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Hydrogen peroxide modulates immunoglobulin expression by targeting the 3'*Igh* regulatory region through an NFκB-dependent mechanism

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Abstract

Reactive oxygen species such as hydrogen peroxide (H_2O_2) appear to play a role in signal transduction in immune cells and have been shown to be synthesized upon antigen-mediated activation and to facilitate cellular activation in B- and T-cells. However, an effect of H_2O_2 on B-cell function (i.e. immunoglobulin (Ig) expression) has been less well-characterized. The effects of H_2O_2 exposure on lymphocytes may be partly mediated by oxidative modulation of the NFκB signal transduction pathway, which also plays a role in Ig heavy chain (*Igh*) gene expression. *Igh* transcription in B lymphocytes is an essential step in antibody production and is governed through a complex interaction of several regulatory elements, including the 3'*Igh* regulatory region (3'*Igh*RR). Utilizing an *in vitro* mouse B-cell line model, this study demonstrates that exposure to low μM concentrations of H_2O_2 can enhance 3'*Igh*RR-regulated transcriptional activity and *Igh* gene expression, while either higher concentrations of H_2O_2 or the expression of a degradation resistant inhibitory κB (IκBα super-repressor) can abrogate this effect. Furthermore, suppressive H_2O_2 concentrations increased protein levels of the p50 NFκB sub-unit, IκBα, and an IκBα immunoreactive band which was previously characterized as an IκBα cleavage product exhibiting stronger inhibitory function than native IκBα. Taken together, these observations suggest that exposure of B lymphocytes to H_2O_2 can alter *Igh* transcriptional activity and Ig expression in a complex biphasic manner which appears to be mediated by NFκB and altered 3'*Igh*RR activity. These results may have significant implications to disease states previously associated with the 3'*Igh*RR.

Keywords: *ROS, IκBα, 3'*Igh*RR, Ig, B lymphocytes.*

Abbreviations: Igh, *immunoglobulin heavy chain gene; 3'*Igh*RR, 3'*Igh *regulatory region; IκBαAA; IκBα super-repressor with two serine to alanine mutations; IKK, IκB kinase; ΔN-IκBα, IκBα lacking the N-terminal; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin;* V_H*, variable heavy chain.*

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Introduction

Reactive oxygen intermediates (ROI) are oxygen containing compounds that readily react with a wide array of biological substrates. There are multiple forms of ROI (e.g. O_2^-, OH^-, H_2O_2) produced from a number of disparate sources including cellular metabolism, enzymatic reactions, xenobiotics and inducible enzymes [1]. The influence of ROI on cellular function ranges from activation to cytotoxicity. The nature of the effect depends on the concentration and form of ROI, as well as the cellular capacity to neutralize them. H_2O_2 is one of the most predominant forms of ROI. It is a relatively stable intermediate and its electrical neutrality allows it to diffuse through a lipid bi-layer making it a readily available inter-compartmental and/or intercellular oxidant. The concept of ROI acting as a functional element of the cell has been well demonstrated in the immune system. For example, upon antigen-mediated activation, immune cells have been found to synthesize ROI (reviewed in [2]). Furthermore in immune cells such as B and T lymphocytes, H_2O_2 can facilitate cellular activation [3–5].

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Moreover, it has been found that xenobiotic-induced ROI can modulate cellular signalling pathways in human lymphocytes [6,7]. While the molecular basis for H_2O_2-mediated modulation of lymphocyte signal transduction and gene transcription likely involves oxidative modification of signal transduction proteins and transcription factors, the effects of H_2O_2 on these processes have not been fully explored. One such pathway that is modulated by H_2O_2 and has been found to play a primary role in lymphocyte activation is the NFκB signalling pathway [8].

NFκB activation canonically occurs when a stimulus activates an inhibitory κB kinase (IKK) which targets an IκB for proteolytic degradation, thus liberating the NFκB transcription factors (e.g. p50/RelA heterodimer) to translocate to the nucleus and facilitate transcription. The effects of H_2O_2 on NFκB activation have been studied in multiple cell types, with results ranging from no effect to a concentration-dependent activation or inhibition [8]. An early study showed NFκB binding to κB motifs and activation of a NFκB-mediated reporter following treatment of a T-cell line with μM H_2O_2 concentrations [4]. A number of subsequent studies have supported this finding [9] and, although not as widely investigated, studies have shown that H_2O_2 treatment of B lymphocytes can modulate NFκB activation and cellular function at similar (μM) concentrations [10]. Additionally, NFκB transcription factors, which were originally discovered in B lymphocytes, are transcriptional regulators of the *Igh* gene [11,12]. The IgH, an essential protein component of the antibody molecule, facilitates the antibody's ability to recognize and interface with antigen as well as with other immune factors and cells. *Igh* transcription is initiated at the variable heavy chain (V_H) promoter and is regulated by several transcriptional regulatory regions, including the intronic enhancer (Eμ) and a regulatory region 3' of the α constant region termed the 3'*Igh* regulatory region (3'*Igh*RR) (Figure 1). While the Eμ is involved in regulating recombination of the variable region and plays a role in *Igh* transcription, deletion of the 3'*Igh*RR severely impairs transcription and class switch recombination of the *Igh* gene, underscoring its functional significance in *Igh* gene regulation [13,14]. The mouse 3'*Igh*RR mediates transcriptional control through at least four hypersensitive sites (hs3a; hs1,2; hs3b; hs4) which interact with specific transcription factors including NFκB [15,16].

Given the relationship between NFκB, 3'*Igh*RR and *Igh* we wanted to test the hypothesis that H_2O_2, either by itself or in conjunction with a B-cell activating

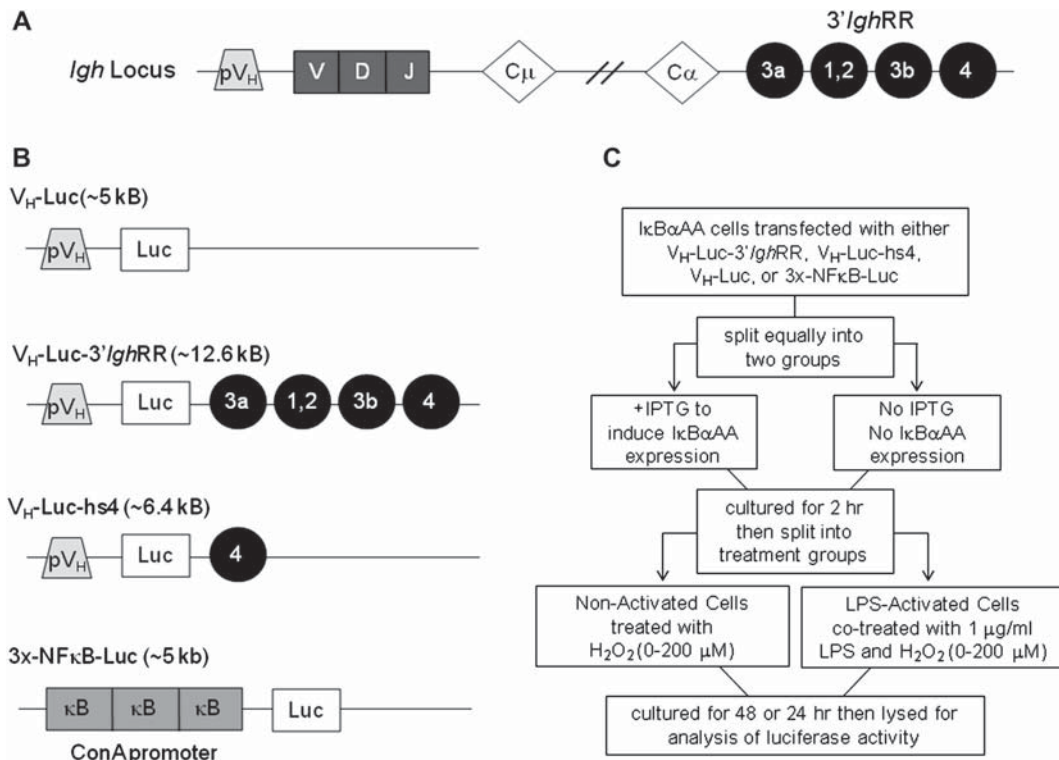

Figure 1. Schematic of the *Igh* locus, luciferase reporter constructs and experimental design. (A) Simplified diagram of a rearranged mouse *Igh* locus which includes the variable heavy chain promoter (pV_H) and the four enhancers (hs3a; hs1,2; hs3b; hs4) of the 3'*Igh*RR. 'VDJ' represents the variable region of *Igh* which encodes the antigen binding site. 'C' represents the constant regions of *Igh*; only Cμ and Cα are depicted which encode the heavy chain for the IgM and IgA isotypes, respectively. (B) Schematic of luciferase (Luc) reporters for the unregulated *Igh* promoter (V_H-Luc), the regulated *Igh* promoter (V_H-Luc-3'*Igh*RR and V_H-Luc-hs4) and 3x-NFκB-Luc which contains a Conalbumin A (ConA) promoter sequence with three NFκB DNA binding consensus sequences (κB). (C) Schematic of the experimental design for the transfection studies.

