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REDOX REGULATION OF NF-κB ACTIVATION BY HYDROGEN PEROXIDE AND EFFECTS ON GENE EXPRESSION

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Resumo

O peróxido de hidrogénio (H_2O_2) é uma molécula pequena que difunde com relativa facilidade através das membranas. Trata-se de um oxidante relativamente fraco, reagindo especificamente com determinados alvos moleculares, nomeadamente com os resíduos de cisteína reactivos das proteínas. Devido a estas propriedades, o H_2O_2 é uma das espécies reactivas de oxigénio (ERO) com potencialidade para participar em vias de sinalização. O hipotético desencadeamento de uma via de sinalização, ou a sua modulação, ocorre com um aumento temporário das concentrações de H_2O_2 que pode ocorrer, por exemplo, durante a resposta inflamatória. Esta resposta é um mecanismo fisiológico desencadeado face a uma lesão e tem como característica o aumento local de citocinas pro-inflamatórias, quimiocinas e leucócitos (neutrófilos e macrófagos). A formação de ERO pelos leucócitos é crucial para a remoção de patogéneos invasores. O H_2O_2 é um intermediário de uma espécie bastante reactiva, o ácido hipocloroso, cuja reacção é catalisada pelo mieloperoxidase. Contudo, parte do H_2O_2 formado consegue difundir-se e atingir células da vizinhança, como células endoteliais e epiteliais. Uma vez estimuladas, células epiteliais produzem mediadores importantes para o processo, apresentando um papel activo na resposta inflamatória. Esta resposta é garantida pela activação do factor de transcrição NF-κB.

O NF-κB constitui um elemento fundamental para a resposta inflamatória, imunidade inata e adaptativa, dado que regula a expressão de genes que codificam para citocinas, quimiocinas, factores de crescimento, moléculas de adesão celular e imunoreceptores. Para além disso, o NF-κB também regula a expressão de genes relacionados com a apoptose e proliferação celular. A família NF-κB/Rel é composta por diversas proteínas (p65, c-Rel, RelB, p50 e p52) que se podem complexar formando homo ou heterodímeros. O heterodímero mais comum é constituído pelas subunidades p50/p65, sendo por vezes denominado de NF-κB clássico, embora outros dímeros também sejam relevantes nas células. Em células não estimuladas, o NF-κB mantém-se latente no citosol pela ligação a proteínas inibitórias: IκBs, as quais são degradadas face a um estímulo adequado levando à libertação do NF-κB e translocação para o núcleo. A activação dos genes alvo ocorre por intermédio de locais κB existentes nas regiões promotoras/*enhancers* dos mesmos, cuja sequência de *consensus* para o NF-κB clássico é GGGRNNYYCC (R para purina, Y para pirimidina e N para qualquer base).

Desde 1991 que foi estabelecida uma relação entre o H_2O_2 e o NF-κB, propondo o H_2O_2 como um mensageiro comum da via do NF-κB. Esta hipótese não foi validada e neste momento é certo que o H_2O_2 participa na via de activação do NF-κB, não sendo contudo o seu papel consensual. Existem trabalhos que referem que o NF-κB é activado na presença de H_2O_2, cuja resposta é mais lenta do que a observada com indutores clássicos, e outros que mostram uma inibição da via por acção nos vários elementos envolvidos. Assim, colocou-se como hipótese de trabalho que a razão para as observações contraditórias sobre o papel do H_2O_2 na via do NF-κB têm origem, em grande parte, na metodologia de exposição das células a este oxidante. Usualmente, as células são sujeitas a uma adição única e inicial de H_2O_2 – adição *bolus* – e devido ao seu consumo catalisado pelos enzimas antioxidantes torna-se necessário recorrer a uma concentração de H_2O_2 mais elevada (tipicamente 100 μM – 1 mM) para se conseguir observar efeitos sinalizadores. Todavia, este excesso de H_2O_2 pode estar na origem de danos oxidativos adicionais nos componentes celulares e consequentemente desencadear outros processos celulares envolvidos na defesa antioxidante, mascarando o hipotético potencial sinalizador e fisiológico do H_2O_2 na via do NF-κB. Mais, não é conhecida a concentração real que está a produzir os efeitos observados. Deste modo, o objectivo global deste projecto baseou-se no estudo da activação do NF-κB em condições que mimetizam a formação fisiológica de H_2O_2 durante a resposta inflamatória. Utilizou-se o método do estado estacionário (s.s.) que se baseia numa adição inicial da concentração desejada de H_2O_2 conjuntamente com o enzima glucose oxidase numa actividade adequada, o qual vai produzir continuamente H_2O_2 ao longo do tempo e contrabalançar o consumo de H_2O_2 pelas células, mantendo uma concentração estacionária de H_2O_2. O s.s. é um sistema calibrado e controlado, sendo possível utilizar doses mais baixas de H_2O_2, mantendo-as constantes durante o tempo de ensaio pretendido.

No capítulo II é feito um estudo comparativo das várias metodologias de exposição das células ao H_2O_2 na activação do NF-κB, medidos pelos níveis nucleares da subunidade p65 do NF-κB, a qual contém um domínio de transactivação do DNA. Em duas células epiteliais, MCF-7 e HeLa, observou-se que 25 μM de H_2O_2 em s.s. não é capaz de activar a via do NF-κB. Contudo, quando adicionado em simultâneo com o TNF-α, um indutor clássico, o H_2O_2 aumentou a translocação do NF-κB para o núcleo. Em oposição, a adição *bolus* de 1 mM de H_2O_2 levou a uma diminuição da translocação induzida pelo TNF-α, demonstrando a importância do método de adição de H_2O_2 às células e validando o método do s.s. O aumento

de NF-κB no núcleo pelo H_2O_2 s.s. levou também a um aumento da expressão de citocinas pro-inflamatórias (e.g. IL-8, MCP-1 and TNF-α), mas também de mediadores anti-inflamatórios (HO-1 e IL-6), indicando um papel duplo do H_2O_2 na regulação do NF-κB.

A activação do NF-κB e a sua translocação para o núcleo pressupõe a degradação das subunidades inbitórias IκBs pelo proteassoma 26S. Assim, o aumento de p65 nuclear induzido pelo H_2O_2 foi abordado ao nível dos IκBs, cujas proteínas maioritárias são o IκB-α, IκB-β e IκB-ε (capítulo III), tendo-se também analisado os níveis da subunidade c-Rel que pode formar homodímeros e heterodímeros com a p50 e a p65 e que contém um domínio de transactivação. Nas células MCF-7, a adição simultânea de H_2O_2 e TNF-α levou a uma forte e sustentada degradação do IκB-α, estando de acordo com o aumento de translocação das subunidades p65 e c-Rel para o núcleo. Este aumento de degradação do IκB-α resultou do aumento da actividade do proteassoma 26S observado na presença simultânea de H_2O_2 e TNF-α, o qual é provavelmente devido a uma modificação oxidativa. O IκB-β não respondeu à presença de H_2O_2. Contudo, para tempos de incubação curtos, os níveis de IκB-ε no citosol aumentaram na presença de H_2O_2, embora não se tenham observado efeitos nos níveis de p65 e c-Rel. Nas células HeLa tratadas com H_2O_2 e TNF-α, observou-se um aumento de degradação do IκB-α quando comparado com tratamento com TNF-α sózinho, o qual não resultou do aumento da actividade do proteassoma, indicando que existe outra possível regulação da via do NF-κB pelo H_2O_2. Contrariamente às células MCF-7, o H_2O_2 não estimulou a translocação da c-Rel para o núcleo; pelo contrário, a tempos curtos inibiu o efeito do TNF-α. Curiosamente esse efeito encontra-se de acordo com o aumento dos níveis de IκB-ε, indicando a existência de complexos c-Rel-IκB-ε nas células HeLa. A p65 e a c-Rel têm afinidades diferentes para regiões κB de genes alvo diferentes e consequentemente, dependente do tipo de célula, a expressão de genes com locais κB com mais afinidade para a c-Rel encontra-se favorecida (células MCF-7) ou inibida (células HeLa).

Os resultados anteriores indicaram que o aumento de p65 e, eventualmente, c-Rel no núcleo devido à adição de H_2O_2 a células tratadas com TNF-α podem estar na origem de uma activação preferencial de alguns genes, em detrimento de outros. Deste modo, colocou-se a hipótese desta indução selectiva estar relacionada com a afinidade das sequências κB para o NF-κB, dado que a mudança de apenas um resíduo de aminoácido na sequência de *consensus* pode levar a afinidades bastante diferentes (capítulo IV). Células HeLa foram transfectadas

com plasmídeos contendo três diferentes regiões κB, acoplados ao gene da luciferase. A expressão da luciferase em resposta a aumentos de concentrações de TNF-α foi distinta para os três plasmídeos, definindo-se uma região de alta (maior expressão de luciferase), média e baixa afinidades. Com a adição simultânea de H_2O_2, observou-se um aumento da expressão da luciferase em células transfectadas com o plasmídeo de baixa afinidade, mesmo para concentrações elevadas de TNF-α (limite máximo 25 ng mL^{-1}). Este estímulo foi observado no plasmídeo de média afinidade, mas para concentrações de TNF-α inferiores (limite máximo de 2 ng mL^{-1}), diminuindo esse limite de concentração de TNF-α para o plasmídeo de alta afinidade (0,08 ng mL^{-1}). Elaborou-se um modelo matemático que reproduz as observações experimentais e que mostra que o aumento de NF-κB nuclear induzido pelo H_2O_2 é importante para genes com baixa afinidade para o NF-κB, dado que genes de alta afinidade já têm as regiões κB ocupadas pela indução causada pelo TNF-α, sendo portanto insensíveis ao efeito do H_2O_2.

Em conclusão, demonstrou-se que o H_2O_2 apresenta um papel duplo na regulação da inflamação, pela modulação selectiva da expressão de genes pro- e anti-inflamatórios, dependente da linha celular.

Palavras Chave:

- NF-κB
- Peróxido de hidrogénio
- Inflamação
- Regulação redox
- Expressão génica

Summary

Evidences suggest that hydrogen peroxide (H_2O_2) is implicated in the regulation of the transcription factor NF-κB. However, no consensus exists regarding the role played by H_2O_2 since both inhibitory and stimulatory effects have been reported. It was hypothesized that these contradictory data are due to the methodology used to expose cells to H_2O_2. Usually, H_2O_2 is added at the beginning of the experiment – bolus addition – in high concentrations of H_2O_2 needed because of its rapid consumption by cells. Therefore the real [H_2O_2] that is exerting the effects is not known and the high initial dose masks a signaling role for H_2O_2 and often causes elevated oxidative damages. In this work a different methodology was used – the steady-state (s.s.) titration whereby the use of glucose oxidase allows cells to be exposed to constant, lower and nearer to the physiological H_2O_2 concentrations.

NF-κB is a key regulator of the inflammatory process, innate and adaptive immunity. In this work [H_2O_2] in s.s., similar to those generated at inflammation sites, modulated positively NF-κB activation induced by the pro-inflammatory cytokine TNF-α. H_2O_2 increased the degradation of IκB-α, the major inhibitor of NF-κB and, in MCF-7 cells, this caused higher levels of nuclear NF-κB-containing p65 and c-Rel proteins. In HeLa cells, c-Rel proteins were retained in the cytosol probably by IκB-ε. The higher NF-κB levels in the nucleus induced by H_2O_2 and TNF-α together were predicted to be important for the expression genes with low/medium-affinity κB sites, which is in accordance with the selective gene expression observed. In MCF-7 and HeLa cells treated with TNF-α, H_2O_2 up-regulated both pro- and anti-inflammatory NF-κB-dependent genes. Therefore, it was proposed a dual regulatory role for H_2O_2 in inflammation by simultaneously exacerbating inflammation through higher levels of pro-inflammatory mediators and by attenuating possible adverse effects through induction of anti-inflammatory genes expression.

Keywords:
- NF-κB
- Hydrogen peroxide
- Inflammation
- Redox regulation
- Gene expression

Abbreviation list

AP-1 – Activation protein-1

ASK1 – Apoptosis signal-regulating kinase 1

BAFF – B-cell activating factor

BCL-3 – B-cell lymphoma 3

BCR – B-cell receptor

cAMP – 3'-5'-cyclic adenosine monophosphate

CBP – CREB binding protein

CGD – Chronic granulomatous disease

ChIP – Chromatin Immunoprecipitation

CKII – Casein kinase II

CO – Carbon monoxide

COPD – Chronic obstructive pulmonary disease

COX-2 – Cyclooxygenase-2

CREB – cAMP response element-binding

CYLD – Cylindromatosis

DD – Death domain

DMSO – Dimethyl sulfoxide

DTT – Dithiothreitol

ECM – Extracellular matrix

Egr-1 – Early growth reponse-1

ELC – Epstein-Barr virus-induced molecular 1 ligand

ERK – Extracellular signal-regulated protein kinase

GPR89 – G-protein coupled receptor 89

GPx – Glutathione peroxidase

GSH – Glutathione, reduced form

GSK3β Glycogen-synthase 3β

GSSG – Glutathione, oxidized form

H_2O_2 – Hydrogen peroxide

$[H_2O_2]_{ss}$ – H_2O_2 in steady-state

HAT – Histone acetyltransferase activity

HDAC – Histone deacetylase

HIV-1 – Human immunodeficiency virus type 1

HLH – Helix-loop-helix

HO-1 – Heme oxygenase 1

$HO^{\bullet}$ – Hydroxyl radical

HSF-1 – Heat shock factor 1

HSP – Heat shock proteins

ICAM-1 – Intracellular adhesion molecule 1

IFN – Interferon

IκB – Inhibitor of NF-κB

IKK – IκB-Kinase

IKAP – IKK complex-associated protein

IL-1 – Interleucine 1

iNOS – Inducible nitric oxide synthase

IRAK – IL-1-receptor-associated kinase

JNK – c-Jun N-terminal kinase

κBl – Low-affinity κB site

κBm – Medium-affinity κB site

κBh – High-affinity κB site

LMB – Leptomycin B

LPS – Lipopolissacharide

LRR – Leucine-rich repeats

LTβR – Lymphotoxin β receptor

LZ – Leucine zipper

MALT – Mucosa-associated lymphoid tissue

MAPK – Mitogen-activated protein kinase

MCP-1 – Monocyte chemotactic protein 1

MEF – Mouse embryonic fibroblasts

MEKK – MAP/ERK kinase kinase

MSK – Stress-activated protein kinase

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MYD88 – Myeloid differentiation primary response gene 88

NAC – *N*-acetyl-L-cysteine

NADH – Nicotinamide adenine dinucleotide, reduced form

NAD^+ – Nicotinamide adenine dinucleotide, oxidized form

NADPH – Nicotinamide adenine dinucleotide phosphate, reduced form

$NADP^+$ – Nicotinamide adenine dinucleotide phosphate, oxidized form

NAK – NF-κB-activating kinase

NEMO – NF-κB essential modulator

NF-κB – Nuclear factor-κB

(NF-κB|κB) – NF-κB and κB site complex

NIK – NF-κB inducing kinase

NOX – NADPH oxidase

NOX_{ph} – Phagocyte NOX

NRF-2 – NF-E2-related factor-2

O_2 – Dioxygen

$O_2^{\bullet -}$ – Superoxide radical

p38 – Mitogen-activated protein kinase

PAMPS – Pathogen-associated molecular patterns

PBS – Phosphate buffer saline

p/CAF – p300/CBP associated factor

PDTC – Pyrrolidine dithiocarbamate

PI3K – Phosphaditylinositol 3-kinase

P-IκB-α – Phosphorylated form of IκB-α

PKA – cAMP-dependent protein kinase

PKC – Protein kinase C

PMA – Phorbol 12-myristate 13-acetate

PMSF – phenylmethylsulphonyl fluoride

PP2A – Protein phosphatase 2A

Prx – Peroxiredoxin

RHD - Rel-homology domain

RIP – Receptor interacting protein

ROS – Reactive oxygen species

RSH – Thiol

RSK – Ribosomal subunit kinase

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SH – Sulfhydryl group

SI – Supplementary information

SLC – Secondary lymphoid tissue chemoline

SOD – Superoxide dismutase

SODD – Silencer of death domain

SRC-1 – Steroid co-activator-1

SS – Disulfide bond

s.s. – Steady-state

TAB – TAK-binding protein

TAD – Transactivation domain

TAK – TGF-β-activated kinase

TCR – T-cell receptor

TGF-β – Transforming growth factor-β

TICAM TIR-domain-containing adaptor molecule

TIR – Toll/IL-1 receptor

TIRAP – TIR-containing adaptor protein

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor-α

TNF-R – TNF-α receptor

TRAF – TNF-R associated factor

TRAM – TRIF-related adaptor molecule 2

TRIF – TIR-containing adaptor inducing IFNβ

Trx – Thioredoxin

TSA – Trichostatin A

UV – Ultraviolet light

Nucleotide abbreviations:

G – Guanine

C – Cytosine

T – Thymine

A – Adenine

N – Any nucleotide

Y – Pyrimidine

R – Purine

Three-letter amino acid code

Lys – Lysine

Ser – Serine

Cys - Cysteine

Chapter I - General Introduction

Part 1 - The NF-κB pathway

1. Prologue

In 1986, the transcription factor nuclear factor-κB (NF-κB) was discovered by Sen and Baltimore[1] in B cells. It was named also according to its nuclear localization and binding to elements of the enhancer of the κ-light chain of immunoglobulin gene. Independent studies of the dorsal protein from *Drosophila melanogaster*[2], the proto-oncogene c-Rel and its retroviral counterpart V-Rel[3] soon related these proteins with each other and it was found that they belong to the same family: the NF-κB or Rel family[4]. These proteins share highly conserved similarities at the N-terminal side, named the Rel-homology domain (RHD) but are almost completely divergent within the C-terminal side, which could attribute specific functions to the proteins. NF-κB was identified as a heterodimer, with the p50 subunit as the common element. p50 could bind to c-Rel, but also to another protein, p65 (RelA) to which an important role was attributed when it was identified that the complex NF-κB/DNA observed in electrophoretic mobility shift assays (EMSA) was the p50/p65/DNA complex[5]. At the time, it had already been shown that NF-κB was present in other cell types and was not restricted to B-cells, but in those other cell types it was localized in the cytosol and phorbol esters could lead to its translocation to the nucleus with the consequent binding to the target enhancers. These findings directed to the discovery of the NF-κB inhibitory proteins (IκBs), whose function is to keep NF-κB sequestered in the cytosol[6,7]. The target enhancers/promoters of NF-κB included genes such as interleucine 2 (IL-2), IL-6, intracellular adhesion molecule 1(ICAM-1) and class I MHC, all of which involved in the inflammatory and immune response[8]. The importance given to NF-κB increased as it was shown to respond to typical immunological stimuli, such as tumor necrosis factor (TNF-α), IL-1 and lipopolysaccharide (LPS). In mammals, it became clear that NF-κB is an inducible factor with a specific regulation and a central role in the inflammatory and immune response. Moreover, NF-κB activation does not require new protein synthesis allowing a fast response in the presence of an inducer[8,9].

