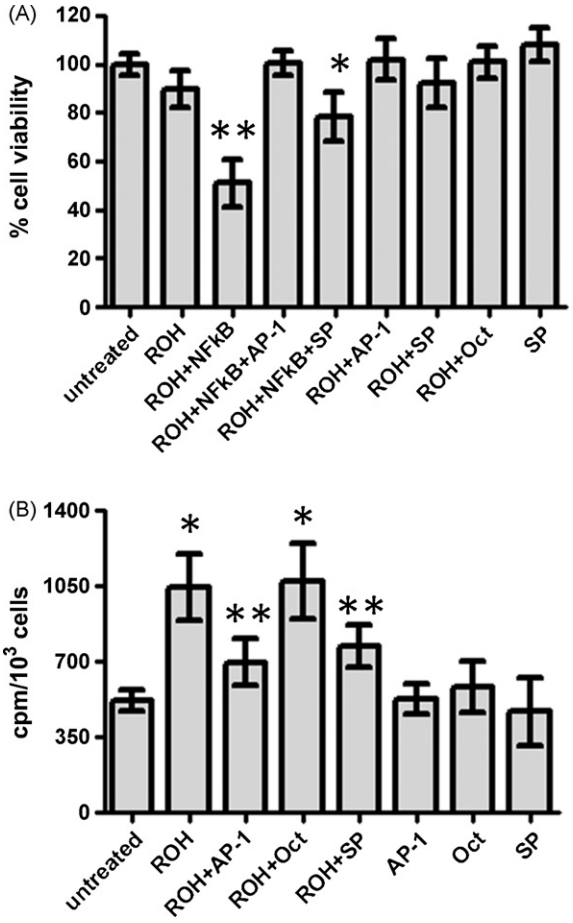

Fig. 5 – Persistent JNK–AP-1 pathway activation mediates the decreases in cell viability following treatment with ROH plus NFκB inhibitors. (A) AP-1 DNA decoy and the JNK1/2 inhibitor SP600125 pre-treatment prevented the decrease in cell viability promoted by NFκB inhibition (NFκB decoy) in 7 μM ROH treatment. (B) ROH induces proliferation through of JNK–AP-1 pathway activation in the presence of NFκB signaling. Cell proliferation was assessed by radiolabeled thymidine incorporation after 24 h of 7 μM ROH incubation. Legends: ROH, retinol 7 μM; NFκB lanes, NFκB decoy; AP-1 lanes, AP-1 decoy; Oct, Oct2A decoy; SP lanes, SP600125 (10 μM). *Different from untreated cells, **different from untreated and from ROH-treated cells. Representative of three experiments (n = 3).

NFκB-dependent manner [8]. In our model, 7 μM ROH induced a dose-dependent increase in SOD2 activity and immunocontent as assessed after 24 h treatment (Fig. 6A). The evaluation of time-course effect of retinol upon SOD2 levels showed that treatment with 7 μM retinol increased SOD2 protein and activity as early as 6 h after retinol addition to Sertoli cells (not shown). Inhibition of NFκB with 10 μM BAY-117082 or DNA decoy ODNs blocked the increase in SOD2 activity and protein (Fig. 6B). Decoy to a non-related sequence Oct2A did not present any effect on ROH-induced SOD2 protein and activity. Pre-incubation with 50 μM Trolox or 1 mM NAC prevented ROH-induced SOD2.