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stimulus, could modulate *Igh* transcriptional activity via the 3'*Igh*RR in an NFκB-dependent manner. Utilizing luciferase reporter constructs transiently expressed in the CH12IκBαAA mouse B-cell line, we determined that H_2O_2 can modulate 3'*Igh*RR-regulated V_H-promoter activity in a concentration-dependent, biphasic manner. This effect appears to be in-part mediated through the NFκB signalling pathway. When the reporter results were compared to the functional end-point of *Igh* transcription we found that H_2O_2 can modulate *Igh* transcription in a manner similar to that of the reporter constructs. These results suggest that Ig production and B-lymphocyte function could be altered following exposure to intrinsic (e.g. physiologic) or extrinsic (e.g. xenobiotic) sources of ROI and provide insight into the potential mechanisms for this effect. Furthermore, the 3'*Igh*RR has been associated with several human immune-related diseases that are also associated with increased levels of ROI [17–20], thus the results of the present study support a link between ROI (whether induced physiologically or by xenobiotics), altered 3'*Igh*RR activity and the initiation and progression of specific disease states.

Methods

Chemicals and reagents

Hydrogen peroxide (H_2O_2) solution (30 wt.% in water), IPTG (isopropyl β-D-1-thiogalactopyranoside) and LPS (*Escherichia coli*) were purchased from Sigma Aldrich (Milwaukee, WI). Chemicals were diluted in either water (H_2O_2, IPTG) or 1 × PBS (LPS).

Cell line

The CH12IκBαAA B-cell line was developed and provided by Dr Gail Bishop [12] and is a variant of the CH12.LX cell line which was derived from the murine CH12 (surface Ig^+ and $CD5^+$) mature B-cell lymphoma [21]. The CH12IκBαAA cell line stably expresses an IPTG-inducible IκBα super-repressor protein (IκBαAA) which contains a stable insertion of an inducible transgene that expresses a degradation resistant IκBα super-repressor (IκBαAA) which constitutively sequesters NFκB transcription factors in the cytoplasm and is resistant to negative feedback regulation by NFκB/Rel proteins [12]. Cells were grown as previously described [22]. Cell viability (mean viability from three separate experiments) was determined by trypan blue exclusion using a Beckman Coulter ViCell instrument (Beckman Coulter, Brea, CA).

Transient transfection and luciferase assay

The *Igh* luciferase reporter plasmids were provided by Dr Robert Roeder (Rockefeller University, New York, NY) and included a promoter alone control containing a V_H-promoter upstream of the luciferase gene and reporters containing the upstream V_H-promoter and either the 3'*Igh*RR or the hs4 enhancer downstream of the luciferase gene (Figure 1B). Plasmids were constructed using a pGL3 basic luciferase reporter construct (Promega, Madison, WI) as described previously [23]. The 3x-NFκB reporter was provided by Dr R. T. Hay and contains a promoter with three consensus NFκB binding motifs derived from the Ig κ-chain promoter and a Conalbumin transcriptional start site upstream of the luciferase gene [24].

Transient transfections were performed by electroporation at 250 volts, 150 µF and 75 ohms, as previously described [22]. For each plasmid, multiple transfections were pooled, seeded at 2.0×10^5 cells/ml and then divided into two equal portions. One portion was treated with 100 µM IPTG for 2 h to activate the IκBαAA transgene. The other portion was cultured without IPTG to provide a control that lacked IκBαAA expression (Figure 1C). After 2 h the cells were treated with H_2O_2 (0–200 µM) in the absence or presence of an LPS (1 µg/ml) co-treatment, seeded in triplicate into 12-well plates and cultured for 24 or 48 h in 5% CO_2 at 37°C. After the 24- or 48-h incubation period, cells were lysed with 1 × reporter lysis buffer (Promega) and then immediately frozen at -80°C. Measurement of luciferase enzyme activity was performed as previously described [22] and represented as relative light units or fold-change relative to the 0 µM H_2O_2 control.

RNA isolation

CH12IκBαAA cells treated with 1 µg/ml LPS and/or 0–100 µM H_2O_2 were plated (2.0×10^5 C/ml; 5.0 ml/well) in triplicate and incubated for 48 h at 5% CO_2 and 37°C. Cells were harvested and resuspended in 500 µl of Tri-Reagent (Sigma Aldrich) and stored at −80°C. Samples were thawed on ice then centrifuged at 12 000 × g for 10 min to remove genomic DNA. The supernatant was mixed with 10% v/v 1-bromo-3-chloropropane (Acros, Geel, Belgium) and the aqueous phase was isolated using phase lock gel tubes (5 Prime, Gaithersburg, MD), then mixed with 250 µl of isopropanol followed by centrifugation at 12 000 × g for 8 min. RNA pellets were washed in 75% ethanol, resuspended in nuclease free water and quantified using a NanoDrop ND1000 (Thermoscientific, Wilmington, DE).

cDNA synthesis and real-time PCR

One microgram of total RNA was reverse transcribed into cDNA using the Taqman RT reagent kit (ABI, Branchburg, NJ), as suggested by the manufacturer's instructions. SYBR®Green Real-Time PCR was utilized to amplify *µIgh* and β-actin (endogenous control

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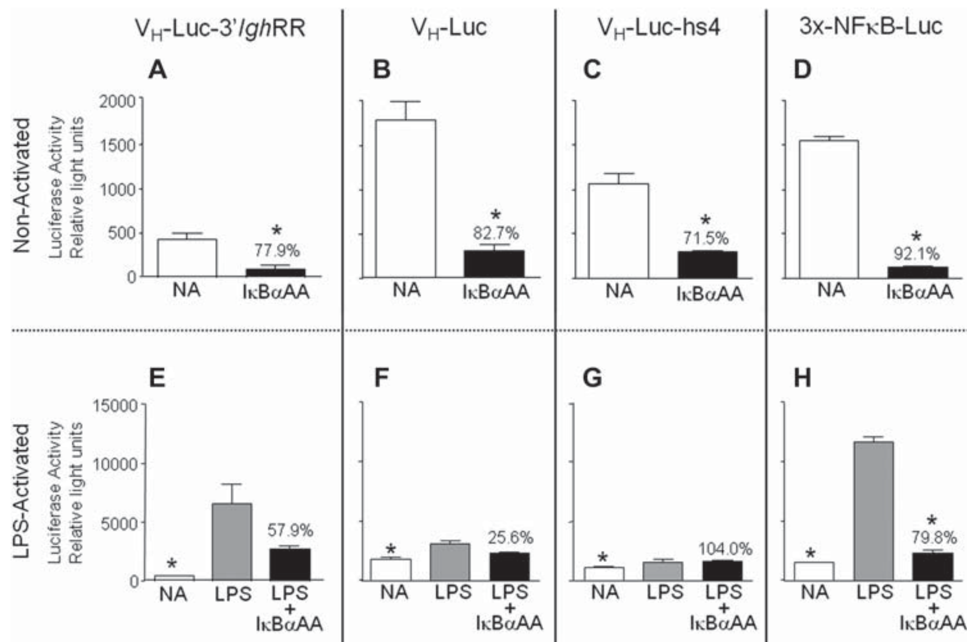

Figure 2. NFκB-mediated activity and 3'*Igh*RR-regulated and unregulated V_H-promoter activity is activated by LPS and inhibited by IκBαAA expression in CH12IκBαAA cells. Cells transiently transfected with either the V_H-Luc-3'*Igh*RR (A and E), V_H-Luc (B and F), V_H-Luc-hs4 (C and G) or 3x-NFκB-Luc (D and H) reporter plasmids were cultured at 2.0×10^5 cells/ml for 2 h in media alone or with IPTG to activate the IκBαAA super-repressor. The cells were then cultured for an additional 48 h in the absence (A–D) or presence (E–H) of LPS (1.0 μg/ml). 'NA' denotes naïve (untreated) cells. Luciferase enzyme activity is represented on the *y*-axis as relative light units (mean ± SEM). The results are representative of 3–7 separate experiments ($n = 3$ for each treatment group). Percentage inhibition (or activation for panel G) by IκBαAA relative to NA (A–D) or LPS (E–H) controls is indicated. Statistical differences between NA and IκBαAA (A–D) were determined by a 2-tailed *t*-test and between LPS and LPS + IκBαAA or NA (E–H) were determined by a 1-way ANOVA with a Dunnett's post-hoc test; $^*p < 0.05$.

to normalize cDNA concentrations) transcripts from the reverse transcribed cDNA. The primer sequences were as follows: *μIgh* forward primer (FP), 5'TCTGCCTTCACCACAGAAGA3'; *μIgh* reverse primer (RP), 5'GCTGACTCCCTCAGGT TCAG3'; β-actin FP, 5'GCTACAGCTTCACCACCACA3'; β-actin RP, 5'TCTCCAGGGAG GAAGAGGAT3'. cDNA (5 ng) was mixed with 2 × SYBR®Green Master Mix, 5.0 μM of both FP and RP and RNase/DNase free water to reach a total reaction volume of 25 μl. The PCR was performed in an ABI 7500 and the cycling conditions were: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation curve following the PCR reaction verified a single PCR product size and no genomic DNA contamination. The results of the PCR amplification were analysed using the SDS 2.0 software to determine relative quantification (RQ) values (i.e. fold-change).