2. The Rel/NF-κB proteins

It is possible to divide the mammalian NF-κB family in two subfamilies: the Rel proteins and the NF-κB proteins (Figure 1). The Rel subfamily includes c-Rel, RelB and p65 (RelA)[10-12]. They all contain C-terminal transactivation domains (TADs) to initiate transcription, which often are not conserved between species[10-12]. The NF-κB subfamily includes NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100). The RHD, which is present in all NF-κB/Rel proteins, has approximately 300 amino acids and contains two Ig-like folds connected by a flexible linker region that binds to DNA. The N-terminal loops are responsible for sequence-specific recognition and the C-terminal loops are responsible for dimerization with other Rel/NF-κB proteins. Dimerization is mediated by extensive hydrophobic interactions along the interface surface, which is formed by a three-stranded β-sheet packing against a similar sheet in the opposite molecule[9].

The NF-κB1 p105 and NF-κB2 p100 contain multiple copies of ankyrin repeats at their C-terminal end and can retain Rel proteins in the cytosol. Proteolytic processing or, eventually arrested translation, produces the DNA-binding proteins p50 and p52, respectively[10,11].

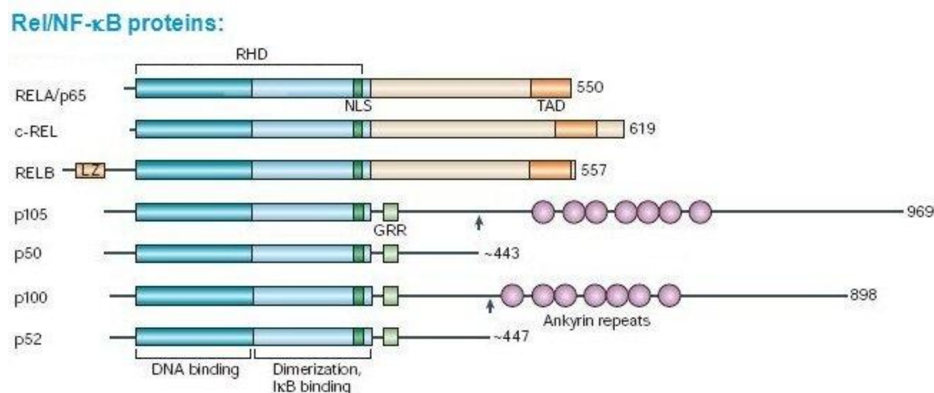


Figure 1 – Schematic representation of the Rel/NF-κB family. Rel/NF-κB proteins all contain the Rel-homology domain (RHD), whose N-terminal portion interacts with the κB enhancers of the DNA and whose C-terminal mediates dimerization with other Rel/NF-κB proteins and binding with the IκBs that mask the nuclear localization signal (NLS). Only Rel proteins possess the C-terminal transactivation domain (TAD) capable of activating transcription. The NF-κB proteins p105 and p100 contain the ankyrin repeats and, after proteolytic processing, originate p50 and p52, respectively. The glycine-rich regions (GRR) are important for the processing. LZ – Leucine zipper. Adapted from[12].

Although it is not certain whether additional Rel/NF-κB genes occur in mammalian genomes, no new members have been identified yet. In *Drosophila* three Rel proteins have been identified: Dorsal (controls the dorsoventral polarity), Dif and Relish. Interestingly, Dif appears to be the fly equivalent of the p65 protein because it is inducible active and up-

regulates transcription of insect immunity genes[13]. So, probably, not only NF-κB proteins, but also their signaling system is evolutionary conserved. Vertebrates and insects belong to the bilaterians (bilateral symmetry) and because the RHD is an evolutionary conserved sequence, researchers have attempted to find NF-κB-like proteins in ancient phylogeny organisms to give new insights about evolution. It was recently shown the presence of RHD-containing genes in the *Nematosstella Vectensis* (Nv), a sea anemone (radial symmetry)[14]. Nv-NF-κB is very similar to vertebrates NF-κB proteins, but lacks the C-terminal ankyrin repeats. The sponges are ancient organisms (asymmetrical) and an ancestral NF-κB was identified in the demosponge *Amphimedon queenslandica*[15]. NF-κB is expressed during embryogenesis of the demosponge, and so a role in development is plausible. Interestingly, this ancient NF-κB possesses ankyrin repeats, meaning that the bilaterians lost the ankyrin repeats of NF-κB during evolution[15].

Active NF-κB is a dimer and hypothetically, Rel and NF-κB proteins can all form dimers with each other, originating homo and heterodimers, but observations lead to the conclusion that certain dimers do not exist. As already referred, the dimer p50/p65 was the first one to be identified and it is the most abundant in the majority of cell types[8,9]. However, in hematopoietic cells and lymphocytes c-Rel is expressed in higher levels than p65[11]. RelB dimerizes only with p50 and p52; p50 homodimers are quite abundant, but as they do not contain the TAD they can function as inhibitors of transcription[16,17].

Since p50 is present in different NF-κB complexes it was predicted that p50 knockout would be lethal. Nevertheless, mice lacking the p50 protein develop normally although they exhibit defects in immune responses involving B cells: B cells cannot differentiate in response to LPS and the production of antibodies is impaired. Interestingly the antiviral IFN-β is up-regulated in these conditions suggesting a repressive role of p50 in its expression, probably through p50 homodimers. In contrast, p65 null mice exhibit a dramatic phenotype-embryonic lethality due to extensive liver apoptosis, reflecting the crucial importance of this subunit[8,13].

c-Rel knockout mice develop normally, but T and B cells are not responsive to certain mitogenic stimulations. The absence of RelB shows defects in immune function and hematopoiesis with a severe phenotype including thymic atrophy, splenomegaly and bone marrow hyperplasia. The different phenotypes resulting by the lack of different Rel/NF-κB proteins helped to understand their specific roles in different cellular processes and specially

that the absence of one subunit cannot be completely complemented by the other family members[8,13].

The heterodimer p50/p65 is the most abundant form of NF-κB and its activation pathway is the best understood so, generally, the term NF-κB refers to the heterodimer p50/p65.

3. The IκB proteins

The IκB proteins bind to NF-κB and prevent the latter to translocate to the nucleus and bind to DNA. The IκB family is composed by IκB-α, IκB-β, IκB-ε, IκB-γ and BCL-3 (B-cell lymphoma 3) (Figure 2). The precursors NF-κB1 p105 and NF-κB2 p100 can be included in the group because they all possess the typical ankyrin repeats. *Drosophila* contains a similar protein called cactus. The six or seven ankyrin repeats are in a helical conformation and mediate the binding to the C-terminal Ig-like folds of the RHD of Rel/NF-κB proteins masking their nuclear localization signal (NLS) and therefore preventing their translocation to the nucleus[9]. IκB-α and IκB-β associate predominantly with p50/p65 and p50/c-Rel heterodimers, whereas IκB-ε interacts only with p65- and c-Rel-containing complexes[13]. BCL-3 is a IκB-like protein predominantly located in the nucleus that interacts with both p50 and p52 homodimers[13]. It is a controversial protein because it has been shown that it could both activate[18,19] or inhibit[17,19] transcription when bound to the p50 or p52 homodimers. Little is known about IκB-γ, which is detected only in lymphoid cells and its sequence is identical to the C-terminal region of p105. Its role is probably limited to the binding and inhibition of p50 or p52 homodimers[13,20].

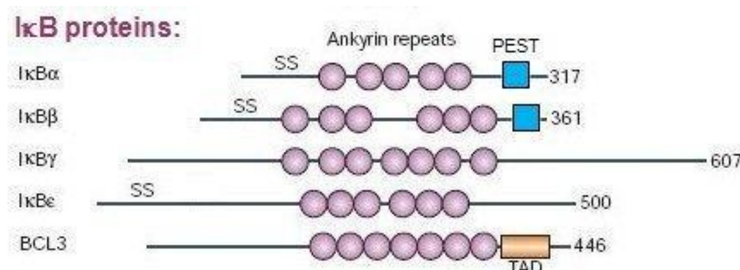


Figure 2 – The IκB family of proteins. The common characteristic of the IκB proteins is an ankyrin-repeat domain, which mediates the assembly with Rel proteins and masks the nuclear localization signal (NLS) of NF-κB. Phosphorylation of two serine residues (SS) triggers degradation of the IκBs and frees NF-κB. BCL-3 is included in this family because of the ankyrin repeats although it has transcriptional-activation properties. The PEST (proline, glutamic acid, serine and threonine) region allows a fast constitutive degradation of IκB-α and IκB-β. Adapted from[12].

IκB-α, followed by IκB-β were the first NF-κB inhibitory proteins to be identified and rapidly their major function was found to be to keep Rel/NF-κB latent in the cytosol of the cells[7,21]. IκB-α and IκB-β have at their C-terminal end a PEST domain, which is a rich region in the proline (P), glutamic acid (E), serine (S) and threonine (T) amino acids. The presence of PEST domains in many proteins predicts a rapid protein turnover. Deletion of the PEST region increases the half-life of IκB-α because it abolishes a constitutive phosphorylation within this region catalyzed by casein kinase II (CKII)[22].

IκB-α, IκB-β and IκB-ε contain N-terminal regulatory regions that are targeted after a stimulus for a subsequent degradation. This is the key step in NF-κB activation and it will be explained below.

4. The inducible activation of NF-κB

NF-κB responds very rapidly in the presence of specific signals. There is a vast list of NF-κB inducers that can be divided in different major groups: cytokines, such as TNF-α or IL-1; bacterial and viral infection, such as LPS, viral proteins and dsRNA; and physical and chemical stress caused by UV light and reactive oxygen species (ROS)[13]. The NF-κB-responsive genes are also related the inflammatory and innate and adaptive immune responses (e.g. cytokines, chemokines, adhesion molecules, cellular receptors) (see Box 1), but also with proliferation and apoptotic processes[23]. NF-κB is a ubiquitous transcription factor. If in one hand, NF-κB is considered a primary defense of the organisms, on the other hand, if NF-κB regulation is somehow corrupted, it can be responsible for numerous diseases, such as inflammatory diseases (e.g. rheumatoid arthritis, asthma) and cancer[24]. Since NF-κB can protect cells from apoptosis and induce cell proliferation, therapies involving the down-regulation of NF-κB are being used to stop tumor development[25]. This highlights that a tight regulation of NF-κB is essential for its correct functionality.

There are two well-described pathways of NF-κB activation: the canonical or classical pathway and the non-canonical or alternative pathway, which are dependent and independent on IκB proteins, respectively. The alternative pathway is primarily related with the adaptive immunity and with lymphoid organ development[26] and will be discussed in section 4.2.

Box 1 – Inflammation and immune response
Phagocytic macrophages are able to recognize surface receptors in invading pathogens and are able to start an innate immune response, which is considered the first line of defense of the organism. Activated macrophages are able to kill pathogens and secrete cytokines that "activate" cells bearing appropriate receptors and chemokines that attract more effector cells, such as neutrophils and monocytes, from the bloodstream. The permeability of the blood vessels is increased, allowing leucocytes, fluid and other mediators to enter the tissues. This situation is known as inflammation. The first definition of inflammation was: *rubor*, *calor*, *dolor*, *tumor* (redness, heat, pain and swelling). In activated leucocytes, endothelial and epithelial cells, NF-κB is induced as an active effector of the inflammatory process. The adaptive immunity is initiated after 4-7 days if the infection is not resolved by the innate response. Adaptive immunity is a specific response with immunological memory. B- and T-lymphocytes constitute the effector cells. After the encounter of the pathogen T cells proliferate and differentiate in antigen-specific effector cell, while B cells in antibody-secreting cells[23].

4.1 The classical pathway

Classically, NF-κB (p50/p65) is kept latent in the cytosol bound to IκBs (Figure 3). The prototypical activators TNF-α, LPS and IL-1 induce rapid degradation of IκB-α by the 26S proteasome, freeing NF-κB. This degradation is a result of a signal cascade that starts at specific membrane receptors (Figure 3).

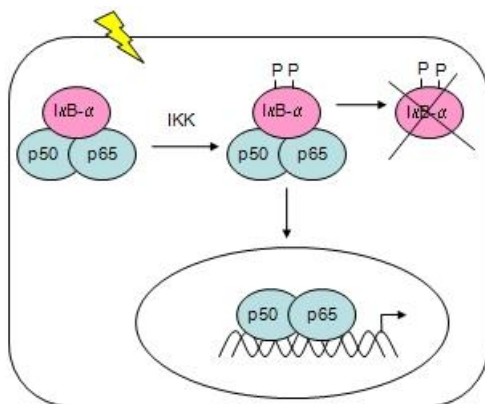


Figure 3 – Simplified representation of the classical NF-κB pathway. p50/p65, which represents the most common NF-κB heterodimer in cells, is sequestered by IκB-α. A specific signal activates IKK that phosphorylates (P) IκB-α in specific locations thereby mediating its degradation. NF-κB is able to translocate to the nucleus and bind to specific target genes.

In 1989, Shirakawa *et al.* found that NF-κB could be activated *in vitro* by treatment of pre-B cells with two protein kinases: cAMP-dependent protein kinase (PKA) and protein kinase C (PKC)[27]. This was the first line of evidence that the inhibitory proteins could be regulated by phosphorylation. Two crucial serines: Ser32 and Ser36 within IκB-α were identified as the targets of phosphorylation causing the subsequent degradation, because point mutations on the residues abolished further degradation of IκB-α. Same results were obtained by using different stimuli: TNF-α, phorbol 12-myristate 13-acetate (PMA) and okadaic acid indicating as well the activation of a common kinase[28]. The IκB kinases were first identified by DiDonato *et al.*[29] and nowadays they are well characterized. They are organized in a

complex: the IκB-Kinase complex (IKK complex) and responsible for the phosphorylation of two conserved serine residues present in the IκB-α, IκB-β (Ser19 and Ser23) and IκB-ε (Ser18 and Ser22) (Figure 2). The resulting motif is highly conserved among the IκB family and is recognized by the ubiquitination machinery (see Box 2), which includes the binding of the β-TrCP-SCF complex (or the $E3^{I\kappa B}$ ubiquitin ligase complex) and results in ubiquitination of specific lysines of IκB (Lys 21 and Lys 22 for IκB-α[30]). The polyubiquitination chain is formed by linkages on Lys48, and further recognition by the 26S proteasome direct IκBs degradation[31,32]. Proteolytic processing of p105 to p50 appears to be largely a constitutive process and p50 subunits can heterodimerize, but as well homodimerize. However, apart from the IκBs, NF-κB dimers can also bind to the p105 form, especially the p50 homodimers[33]. Under different stimuli, such as TNF-α, IL-1 and PMA, p105 is also phosphorylated by the IKK and is targeted for complete degradation by the proteasome, just like the other IκBs[33,34].