4. Discussion

NFκB is a transcription factor consisting of a heterodimer of p65/p50 retained in the cytoplasm as an inactive tertiary complex associated with inhibitory proteins known as IκBs. After specific stimuli as for example tumor necrosis factor alpha (TNFα), IκB phosphorylation by IKKs leads to proteasome degradation of IκB, releasing NFκB to the nucleus [24]. Diverse stimuli as plasma membrane receptor-mediated mechanisms and alterations in the intracellular redox state may induce NFκB activation [24,25]. Once in nucleus, the mechanisms by which NFκB controls cell survival in pro-oxidant environments is to enhance transcription of anti-apoptotic and antioxidant genes, including Bcl-xL c-FLIP, XIAP, SOD2, glutathione S-transferase, and ferritin heavy chain [9,10]. The modulation of the intracellular redox status seems to play an important role in understanding the protective functions of NFκB in our model. ROH alone increased RS production and lipoperoxidation. However, inhibition of NFκB with the pharmacological IKK inhibitor BAY-117082 or DNA decoy ODNs potentiated ROH-induced RS, TBARS formation, and it induced decreases in cell viability. The decrease in cell viability was mediated by a prolonged activation of JNK–AP-1 pathway, since ROH in the presence of NFκB inhibitors, besides to promote persistent RS, induced persistent activation of JNK–AP-1, and pre-treatment with JNK1/2 inhibitor or DNA decoy to AP-1 blocked the decrease in viability. In addition, antioxidant pre-treatment inhibited the persistent activation of JNK1/2 and AP-1 suggesting that NFκB controls JNK–AP-1 activation by modulating intracellular redox state. It is important to note that NFκB was activated through alterations in intracellular redox state, since ROH increased RS and NFκB activation, and pre-treatment with antioxidants inhibited both RS production and NFκB activation. Taken in consideration that NFκB was activated by RS-dependent mechanisms, and its activation was pivotal to counteract oxidative stress at later time points of ROH treatment, data suggest that NFκB mediates adaptative responses to pro-oxidant effects of ROH.

In Sertoli cells, we have characterized that ROH at concentrations higher than physiological (ROH $\geq$ 5 μM) induces dysfunction in mitochondrial electron transfer system leading to increases in the rate of mitochondrial superoxide formation [15,26,27]. The effect of ROH on mitochondrial superoxide formation was also evidenced in brain mitochondria isolated from ROH supplemented rats [28], and in liver isolated mitochondria incubated with ROH in vitro [29]. In previous studies, we reported that incubation with electron transfer inhibitors as rotenone blocked ROH-induced RS formation and JNK1/2 activation in Sertoli cells [15]. In the presence of NFκB inhibition, the potentiating of RS formation in ROH-treated cells was attenuated by rotenone addition (Fig. 3A), suggesting that mitochondria act as a primary source of RS. Mitochondria-generated RS, including superoxide anions and hydrogen peroxide, may act as signaling intermediates or damaging agents depending on their intracellular concentrations [1]. In mitochondria, the formed superoxide anions are significantly eliminated by manganese SOD2 in order to avoid its accumulation [23,30,31]. It has been well established that decreases in, or SOD2 knockout/knockdown,