Protein isolation for Western blot analysis

CH12IκBαAA cells treated with 1 μg/ml LPS and/or 0–200 μM H_2O_2 were harvested at 24 and 48 h, washed once with 1 × PBS, then re-suspended in 150 μl of mild lysis buffer (150 mM NaCl, 10 mM sodium phosphate pH 7.2, 2 mM EDTA and 1% Nonidet P-40) and frozen at −80°C for at least 1 h. Lysates were thawed on ice and centrifuged at 14 000 rpm for 5 min; whole cell lysate was removed from the pelleted cell debris, quantified using the Bio-Rad Protein Assay (Bio Rad, Hercules, CA) and frozen at −80°C.

Western blot analysis

Whole cell lysates were thawed on ice and 50 μg of protein was run on a 10% polyacrylamide gel at 200 volts for 30–40 min. The proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) at 1.0 amp for 1 h. Membranes were blocked overnight at 4°C in 3% BSA (bovine serum albumin)/TTBS (tris-buffered saline with 0.05% tween-20), then incubated overnight at room temperature with either mouse anti-β-actin (Sigma Aldrich) at a 1:10 000 dilution, rabbit anti-IκBα [sc-371 (C-21), Santa Cruz, Santa Cruz, CA] at a 1:1000 dilution or mouse anti-p50 (sc-114, Santa Cruz) at a 1:200 dilution. Prior to and after a 1 h incubation at ~ 20°C with the appropriate horse-radish-peroxidase-conjugated secondary antibody (goat anti-mouse at 1:8000 or goat anti-rabbit at 1:2500), the membrane was washed four times in TTBS at 10 min intervals. All antibodies were diluted in 3% BSA/TTBS and the proteins of interest were detected using the Pierce ECL

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substrate (Thermoscientific Pierce, Waltham, MA) and a Fuji LAS-3000 Bioimager (Tokyo, Japan).

Statistical analysis

In Figures 3, 5 and 7, the mean (n = 3) was determined for each treatment group and the means generated from several experiments were then averaged and transformed to fold-change (mean ± SEM) with the 0 μM H_2O_2 control set to 1. A statistical difference in the fold-change between treatment groups and the control was determined by 1-way ANOVA with a Dunnett's post-hoc test. Statistical differences in the fold-effect between the non- and IκBαAA-expressing cells (Figures 3 and 5) or between the reporters (Figures 3 and 7) were determined by a 2-way ANOVA with a Bonferroni post-hoc test. Figures 2, 4 and 6 represent the mean ± SEM for each treatment group (n = 3) of a representative experiment and significance was determined by either a two-tailed t-test (Figures 2A–D and 6A–D) or a 1-way ANOVA with a Dunnett's post-hoc test (Figures 2E–H, 6E–H and 4).

Results

IκBα inhibits transcriptional activity of the 3'IghRR-regulated V_H-promoter

To determine if H_2O_2 modulates *Igh* transcriptional activity via the 3'*Igh*RR in an NFκB-dependent manner, we utilized a luciferase reporter transcriptionally regulated by the V_H-promoter and the 3'*Igh*RR as well as the CH12IκBαAA mouse B-cell line which contains a stable insertion of an inducible transgene that expresses a degradation resistant IκBα super-repressor (IκBαAA). The IκBαAA constitutively sequesters NFκB transcription factors in the cytoplasm [12]. Since our previous studies have shown that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a known disrupter of B-cell function (reviewed by [25] and an inducer of ROI [26,27]), can enhance NFκB binding within the hs4 enhancer as well as transcriptional activity of an hs4 luciferase reporter [28,29], we also investigated the effects of H_2O_2 on the hs4 enhancer. Furthermore, to express Ig and secrete antibodies, B lymphocytes must first be stimulated. Therefore, we utilized the polyclonal B-cell activator and TLR-4 ligand LPS which is a major component of bacterial cell walls.

CH12IκBαAA cells were transiently transfected with reporter constructs regulated by a V_H-promoter alone (V_H-Luc) or the V_H-promoter in conjuction with either the 3'*Igh*RR (V_H-Luc-3'*Igh*RR) or the hs4 enhancer (V_H-Luc-hs4) (Figure 1B). The transfected cells were cultured for 48 h in the absence or presence of IκBαAA and/or cellular activation (Figure 1C). A 48 h treatment period was previously determined to be an optimal time to achieve LPS-induced activation of the 3'*Igh*RR luciferase reporter [22,29]. Utilizing the same treatment conditions (Figure 1C), the level of NFκB activity in our cell line model was also assessed by measuring the transcriptional activity of a 3x-NFκB reporter plasmid (3x-NFκB-Luc) (Figure 1B).

In the non-activated cells, IκBαAA expression significantly inhibited the activity of the regulated (3'*Igh*RR and hs4) and unregulated promoter (V_H) and the 3x-NFκB reporter (Figures 2A–D). In LPS-activated cells, 3'*Igh*RR activity increased 15.2-fold over the NA control group, and consistently exhibited higher overall levels of luciferase activity compared to LPS-induced V_H and hs4 reporter activity (Figures 2E–G). IκBαAA expression decreased the LPS activation of the 3'*Igh*RR reporter by 57.9% (Figure 2E). Interestingly, V_H and hs4 were only modestly activated (<2-fold) by LPS. Furthermore, IκBαAA expression induced a modest, non-significant inhibition of LPS-induced V_H activity and no effect on LPS-induced hs4 activity which contrasted with the significant inhibition of both V_H and hs4 basal activity (compare Figures 2F and G to 2B and C). Consistent with LPS induction of NFκB-mediated transcriptional activity, LPS significantly activated the 3x-NFκB reporter (7.5-fold), which was inhibited by 79.8% with the co-expression of IκBαAA (Figure 2H). Taken together, these results demonstrate that LPS induction of transcriptional activity is primarily mediated through the 3'*Igh*RR not the V_H-promoter alone and that the hs4 enhancer is not sufficient to optimally mediate LPS-induced activation of the 3'*Igh*RR. There also appears to be a significant NFκB component to these effects since IκBαAA expression partially inhibits LPS-induced activation of the 3'*Igh*RR-regulated and unregulated V_H-promoter.

H_2O_2 differentially modulates the activity of the regulated and unregulated V_H-promoter and μIgh transcription

To explore the effects of oxidative stress (i.e. H_2O_2) on the regulated and unregulated V_H-promoter, we transiently expressed either the 3'*Igh*RR, hs4 or V_H reporters in the CH12IκBαAA cells and treated with varying concentrations of H_2O_2 for 48 h. Luciferase activity was represented as fold-change compared to the 0 μM H_2O_2 control. Lower concentrations of H_2O_2 (30–50 μM H_2O_2) resulted in a significant enhancement of 3'*Igh*RR activity in both the non-activated and LPS-activated cells, albeit to a lesser degree in the non-activated cells, i.e. 2-fold above basal activity vs 3-fold above LPS-induced activity (Figures 3A and B). Non-activated cells also had a much lower luciferase activity compared to LPS-activated cells (Figure 2). Increasing the concentration above 50 μM H_2O_2 resulted in a concentration-dependent decrease in reporter activity in both the non-activated and LPS-activated cells (Figures 3A and B).