Following the degradation of the inhibitors of NF-κB, the NLS of NF-κB is unmasked and thus NF-κB translocates into the nucleus where it binds to promoter/enhancer regions of the target genes, the κB sites, inducing specific gene expression. The canonical consensus sequence for p50/p65 is the following: GGGRNNYYCC (R is purine, Y is pyrimidine and N is any base), which may be different for other NF-κB dimers. For example, the heterodimer c-Rel/p65 selectively binds to a different consensus sequence: HGGARNYYCC (H indicates A, C or T)[35].

Box 2

Ubiquitin is a protein containing 76 amino acid residues that can be covalently attached to lysine residues present in cellular proteins. The ubiquitination mechanism is a three-step chain reaction catalysed by E1 – ubiquitin activation enzyme – which activates ubiquitin in an ATP-dependent reaction, E2 – ubiquitin conjugation enzyme to which the activated ubiquitin is transferred and finally E3 – ubiquitin ligase which catalyses the isopeptide bond formation between the target protein and ubiquitin. Ubiquitin also contains lysine residues, such as Lys48 and Lys63, which mediate the formation of a polyubiquitin chain on target proteins. Lys48-linked polyubiquitin chains lead to the recognition and degradation of proteins, like the IκB proteins, by the proteasome, whereas lys63-linked polyubiquitin chains are involved in signal transduction[32].

4.1.1 The IκB-Kinase (IKK) complex

The native IKK is a multicatalytic complex with an apparent molecular mass of 700-900 kDa composed of three proteins: IKKα or IKK1 (85 kDa), IKKβ or IKK2 (87 kDa) and IKKγ, also known as NEMO (NF-κB Essential Modulator), with 48 kDa. IKKα and IKKβ, which were the first to be identified, are highly homologous (52%) and are the catalytic subunits of the IKK complex[9,25]. The DNA sequence of IKKα was first identified as a putative serine/threonine kinase of unknown function called CHUK and, by using a yeast two-hybrid system, CHUK was shown to interact with another kinase: the NF-κB inducing kinase (NIK)[36]. In addition to the kinase domain at N-terminal end, the IKKα and IKKβ also contain other protein interaction motifs, leucine zippers (LZ) and helix-loop-helix (HLH) at their C-termini. They can form homodimers and heterodimers *in vitro* and purified recombinant forms can directly phosphorylate IκB-α and IκB-β at their proper sites[36]. Although IKKγ/NEMO does not bare a catalytic domain it is crucial for an adequate phosphorylation of the IκBs and functions as a regulatory subunit[37,38]. It is a glutamine-rich protein with several coiled-coil protein interaction motifs, including a LZ motif[38] and it interacts with a domain in IKKβ, designated as the NEMO-binding domain, which regulates the formation of the IKK complex. Similarly to the p65 knockout mice, both IKKβ- and IKKγ-null mice die of extensive liver apoptosis and cells derived from these animals show profound defects in NF-κB activation[25]. IKKβ appears to be the dominant kinase for the canonical stimulus-induced NF-κB activation, whereas IKKα emerges as a versatile kinase with additional functions identified using IKKα-knockout mice, such as in epidermal differentiation[31]. Although the IKKα mutation is lethal, mouse embryonic fibroblasts (MEF) cells from these IKKα-knockout mice exhibit a relatively normal cytokine-induced IκB-α degradation[31]. Cohen *et al.* isolated IL-1-inducible IKK complexes containing IKKα, IKKβ, IKKγ, NIK and p50/p65 but also a 150 kDa protein named IKAP (IKK complex-associated protein)[39]. IKAP was thought to function as a scaffold protein due to its ability to assemble all the other proteins mentioned[39]. Simultaneously with the identification of the IKK complex, strong evidences suggested that it was itself activated by phosphorylation. Treatment of HeLa cells with protein phosphatase 2A (PP2A) inactivated the purified IKK, while the PP2A inhibitor okadaic acid, reverted this effect[29]. Extensive work has been done to understand the mechanism of IKK activation. It is important to mention that recently the existence of distinct IKK complexes that do not contain the three typical subunits was demonstrated. A PMA-

inducible IκB kinase complex has been described which contains an IKK-related kinase designated as IKKε, which is identical to another kinase named IKK-i[40].

4.1.2 IKK activation

Globally, the IKK complex is the convergent point of the inducible activation of the canonical NF-κB pathway by different stimuli. As so, there are many candidates with the ability to phosphorylate IKK. MAP3 kinases, such as NIK, mitogen activated protein/ERK kinase kinase (MEKK) 1 and MEKK3, the transforming growth factor (TGF)-β activating kinase 1 (TAK1) and NF-κB-activating kinase (NAK) can all phosphorylate the IKK complex and induce NF-κB activation when overexpressed or when assayed *in vitro*[31]. However, neither NIK nor MEKK1 seem to be physiological activators because MEF cells isolated from NIK- and MEKK1-knockout mice display normal NF-κB activation in response to TNF-α[31]. Cell stimulation with TNF-α enhances phosphorylation of all three IKK subunits, although consistently with the genetic studies referred about IKK knockouts, mutation of critical Ser176 and Ser180 located at the activation loop within the catalytic domain of IKKα had no effect on TNF-α or IL-1-induced IKK activity. Therefore, even though phosphorylation of the IKKα activation loop is undoubtedly important for its activation, it is not essential for activation of the IKK complex by pro-inflammatory stimuli. In opposition, replacement of the corresponding serines with alanines within the activation loop of the kinase domain of IKKβ completely prevented IKK activation[9].

Dependently on the inducer, different signal cascades are triggered and converge to IKK phosphorylation. Next, some examples of IKK activation by different stimuli are presented.

4.1.3 From cell receptors to IKK activation

TNF-α is a multifunctional cytokine involved in apoptosis, cell survival, inflammation and immunity[41]. TNF-α was first identified in 1975 and was named according to its capacity to caused hemorrhagic necrosis of mice tumors. It can bind to two distinct surface receptors: the TNF-R1, which is ubiquitously expressed, and the TNF-R2, which is found in higher quantities in immune system cells and that is highly regulated[42]. Signaling through the TNF-R1 can activate both the NF-κB pathway (Figure 4) and apoptosis, through activation of

caspases[41]. It is not completely understood how TNF-R1 is able to regulate this dual-signaling.

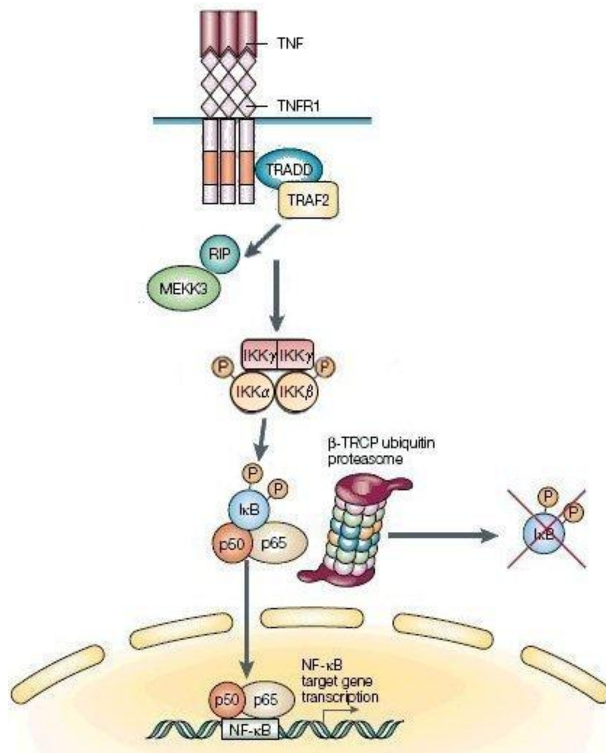


Figure 4 – NF-κB activation by TNF-α. TNF-α binds to TNF receptor 1 (TNFR1), resulting in the trimerization and recruitment of the adaptor protein TNF receptor-associated death domain TRADD, which in turn interacts with the C-terminal of TNF-R associated factor 2 (TRAF2) and the receptor interacting protein (RIP). TRAF2 is responsible for the recruitment of the IKK complex and it interacts with IKKα and IKKβ subunits through their LZ motif, while RIP functions as an adaptor molecule and interacts with IKKγ and with the downstream signaling components, such as the mitogen activated protein/ERK kinase kinase 3 (MEKK3). The next steps are a convergence point with the other activation-pathway and characterized by phosphorylation of IκB and its degradation, followed by NF-κB (p50/p65) translocation to the nucleus. Adapted from[11].

Most TNF-α biological functions are initiated through binding to TNF-R1, which triggers trimerization of the receptors and the release of an inhibitory protein, silencer of death domain (SODD), from the intracellular domain of TNF-R1. The now exposed intracellular death domain (DD) is recognized by the TNF receptor-associated death domain (TRADD), which acts as a scaffold protein that recruits the receptor interacting protein (RIP) and the TNF-R associated factor 2 (TRAF2)[43]. TRAF2, RIP and IKKγ are probably modified by Lys63-linked polyubiquitination in response to TNF-α[32]. TRAF proteins are ubiquitin E3 ligases and there are evidences that suggest that TRAF2 is capable of self-ubiquitination[44] and/or inducing RIP ubiquitination[43]. Some studies have shown that IKKγ preferentially binds to polyubiquitinated RIP[43]. A recent work described that TRAF family member associated NF-κB activator (TANK) interacts with the IKK complex in response to TNF-α[45]. Interestingly TANK is subsequently phosphorylated by IKKβ in a RIP-dependent manner and this modification decreases the association of RIP with IKKγ, thus down-regulating the TNF-α induction[45].

There are other cell receptors able to trigger NF-κB activation. The Toll-like receptors (TLRs) and IL-1 receptors share a similar pathway of NF-κB activation (Figure 5). TLRs are

transmembrane proteins composed of N-terminal rich extracellular leucine-rich repeats (LRRs) that recognize pathogen-associated molecular patterns (PAMPs) and induce pro-inflammatory proteins and co-stimulatory molecules that trigger innate and adaptive immunity[19]. Each member of the TLR family detects distinct PAMPs leading to differences in the downstream signaling cascade. For example, bacterial LPS are recognized by TLR4, while bacterial lipoproteins are recognized by TLR2. TLRs have a C-terminal cytosolic domain, similar to the IL-1 receptor, known as the Toll/IL-1 receptor (TIR) domain, which interacts with a set of adapter proteins[46]. Four of these adapter proteins have been identified: myeloid differentiation primary response gene 88 (MYD88), TIR-containing adaptor protein/MYD88 adaptor-like (TIRAP/MAL), TIR-containing adaptor inducing interferon-β (IFN-β)/TIR-domain-containing adaptor molecule 1 (TRIF/TICAM1) and TRIF-related adaptor molecule (TRAM)/TICAM2[46]. With the exception of TLR3, MYD88 is universally recruited to all receptors and, in some cases (TLR5, TLR7 and TLR8), is the only adaptor molecule. TLR2 also uses TIRAP to bridge for MyD88 interaction while TLR4 uses the four adaptors[46]. Subsequently these adaptors recruit and activate members of the IL-1-receptor-associated kinase (IRAK) family. Figure 5 describes in detail NF-κB activation through the TLR4 receptor.

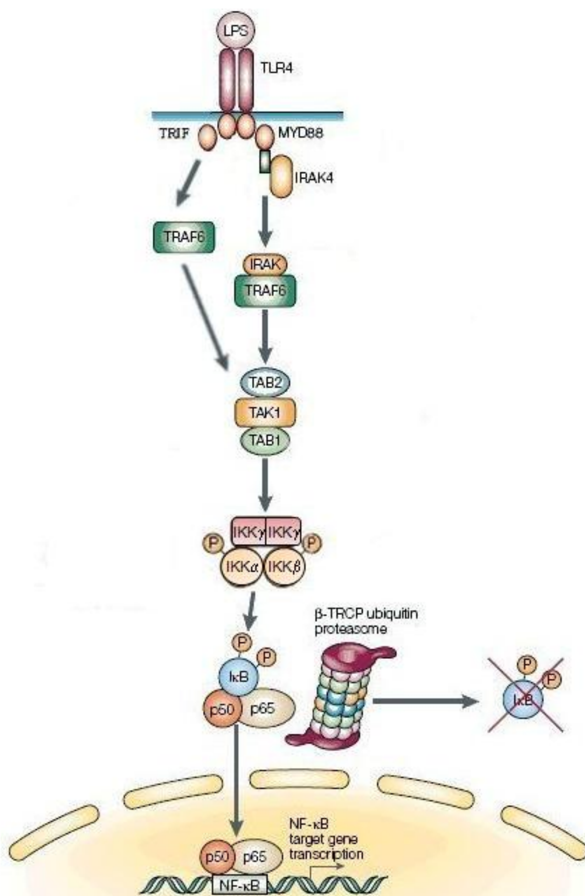


Figure 5 – NF-κB activation by LPS/IL-1. Binding of LPS to TLR4 recruits the four Toll/IL-1 receptor (TIR) domains, including the TIR-containing adaptor inducing interferon-β (TRIF) and the myeloid differentiation primary response gene 88 (MYD88) represented in the figure. MYD88 engages IL-1-receptor-associated kinase-1 (IRAK-1) that is phosphorylated by the already active IRAK-4 leading to its dissociation from MYD88 and to an interaction with the downstream TRAF6 protein. Just like TRAF2, TRAF6 is a E3 ubiquitin ligase. Together with E2, Ubc13 and Uev1A, TRAF6 promotes the Lys63-linked polyubiquitination of itself and IKKγ leading to the recruitment of a protein kinase complex containing TAK1 and TAK1-binding proteins 1 and 2 (TAB1/2). Subsequently the IKK complex is activated. TLR4 can also activate NF-κB through a TRIF-dependent pathway which involves the direct binding of TRIF to TRAF6, converging after to the MYD88 pathway[11,19,46]. Adapted from[11].

The two signaling pathways described are the ones most studied and are the major contributors for NF-κB activation in different cell types. Nevertheless, B and T lymphocytes have special receptors, B-cell receptors (BCR) and T-cell receptors (TCR) respectively, to sense antigens and consequently to activate NF-κB. The binding of an antigen to the TCR leads to an immediate activation of protein tyrosine kinases which leads to the activation of PKCθ. It then binds to Bcl10 that in turn is phosphorylated by a RIP2 protein leading to interaction with the mucosa-associated lymphoid tissue 1 protein (MALT1). Then, Bcl10 and MALT1 induce the Lys63-linked polyubiquitination of IKKγ, probably through TRAF6. The engagement of the BCR is very similar, depending as well on Bcl10/MALT1[19,32].

4.1.4 Down-regulation of IKK activation

As expected for an inducible pathway, negative regulators are also activated to attenuate or terminate the initial activation signal of IKK. Since ubiquitination plays an important role in the cascade until IKK activation, de-ubiquitinating enzymes have the opposite role. At least three de-ubiquitinating enzymes have been identified:

- The zinc-finger protein A20, which belongs to the ovarian tumor (OTU) family of proteins, contains two short conserved sequences with putative catalytic cysteine and histidine residues. The functional OTU domain of A20 hydrolyses polyubiquitin chains either with Lys48 or Lys63 linkages[47]. A20 inhibits IKK by removing the essential Lys63-linkages present in RIP and, interestingly, by subsequently catalyzing the Lys48-linked polyubiquitination which triggers RIP degradation. A20 also catalyses the de-ubiquitination of IKKγ Lys63-linkages and it interacts with TRAF molecules[43]. Both the mRNA and the protein levels of A20 increase after NF-κB activation, which reinforces its important role in NF-κB down-regulation. A global de-ubiquitination is not observed in these conditions and so A20 is probably specific for the target protein.
- The tumor suppressor cylindromatosis (CYLD), which interacts with IKKγ, TRAF2 and TRAF6, has a de-ubiquitinating activity for Lys63-ubiquitin linkages, thus inhibiting IKK activation. Family CYLD is an autosomal dominant predisposition to tumors of skin appendages called cylindromas, which are formed by loss of de-ubiquitinating activity of CYLD[48,49].

- The recently identified Cezanne, which is a cysteine protease and is responsible for the de-ubiquitination of RIP and consequently for the down-regulation of IKK activity and NF-κB activation[50].