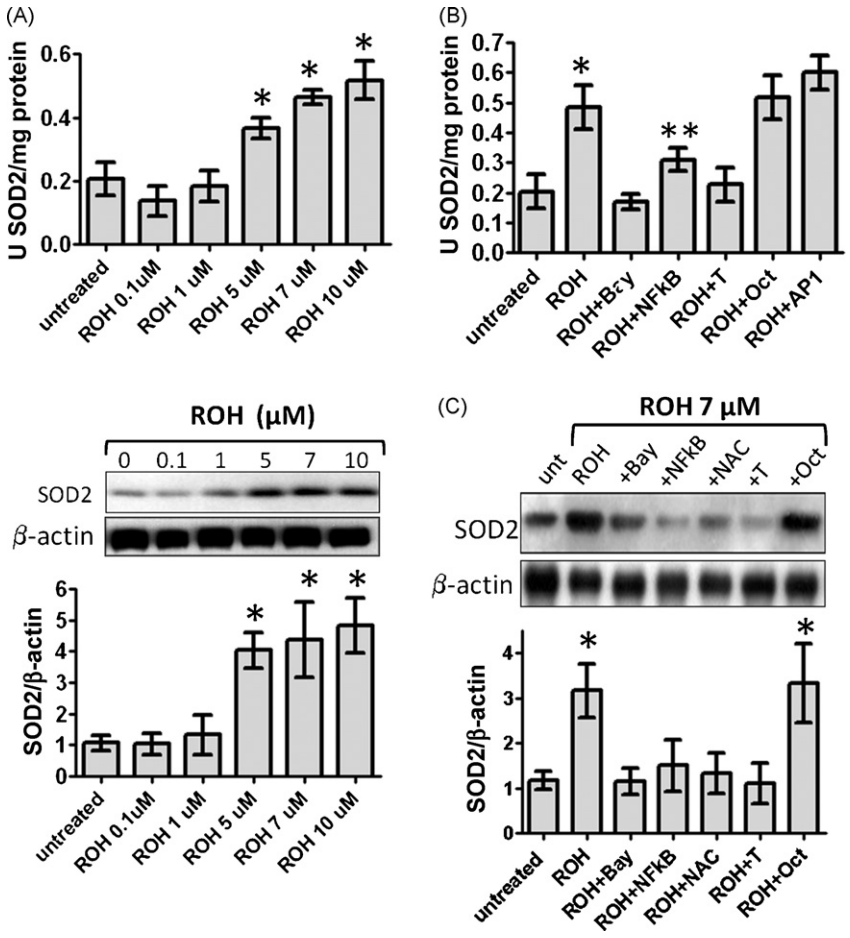

Fig. 6 – ROH increases SOD2 activity and protein through an NFκB-dependent mechanism. (A) Effect of different ROH concentrations (μM) on SOD2 activity and immunocontent at 24 h. (B) Effect of pre-treatment with 50 μM Trolox, 10 μM BAY-117082, or NFκB decoy on SOD2 protein and activity at 24 h of treatment. Legends: ROH, retinol 7 μM; NFκB lanes, NFκB decoy; Oct, Oct2A decoy; NAC lanes, N-acetylcystein (1 mM); AP-1 lanes, AP-1 decoy; Bay, BAY-117082 (10 μM). *Different from untreated cells, **different from untreated and from ROH treated cells. Representative of three experiments (*n* = 3).

increase oxidative stress, biomolecules damage and cellular sensitivity to oxidants [32]. In our model, ROH increased SOD2 protein and activity through an NFκB-dependent mechanism. Considering that ROH-induced RS are formed significantly in mitochondria, and that SOD2 play a role in mitochondrial RS detoxification, its plausible that NFκB dependent increases in SOD2 play a function in inhibiting ROH-induced RS accumulation and the persistent JNK–AP-1 pathway activation [9,23]. Corroborating, a recent study demonstrated that retinoic acid increases SOD2 through NFκB-dependent mechanisms in neuroblastoma cells [8]. Besides upregulating SOD2, it was recently reported that NFκB may suppress RS accumulation and downregulate the activation of JNK pathway by increasing ferritin heavy chain expression – a primary iron storage factor – as a mediator of antioxidant and protective activities of NFκB [9,10].

MAPK cascades are activated by several cellular stresses, and are involved in various biological responses such as differentiation, proliferation, and cell death [25,33,34]. Several lines of evidence suggested that transient MAPK activation is associated with cell proliferation or differentiation, whereas prolonged MAPK activation may promote cell death [2–4,35]. The relation between persistent or transient activation of MAPKs and its consequences on cell death/proliferation are well characterized on prolonged JNK activation observed during TNFα-induced cell death in cell types lacking NFκB signaling [2,9,36–38]. Cells lacking p65 and IKKb show increased sensitivity to TNF-induced cell death. In the absence of NFκB signaling, TNF promotes persistent mitochondrial RS formation and sustained stimulation of JNK1/2, which mediates necrosis. Treatment with antioxidants prevented both persistent JNK activation and cell death [2].