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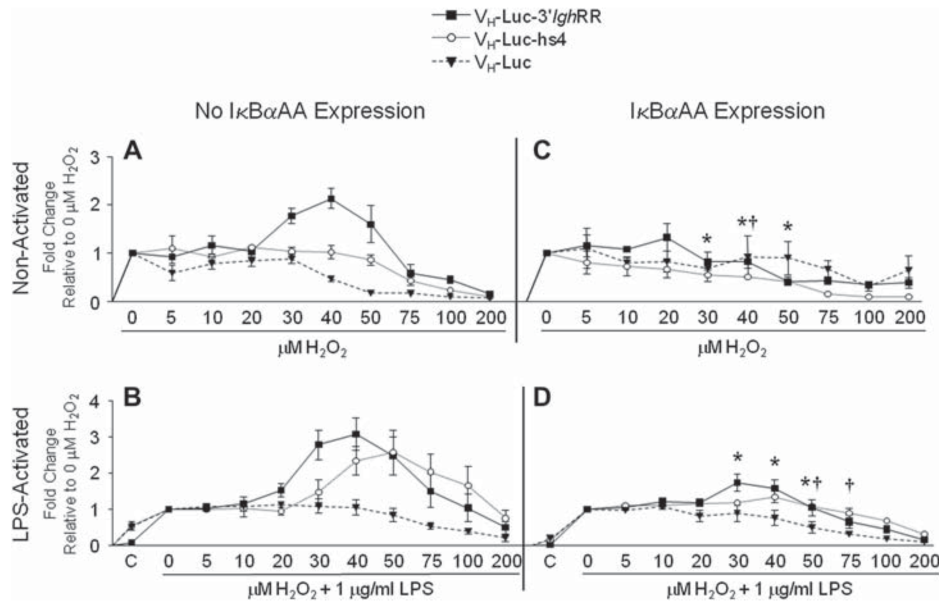

Figure 3. Hydrogen peroxide modulates 3'*Igh*RR-regulated V_H-promoter activity in an IκBα-dependent manner. CH12IκBαAA cells transiently transfected with the V_H-Luc-3'*Igh*RR (■) or V_H-Luc-hs4 (○) or V_H-Luc (▼) reporter plasmids were cultured at 2.0×10^5 cells/ml for 2 h in media alone (A and B) or with IPTG to activate the IκBαAA super-repressor (C and D). The cells (with or without IPTG) were then cultured for 48 h with varying concentrations of H_2O_2 (0–200 μM) in the absence (A and C) or presence (B and D) of LPS (1.0 μg/ml). 'C' denotes the non-activated control. Results are represented as fold-change (mean ± SEM) relative to the respective 0 μM H_2O_2 control and were generated from 3–7 separate experiments (n = 3 for each treatment group). Statistical differences of the H_2O_2 treatment groups compared to 0 μM H_2O_2 were determined by a 1-way ANOVA with a Dunnett's post-hoc test and resulted in significance ($p < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: (A) V_H-Luc-3'*Igh*RR: 30, 40 and 200 μM; V_H-Luc: 5 and 40–200 μM; V_H-Luc-hs4: 75–200 μM. (B) V_H-Luc-3'*Igh*RR: 30–50 μM; V_H-Luc: 100–200 μM; V_H-Luc-hs4: 50 μM. (C) V_H-Luc-3'*Igh*RR: 100–200 μM; V_H-Luc-hs4: 50–200 μM. (D) V_H-Luc-3'*Igh*RR: 30, 40 and 200 μM; V_H-Luc: 50–200 μM; V_H-Luc-hs4: 40 and 200 μM. Statistical differences between treatment groups of no IκBαAA (A and B) and IκBαAA (C and D) expressing cells were determined by a 2-way ANOVA with Bonferroni post-hoc test. *$p < 0.05$ for V_H-Luc-3'*Igh*RR and † $p < 0.05$ for V_H-Luc-hs4 relative to corresponding treatment group. Statistical differences compared to the V_H-Luc reporter were determined by a 2-way ANOVA with a Bonferroni post-hoc test and resulted in significance ($p < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: (A) V_H-Luc-3'*Igh*RR: 30–50 μM; V_H-Luc-hs4: 50 μM. (B) V_H-Luc-3'*Igh*RR: 30–50 μM; V_H-Luc-hs4: 50–75 μM). (D) V_H-Luc-3'*Igh*RR: 30–50 μM.

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Interestingly, V_H did not exhibit H_2O_2-mediated enhancement in the non-activated and LPS-activated cells, but did exhibit a concentration-dependent inhibition by H_2O_2 (Figures 3A and B). Since enhancement of transcriptional activity by lower concentrations (30–50 μM) of H_2O_2 occurs only with the regulated V_H-promoter and inhibition of promoter activity by higher concentrations of H_2O_2 occurs with the regulated and unregulated V_H-promoter, these results suggest that the 3'*Igh*RR mediates the H_2O_2-induced activation and higher concentrations of H_2O_2 directly inhibit V_H-promoter activity.

H_2O_2 is known to cause oxidative stress and cell death, therefore it is important to consider the effects of H_2O_2 on cell viability. Following the 48 h treatment, there was a concentration-dependent effect of H_2O_2 on cell viability of the non-activated cells as measured by trypan blue exclusion. The 40 μM H_2O_2 treatment resulted in 87.0 ± 2.2% cell viability while the 100 and 200 μM H_2O_2 treatments resulted in cell viabilities of 57.6 ± 2.8% and 46.2 ± 4.9%, respectively. The effect of H_2O_2 treatment on cell viability of the LPS-activated cells was minimal, with 94.8 ± 0.6% cell viability following the 40 μM H_2O_2 treatment, 85.8 ± 2.5% viability with the 100 μM H_2O_2 treatment and 79.1 ± 3.2% viability with the 200 μM H_2O_2 treatment. Clearly, due to the marked decrease in cell viability, cytotoxicity cannot be ruled out as a major contributor to the inhibitory effect of the 100 and 200 μM H_2O_2 treatments on reporter activity in non-activated cells. In contrast, the effect of 100 and 200 μM H_2O_2 treatments on the viability of LPS-activated cells is much less pronounced and most likely does not play a significant role in the inhibitory effect of higher H_2O_2 concentrations on reporter activity; however, we cannot rule out the initiation of early apoptotic events not detectable by trypan blue exclusion that could influence reporter activity.

We examined hs4 reporter expression under the same H_2O_2 treatment conditions. In a manner similar to the 3'*Igh*RR, LPS activation of the hs4 reporter was enhanced by lower concentrations of H_2O_2 (40–75 μM) and this enhancement appeared to be abrogated by higher concentrations of H_2O_2 (100–200 μM) (Figure 3B). Notably the enhancement of the hs4 reporter occurred at a slightly higher H_2O_2 concentration range (Figure 3B). Furthermore, in marked contrast with the 3'*Igh*RR reporter, the non-activated hs4 reporter was not activated by H_2O_2 (Figure 3A).

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However, similar to the 3'*Igh*RR reporter the basal activity of the hs4 reporter was significantly inhibited by the 75-200 μM H_2O_2 treatments (Figure 3A). Taken together, these results suggest that the hs4 enhancer plays a significant but partial role in the H_2O_2 enhancement of 3'*Igh*RR-mediated transcriptional activity in LPS-activated cells and has no role in non-activated cells. Therefore, other enhancer elements (i.e. hs3a, hs1,2 or hs3b) within the 3'*Igh*RR appear to play a part in the enhancement of 3'*Igh*RR-mediated transcriptional activity in non-activated cells and to contribute to the greater sensitivity of 3'*Igh*RR as compared to hs4 to H_2O_2.

The effect of H_2O_2 treatment on the expression of the endogenous *μIgh* (encodes the heavy chain protein of IgM) was measured by real-time PCR in the CH12IκBαAA cell line to assess whether or not the 3'*Igh*RR reporter constructs exhibited an expression profile representative of an endogenous locus. A 40 μM H_2O_2 co-treatment significantly enhanced the 2-fold LPS-mediated induction of *μIgh* expression, while the 100 μM H_2O_2 co-treatment resulted in a modest inhibition. These results correlate well with the biphasic effect of H_2O_2 observed with the 3'*Igh*RR and hs4 reporters (compare Figures 4 and 3). Furthermore, with the expression of IκBαAA the 40 μM H_2O_2 co-treatment did not result in an enhancement of LPS-induced *μIgh* expression (Figure 4B). However, in non-activated cells, H_2O_2 treatment did not generate a significant enhancing effect, but there was a significant inhibition of basal expression with the higher concentrations of H_2O_2 (compare Figures 4 and 3). A lack of a stimulatory effect on *μIgh* expression in the non-activated cells is not altogether surprising considering the very low overall activity of the 40 μM H_2O_2-induced 3'*Igh*RR reporter in non-activated cells compared to activated cells. For example, LPS induced an ~ 20-fold increase (above basal) in 3'*Igh*RR activity (~ 500 RLU to ~ 10 000 RLU) which corresponded to an ~ 2-fold increase in endogenous *μIgh* levels (Figures 2 and 4). In comparison, 40 μM H_2O_2 only induced an ~ 2-fold increase (above basal) in 3'*Igh*RR activity (~ 500 RLU to ~ 1000 RLU) in non-activated cells (Figure 3). Therefore, it is unlikely that this low induction in transcriptional activity would produce a biologically significant effect in endogenous *μIgh* expression.

Inhibition of NFκB/Rel activity by IκBαAA abrogates the stimulatory but not the inhibitory effect of H_2O_2 on the 3'IghRR- and hs4-regulated V_H-promoter

NFκB proteins can be functionally modulated by changes in the redox state of the cell and may also be involved in regulating 3'*Igh*RR activity through interactions with NFκB DNA binding sites within the 3'*Igh*RR [15]. Correspondingly, IκBαAA expression suppressed the H_2O_2-mediated enhancement of 3'*Igh*RR and hs4 in LPS-activated cells (Figure 3D) and eliminated the H_2O_2-enhanced 3'*Igh*RR activity in the non-activated cells (Figure 3C), suggesting a prominent role of NFκB/Rel proteins in these effects. IκBαAA expression did not appear to significantly alter the inhibitory profile of the regulated and unregulated V_H-promoter activity observed with higher concentrations of H_2O_2 (Figure 3).