4.2 The alternative pathway of NF-κB activation

The non-canonical or alternative pathway of NF-κB activation was described in 2001 by Senftleben *et al.*[51] and it is probably involved in the development and maintenance of secondary lymphoid organs, which are related with the adaptive immunity. As referred earlier, experiments with MEF-knockout cells showed that IKKα is dispensable for the classical activation of NF-κB, while IKKβ is essential, and that IKKα is involved in processes that are not dependent on IKK activity or in NF-κB activation, e.g. skeletal morphogenesis and epidermis differentiation. Therefore, these observations raised the question if IKKα could have another NF-κB-related function that was masked because of the perinatal lethality of IKKα-knockout mice[51]. IKKα is required for B cell maturation and for the formation of secondary lymphoid organs (bone marrow, spleen and lymph nodes). The proposed model (Figure 6) involves NIK activation, which by having the ability to bind both the IKKα and p100 is essential for the recruitment of IKKα homodimers to p100[52]. Two serine residues (Ser866 and Ser870) within the C-terminal end of p100 were identified as critical for this interaction. Interestingly, other serine residues present in both the N- and C-terminal ends of p100 are further targets for the IKKα phosphorylation which by triggering the ubiquitination and proteolytic processing leads to the release of the inhibitory ankyrin repeats[52]. p100 is most commonly associated with RelB and activation of this alternative pathway results in nuclear translocation of p52/RelB[53]. A distinct set of stimuli from those that activate the canonical pathway have been found and include the lymphotoxin β (LTβ), the CD40 ligand and the B-cell-activating factor (BAFF). Moreover, the alternative NF-κB pathway controls a distinct genomic response, which includes chemokines such as the secondary lymphoid tissue chemoline (SLC/CCL21), the Epstein-Barr virus-induced molecular 1 ligand (ELC/CCL19) and the stromal cell-derived factor 1a (SDF-1/CXCL12) and cytokines, such as the BAFF proteins[54]. Because NIK is essential for the transduction signal in the non-canonical pathway, extensive work has been done to try to understand the mechanism of its activation. A role for this non-canonical pathway has been clearly

established in B-cell maturation and immunity, and new reports show as well the activation of this pathway in airway epithelial cells in response to virus infection[54].

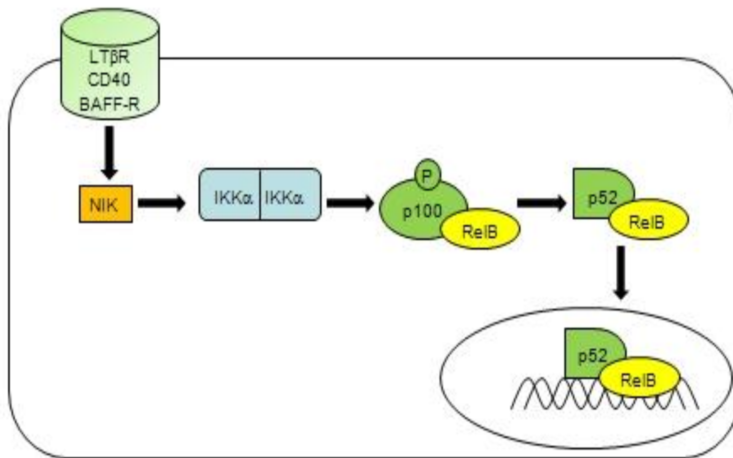


Figure 6 – The alternative pathway of NF-κB activation. Members of the TNF-α receptor family: CD40, lymphotoxin β receptor (LTβR) and B-cell-activating factor-receptor (BAFF-R) cause sequential activation of NF-κB-inducing kinase (NIK) and IKKα. The latter phosphorylates p100 resulting in its polyubiquitination and processing to p52. This allows the translocation of p52/RelB to the nucleus.

5. Regulation of NF-κB activation

Given the biological importance of the NF-κB transduction pathway, it is not surprising that it is subjected to a multi-level regulation. Mostly, post-translational modifications are implicated in the signal transduction pathway, including ubiquitination, phosphorylation and acetylation. The polyubiquitination of Lys48 and Lys63 residues and phosphorylations, already described as important signaling effectors, take place both at the phase where degradation of the IκB proteins occur and at the phase where upstream kinases act. The ultimate role of NF-κB/Rel proteins is after translocation to the nucleus to bind to promoter/enhancers of specific genes and activate gene expression. The question addressed below is how regulated the response of the different components of the NF-κB pathway after activation of the IKK and consequent IκB degradation, so that a concerted action occurs and gene expression is efficiently regulated.

5.1 Regulation by the IκB proteins

One of the first feed-back mechanisms identified in the NF-κB pathway was the autoregulation of IκB-α activity[55,56]. It was shown that after the degradation of IκB-α and the subsequent translocation of NF-κB into the nucleus, new IκB-α is rapidly synthesized due to the presence of three κB sites in the IκB-α promoter[57]. Therefore, soon a role as an important

down-regulator of NF-κB activation was attributed to IκB-α. Typically, within the first 30 minutes of NF-κB activation, almost all IκB-α is degraded, with a new pool of IκB-α appearing after that time frame. The newly synthesized IκB-α accumulates in the nucleus of TNF-α- and IL-1-activated cells[58] and can remove NF-κB from its binding to DNA, thus terminating NF-κB-dependent gene expression. Efforts were done to understand the mechanism of this regulation: the mRNA of IκB-α is translated in the cytosol, so IκB-α has to translocate to the nucleus (Figure 7).

IκB-α does not bear a traditional NLS but is able to translocate to the nucleus trough a region within the first and/or second ankyrin repeat[59,60]. After binding and removal of NF-κB from the DNA, the exportation of the complex NF-κB/IκB-α is mediated by a nuclear export sequence (NES) present in IκB-α[59,61], thus terminating the NF-κB signaling. The presence of a NES is compatible with the fast translocation out of the nucleus, which otherwise is a slow process.

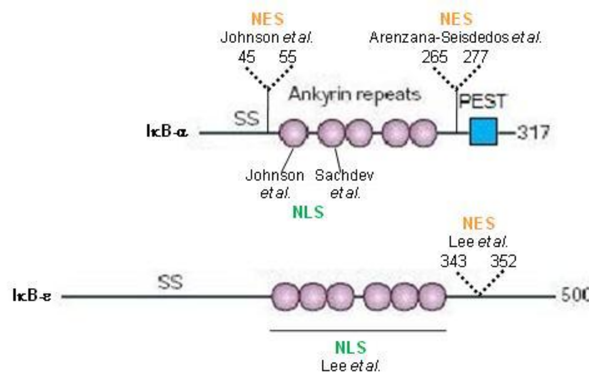


Figure 7 – Nuclear localization signal (NLS) and nuclear export signal (NES) identified in IκB-α and IκB-ε. Sachdev *et al.* demonstrated that the second ankyrin repeat in IκB-α functions as a nuclear import sequence[60], while Johnson *et al.* identified the NLS within the first repeat[59]. Arenzana-Seisdedos *et al.* described that IκB-α promotes the transport of NF-κB complexes back to the cytosol through a nuclear export signal (NES) present in its C-terminal domain, whereas Johnson *et al.* reported the existence of a NES within the N-terminal end of IκB-α. This N-terminal NES is not masked with the binding of NF-κB, which suggests that the apparent cytosolic localization of the complexes is a result of NES, rather than the masking of the NLS on Rel/NF-κB proteins. IκB-ε also shuttles between the cytosol and the nucleus and therefore bears an NLS and NES[62].

In unstimulated cells, a small nuclear pool of NF-κB/IκB-α was identified, which suggests a rapid response to the signaling cascade in the presence of an inducer and consequently, a more efficient triggering of the early-responsive genes expression[59]. This assumption is supported by the existence of proteasomes in the nucleus that can degrade the IκB proteins[59]. Carlotti *et al.* determined the kinetics of this NF-κB/IκB-α shuttling by analyzing the differences observed when the cells are stimulated by IL-1 or are kept unstimulated[63]. Using

the specific inhibitor of nuclear export leptomycin B (LMB) they observed an import of NF-κB and IκB-α to the nucleus in unstimulated cells that did not induce the expression of NF-κB-target genes, as observed for cells treated both with IL-1 and LMB. The constitutive nuclear import might be a result of the dissociation of the NF-κB/IκB-α complex, which uncovers the respective NLSs[63,64]. Import of IκB-α is faster than the import of p65 probably because of the non-classical NLS of IκB-α[64]. However, based on the crystallographic analysis of p50/p65 complexed with IκB, Malek *et al.* showed that IκB-α only masks the p65 NLS, leaving the p50 NLS free which results in the shuttling of the whole complex between the nucleus and the cytosol[65]. Although with some divergences, those works were important to prove that the NF-κB/IκB-α complex is a dynamic nucleocytosolic complex, rather than a static complex latent in the cytosol.

Besides IκB-α, the other members of the IκB family also have important regulatory roles. IκB-β was identified soon after IκB-α and although they share the same function of sequestering NF-κB, molecular differences were rapidly reported, namely the presence of a phosphorylation crucial for IκB-β capacity to bind and sequester NF-κB in the cytosol and for its weak capacity for inhibiting NF-κB-DNA binding[21]. Furthermore, following degradation of the first IκB-β pool, the *de novo* form of IκB-β is basally unphosphorylated and it can still bind to NF-κB, but without masking the NLS of NF-κB, thus resulting in NF-κB/IκB-β translocation to the nucleus and binding to DNA. This regulation allows a persistent NF-κB activation by preventing at the same time the binding of NF-κB to IκB-α[66]. The PEST region of IκB-β is proposed to be the location for its basal phosphorylation, probably resulting from the CKII catalytic activity. A deletion on the PEST region of IκB-β gave rise to a protein stably associated with NF-κB without any inhibitory effects, just like it is observed for the unphosphorylated form of IκB-β[67]. It is assumed that IκB-α is a stronger inhibitor than IκB-β[67,68]. Tran *et al.* suggested the need of a more negatively charged protein to efficiently sequester NF-κB. IκB-α bears more acidic amino acid residues than IκB-β, and so the phosphorylation by CKII on the PEST region might be crucial for its efficient role. The ankyrin repeats are indispensable for association with NF-κB but are not sufficient for the inhibition of NF-κB/DNA binding[67].

One major difference between IκB-α and IκB-β is that the regulation of NF-κB by IκB-α is fast and transient while that by IκB-β is persistent. According to Schmidt *et al.*[69], the

formation of the complexes IKK/NF-κB/IκB-α and IKK/NF-κB/IκB-β is regulated by different upstream kinases: MEKK3 and MEKK2, respectively, which have different activation kinetics and, consequently, are the responsible for the different degradation kinetics.

Unlike IκB-α, IκB-β is not fully degraded in response to most stimuli. Hence, Chen *et al.* identified a pool of IκB-β bound to NF-κB dimers and to a κB-Ras protein, which masks the serine residues targeted for phosphorylation in IκB-β[70]. The IκB-β-NF-κB-Ras complex was identified as an inhibitor of NF-κB transcriptional activity by a yeast two-hydrid assay. This ternary complex co-exists with the normal IκB-β/NF-κB heterodimer in unstimulated cells, and is more resistant to degradation, which might explain the differences of IκB-β degradation for various stimuli[70]. Probably only specific inducers can liberate the IκB-β/NF-κB heterodimer from the IκB-β-NF-κB-Ras complex and, consequently, lead to a total IκB-β degradation.

IκB-ε is degraded with slower kinetics than IκB-α. Unlike IκB-α and IκB-β, IκB-ε contains a cluster of serine residues at the N-terminal and lacks a PEST domain at the C-terminal, which suggests differences in function (Figure 7). Similarly to IκB-α, the IκB-ε also shuttles to the nucleus mediated by its ankyrin repeat domain, but with slower kinetics when compared with IκB-α[62]. It also bears a canonical NES which mediates its export back to the cytosol. The expression of IκB-ε is induced by NF-κB, and the newly synthesized IκB-ε can displace the Rel proteins from the DNA and is able to shuttle between the cytosol and the nucleus, supporting a post-induction role in the repression of the Rel protein dimers[62]. Overall, IκB-ε and IκB-α have similar roles, although IκB-ε is less effective and regulates predominantly other Rel/NF-κB dimers that guarantee non-overlapping functions.

BCL-3 has ankyrin repeats, however, unlike the other IκBs, BCL-3 is a nuclear transcriptional co-activator and is recruited to the promoter through direct binding to p50 or p52 homodimers. These NF-κB dimers are generally considered as inhibitors of the transcriptional activity, because they do not have TADs, but in the presence of BCL-3 they are transformed in potent activators of transcription[25].

5.1.1 The oscillatory response of NF-κB

Cell stimulation with TNF-α initiates a signaling cascade already described and culminates with the binding of NF-κB to the DNA and activation of gene expression. By following the nuclear levels of NF-κB along time, it was possible to recognize two phases of activation. Using computational modelling Hoffmann *et al.*[71] suggested that the response was oscillatory. They tested this hypothesis by using MEF cells with knockouts in two of the IκB isoforms (IκB-α, IκB-β and/or IκB-ε) to keep only one of them in the system (Figure 8). TNF-α stimulation of MEF cells containing only the IκB-α isoform resulted in a highly oscillatory NF-κB response. In contrast, in cells harboring only IκB-β or IκB-ε the nuclear NF-κB increased monotonically to a plateau. IκB-α mediates a rapid NF-κB activation and a strong negative feed-back regulation. When the induction-signal is persistent, it will result in an oscillatory NF-κB activation profile. IκB-β and IκB-ε response to IKK activation is slower and act to dampen the long-term oscillations of the NF-κB response[71].

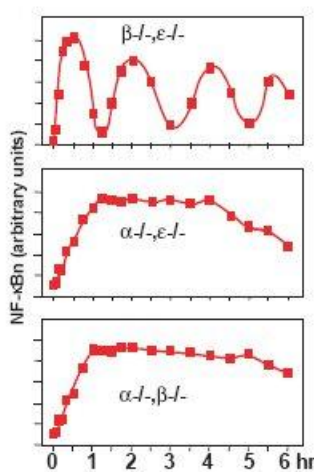


Figure 8 – Quantification of nuclear NF-κB obtained in knockout-MEF cells. The first, second and third panels represent the MEF cells only expressing IκB-α, IκB-β and IκB-ε, respectively. Image from[71].

As happens with IκB-α, IκB-ε expression is also regulated by NF-κB and chromatin immunoprecipitation assays showed that NF-κB bounds directly to the IκB-ε promoters[72]. Kearns *et al.* confirmed the results, however they showed that there is a delay in IκB-ε mRNA production when compared with IκB-α. This delay turns the *de novo* synthesis of IκB-ε in an antiphase of negative feedback with IκB-α, which could be responsible for the dampening of the oscillations[73]. Importantly, they suggested that for longer periods of time , IκB-ε is responsible for NF-κB removal from the nucleus[73]. Those findings are in accordance with the similar characteristics found in IκB-α and IκB-ε. Although the role of IκB-β is not

well understood, IκB-β is necessary for the overall oscillatory profile of NF-κB activation in wild-type cells[73].

In conclusion, the three more abundant IκB found in cells probably function in a coordinated fashion so that a highly dynamic and regulated profile of NF-κB response is obtained. Ultimately, this oscillatory profile is important to regulate differentially gene expression, as explained below.

5.2 Transcriptional regulation of NF-κB

NF-κB is a ubiquitous transcription factor responsible for the expression of a vast list of genes with different and sometimes opposite functions. Classicaly, NF-κB activity is considered to be regulated by signal-induced IκB degradation. However, degradation of IκBs and nuclear translocation of NF-κB are not sufficient to promote a maximal NF-κB transcriptional response.

NF-κB-dependent gene expression can be divided temporally, with sets consisting of early-, middle- and late-responsive genes[71,74]. Early-expression genes encode for cytokines (IL-6, IL-8) and negative regulators of NF-κB (IκB-α, A20) and late-expression genes encode for cell surface adhesion molecules (ICAM-1), signaling adapter molecules (TRAF1/3) and p100/NF-κB2[74]. Middle expression genes include IκB-ε, BCL-3 and TRAF2[74]. It has been suggested that the dampened oscillatory response of NF-κB is important for the expression of late genes, since a short incubation with TNF-α is enough to trigger the expression of early genes. The level of nuclear NF-κB is crucial for the control of gene expression, but other factors are needed to account for the overall regulation. Gene activation is a multistep process involving a very large number of proteins functioning in discrete complexes[75]. Rel/NF-κB proteins bind to DNA in a sequence specific manner and mark a gene for activation or repression by recruitment of co-activators or co-repressor proteins (see Box 3), respectively. A combination of direct post-translational modifications, essentially phosphorylations and acetylations of the NF-κB complex and of the histones that surround various NF-κB target genes, and the affinity of the κB sequence toward the NF-κB complex dictate the overall expression status.

Box 3 – The histones

An extent list of co-activators and co-repressors determines the fate of gene expression. They bear different enzymatic activities that modify histones and transcription factors. Histone proteins: H1, H2, H3 and H4 form nucleosomes associated with DNA and are fundamental components of chromatin. The packaging of chromatin plays an active role in transcriptional regulation by interfering with the accessibility to the transcription factors. In response to stimuli, remodeling of chromatin is triggered by histone modification and enables interaction of the transcription factors with gene promoters/enhancers. The best characterized modification of histones is acetylation, but phosphorylation, methylation and ubiquitination are also included. Acetylation of specific lysines within the N-terminal tails of the histones promotes chromatin unwinding and binding of the transcription factors. Co-activators that have acetyltransferase activity are named HATs (histone acetyltransferases) and include the CBP/p300 protein. In opposition, the co-repressors include proteins that have deacetylase activity known as HDAC (histone deacetylases). Nowadays it is known that these enzymes are not histone specific and can also modify transcription factors[75].

5.3 Phosphorylation of the Rel/NF-κB proteins

Among members of the Rel family, p65 is one of the principal targets of phosphorylation by various kinases present both in the nucleus and in the cytosol (Figure 9).