Corroborating with these studies, data presented here show that ROH could induce different cell fates depending on presence or absence of NFκB signaling, and duration of JNK pathway activation plays a role in these events. Upon NFκB inhibition, we determined a persistent (up to 24 h) increase in RS formation, which induced a prolonged stimulation of JNK–AP-1 pathway and JNK–AP-1-dependent decreases in cell viability. In these conditions, treatment with the antioxidant Trolox, JNK1/2 inhibitor, and DNA decoy to AP-1 attenuated both ROH-induced JNK–AP-1 pathway activation and decreases in cell viability. In contrast, upon normal conditions (i.e. with active NFκB signaling) ROH induced a transient

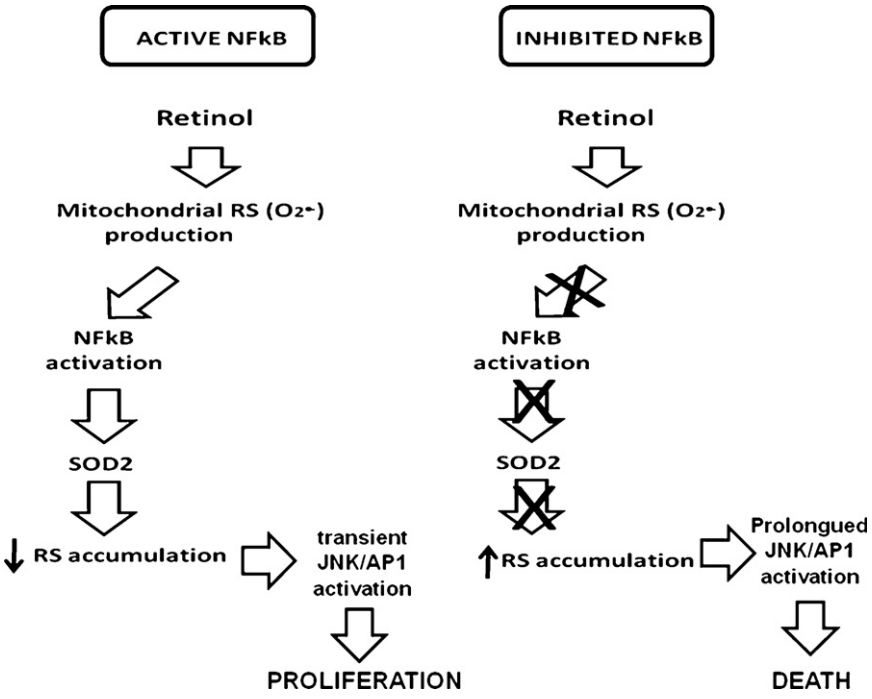

Fig. 7 – Schematic representation of ROH pro-oxidant effects in the presence or absence of NFκB signaling in Sertoli cells.

increase in RS formation, lower levels of oxidative damage, and no significant alterations in cell viability compared to that observed upon NFκB inhibition. In presence of NFκB, signaling was also observed that a transient activation of JNK–AP-1 pathway mediates proliferation, since inhibition with SP600125 or DNA decoy to AP-1-inhibited ROH-induced proliferation (Fig. 7). Thus, data indicate that a prolonged activation of JNK–AP-1 induces decreases in cell viability, while a transient activation leads to proliferation, and NFκB play a role in modulating these differential effects. This dual effect of JNK1/2 modulating proliferation and apoptosis also has been demonstrated during UVC-induced oxidative stress [4]. Data from others [2,4,22,39,40] and data presented here suggest that NFκB-mediated control of JNK1/2 signaling, and the consequences of prolonged/transient JNK activation on cell proliferation/apoptosis seems to represent a common mechanism among different pro-oxidant agents as UVC, TNF, and vitamin A.

Taken together, data suggest that NFκB plays important functions in modulating the duration of an oxidative insult, the duration of activation of redox-sensitive pathways as JNK–AP-1 and, consequently, the cellular fates in stressor environments. The events observed in this work could be useful not only to understanding vitamin A actions in biological systems but also to understand the NFκB function as an important factor involved in cellular resistance to oxidants and other cytotoxic agents [37,38,41], and show the inhibition of NFκB as mechanism to enhance cytotoxicity of oxidant agents.

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