To further characterize the role of NFκB/Rel proteins in the H_2O_2-mediated enhancement of the hs4 and 3'*Igh*RR luciferase reporters, we evaluated the effect of H_2O_2 on the 3x-NFκB reporter under the same treatment conditions. Similar to the hs4 and 3'*Igh*RR reporter constructs, lower concentrations (30–50 μM) of H_2O_2 enhanced LPS activation of the 3x-NFκB reporter; whereas, higher concentrations (75–200 μM) either lacked an effect or inhibited LPS activation (compare Figures 5B and 3B). In contrast

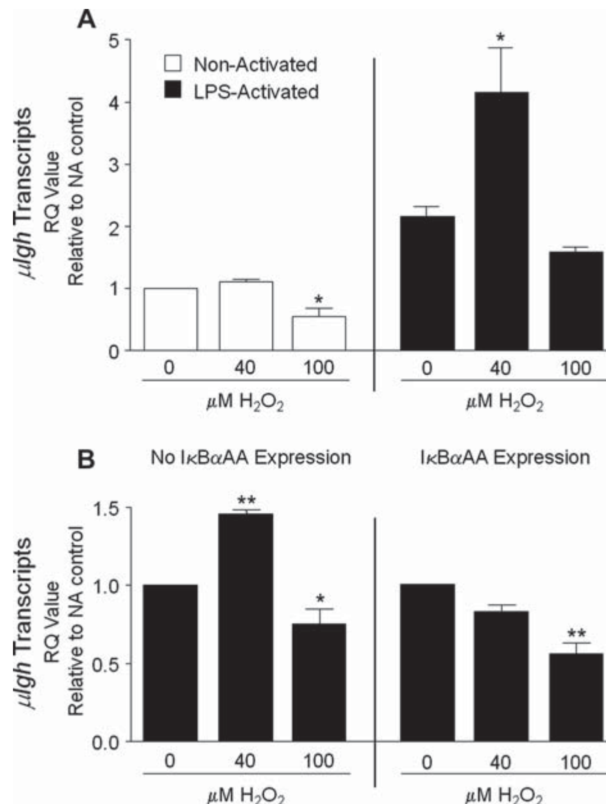

Figure 4. Hydrogen peroxide affects *μIgh* transcript levels. Total RNA was extracted from CH12IκBαAA cells (2.0×10^5 cells/ml) that were cultured for 48 h. Prior to RNA isolation cells were (A) treated for 48 h with 0, 40 or 100 μM H_2O_2 in the absence or presence of 1.0 μg/ml LPS or (B) pre-treated for 2 h in media alone or with IPTG to activate the IκBαAA super-repressor then treated for 48 h with 0, 40 or 100 μM H_2O_2 in the presence of 1.0 μg/ml LPS. One microgram of total RNA was reverse transcribed to cDNA and 5 ng of total cDNA was utilized to amplify *μIgh* and β-actin via SYBR®Green real-time PCR. The results are expressed as relative quantification (RQ) value compared to the 0 μM H_2O_2 control. The data is representative of at least two separate experiments ($n = 3$ for each treatment group). Statistical differences compared to the respective 0 μM H_2O_2 control were determined by a 1-way ANOVA with a Dunnett's post-hoc test; $^*p < 0.05$ or $^{**}p < 0.01$.

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with the 3'*Igh*RR but similar to the hs4, the 3x-NFκB expression profile in non-activated cells did not exhibit an H_2O_2-mediated enhancement but did show a concentration-dependent inhibition, albeit the 3x-NFκB expression was inhibited by a slightly wider range of concentrations (compare Figures 5A and 3A). Taken together these results suggest that, during LPS activation, H_2O_2 can elicit a concentration-dependent biphasic effect on 3'*Igh*RR and hs4 transcriptional activity that is in-part directly mediated by NFκB. Conversely, the contrast between the 3x-NFκB and 3'*Igh*RR profiles suggests that the H_2O_2-mediated enhancement of 3'*Igh*RR activity in non-activated cells may not involve direct regulation by NFκB or may require additional transcription factors to facilitate H_2O_2-mediated NFκB binding. Indeed there are binding sites for several other transcription factors within the 3'*Igh*RR enhancers [16] which may mediate the transcriptional activity in non-activated cells.

H_2O_2 and LPS exhibit temporally-dependent effects on transcriptional activity

Interestingly, NFκB activation of B lymphocytes by LPS occurs rapidly and is likely initiated by LPS-induced IκBα degradation, which occurs in 1–1.5 h ([30] and data not shown). Therefore, optimal induction of 3'*Igh*RR by LPS at 48 h may involve secondary effects. Subsequently, we evaluated the temporal effects of IκBαAA expression on the basal and LPS-induced transcriptional activation of the regulated and unregulated V_H-promoter. Similar to the 48 h time point, IκBαAA expression inhibited the basal activity of all the reporters at 24 h (Figures 6A–D and Figures 2A–D). LPS activation resulted in a less than 2-fold activation of V_H and hs4 at 24 h which was comparable with the expression profile of those plasmids at 48 h (compare Figures 6F and G to 2F and G). Expression of IκBαAA inhibited LPS-activation of the V_H reporter by 36.9% at 24 h vs 25.6% at 48 h (compare Figures 6F and 2F). Notably, LPS activation of the hs4 reporter was not inhibited by IκBαAA expression at 24 or 48 h (compare Figures 6G and 2G). The most prominent difference between the two time points is with the 3'*Igh*RR reporter which exhibited only a 2-fold activation by LPS at 24 h vs the 21.1-fold activation at 48 h (compare Figures 6E and 2E). Interestingly, at 24 h IκBαAA expression did not inhibit the modest LPS-induced activation of the 3'*Igh*RR reporter but instead trended toward a non-significant enhancing effect, which contrasted with the 72.2% inhibition observed at 48 h (compare Figures 6E and 2E). For the 3x-NFκB reporter, LPS induced a 2.6-fold activation at 24 h vs a 7.5-fold activation at 48 h. At both time points IκBαAA expression significantly inhibited LPS-induced 3x-NFκB reporter activity, i.e. 88.1% for 24 h and 79.8% for 48 h (compare Figures 6H and 2H). In

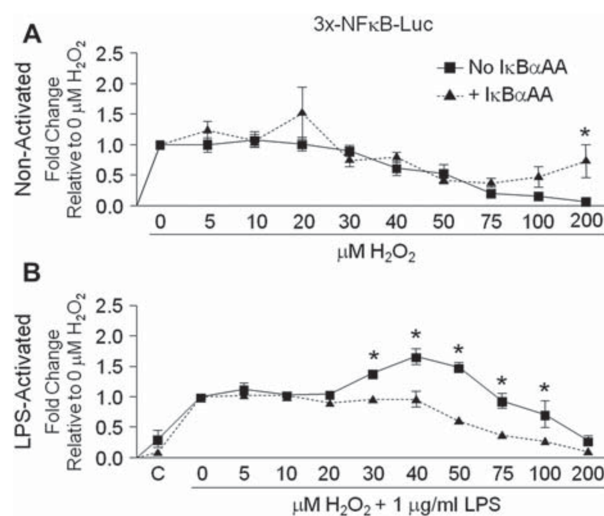

Figure 5. Hydrogen peroxide modulates 3x-NFκB-Luc reporter activity. CH12IκBαAA cells transiently transfected with the 3x-NFκB-Luc reporter plasmid were cultured at 2.0×10^5 cells/ml for 2 h in media alone (■ No IκBαAA) or with IPTG to activate the IκBαAA super-repressor (▲ + IκBαAA). The cells (with or without IPTG) were then cultured for 48 h with varying concentrations of H_2O_2 (0–200 μM) in the absence (A) or presence (B) of LPS (1.0 μg/ml). Results are represented as fold-change (mean ± SEM) relative to the respective 0 μM H_2O_2 control and were generated from five separate experiments for non-activated cells and three separate experiments for the LPS-activated cells ($n = 3$ for each treatment group). Statistical differences of the H_2O_2 treatment groups compared to 0 μM H_2O_2 were determined by a 1-way ANOVA with a Dunnett's post-hoc test and resulted in significance ($p < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: (A) 'No IκBαAA': 50–200 μM H_2O_2; ' + IκBαAA': no significance. (B) 'No IκBαAA': 40, 50 and 200 μM H_2O_2; ' + IκBαAA': 50–200 μM H_2O_2. $^*p < 0.05$ represents statistical differences between 'No IκBαAA' and ' + IκBαAA' as determined by a 2-way ANOVA with a Bonferroni post-hoc test.

summary, LPS does not appear to optimally activate 3'*Igh*RR activity at 24 h and, given the diverging effects of IκBαAA on the 3'*Igh*RR and hs4 reporters at 24 vs 48 h, the 48 h transcriptional effects are likely influenced by delayed or secondary IκBα/NFκB signalling processes.