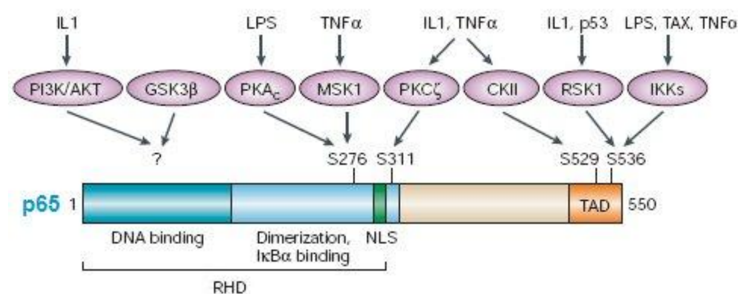


Figure 9 – **Phosphorylations identified on p65 induced by different stimuli**. Ser276 and Ser311 within the RHD and Ser529 and Ser536 in the TAD constitute the four serine residues phosphorylated identified in p65, since the target of phosphaditylinositol 3-kinase (PI3K)/Akt and glycogen-synthase kinase 3β (GSK3β) remain unidentified. Ser276 is phosphorylated by PKAc and mitogen- and stress-activated protein kinase-1(MSK1), Ser311 by PKCζ, S529 by CKII and S536 by ribosomal subunit kinase-1 (RSK1) and IKKs. TAX – human T lymphotrophic virus-1 (HTLV1)-encoded TAX proteins. Image from[12].

The first report is from Zhong *et al.* who discovered that in LPS-treated cells the Ser276 residue of p65, which is located in the RHD, is phosphorylated in a reaction catalyzed by PKAc[76]. PKAc is maintained in an inactive state in a NF-κB/IκB complex and, upon an IκB-induced degradation stimulus, is released in order to catalyze p65 phosphorylation[76]. This modification increases the transactivation potential of NF-κB. Inhibiting PKAc activity does not affect significantly the dissociation of the NF-κB/IκB complex and the translocation and binding of NF-κB to DNA, but reduces dramatically NF-κB ability to drive transcription [76].

In TNF-α-treated cells the mitogen- and stress-activated protein kinase-1 (MSK1) also catalyses Ser276 phosphorylation[77]. This kinase is located in the nucleus and it is activated by both p38 and the extracellular signal-related kinase (ERK) mitogen-activated protein kinases (MAPKs) in response to TNF-α[77]. So, with two different stimuli, LPS and TNF-α, the same serine residue of p65 is phosphorylated both in the cytosol and the nucleus. It is not known whether the phosphorylation mechanisms are mutually exclusive but, importantly, they both lead to an increased transcriptional activity of NF-κB. Two other phosphoacceptor sites were found in response to TNF-α – Ser311[12] and Ser529[78,79] – and they both enhance the overall transcriptional response.

In addition, phosphorylation of p65 at Ser536, which is located at the C-terminal TAD, is catalyzed by a variety of kinases[79]. IKKα and IKKβ were the first putative kinases identified and the upstream kinases involved are the phosphaditylinositol 3-kinase (PI3K) and Akt[80,81]. However, these observations are not consensual and others showed a stimulus-dependent activation[82]. In IL-1-induced cells, IKKα induced phosphorylation of Ser536 and was activated by the PI3K-Akt pathway, while in TNF-α-induced cells, phosphorylation of Ser536 was dependent on IKKβ, which is activated by PI3K/Akt and the mitogen-activated protein kinase p38 (p38) pathways[82]. Other candidates which may also account for the total Ser536 phosphorylation include IKKε and TBK1 kinases[83]. The ribosomal subunit kinase-1 (RSK1) is induced by the p53 tumor suppressor and was identified as another kinase able to modified the Ser536 of p65 and consequently to lower the affinity of p65 for IκB-α[12].

c-Rel is also regulated by phosphorylation and Ser471 has been identified as a target[84]. A mutation at Ser471 within the c-Rel TAD abolishes the ability of Jurkat T cells to respond to TNF-α-mediated NF-κB activation[84]. RelB is also subjected to phosphorylations, namely on Ser368, which seem to be essential for both RelB dimerization and p100 stabilization[79].

Overall, although some divergent results are found in the literature, there is an agreement that the phosphorylations of Rel proteins increase their transactivation potential. Anrather *et al.*[85] proposed that the distinct p65 phosphorylations identified modulate transcriptional activity in a promoter-specific context. By using several p65 serine to alanine mutants, they observed that the transcriptional activity of hypophosphorylated p65 is not globally decreased but is rather dependent on the specific κB sequence[85]. For example, the IL-8 and IL-2Rα reporter constructs did not respond in mutated-p65 cells, induction was only observed with wild-type p65, and constructs were included in a group of highly asymmetric κB sites bearing the

following consensus sequence: GGRWWWYYYY (W – A or T). In the second group (IκB-α, ICAM-1, IL-6) C-terminal serine residues were essential for a proper plasmid transactivation and the consensus sequence was KGRAHWTYCC (K – G or T; H – not G). Finally, a third group (MHC class I), with a palindromic κB sequence (GGGRATTYCC) was insensitive to any serine mutation[85]. These observations might have important physiological implications. As described, different signals induce different phosphorylations and, consequently, restrict the gene-expression profile in a κB-sequence dependent manner. The importance of the κB site regulation is developed below.

5.4 Acetylation of the Rel/NF-κB proteins

Perkins *et al.*[86] were the first to identify an interaction between the C-terminal end of p65 with both the cAMP response element-binding (CREB)-binding protein (CBP) and p300. Other studies revealed that p65-dependent gene expression requires other co-activators, such as the steroid receptor co-activator-1 (SRC-1) and the p300/CBP associated factor (p/CAF), confirming the importance of p300/CBP interaction[87]. It is important to note that SRC-1 specifically binds to the p50 subunit and not to p65[88]. These co-activators have multiple binding domains that ensure interaction between them and the transcription factor and, simultaneously, they have a histone acetyltransferase activity (HAT). For example, the central portion of p300/CBP possesses an intrinsic acetyltransferase activity, the C-terminal region binds to other co-activators such as SRC-1 and, more specifically, the cysteine/histidine rich region 3 (CH3) interacts with p/CAF and RNA polymerase II. The N-terminal end interacts directly with the p65 subunit[87,89]. So, it is clear that multiple co-activators are required for NF-κB-dependent gene expression[87]. The participation of co-repressors was also studied. By using trichostatin A (TSA), an inhibitor of the histone deacetylases (HDACs), it was possible to observe an increased expression of a stably integrated NF-κB-dependent reporter gene, both in unstimulated and TNF-α-treated cells[90]. These results were due in part to a hyperacetylation of the promoter region. HDAC1 and HDAC2 interact with each other and are typically found as components of large co-repressor complexes such as the mSin3. HDACs not only deacetylate chromatin, but also p65 is also deacetylated. However there are contradictory observations whether it is HDAC1 or HDAC2 that interacts with p65[90-92].

All these studies rely on overexpressed p65 and co-activators. Chen *et al.*[93] were the first to show that in response to TNF-α, endogenous p65 is acetylated in the nucleus by CBP and p300 (Figure 10). This acetylated form binds weakly with IκB-α. However, the subsequent HDAC3-catalyzed deacetylation of p65 promotes simultaneously the binding of p65 with the newly synthesized IκB-α and their shuttling to the cytosol[93]. They actually proposed that the shuttling of NF-κB/IκB-α was regulated by a reversible acetylation of Lys218, Lys221 and Lys310[94]. It was suggested that acetylated Lys221 enhances the binding affinity of the NF-κB complex for the κB site and, *per se* or in combination with acetylated Lys218, these modifications impair the assembly with IκB-α[94].

On the other hand, Kiernan *et al.*[92] observed that acetylation of Lys122 and Lys123 of p65 decreases the transcriptional activity and facilitates the exportation of the dimer with IκB-α back to the cytosol (Figure 10)[92].

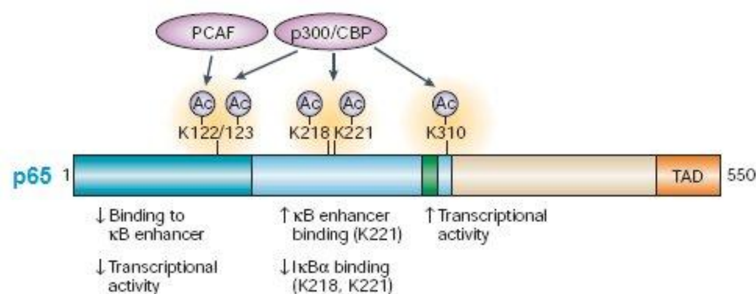


Figure 10 – Acetylation target lysine residues (K) on p65. Acetylation (Ac) of Lys218, Lys221 and Lys310 is favorable for gene expression. Conversely, acetylation of Lys122 and Lys123 decreases the overall transcription. TAD – transactivation domain. Image from[12].

In vivo, overexpression of p300 increases p50 acetylation levels[88]. Lys431, Lys440 and Lys441 in p50 were shown to be acetylated *in vitro*, but a specific role for these acetylations was not demonstrated because of the inability of generating stable mutants.

Zhong *et al.*[95] introduced a relationship between the phosphorylation and acetylation modifications. Unstimulated cells have p50 homodimers associated with HDAC-1 that bind to DNA and repress NF-κB-dependent gene expression. Following stimulation, p50/p65 heterodimers containing a phosphorylated p65 enter the nucleus, displace p50/HDAC1 and then CBP and p300 activate transcription[95]. This proposed mechanism ensures that only the stimulus-activated heterodimer can induce gene expression and emphasizes the importance of the phosphorylation status to drive gene expression.

5.5 The nuclear role of IKKα

Although IKKα is intimately linked to the alternative pathway of NF-κB activation, recent discoveries found a novel function in the regulation of the canonical pathway. IKKα, but not IKKβ[64], shuttles constitutively between the cytosol and the nucleus, indicating a possible nuclear role for IKKα. The nuclear levels of IKKα increase in the presence of TNF-α and then IKKα is recruited to promoters of NF-κB-dependent genes, such as IκB-α, IL-8 and IL-6[96,97]. Subsequently IKKα is responsible for the phosphorylation of histone H3 at Ser10, which was confirmed using MEF cells lacking IKKα where phosphorylation was completely abolished[96,97]. Importantly, this specific phosphorylation recruits CBP that is responsible for Lys14 acetylation of histone H3 thus facilitating DNA transactivation[96,97]. In the same line of evidences, studies using chromatin immunoprecipitation (ChIP) revealed that IKKα interacts strongly with the N-terminal end of CBP[96,97]. IKKβ and IKKγ, the latter with a slower kinetics, also respond to TNF-α stimulation and translocate to the nucleus, but their nuclear functions remain unknown.

5.6 The κB site

Functional NF-κB dimers bind to their cognate DNA-binding element, the κB site, at promoters of NF-κB-responsive genes. Classical NF-κB (p50/p65) binds to a conserved nucleotide sequence forming a decamer (GGGRNNYYCC), although some κB sites that do not match the conserved sequence have been identified. Martone *et al.*[98] mapped the NF-κB-binding sites within human chromosome 22 and found that, in response to TNF-α, p65 not only binds to almost half of the genes up-regulated by TNF-α, but also binds to genes down-regulated and un-regulated by TNF-α. This indicates that in a given cell, NF-κB binds to a large number of target genes, including those that may be functionally significant in other cell types or under different conditions, confirming that the binding is not sufficient to drive expression. So, what are the requirements for a correctly driven gene expression? Leung *et al.*[99] showed that the κB sequence not only determines the binding of NF-κB but, more importantly, it also determines the binding of the co-activators. MCP-1 and IP-10 bear two κB sites and one of them differs only in one nucleotide. However, MCP-1 is activated by both p50/p65 heterodimers and p65 homodimer, whereas IP-10 can only be activated by p50/p65 heterodimers[99]. The NF-κB conformation when bound to DNA is dependent on the

κB sequence and determines the efficient recruitment of the co-activators. Along these lines, the IκB-β promoter which has a κB site, is not strongly induced by NF-κB because the conformation of the promoter-bound NF-κB is probably unable to efficiently recruit TFIIH and, consequently, RNA polymerase II[100]. Therefore, the κB site itself affects the configuration of the Rel/NF-κB complexes after binding to the κB site, determining the cofactors needed for gene activation[99,100].

Different Rel/NF-κB dimers may have a different affinity to the same κB site[101], but different κB sites may also have a different affinity toward the same heterodimer[102]. Udalova *et al.*[102] developed a method to predict the effects of single-nucleotide polymorphisms within the κB sites, showing that a single modification of a nucleotide can change abruptly the affinity of NF-κB to a κB site. Thus, it is expected that, for limiting amounts of NF-κB, the affinity of the competing κB sites toward NF-κB are relevant for gene expression. Accordingly, DNA binding affinity is a significant determinant for the intranuclear mobility of a transcription factor[103]. NF-κB "spends" more time bound to high affinity sites, which explains the higher transcriptional activity[103]. However, contrary to what was initially thought, NF-κB does not make long-lasting contacts with high-affinity κB sites[104]. NF-κB is immobilized onto high-affinity binding sites only transiently and complete NF-κB turnover occurs in less than 30 s[104]. In that sense, maintenance of the κB site occupancy requires the continuous supply of NF-κB dimers and is sensitive to changes in the nuclear level of the dimers[104]. The enhanceosome should probably be regarded as a dynamic framework, instead of the previously accepted concept of a very stable complex[104].

Part 2 - Hydrogen Peroxide

1. Reactive oxygen species (ROS) – *"Les enfants terribles"*

The first forms of life evolved on earth under a atmosphere where dioxygen (O_2) was not present[105]. However, about 3 million years ago photosynthesis changed the course of evolution, because the cyanobacteria developed the capacity to split water to produce hydrogen atoms (H) to reduce CO_2. The waste product of the reaction was O_2, a highly toxic element for the anaerobic organisms that lived on earth[105]. Some organisms remained anaerobic and restricted to environments where O_2 could not penetrate, but others adapted to O_2 by developing protection against its toxicity: the antioxidants. Some of those organisms then evolved to aerobic respiration, which is far more efficient in ATP production[106].

Experiments in the middle 1900 have proved O_2 toxicity. Animals exposed to O_2 pressures above two or three atmospheres (atm) developed generalized convulsions as first symptoms, followed by severe pulmonary damage[107]. Then, Gerschman *et al.* proposed that O_2 toxicity was due to increases in oxidizing free radicals up to toxic levels in cells and tissues[107]. In 1968, by discovering the enzyme superoxide dismutase, Fridovich and McCord demonstrated that the free radical superoxide anion ($O_2^{\bullet-}$), a toxic by-product of O_2, was formed *in vivo*[106]. Nowadays, other oxygen-centered free radicals formed from O_2 *in vivo* are well characterized and, besides $O_2^{\bullet-}$, it includes hydroxyl radical ($HO^{\bullet}$), perhydroxyl radical ($HO_2\bullet$), peroxyl radical ($ROO^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$). Apart from these radicals, O_2 metabolism also produces non-radical molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), so globally all these elements have been named reactive oxygen species (ROS)[108]. ROS are oxidizing molecules having the potential of damaging proteins, lipids and nucleic acids, either directly or indirectly. These modifications can lead to cell death or induce differentiation and proliferation, which can culminate in diseases such as cancer. The physiological production of ROS results from metabolic processes that are essential to the cell and antioxidants have as function their elimination to avoid cellular damage. Oxidative stress arises when there is a disturbance in the oxidant-antioxidant balance, in favor of the oxidants, leading to potential damage[109]. Antioxidants include non-thiol compounds, essentially vitamins (ascorbate, tocopherol) and polyphenols; sulfhydryl-containing compounds, such as glutathione (GSH) and thioredoxin (Trx); and enzymes, such as

superoxide dismutases (SOD), catalase, glutathione peroxidases (GPx) and peroxiredoxins (Prx)[110].

Until the late 1900's, ROS were seen as dangerous molecules for the organism that needed to be eliminated. With the discovery of the first systems regulated by ROS, it became clear that at low concentrations, ROS can participate in signal transduction.

2. Hydrogen Peroxide metabolism

Louis Jacques Thénard discovered H_2O_2 in 1818 and H_2O_2 rapidly gained attention because of its industrial applications[111]. H_2O_2 is the most abundant ROS in aerobic organisms, including plants. Inside the cells, H_2O_2 exists in a steady-state level between 10^{-9}-10^{-7} M[107]. The biological source of H_2O_2 is diverse, however it is mostly produced from the dismutation of $O_2^{\bullet-}$ (equation 1):

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \text{ (equation 1)}$$

The spontaneous dismutation of $O_2^{\bullet-}$ has a rate constant of $\sim 2\times10^5$ $M^{-1}.s^{-1}$ at physiological pH, however the existence of enzymes – SODs – that catalyze the reaction improves greatly the process: 2×10^9 $M^{-1}.s^{-1}$. SODs are classified according to their metal ion content and localization: CuZnSOD that is largely located in the cytosol, MnSOD that localizes in mitochondria and FeSOD, which is found in bacteria. A different form of CuZnSOD can also be found extracellularly in the plasma[108].

In chemical terms, H_2O_2 is poorly reactive: it can act as a mild oxidizing or as a mild reducing agent, but it does not oxidize most biological molecules including lipids, DNA and proteins, except for those proteins with highly reactive sulfhydryl groups (discussed further)[112]. Nevertheless, H_2O_2 is seen as a threat to organisms. The importance of keeping H_2O_2 in low levels arises from its interaction with transition metals, mostly reduced iron (Fe^{2+}) or copper (Cu^+) ions and the consequent formation of the extremely reactive $HO^\bullet$ radical, through the Fenton reaction (equation 2) or directly by exposure to ultraviolet light[112]. *In vivo*, iron is kept protected in heme proteins, such as hemoglobin, transferrin, ferritin and lactoferrin, but H_2O_2 can induce the liberation of iron from some of those proteins[112].

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complexes} \rightarrow Fe^{3+} + HO^\bullet + HO^- \text{ (equation 2)}$$

Thus, to avoid oxidative stress from H_2O_2, it is necessary to keep it at low levels, which is guaranteed by specific enzymes.

2.1 Consumption of H_2O_2

H_2O_2 is relatively stable *in vivo*, for example, in lymphocytes, H_2O_2 half-life is 1 ms, while $O_2^{\bullet -}$ is 1 μs[108].

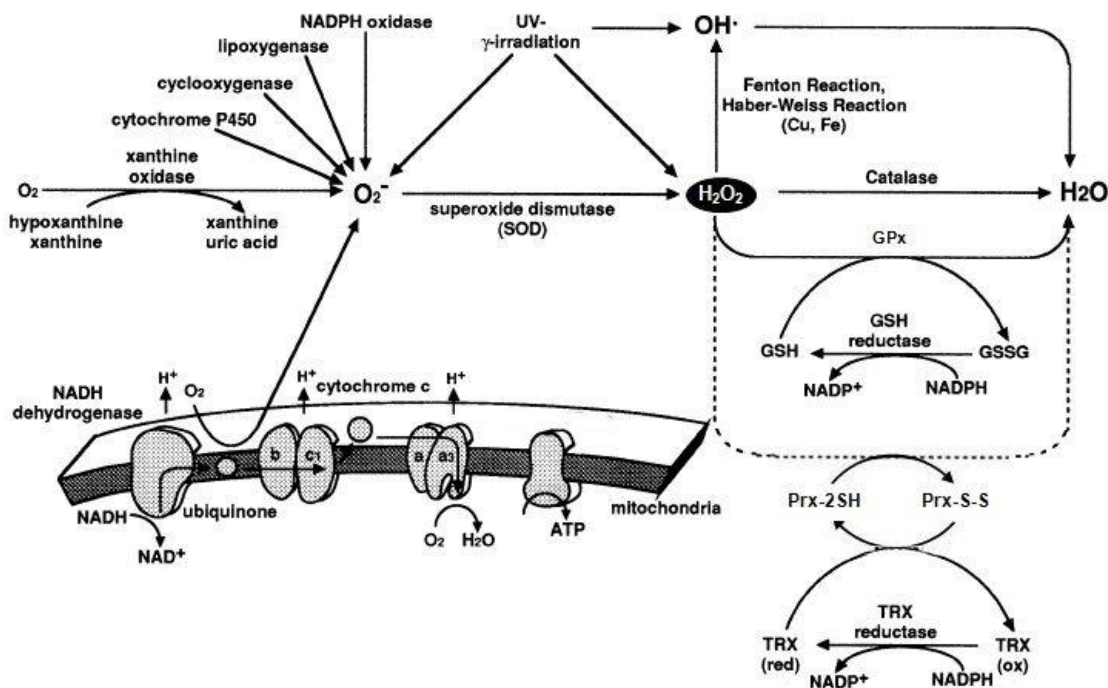
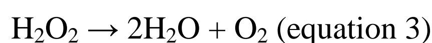


Figure 11 – Production and consumption of H_2O_2. H_2O_2 is mainly formed by the dismutation of $O_2\cdot^-$, which in turn is produced by several pathways indicated in the figure. Catalase, Gpx and Prx are responsible of H_2O_2 detoxification. Adapted from[113].

The main enzymes responsible for decomposition of H_2O_2 are catalase and GPx (Figure 11). At the same time that Thénard identified H_2O_2, he also suggested that a substance was able to decompose it. In 1900, Oscar Loew named the substance catalase and found its presence in plants and animals. Catalase is virtually present in all cells, in low levels in the cytosol and principally localized in subcellular organelles called the peroxisomes[107,114]. Peroxisomes exist at higher levels in the liver and kidneys[107]. Catalase contains a Fe^{3+}-protoporphyrin in its active center, and the overall reaction is the following:



$$H_2O_2 \rightarrow 2H_2O + O_2 \text{ (equation 3)}$$

Actually, the reaction can be divided in two steps with the formation of an intermediate, named compound I and identified in 1947 by Chance[107].

Gordon C. Mills discovered the classical GPx in 1957, as an enzyme able to prevent the oxidation of hemoglobin by hydrogen peroxide[115]. The specific GPx substrate, the tripeptide glutathione (GSH – γ-glutamyl-cysteinyl-glycine) was already known since 1921. Apart from the classical GPx, there are three other major GPx isoenzymes: gastrointestinal GPx, plasma GPx and phospholipid hydroperoxide GPx (PHGPx)[116]. Classical GPx is ubiquitously distributed in the organisms and it is located in the cytosol, the mitochondrial matrix, endoplasmic reticulum and nuclei, and catalyses the reduction of intracellular H_2O_2 and some organic hydroperoxides (ROOH) by GSH. PHGPx is mostly located in the membrane, but also nucleus, mitochondria and cytosol and can contribute for the removal of intracellular H_2O_2[116]. In general, GPx is the most important peroxidase for H_2O_2 removal in mammals[114] In 1969, Pinto and Bartley described the glutathione redox cycle[117] (Figure 12):

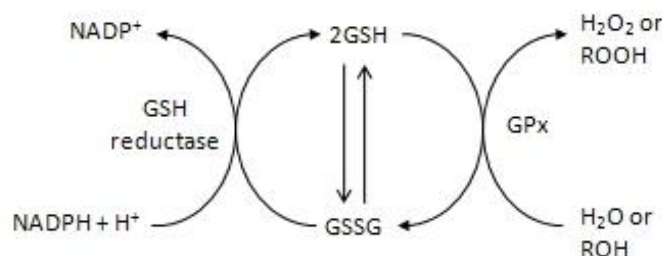


Figure 12 – The glutathione redox cycle. GPx catalyses the reduction of H_2O_2 and organic hydroperoxides (ROOH) by GSH to water (H_2O) and alcohols (ROH), respectively. The oxidized glutathione, GSSG, formed in this reaction is reduced back to GSH at the expense of nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction catalyzed by GSH reductase. Adapted from[117].

Catalase and GPx are the “classical” enzymes for H_2O_2 detoxification, but a new class of enzymes may contribute for the total removal of H_2O_2. Prx have a different reaction mechanism than GPx. While GSH is a substrate for GPx, the typical Prx contain two reduced cysteine residues in its active center, which are responsible for hydroperoxide reduction. The intramolecular disulfide formed is then reduced by thiols, such as Trx. Prx are primarily located in the cytosol, but can also be found in mitochondria, peroxisomes and nucleus. Six isoforms have already been identified in mammalian cells[118].

2.2 Production of H_2O_2

Production of H_2O_2 can occur in different subcellular localizations: mitochondria, peroxisomes, cytosol and plasma membrane (Figure 11). The major contributor of $O_2^{\bullet -}$

production, which is then converted to H_2O_2, was identified by Boveris and Chance in 1970[107] as being the electron transport in the mitochondria. $O_2^{\bullet-}$ is produced through one electron reduction of O_2 by a iron-sulfur cluster at the level of nicotinamide adenine dinucleotide (NADH) dehydrogenase (Complex I) and by an ubisemiquinone radical at the level ubiquinonol-cytochrome bc_1 reductase (Complex III)[107,108].

Peroxisomes contain different oxidases that catalyze several reactions that may produce H_2O_2 directly as a side-product, such as D-amino-acid oxidase, L-α-hydroxyacid oxidase, fatty acyl-CoA oxidase and urate oxidase, which explains the high content of catalase in these organelles[107]. The endoplasmic reticulum and mitochondria also contribute for $O_2^{\bullet-}$ and H_2O_2 formation through cytochrome P_{450}. Finally, cytosolic enzymes, such as xanthine oxidase, contribute to the cellular production of H_2O_2[107,108]. Overall, H_2O_2 is produced as a byproduct of aerobic metabolism. The adequate localization of H_2O_2-removing enzymes, catalase and GPx, prevents high intracellular levels of H_2O_2.

The first enzyme identified as producing deliberately ROS, including H_2O_2, was the phagocyte NADPH oxidase (NOX_{ph}, see box 4), during the respiratory burst of leucocytes[119]. ROS appear as the first line of defense against the pathogens because of their toxicity. Following the contact between the phagocyte and an opsonized particle, NOX_{ph} is activated and reduces the extracellular O_2, at the expense of intracellular NADPH, resulting in the production of $O_2^{\bullet-}$. After the integration of the pathogen by the membrane and the formation of the phagosome, phagocyte granules containing SOD and myeloperoxidase will fuse and contribute for the formation of hypochlorous acid and $HO^{\bullet}$[120,121]. Importantly, part of the $O_2^{\bullet-}$ dismutates to H_2O_2 and diffuses out of the phagosome, staying in the extracellular compartment[122]. This leakage was first interpreted as a local damaging process, contributing for tissue inflammation.

During the 1990s sensitive assays improved the intracellular detection of H_2O_2, and other ROS, allowing the identification of other possible sources of H_2O_2[120]. In 1999, a first homologous protein to the gp91 was identified in a colon epithelial cell and

Box 4 - The phagocyte NADPH oxidase (NOX_{ph})
In unstimulated phagocytes, the catalytic subunit gp91 and the regulatory subunit p22 form an inactive complex, known as the flavocytochrome b_{558}. It is mostly located in the membranes of intracellular granules, although ~5% is located in the membrane of the phagocytes. Exposure of cells to microorganisms or inflammatory mediators results in the assembling of other cytosolic subunits to the flavocytochrome b_{558}. Those subunits are regulators of the gp91 activity and include the p40, the p47, the p67 and the small GTPase RAC subunits. The complete assembly originates an active NADPH oxidase capable of producing $O_2^{\bullet-}$[119,120].

named NOX1. Nowadays, seven different NOX have been identified in different tissues[120]. The exact function of these extracellular sources of $O_2^{\bullet-}$ and H_2O_2 is not clearly understood, but they might work similarly to the leucocytes in the defense of the organism, although a signaling role may not be excluded.

3. The intracellular redox state

It is important to consider that redox reactions occur not only during antioxidant response to oxidative stimuli, but as the continuous fine adjustment of a network of redox couples that ultimately defines the redox potential of the cell[110]. Because of the oxidizing environment, the redox status existing inside the cells is tightly controlled and remains relatively constant unless cells are exposed to drastic oxidizing conditions[123]. Enzyme activation, DNA synthesis, cell cycle regulation, transcriptional activation of specific genes and programmed cell death are all biological events dependent on the redox state of the cell[123].

In the 1980s, as the concept of oxidative stress was biochemically defined, a link between cellular redox properties and GSH began to emerge[124]. Thomas and co-workers showed that GSH may interact in a reversible manner with the sulfhydryl groups of the cysteine residues of many cellular proteins during boosts of oxidative stress[124]. GSH participates in redox reactions through the reversible oxidation of its active sulfhydryl, by forming intermolecular disulfide bonds (GSSG form) (Figure 12). The reversible reduction of the disulfide is catalyzed by the enzyme glutathione reductase, which is dependent on the NADPH pool for the source of electrons. Without doubt, GSH functions as the major “redox-buffer” of the cell[123]. In unstressed cells, the majority of this redox regulator is in its reduced form and its cellular concentration is between 0.5 and 10 mM, depending on the cell type[123].

Apart from GSH, Trx also participates in the maintenance of a cellular reducing environment, especially in the nucleus. This reducing nuclear environment is necessary to assure DNA synthesis and transcription. Trx is a small multifunctional protein with two redox-active cysteine residues within a conserved active site (Cys-Gly-Pro-Cys)[125]. As a dithiol $-(SH)_2$, it usually forms intramolecular disulfide bonds and its reversible reduction by NADPH is catalysed by thioredoxin reductase[126]. Importantly, H_2O_2 induces the expression of Trx[127]. Hence, the cytosol and nuclei of cells are maintained under a strong reducing environment essentially by the contribution of the couples GSH/GSSG, $Trx(SH_2)$/TrxSS and $NADPH/NADP^+$ [110].

4. Oxidative modifications of cellular proteins

Due to its relatively low reactivity H_2O_2 is able to react rather specifically[111]. The term critical cysteine is used to define a reactive cysteine residue within a protein. Unlike the majority of cysteine residues in proteins, reactive cysteine residues have a low p*K*a and are in the thiolate form (S^-) at physiological pH[128]. These cysteine residues of proteins are targeted by H_2O_2, and their oxidation can alter the protein structure and function. The sulfhydryl group (-SH) of a single cysteine residue of a protein may be oxidized to form a sulfenic acid (-SOH), which is generally unstable and can either react with a nearby thiol (discussed below) or being further oxidized to sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) acids (Figure 13). Sulfinic and sulfonic acids were viewed as irreversible protein modifications until the discovery of sulfiredoxin, first in *Saccharomyces cerevisiae*, which is able to reduce the cysteine-sulfinic acid in Prx[129,130]. GSH can reduce the sulfenic acid, by a process called S-glutathionylation, a particular case of S-thiolation, which consists in the formation of a mixed disulfide involving the SH group of GSH and the SH group of the oxidized protein. It is a reversible process that occurs under physiological conditions, but it is an early cellular response to oxidative stress and affects the cellular redox state[125,126]. Protein mixed disulfides are efficiently reduced by the enzyme glutaredoxin, a reaction dependent on the NADPH pool[125]. A minor contribution of Trx also exists.

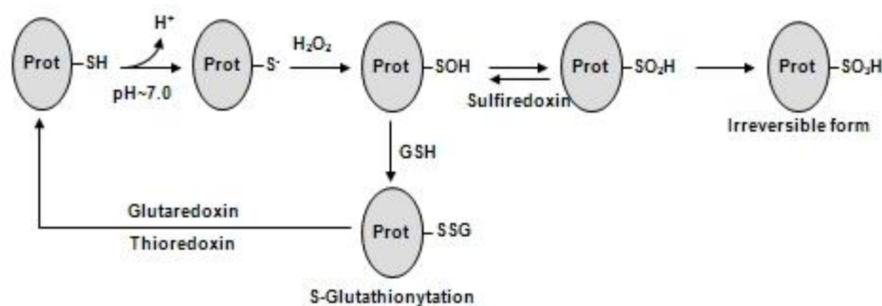


Figure 13 – Oxidation of critical sulfhydryl groups on proteins. H_2O_2 is able to oxidize critical sulfhydryl groups (S^-) on proteins (Prot) forming sulfenic (-SOH), sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) acids. Sulfenic products may react with GSH – S-glutathionylation – and be reduced by glutaredoxin. Further oxidation of the sulfenic acid form originates the sulfinic form, which might be reduced by sulfiredoxin, and then the irreversible sulfonic acid form.

A recent work from Cumming et al.[131] showed that 10 mM H_2O_2 for 5 minutes increased 14 times the cellular content of mixed disulfides, with only a 3 fold increase in GSSG content, relatively to untreated cells.

The presence of two (or more) cysteine residues within the same protein may be oxidized by H_2O_2 to form an intramolecular disulfide bond(s) (Figure 14). This modification alters the

conformation of the protein and consequently its function[125]. GSH and Trx can directly reduce the disulfide bonds through their reversible oxidation.

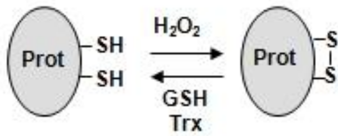


Figure 14 – Formation of intramolecular disulfide bonds in proteins induced by H_2O_2.

5. H_2O_2 regulates cellular signaling systems

Although H_2O_2 was first known for its cytotoxic effects, nowadays its involvement in signal transduction pathways is well established. H_2O_2 is an effective signaling molecule because of the characteristics mentioned before, including its fast production, relative stability *in vivo* and reaction with specific protein targets[111,132]. Essentially, the concentration of H_2O_2 determines the cellular events in which it participates, such as apoptosis, proliferation, cell adaptation and regulation (Figure 15). Next, some of the regulatory pathways where H_2O_2 is involved are described.