Since the above results clearly demonstrate time-dependent effects, reporter activity following H_2O_2 treatment was also analysed at 24 h. In marked contrast to the 48 h results (Figures 3 and 5), there was no H_2O_2-mediated enhancement of basal or LPS-induced activity of the 3'*Igh*RR, hs4 or 3x-NFκB reporter constructs at 24 h (Figures 7A and B). However, an H_2O_2 concentration-dependent inhibition was observed for all reporter constructs. Notably there was significantly weaker inhibition of the 3'*Igh*RR when compared to the V_H in the non-activated (40 μM H_2O_2) and LPS-activated (40–100 μM H_2O_2) cells. The hs4 and 3x-NFκB reporters also demonstrated a weaker inhibition by H_2O_2 as compared to V_H, but only significantly in the non-activated cells (Figures 7A and B). Additionally, H_2O_2 induced a slightly greater but non-significant decrease in cell

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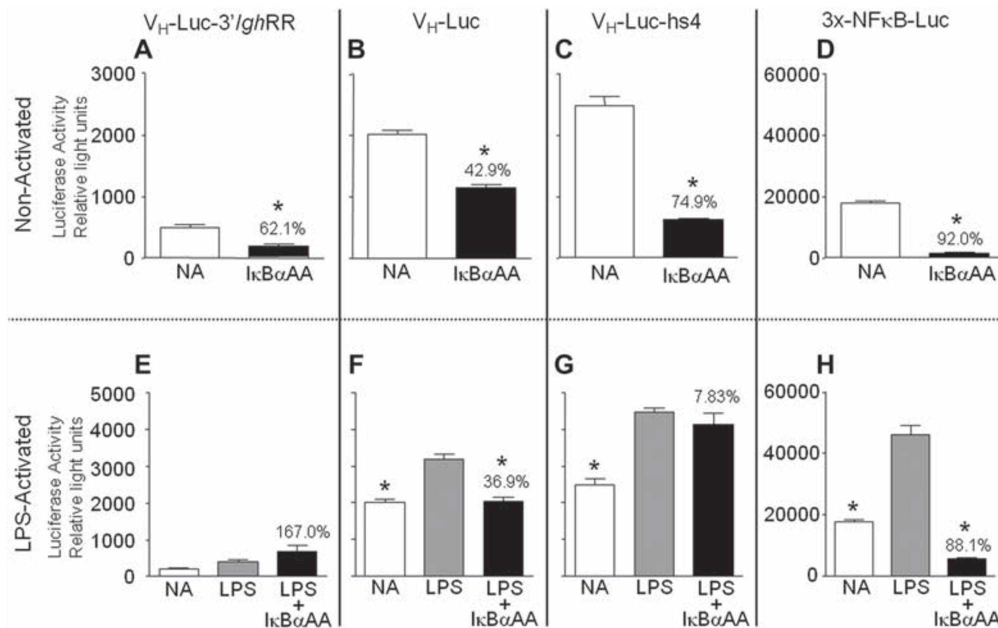

Figure 6. LPS activation and IκBαAA expression result in differential effects on reporter activity at 24 h. CH12IκBαAA cells transiently transfected with the V_H-Luc-3'*Igh*RR (A and E), V_H-Luc (B and F), V_H-Luc-hs4 (C and G) or 3x-NFκB-Luc (D and H) reporter plasmids were either cultured at 2.0×10^5 cells/ml for 2 h in media alone or with IPTG to activate the IκBαAA super-repressor. The cells were then cultured for 24 h in the absence (A–D) or presence (E–H) of LPS (1.0 μg/ml). 'NA' denotes naïve (untreated) cells. Luciferase enzyme activity is represented on the *y*-axis as relative light units (mean ± SEM). The results are representative of three experiments for each reporter ($n = 3$ for each treatment group). Percentage inhibition (or activation for panel E) by IκBαAA relative to NA (A–D) or LPS (E–H) controls is indicated. Statistical differences between NA and IκBαAA (A–D) were determined by a 2-tailed *t*-test and statistical differences between LPS and LPS + IκBαAA or NA (E–H) were determined by a 1-way ANOVA with Dunnett's post-hoc test; $^*p < 0.05$.

viability in non-activated cells at 24 h compared to 48 h (77.6 ± 7.7% vs 87.0 ± 2.2% for 40 μM H_2O_2; 48.0 ± 5.4% vs 57.6 ± 2.8% for 100 μM H_2O_2; and 42.1 ± 4.9% vs 46.2 ± 4.9% for 200 μM H_2O_2). However, in LPS-activated cells at 24 h vs 48 h, H_2O_2 treatment had little effect on cell viability (94.4 ± 1.4% vs 94.8 ± 0.6 for 40 μM H_2O_2; 93.6 ± 0.9% vs 85.8 ± 2.5% for 100 μM H_2O_2; and 86.5 ± 2.1% vs 79.1 ± 3.2% for 200 μM H_2O_2). For all of the reporters IκBαAA expression did not alter the inhibitory effect of H_2O_2 at 24 h (data not shown). Therefore, it appears that the H_2O_2-mediated enhancement vs inhibition are differentially regulated with the enhancement being specifically mediated by the 3'*Igh*RR and hs4 in a temporal and IκBα/NFκB-dependent fashion as further supported by similar results obtained with the 3x-NFκB reporter. Furthermore, cytotoxicity may play a role in the inhibitory effect of H_2O_2 on luciferase activity in non-activated cells. However, it is unlikely that the inhibitory effect of H_2O_2 in LPS-activated cells is mediated by cytotoxicity, although initiation of early apoptotic events cannot be ruled out.

H_2O_2 temporally modulates IκBα and p50 protein expression

The H_2O_2-mediated enhancement of 3'*Igh*RR and hs4 expression and its inhibition by IκBαAA suggests that NFκB plays a role in this effect, which is further substantiated by a similar H_2O_2-mediated enhancement of LPS-activated 3x-NFκB transcriptional activity. These observations led us to explore the effects of H_2O_2 on IκBα, as it is a key regulator of NFκB activity and its expression can be modulated by ROI. The phosphorylation of IκBα and subsequent activation of NFκB by H_2O_2 at the 1–2 h time point has been demonstrated, but little is known about the effects of H_2O_2 exposure on IκBα expression at later time points [9]. Given that the H_2O_2-mediated enhancement of 3'*Igh*RR, hs4 and 3x-NFκB expression was not observed until the 48 h time point and that the inhibitory effect at higher concentrations of H_2O_2 observed at 24 h was maintained through the 48 h time point, IκBα expression was examined at 24 and 48 h to determine how IκBα expression correlates with the effect on reporter activity.

Western blot analysis with an anti-IκBα antibody yielded a doublet with the bottom band representing IκBα (~ 37 kDa) and the top band most likely IκBβ since the top band appears to migrate at the correct size for IκBβ (~ 43 kDa) and the anti-IκBα antibody is cross-reactive with the β isoform (Figures 8A and B). The co-treatment of LPS with 100 or 200 μM H_2O_2 resulted in increased IκBα at 48 h (Figure 8B). A third, faster migrating IκBα immunoreactive band (~ 34 kDa) appeared prominently in both the non-activated and LPS-activated cells at 24 h and in only the LPS-activated cells at 48 h (Figures 8A and B). It was not present in any treatment group at 1 h (data

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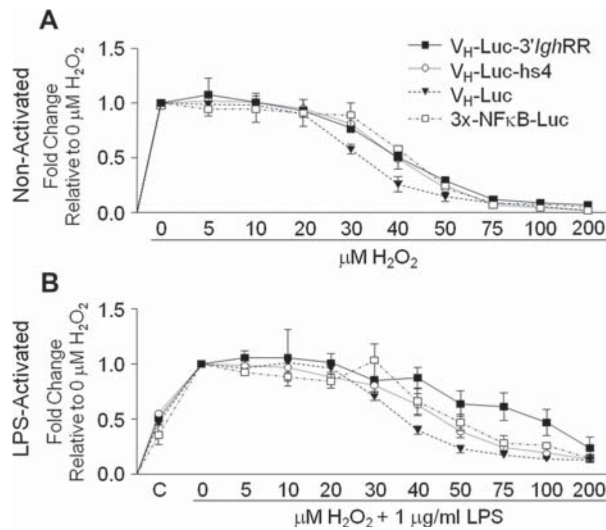

Figure 7. Hydrogen peroxide inhibits reporter activity at 24 h. CH12IκBαAA cells transiently transfected with either the V_H-Luc-3'*Igh*RR (■), V_H-Luc-hs4 (○), V_H-Luc (▼) or 3x-NFκB-Luc (□) reporter plasmids were cultured at 2.0 × 10^5 cells/ml for 2 h in media alone (A and B) or with IPTG to activate the IκBαAA super-repressor (data not shown). The cells were then cultured for 24 h with varying concentrations of H_2O_2 (0–200 μM) in the absence (A) or presence (B) of LPS (1.0 μg/ml). 'C' denotes the non-activated control. Results are represented as fold-change (mean ± SEM) relative to the respective 0 μM H_2O_2 control and statistical differences of the H_2O_2 treatment groups compared to 0 μM H_2O_2 were determined by a 1-way ANOVA with a Dunnett's post-hoc test and resulted in significance ($p < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: (A) V_H-Luc-3'*Igh*RR: 40–200 μM; V_H-Luc: 30–200 μM; V_H-Luc-hs4: 40–200 μM; 3x-NFκB-Luc: 40–200 μM. (B) V_H-Luc-3'*Igh*RR: 100–200 μM; V_H-Luc: 30–200 μM; V_H-Luc-hs4: 30–200 μM; 3x-NFκB-Luc: 40–200 μM. Statistical differences compared to the V_H-Luc reporter were determined by a 2-way ANOVA with a Bonferroni post-hoc test and resulted in significance ($p < 0.05$; not represented on the graph) at the following H_2O_2 concentrations: (A) V_H-Luc-3'*Igh*RR: 40 μM; V_H-Luc-hs4: 30 and 40 μM; 3x-NFκB-Luc: 30 and 40 μM. (B) V_H-Luc-3'*Igh*RR: 40–100 μM; 3x-NFκB-Luc: 30 μM. Results were generated from three separate experiments (n = 3 for each treatment group).

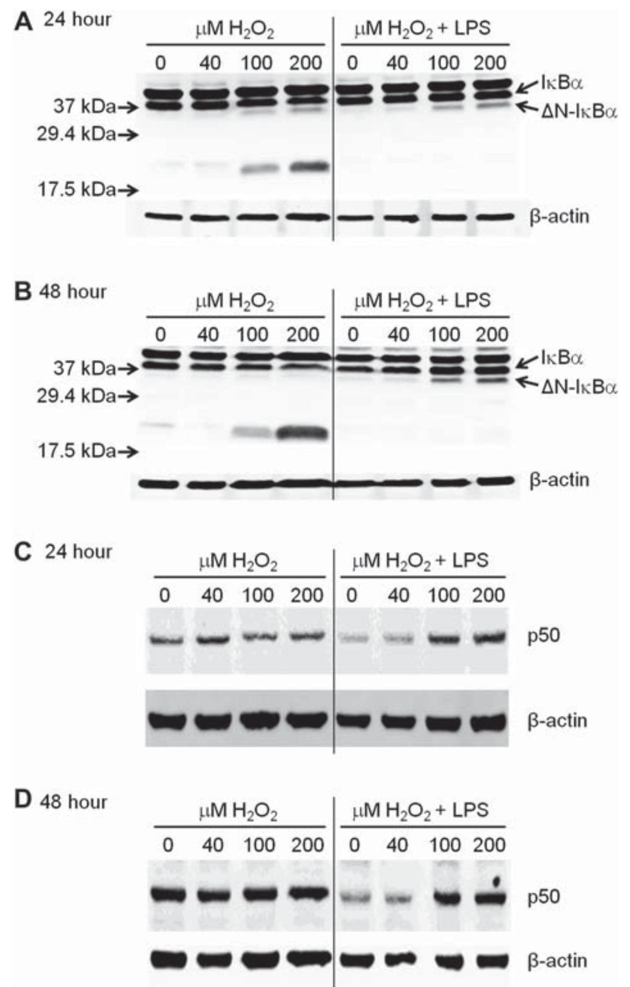

Figure 8. Hydrogen peroxide alters IκBα and NFκB/p50 protein expression. CH12IκBαAA cells (2.0 × 10^5 cells/ml) were cultured for 24 h (A and C) or 48 h (B and D) with varying concentrations of H_2O_2 (0, 40, 100 and 200 μM) in the absence or presence of LPS (1.0 μg/ml). Cells were harvested in mild lysis buffer and 50 μg of total protein from each treatment group was subjected to 10% SDS-PAGE electrophoresis, transferred to a PVDF membrane and probed with anti-IκBα (sc-371) (A and B), anti-p50 (sc-114) (C and D) or anti-β-actin antibody (A–D). The immunoreactive bands for IκBα and ΔN-IκBα (~ 34 kDa band) are indicated by arrows.

not shown). Additionally, there was a similar but much lighter band with the 40 μM H_2O_2 treatment which was comparable in intensity to the respective 0 μM H_2O_2 control (Figures 8A and B). This ~ 34 kDa band may represent ΔN-IκBα, a caspase-mediated cleavage product of IκBα. Interestingly, ΔN-IκBα has been shown to act as a degradation resistant form of IκBα and to inhibit NFκB/DNA interactions and NFκB-mediated transcriptional activity [31]. Furthermore, the expression of ΔN-IκBα appears to be stronger at both the 24 and 48 h time points in all treatment groups with LPS stimulation (Figures 8A and B). Moreover, expression of the ΔN-IκBα is greater in the LPS co-treatments with higher concentrations of H_2O_2 (100 and 200 μM) compared to a 40 μM H_2O_2 concentration or LPS alone (Figures 8A and B). Notably, a prominent anti-IκBα immunoreactive band appeared at 48 h and inconsistently at 24 h in the non-activated 100 and 200 μM H_2O_2 treatments which migrated between 17.5–29.4 kDa (Figures 8A and B). IκBα cleavage products of this size have not been well characterized; however, the appearance of this band corresponds with the decreased cell viability seen in non-activated cells treated with 100 and 200 μM H_2O_2, contrasting with the more viable 0 and 40 μM H_2O_2 treatments.

In addition to IκBα, we also evaluated the expression levels of the p50 NFκB sub-unit due to its potential to form inhibitory p50/p50 homodimers. Interestingly, p50 expression was noticeably reduced at 24 h and markedly reduced at 48 h in response to LPS activation which was not significantly altered by the 40 μM H_2O_2 co-treatment (Figures 8C and D). In contrast, p50 protein levels were clearly greater in the LPS-activated cells co-treated with 100 and 200 μM H_2O_2

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at both 24 and 48 h (Figures 8C and D). Taken together, these results suggest that higher concentrations of H_2O_2 can potentially result in a delayed, concentration-dependent increase in p50 and IκBα levels (LPS-activated cells only) as well as an increase in ΔN-IκBα levels. Based on previous studies, the 34 κDa ΔN-IκBα protein can serve as a degradation resistant inhibitor of NFκB [31] and its appearance at 24 h temporally correlates with the concentration-dependent decrease in reporter activity by H_2O_2 (compare Figures 8A and 7). If the combined effect of increased IκBα and ΔN-IκBα and p50 are indeed mediating the inhibitory effect on the 3'*Igh*RR and 3x-NFκB reporters, the concentration-dependent and temporal effect of H_2O_2 on reporter activity and IκBα expression may define the balance between enhancing and inhibitory signals on reporter activity.

Discussion

We have previously identified chemical-induced modulation of 3'*Igh*RR activity that mirrored the effects on endogenous heavy chain mRNA and protein expression [22,28,29,32]. Similarly, the current study demonstrated an H_2O_2-induced concentration-dependent and biphasic effect on LPS-mediated 3'*Igh*RR reporter activity that highly correlated with the effects of H_2O_2 on LPS-induced *μIgh* RNA transcript production (i.e. functional endpoint of endogenous *Igh* transcription). These results also correlated with the biphasic effect of H_2O_2 on LPS-induced Ig protein levels ([22] and data not shown). Therefore, ROI appears to target *Igh* transcriptional activity through the 3'*Igh*RR. There are a host of sources for ROI, including IgM biosynthesis, other cells in the inflammatory process, xenobiotics, ionizing radiation or pharmacologic agents such as the redox cycling anthracyclines (reviewed in [1] and [33]), all of which could target the 3'*Igh*RR and modulate antibody production and B-lymphocyte function.

We further investigated the mechanisms by which ROI (i.e. H_2O_2) exposure can modulate *Igh* transcriptional activity through the 3'*Igh*RR. In contrast to the concentration-dependent biphasic effect of H_2O_2 on 3'*Igh*RR reporter expression and on endogenous *μIgh* transcript levels, the V_H reporter only exhibited an inhibitory effect. These differences suggest that 3'*Igh*RR regulation confers not only a higher concentration-dependent threshold for H_2O_2-mediated inhibition but an enhancement of transcriptional activity at low H_2O_2 concentrations which likely leads to the previously demonstrated enhancement of endogenous Ig protein levels at low H_2O_2 concentrations [22]. However, it should also be noted that the activation of 3'*Igh*RR by H_2O_2 in the absence of stimulation did not appear to translate into an effect on endogenous *μIgh* transcription which is not surprising considering the markedly lower basal activity of the 3'*Igh*RR reporter compared to the LPS-induced activity. Therefore, a stimulatory signal in addition to H_2O_2 exposure is likely required to reach a threshold of 3'*Igh*RR activation to induce transcriptional effects on endogenous *Igh* transcription. Such a threshold effect was also observed in a sub-clone of the Jurkat T-cell line which required co-stimulation with phorbol myristate acetate for 30–50 μM H_2O_2 to enhance NFκB-mediated expression [5].