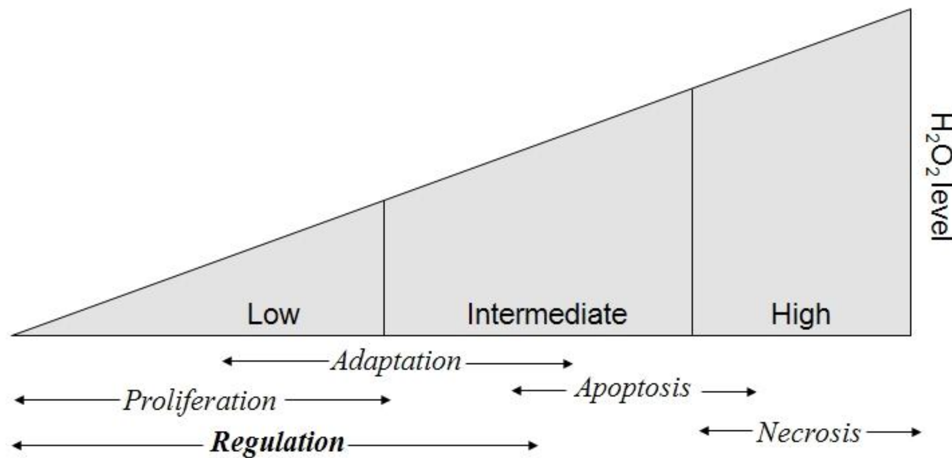


Figure 15 – Metabolic processes dependent on H_2O_2 concentration. The intracellular function of H_2O_2 is determined by its concentration. Concentration is roughly divided in high (mM range) to low (nM to µM range).

H_2O_2 levels are tightly controlled by the H_2O_2-degrading enzymes, which are themselves regulated by their specific cellular localization and expression[133]. In fact, exposure of muscle cells to H_2O_2 increase the transcript levels of GPx and catalase and their respective activity[134]. H_2O_2 is a small molecule, with the ability to cross membrane barriers, enabling the entry of H_2O_2 produced by NOX proteins into cells. It is important to note that the diffusion of H_2O_2 across membrane barriers is not a free process, and gradients across cell

membranes are formed[135]. Estimation of the value of gradient (*R*), i.e. the ratio of the extracellular concentration of H_2O_2 by the intracellular concentration ($[H_2O_2]_{out}/ [H_2O_2]_{in}$), clearly showed the role of the plasma membrane in regulating the entrance of H_2O_2 into cells[135,136].

Signal transduction is strongly influenced by phosphorylation events, which are controlled by cascade kinases. About 500-600 kinases have been identified and can be divided in different sub-families. In eukaryotes, a well-known distinction is between the serine/threonine protein kinases, such as the MAPK, PKC and protein kinase B (Akt), and protein tyrosine kinases (PTK), such as Src family, PI3K and Janus kinase (JAK)[110]. This classification indicates the amino acid residue target for phosphorylation. Similar to the reversibility of oxidative modifications of proteins, the action of specific phosphatases can deactivate phosphorylation signaling[110]. The identification of a cross-talk between redox and phosphorylation processes helps to understand H_2O_2 role in signaling.

5.1 H_2O_2 regulates kinases and phosphatases

Early studies have described a role for H_2O_2 in cellular regulation, even though the H_2O_2 doses applied were not physiological. The first report on a possible regulatory role for H_2O_2 appeared in 1972 when Czech *et al.*[137] demonstrated that 8 mM H_2O_2 markedly increased the uptake of D-glucose by isolated white fat cells, thereby mimicking the insulin effect. Soon after, the same authors showed that components of the glucose transport system must be maintained in its disulfide state for transport activity, suggesting a possible target for H_2O_2 action[137]. Nowadays, it is known that signaling by insulin is mediated by autophosphorylation of the insulin receptor kinase and H_2O_2 doses above 100 μM are able to directly cause this phosphorylation[127] (Figure 16). Lower and physiological H_2O_2 concentrations only up-regulate the insulin effect. Moreover, besides its autophosphorylation capacity, the insulin receptor kinase is able to catalyze the phosphorylation of tyrosine residues in other proteins and H_2O_2 potentiates this effect in insulin-treated cells[138]. In 1976, White *et al.*[139] described another evidence of regulation by H_2O_2, and showed that H_2O_2 could activate guanylate cyclase, in rat lung. Guanylate cyclase is the enzyme responsible for the formation of 3',5'-cyclic guanosine monophosphate (cGMP), which acts as a second messenger. In vascular smooth muscle cells, insulin stimulates the production of $O_2^{\bullet-}$ by NOX. $O_2^{\bullet-}$ is converted to H_2O_2 by SOD which then activates guanylate cyclase[140]. Importantly, the effect of H_2O_2 was only observed in the presence of nitric oxide (NO), a gas known to induce vasodilatation[140].

The mechanism is not well understood, but again, H_2O_2 seems to have a cooperativity effect with the insulin receptor, rather than an individual action.

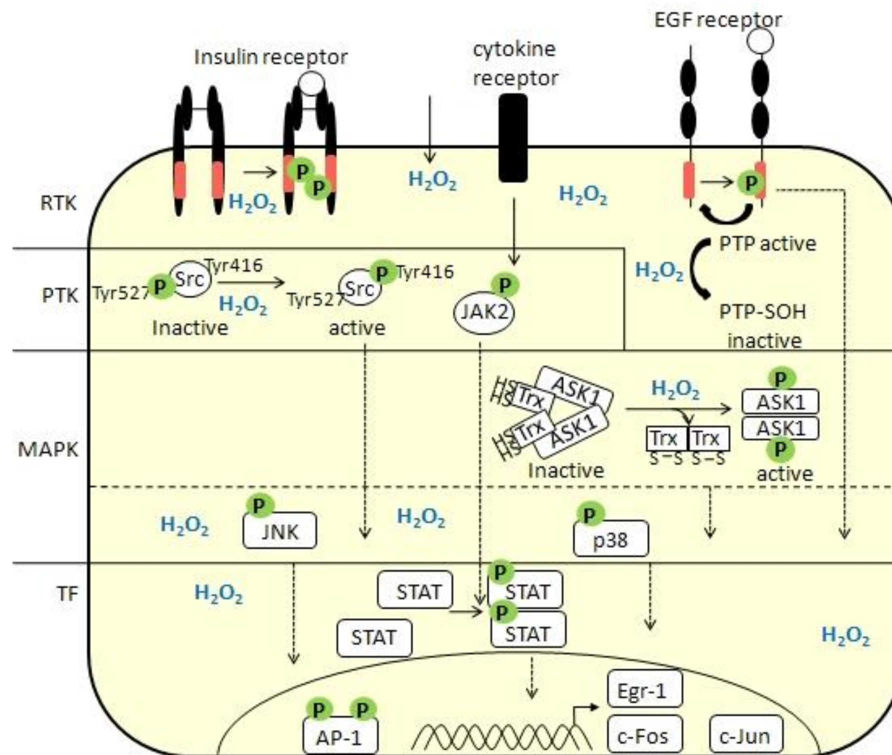


Figure 16 – Redox-sensitive kinases, phosphatases and transcription factors. H_2O_2 is able to mediate a signaling cascade from the receptor-tyrosine kinases (RTK), e.g. the insulin receptor and epidermal growth factor (EGF) receptor, up to the transcription factors, by activating protein tyrosine kinases (PTKs) and mitogen-activated protein kinases (MAPK). H_2O_2 distributed in the cell can also activate directly some kinases. The RTKs are activated by phosphorylation (P), mediated by H_2O_2, which by inhibiting protein tyrosine phosphatases (PTPs) promotes activation of RTKs. Tyr527 phosphorylation maintains Src kinase inhibited. Src kinase under oxidizing conditions is dephosphorylated, thereby activating the kinase. The Janus kinase 2 (JAK2) is typically activated by cytokines and growth factors, but also by H_2O_2 through phosphorylation. JAK2 catalyzes the phosphorylation of the signal transducer and activator of transcription (STAT), which forms activated homodimers. The apoptosis signal-regulating kinase 1 (ASK1) is latent when bound to reduced Trx. c-Jun-N-terminal kinase (JNK) is activated by various upstream kinases and probably by a direct effect of H_2O_2. JNK catalyzes the phosphorylation of the subunits of the activating protein 1 (AP-1), which activates gene expression. H_2O_2 increases the expression of various genes, such as early-growth response 1 (Egr-1), c-Fos and c-Jun[110,141-143].

H_2O_2 is known to induce tyrosine phosphorylation of several proteins[127]. Nevertheless, increased tyrosine phosphorylation by H_2O_2 is a consequence, albeit not exclusive, of the oxidative inhibition of protein tyrosine phosphatases (Figure 16). The extent of protein tyrosine phosphorylation reflects the equilibrium between the actions of PTKs and protein-tyrosine phosphatases (PTPs)[144]. In eukaryotes, PTPs have a central role in controlling signaling events initiated in response to many stimuli, including growth factors and cytokines[133]. Similarly to the insulin receptor, the epidermal growth factor (EGF) also undergoes autophosphorylation after binding of its ligand EGF, to initiate the signaling

cascade. It is possible that this process is mediated by stimulation of a transient H_2O_2 production[144,145]. PTPs contain a reactive cysteine residue (Cys215) subject to redox regulation within its active center. H_2O_2 added extracellularly, or generated by EGF, inhibits PTP1B activity through oxidation of Cys215 to a sulfenic acid intermediate[144-146]. The reversibility of the process is guaranteed by glutathionylation of the sulfenic acid-PTP1B[146], or by the formation of a sulphenyl-amide species[147,148]. The latter is a newly described modification that imposes conformational changes on the enzyme. The mechanism of stimulation of H_2O_2 production allows the rapid activation of the signaling cascade by EGF, followed by a down-regulation through re-activation of the PTPs, thus involving H_2O_2 in cell proliferation.

The Src proteins are non-receptor upstream kinases critically involved in the control of cytoskeletal organization and in the generation of integrin-dependent signaling responses in fibroblasts, including tyrosine phosphorylation[142]. The activity of the Src tyrosine kinases is tightly controlled by the inhibitory phosphorylation of a C-terminal tyrosine residue (Tyr527) of Src, which induces an inactive conformation[142] (Figure 16). Binding of extracellular matrix (ECM) ligands to the integrins causes a rapid dephosphorylation, regulated by different phosphatases in different cell types, which induces a conformational change that promotes an autophosphorylation in another tyrosine (Tyr416) and, consequently, activates the kinase[142]. H_2O_2 (0.15-0.5 mM) induces Src phosphorylation, probably at Tyr416, because it induces an increase of its kinase activity[127]. Giannoni *et al.* proposed that the engagement of integrins by the binding of ECM stimulates ROS production, namely H_2O_2, which peaks after 45 minutes and contributes for a late activation of the kinase. Src undergoes a direct oxidation by ROS, which enhances its kinase activity, and is coherent with an increase in cell adhesion and spreading[142]. Src proteins are also activated by other stimuli and have been shown to be involved in the activation of other kinases, namely the c-Jun-N-terminal kinase (JNK)[149]. JNK is a member of the MAPK implicated in the survival/apoptosis pathways and linked to various stress-responses. It is rapidly activated by H_2O_2 in numerous cell lines and with different stimuli[125,127] (Figure 16). In lung epithelial cells JNK activation by H_2O_2 is dependent on the TNF receptor. Pantano *et al.*[150] observed that exposure to 300 μM H_2O_2 induced the recruitment of the adapter proteins to the TNF-R1, which lead to activation of JNK, leaving the IKK inactivated because H_2O_2 induced RIP degradation[150]. A direct effect on JNK by H_2O_2 is also possible. In non-stressed cells JNK is bound to an inhibitor, identified as the isoform GSTπ, which avoids JNK phosphorylation. In MEF cells, treatment

with 10 μM H_2O_2 leads to a dissociation of the complex GST/JNK, probably by oxidative modifications that were not yet identified, which results in the activation of JNK [110,151].

Elevated levels of ROS, including H_2O_2, contribute to the activation of the apoptosis pathway. JNK and ERK activated by H_2O_2 have different contributions for the apoptotic process[152]. The ERK pathway is activated by various growth factors and closely linked to the regulation of cell cycle. Exposure of HeLa cells for 12 hours with 600 μM H_2O_2 induced 30-40% of apoptosis[152]. ERK was important in the control the survival process, while JNK contributed for apoptosis, suggesting that a dynamic balance between the ERK and JNK pathways is important in determining whether a cell survives or undergoes apoptosis[152]. Which pathway superimposes the other and determines cell fate is a function of H_2O_2 concentration and of the sensitivity to H_2O_2 of a particular cell line. The exact mechanism and the upstream kinases involved in JNK and ERK activation by H_2O_2 is not clear, but it probably involves the apoptosis signal-regulating kinase 1 (ASK1). ASK1 is a MAPK kinase kinase (MAPKKK) activated by various stresses and is implicated in the regulation of cell death, cell differentiation and immune regulation in response to cytokines[153] (Figure 16). In its resting state ASK1 forms a static homo-oligomer through its C-terminal end and the N-terminal end binds to Trx, thereby inhibiting its activity in the absence of oxidative stress[153]. H_2O_2 induces the formation of a disulfide bond at the sulfhydryl groups of Trx, causing its dissociation from ASK1 and, consequently, the autophosphorylation of the threonine residue (Thr838) located in the activation loop of the latter. ASK1 is strongly activated by H_2O_2 or by other ROS-generating stimuli and the ASK1-Trx regulatory mechanism functions as an important redox sensor, converting the signaling due to intracellular redox changes into the signaling of kinase cascades[153]. It is important to note that probably high H_2O_2 concentrations will activate ASK1. 10 mM H_2O_2 clearly activated ASK1 and selectively inhibited MEKK1, which is an upstream kinase involved in survival signaling[154].

PKA is a ubiquitously expressed signaling molecule. In the absence of cAMP, the kinase exists as an inactive tetramer comprised of two catalytic (C) and two regulatory (R) subunits. Upon cAMP binding to the R-subunits, the active C-subunit is released[155]. The C-subunit activation loop contains a threonine (Thr197), which needs to be phosphorylated for optimal enzymatic activity. Interestingly, this phosphorylated threonine is resistant to phosphatase treatment because of a conformational protection induced by a highly reactive cysteine (Cys199)[155]. Down-regulation of the C-subunit PKA is achieved by the oxidation of Cys199, using okadaic acid, probably by forming mixed disulfides, which makes possible to expose

the phosphorylated threonine to the action of phosphatases[155]. While the oxidation of Cys199 seems to inhibit the catalytic activity of PKA, another report shows that the regulatory subunit can be activated by H_2O_2[156]. H_2O_2 (100 μM) induces the formation of an intramolecular disulfide bond between sulfhydryl groups present in the two R-subunits, which increases the affinity of the PKA anchor proteins, which “drives” PKA to its substrates. [156]. Thus, whether PKA is activated or not by H_2O_2 is determined by the modifications induced in both the catalytic and regulatory subunits[156]. Actually, in many cases H_2O_2 has been shown to have a dual-role. The list of reports showing the influence of H_2O_2 in the activity of kinases is extensive and also includes the PI3K and PKC kinases.

5.2 H_2O_2 regulates transcription factors

The redox regulation previously discussed, which starts at the cell receptors and involves the MAPKs family, often culminates in the activation of different transcription factors. In fact, it has been shown that the expression of many genes, such as c-fos, c-jun, intracellular adhesion molecule 1 (ICAM-1) and heme oxygenase 1 (HO-1), increases in the presence of H_2O_2. The expression of such genes may be mediated by transcription factors either in response to the activation of upstream signaling pathways or by direct modulation[113].

The loss of function of transcription factors after the oxidation of critical cysteine residues may be a result of a specific signaling event, but as well can reflect non-physiological oxidizing conditions. A gain of function by formation of disulfide bonds was described by a direct effect of H_2O_2 in the transcription factor OxyR in *Escherichia coli*[157,158]. OxyR activates the expression of bacterial antioxidant enzymes and is kept inactive in the reduced state in the cytosol of the cells. It has two critical cysteine residues (Cys199 and Cys208) that are oxidized in the presence of H_2O_2 and form a disulfide bond, rendering the transcription factor transiently active[157,158]. OxyR induces the expression of several genes coding for antioxidants, including glutaredoxin1 and glutathione reductase, which, by catalyzing the reduction of the disulfide bond are responsible for the turn-off of the transcription factor[157,158].

The transcription factor Yap1 in yeast was named after the similarity of its DNA consensus sequence with the one present in mammalian AP-1 transcription factor[159]. In *Saccharomyces cerevisiae*, Yap1 was shown to be required for the adaptive response to H_2O_2 and the H_2O_2 - dependent activation of Trx2[159]. Yap1 functions as a redox sensor as it is rapidly activated

after exposure to H_2O_2. Delaunay *et al.*[160] showed the involvement of two reactive cysteine residues (Cys303 and Cys598) in the formation of a intermolecular disulfide bond, which changes the conformation of the transcription factor, allowing its activation[160]. Just like it happens for OxyR, Yap1 activation is down-regulated by antioxidant enzymes.