The ability of IκBαAA expression to suppress the H_2O_2-mediated enhancement of the 3'*Igh*RR and hs4 reporters suggests a significant role for NFκB which is further supported by an H_2O_2-mediated enhancement of the 3x-NFκB reporter and inhibition of this enhancement by IκBαAA expression. A significant role of NFκB corroborates a host of studies demonstrating that a wide concentration range of H_2O_2 can induce NFκB activation. Studies with lymphocytes and HeLa cells have demonstrated H_2O_2-induced NFκB DNA binding and/or NFκB-mediated transcriptional activity with H_2O_2 concentrations (30–60 μM) similar to those used in our study [4,34].

Interestingly, the biphasic effect of H_2O_2 on reporter activity and on endogenous *μIgh* expression was limited to the later time point (i.e. 48 h vs only inhibition at 24 h) which was also the ideal time frame for optimal LPS-induced 3'*Igh*RR transcriptional activity. Delayed activation is consistent with the requirement of 2–5 days to achieve optimal Ig secretion in LPS-activated B lymphocytes [35]. The mechanism of this delayed activation may involve a prolonged activation of NFκB/Rel proteins which has been shown to persist for up to 24 h (later time points were not examined) in pre-B cells and immature dendritic cells treated with LPS [36,37]. Furthermore, following the initial rapid activation of p50/RelA NFκB dimers, by 24 h the NFκB proteins p52, RelB and c-Rel were also activated [36]. Krappman et al. [37] also demonstrated a delayed, NFκB-dependent activation of AP-1 and Oct-2 which appeared to be necessary for the NFκB-dependent activation of Igκ light chain; the authors postulated that rapid induction of the p50/RelA NFκB dimer was important to immediate early gene induction, but the delayed activation of the p52/RelB NFκB dimer and of AP-1 and Oct-2 are required to maintain high expression levels of persistently activated genes such as the Igκ light chain [36,37]. Since previous studies have demonstrated a prominent role of NFκB, Oct and AP-1 in the activation of the 3'*Igh*RR [15,38], a similar mechanism as described above for the Igκ light chain may be mediating the delayed NFκB-dependent activation of the 3'*Igh*RR and endogenous *μIgh* as seen in the current study with LPS-activated CH12IκBαAA cells.

Since NFκB activation canonically occurs with rapid (30 min to 2 h) proteolytic degradation of IκBα [30], NFκB activation in the presence of renewed and

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sustained endogenous IκBα expression at the 24 and 48 h time points appears paradoxical. However, IκBα is up-regulated by activation of NFκB and, as mentioned above, a prolonged activation of NFκB/Rel proteins that was sensitive to inhibition by IκBαAA, was demonstrated up to 24 h in pre-B cells and immature dendritic cells treated with LPS despite endogenously expressed IκBα [36,37]. This effect may relate to a delayed activation of other NFκB dimers and NFκB-dependent transcription factors that are only sensitive to IκBα inhibition during the early, transient NFκB activation phase [36,37]. Interestingly, tyrosine phosphorylation of IκBα has been found to decrease its association with RelA in the absence of IκBα degradation [39]. Likewise, H_2O_2 was shown to induce tyrosine phosphorylation of IκBα in the Jurkat T-cell line as well as nuclear translocation of RelA without IκBα degradation, thus potentially explaining how endogenous IκBα expression could occur simultaneously with NFκB activation [9].

Results with the 3x-NFκB reporter and expression of the IκBαAA strongly support a role of IκBα-regulated NFκB proteins in the H_2O_2-mediated enhancement of the reporters, but the mechanism behind the inhibitory effect is less clear. Consistent with the previously reported inhibition of CD40-induced IgM expression by 100 μM H_2O_2 [10], inhibition of V_H, 3'*Igh*RR and hs4 reporter activity at 24 and 48 h was maximal at the 100 and 200 μM treatments and was most pronounced at the 24 h time point. Interestingly, LPS abrogated the inhibitory effect of the 100 and 200 μM H_2O_2 treatments on 3'*Igh*RR and hs4 reporter activity at 48 h and to a lesser extent at 24 h. However, H_2O_2 concentrations greater than 50 μM reversed the enhancing effect of 40 μM H_2O_2 and also resulted in a decrease of H_2O_2-enhanced and LPS-induced Ig expression. The mechanism behind the H_2O_2-mediated reduction in reporter activity and Ig expression is likely multi-faceted and may involve a combination of increased IκBα expression, expression of ΔN-IκBα and decreased cellular viability. Additionally, the inhibition of the unregulated (V_H) and regulated (3'*Igh*RR and hs4) promoter by H_2O_2 at 24 h suggests a common mechanism of action. We speculate that the V_H-promoter is directly targeted for the inhibitory effect, whereas the transcription factor milieu induced by low concentrations of H_2O_2 induces enhancer activity that overrides the inhibitory effect on the V_H-promoter (results summarized in Table I). It is also important to note that the 3'*Igh*RR has been shown to physically interact with the V_H-promoter, creating a unique promoter/enhancer DNA complex that regulates *Igh* transcription [40]. Therefore, the effects on the V_H may be artificial due to the absence of the 3'*Igh*RR and may not be relevant in a functional 3'*Igh*RR-regulated *Igh* locus. Additionally, the initial inhibitory effects of H_2O_2 observed at 24 h may involve p50/p50 homodimer formation, which is substantiated by a study demonstrating that low μM H_2O_2 treatments of a Jurkat T-cell line resulted in protein binding to κB motifs within the κ light chain enhancer that was entirely composed of p50 [4]. Correspondingly, our studies with LPS-activated cells demonstrate increased p50 protein levels relative to the LPS control with the 100 and 200 μM H_2O_2 treatment at 24 and 48 h. Therefore, an H_2O_2-mediated induction or stabilization of p50 may contribute to the inhibitory effect on reporter activity and Ig expression due to the well-established inhibitory effect of p50 homodimers on transcription.

The wide array of potential oxidative forces that can arise from chemical exposure, drug treatment regimens and components of the physiological and pathophysiological immune responses may significantly modulate Ig expression through NFκB regulation of the 3'*Igh*RR and ultimately affect local or systemic humoral immune function. Furthermore, the 3'*Igh*RR has been associated to date with several human immune-related disorders including Burkitt's lymphoma and rheumatoid arthritis [17,18]. Moreover, these same diseases have been associated with oxidative stress and ROI. For example, exogenous or induced H_2O_2 has been found to modulate cell growth and viability of Burkitt's lymphoma cells *in vitro* [19]. Additionally, a decrease in intracellular reductants

Table I. Summary of H_2O_2-mediated transcriptional activity.

		Non-activated		LPS-activated	
		Low $[H_2O_2]$[b]	High $[H_2O_2]$	Low $[H_2O_2]$	High $[H_2O_2]$
Transcriptional activity	24 h	↓ 3'*Igh*RR[a] ↓ hs4 ↓ V_H ↓ NFκB	↓ 3'*Igh*RR ↓ hs4 ↓ V_H ↓ NFκB	− 3'*Igh*RR ↓ hs4 ↓ V_H ↓ NFκB	↓ 3'*Igh*RR ↓ hs4 ↓ V_H ↓ NFκB
	48 h	↑ 3'*Igh*RR − hs4 ↓ V_H ↓ NFκB	↓ 3'*Igh*RR ↓ hs4 ↓ V_H ↓ NFκB	↑ 3'*Igh*RR ↑ hs4 − V_H ↑ NFκB	↓ 3'*Igh*RR ↓ hs4 ↓ V_H ↓ NFκB

[a]Activity (↑, increased; ↓, decreased; −, no change) of luciferase reporters.
[b]Low $[H_2O_2]$, ~ 30–50 μM H_2O_2; High $[H_2O_2]$, ~ 100–200 μM H_2O_2.

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(e.g. the reduced form of glutathione-GSH) has been identified in lymphocytic cells of rheumatoid arthritis patients [20]. Inappropriate 3'*Igh*RR-mediated increases in Ig expression initiated by H_2O_2 or other forms of ROI could be involved in the initiation and progression of autoimmune-related diseases or lymphomas, whereas inhibition of Ig expression may lead to inadequate immunity against pathogens. Understanding ROI's capacity to alter *Igh* expression and potentially Ig-related immune responses will contribute to our understanding of how the exposure to a variety of ROI or ROI-producing compounds may affect immune function and ultimately human health.

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Declaration of interest

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