A regulation involving H_2O_2 signaling has been proposed also for some mammalian transcription factors. The transcription factor activation protein-1 (AP-1) is typically composed by the c-Fos and c-Jun proteins[113,123]. c-Fos and c-Jun are immediate-early genes that bear different DNA *cis*-elements and are expressed by exposure to H_2O_2 (Figure 16). For example, exposure to 100 μM H_2O_2 for 1 h induces maximal expression of c-Jun in aortic smooth muscle cells[161], whereas exposure to 100 μM H_2O_2 for 10 min induces c-Fos expression in cardiomyocytes[162]. Newly synthesized, and some pre-existing, components of AP-1 are activated by phosphorylation. H_2O_2 mediates this activation through regulation of JNK[161-163].

The heat shock factor 1 (HSF-1) is responsible for the expression of heat shock proteins (HSP) and resistance to stress-induced apoptosis[164]. It is activated by numerous stimuli, such as inflammation, heat shock and H_2O_2[164]. Inactive monomers of HFS-1 are activated by formation of homotrimers, which are able to bind to the cognate sites on DNA. Ahn *et al.* [164] showed that H_2O_2 is able to cause this trimerization, probably through a redox reversible mechanism involving formation of a disulfide bond between two-cysteine residues[164]. A previous report, also described the activation and translocation of HSF-1 to the nucleus by H_2O_2[165]. However, it also demonstrated the requirement of Trx to reduce the cysteine residues, otherwise HSF-1 was not able to bind to DNA[165]. These works are not mutually exclusive and both show the importance of redox-regulation.

The early growth response-1 (Egr-1) transcription factor is a nuclear protein that is co-expressed with several growth factor genes. Increase in its expression is important for the survival of cells from apoptosis. Jin *et al.* demonstrated that H_2O_2 increases the expression level of Egr-1, by increasing tyrosine kinase activity[161] (Figure 16).

The redox regulation of NF-κB has been widely studied. As the theme of this work is the investigation of the regulation of NF-κB by H_2O_2, a detailed description of this subject is provided in the next section.

Part 3 – NF-κB and H_2O_2

1. H_2O_2 as the universal mediator of NF-κB activation

NF-κB was the first eukaryotic transcription factor shown to be redox-regulated. In 1991 Herzenberg and coworkers[166] described that intracellular thiols mediate NF-κB activation by TNF-α and PMA. By lowering the levels of GSH, TNF-α-induced NF-κB activation increased, and accordingly, by using *N*-acetyl-L-cysteine (NAC) NF-κB activation decreased[166]. NAC is a radical scavenger and a precursor of glutathione synthesis. Similarly, the antioxidant pyrrolidine dithiocarbamate (PDTC) inhibited NF-κB activation by blocking NF-κB released from IκB-α in cells treated with IL-1 and TNF-α[167]. Then, the work of Schreck *et al.*[168] demonstrated that the NF-κB pathway was activated by adding 150 μM H_2O_2 directly to a subclone of Jurkat T cells, later named Wurzburg cells. The cells used were infected with the human immunodeficiency virus type 1 (HIV-1), whose expression is dependent on NF-κB activation, and H_2O_2 was shown to increase expression of the virus[168]. Interestingly, H_2O_2 did not activate the purified complex NF-κB/IκB *in vitro*, which indicated that the *in vivo* activation was due to a subsequent regulation by H_2O_2, for example changes in the redox state of the cell. These first two works indicated a possible convergent pathway for NF-κB activation by different stimuli, which was dependent on the intracellular redox level. The idea was supported by Anderson *et al.*[169], who added tyrosine phosphorylation as an essential component of this signal transduction pathway. The common pathway was considered universal, regardless to the cell line, and supported by the fact that all NF-κB stimuli shared the ability to produce H_2O_2[170]. So, H_2O_2 or other ROS were proposed as universal second messengers of the NF-κB activation pathway, whose function was inhibited by antioxidants like NAC and PTDC, but also by overexpression of catalase[171].

Soon, several reports began to question the universal pathway of NF-κB activation mediated by ROS. For instance, the direct activation by H_2O_2 was controversial: while in certain cell types, such as Wurzburg, MCF-7 and 70Z/3 pre-B cells, H_2O_2 was an effective inducer of NF-κB; in other, such as Jurkat T cells, HeLa and fibroblasts no activation was observed with H_2O_2 treatment[172]. In addition, in H_2O_2-responsive cells, NF-κB activation proceeds slowly (hour range) after H_2O_2 treatment, contrasting with the rapid response (minute range) by cytokine stimuli[173].

The use of NAC and PDTC to prove the common pathway was re-analyzed and reports showed that they attenuated NF-κB activation independently of their antioxidant potential[174]. Hayakawa *et al.*[174] found that NAC selectively blocked TNF-α-induced signaling by lowering TNF-α affinity to its receptor TNF-R1 and that PDTC interfered with the ubiquitin ligase activity, thereby inhibiting IκB-α degradation. An earlier study from Brennan *et al.*[175] identified a pro-oxidant character of PDTC capable of inhibiting NF-κB activation, which was reversed by addition of 2-mercaptoethanol. Conflicting data using antioxidant enzymes appeared to argue against a universal role for H_2O_2 in NF-κB signaling. Whereas in one study the overexpression of MnSOD in MCF-7 cells completely abolished TNF-mediated NF-κB activation[176], in another study using the same cells, overexpression of MnSOD greatly potentiated TNF-α-dependent NF-κB activation[171]

2. Is NF-κB regulated by H_2O_2?

Even though the universal role for H_2O_2 in the NF-κB pathway was ruled out, studies using direct cell exposure to H_2O_2 continued because parts of the pathway were sensitive to redox changes. H_2O_2 alone or in conjunction with other NF-κB stimulants was used in these studies. The reason for the latter strategy was to simulate somehow inflammatory conditions. The inflammatory environment is rich in cytokines and chemokines, but also in H_2O_2 originating from the respiratory burst. ROS have been related with chronic-inflammatory diseases, but in physiological conditions could they participate in signaling?

Many contradictory data have been published about H_2O_2 participation in the NF-κB pathway. The use of different cell types is an important factor, although the concentration of H_2O_2 employed is crucial, because increased doses can lead to apoptosis and necrosis (Figure 15) and on the other hand low doses are rapidly consumed by the specific enzymes.

Next, some points of regulation by H_2O_2 identified in the NF-κB pathway are presented, taking into account the existing divergent data (Figure 17).

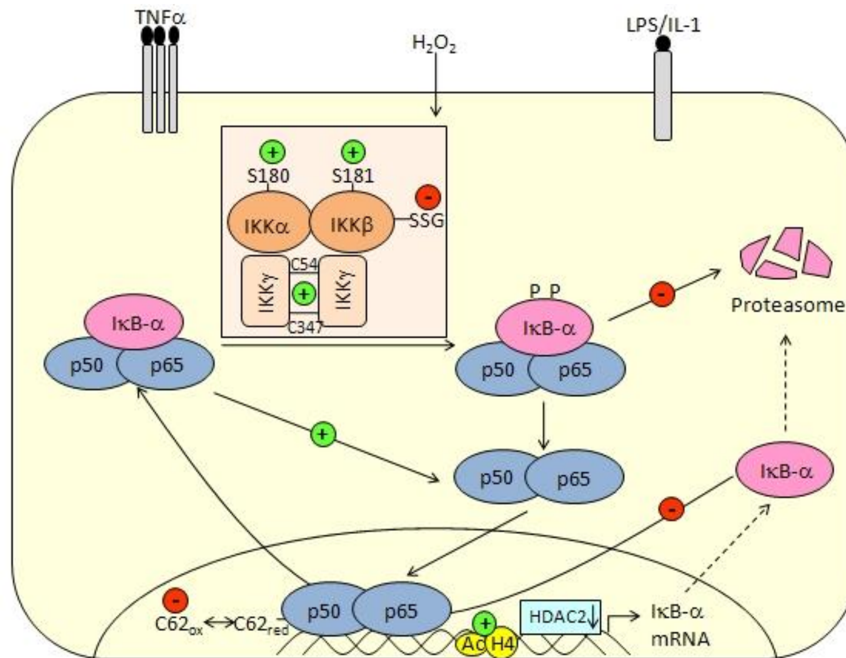


Figure 17 – Regulation of the NF-κB pathway by H_2O_2. H_2O_2 is able to cooperate or inhibit the action of TNF-α, LPS and IL-1 by inducing specific molecular modifications and/or modulate the classical pathway. H_2O_2 targets are represented with the symbols (+) and (-) for actions that stimulate or inhibit the NF-κB pathway, respectively. H_2O_2 increases the activity of the IKK complex through a direct action on the activation loops of IKKα and IKKβ, and by inducing dimerization of IKKγ proteins, whereas S-glutathionylation of Cys179 in IKKβ has the opposite effect. H_2O_2 is able to stimulate the NF-κB pathway by increasing NF-κB translocation to the nucleus without IκB-α degradation, which is in opposition with the reported inhibitory effect of NF-κB translocation because of the decreased catalytic activity of the proteasome by H_2O_2. Once in the nucleus, Cys62 of p50 needs to be reduced to enable NF-κB transactivation of the DNA, which in turn is facilitated by acetylation of histone H4 and decreased activity of HDAC2 by H_2O_2. H_2O_2 is able to inhibit the import of newly synthesized IκB-α thus maintaining NF-κB inside the nucleus for a longer time[50,177-182].

2.1 H_2O_2 and the IKK complex

The ability of H_2O_2 to regulate IKK activity has been investigated by multiple groups. In mouse alveolar epithelial cells, IKK and NF-κB activation were insensitive to H_2O_2 alone[183]. However, 200 μM H_2O_2 markedly decreased the ability of 10 ng mL^{-1} TNF-α to activate the IKK complex, resulting in the prevention of IκB-α degradation and NF-κB activation[183]. Inhibition of the IKK was associated with direct oxidation of the Cys179 in IKKβ, which afterwards suffers S-glutathionylation that is reversed by glutaredoxin-1[179]. In opposition, Kamata *et al.*[177] using HeLa cells showed that 3 mM H_2O_2 slightly activated IKK, although at later time points than TNF-α and consequently originated a sustained activation of NF-κB when H_2O_2 and TNF-α were added together. Moreover, phosphorylation of serine residues, namely Ser180 of IKKα and Ser181 of IKKβ which are located in the activation loops, was essential for H_2O_2-mediated IKK activation[177]. A recent study reported that IKKγ dimers are linked through disulfide bonds formed between Cys54 and Cys347, a requirement for the

correct NF-κB activation. H_2O_2 was able to induce IKKγ dimerization, but a pre-treatment with 200 μM H_2O_2 before the addition of TNF-α prevented IKK activation, probably by interfering with the IKKβ subunit[178].

Alternatively, it is possible that H_2O_2 acts upstream of the IKK. IL-1β-stimulated MCF-7 cells induce H_2O_2 production in a NOX-dependent manner, which in turn facilitates NIK activation through inhibition of phosphatases. The consequent phosphorylation of IKKα by NIK is confirmed by the treatment of cells with 1 mM H_2O_2[184].

2.2 H_2O_2 and the IκBs

In cells where NF-κB is activated by H_2O_2, the fate of IκB-α after the activation of the pathway is somewhat conflicting. With the EL4 mouse lymphoma cell line, Schoonbroodt *et al.*[185] observed that phosphorylation of the Ser32 and Ser36 residues in IκB-α were not required for NF-κB activation by H_2O_2 alone (300 μM), whereas phosphorylation of the Tyr42 residue and the PEST sequence was essential but independent of IKK activity. Phosphorylation of Tyr42 and PEST relies probably on CKII activity[185]. Whether phosphorylated IκB-α on Tyr42 is consequently degraded is not yet consensual[185,186]. The Syk upstream-kinase has been identified as the mediator of Tyr42 IκB-α phosphorylation[186]. It is important to note that Tyr42 phosphorylation was observed in other cell lines, but with other conditions including pervanadate and hypoxia/reoxygenation. A direct H_2O_2 effect on Tyr42 is probably restricted to T cells, even though an independent pathway involving H_2O_2 is still valid. This possibility allows a synergistic effect of H_2O_2 on TNF-α-induced NF-κB via the classical pathway[180,187] (Figure 17). Janssen-Heininger *et al.*[187] did not report IκB-α degradation by H_2O_2 alone in rat lung epithelial cells, but they observed a cooperativity of NF-κB activation for 100 μM H_2O_2 and 10 ng mL^{-1} TNF-α. The activation pathway by H_2O_2 was dependent on Ras, known to be a sensor of oxidative stress. Downstream of Ras they showed that the engagement of MEKK1 and JNK, was essential for the H_2O_2 effect.

Antagonistic effects of H_2O_2 on NF-κB activation were also described. Jaspers *et al.*[181] observed, in TNF-α-induced human bronchial epithelial cells, a stimulation of IKK activity, IκB-α phosphorylation and ubiquitination by H_2O_2 (500 μM). However, NF-κB transactivation was inhibited because no subsequent degradation of IκB-α was reported, which suggested inhibition of the proteasome activity. In LPS-treated neutrophils, 250 μM

H_2O_2 decreased NF-κB-dependent gene expression by inhibiting the proteasome and consequently IκB-α degradation[188].

2.3 H_2O_2 and the transactivation potential of NF-κB

The intracellular redox state can influence the NF-κB pathway. Sulfhydryl modifying agents, such as the oxidant diamide, inhibit *in vitro* NF-κB binding to DNA, while, reducing agents increase DNA binding[189]. The Cys62 residue on the p50 subunit of NF-κB was identified as being responsible for this dependence on oxidation-reduction conditions (Figure 17)[182,190]. Formation of a sulfenic acid by oxidants, followed by S-glutathionylation is the probable modification of Cys62, which inhibits the capacity of NF-κB to bind to DNA[191]. Overexpression of Trx is able to reverse Cys62 oxidation[190]. Endogenous Trx is responsible for the maintenance of reducing conditions within the nucleus, in order to permit NF-κB binding to the DNA[192]. Therefore, different redox requirements in the cytosol and in the nucleus are necessary for appropriate NF-κB activation. Jornot *et al.*[193] illustrated the ability of H_2O_2 to induce NF-κB translocation to the nucleus, but without further transactivation of DNA. In the cytosol, a pro-oxidant signal may activate NF-κB and its translocation to the nucleus but in the nucleus, NF-κB proteins must remain reduced in order for DNA binding to occur. Trx is the key molecule for this compartmental regulation[194].

Oxidative stress is a characteristic of chronic inflammatory diseases, such as asthma, chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis[195]. There is a link between these diseases and enhanced NF-κB activation, and so the effect of H_2O_2 on NF-κB transactivation in a pathological context is widely studied. H_2O_2 (100 µM) was shown to enhance acetylation of histone H4 proteins and to decrease HDAC2 activity and expression, permitting an increased-NF-κB transcription in alveolar epithelial cells[196].

A recent work from Enesa *et al.*[50] reported that H_2O_2 stimulation of IL-1 and TNF-α activation of NF-κB transcription was due in part to the suppression of p65 export, thus prolonging its nuclear localization and binding to DNA. This effect resulted form the enhanced degradation of the newly synthesized IκB-α, which normally goes to the nucleus to remove NF-κB from the DNA. In TNF-α-treated cells, H_2O_2 was also able to inhibit the catalytic activity of cezanne, thus prolonging RIP polyubiquitination and signaling through the IKK.

Part 4 – Scope of the thesis

A relationship between NF-κB activation and H_2O_2 has been established since 1991, but there are still fundamental questions on the subject that remain open.

Working with H_2O_2 is not an easy task. In Figure 15 it is possible to observe how, depending on the dose used, there is a multi-functionality of this small molecule. For sure H_2O_2 is not a classical NF-κB inducer, but there is enough evidence suggesting that it can regulate NF-κB activation. Nevertheless, whether H_2O_2 is a stimulator or inhibitor of the NF-κB activation pathway and what species are subjected to H_2O_2-regulation remains to be elucidated. In that sense, the working hypothesis of this thesis is that the lack of consistent observations is caused by the methodology used to expose cells to H_2O_2. A bolus addition of H_2O_2 consists of a single addition of H_2O_2 at the beginning of the experiment and represents the most commonly used method to expose culture cells to H_2O_2. The disadvantage of this method relies on the cellular consumption of H_2O_2 catalyzed by the antioxidant enzymes (GPx, catalase), which forces the use of high initial doses of H_2O_2 (typically 100 μM-1 mM) and consequently may affect the redox homeostasis of cells and activate death pathways. More, the actual concentration of H_2O_2 that is producing the effects reported is not known. For example, in a study describing an exposure to 100 μM H_2O_2 for 6 hours, in reality it should be noted that 100 μM is the initial concentration and that, depending on the cell type and density, probably H_2O_2 is no longer present in the system after 30 min.

Therefore, the aim of this thesis is to investigate the NF-κB activation pathway by H_2O_2 using a controlled and calibrated method to expose the cells to H_2O_2: the steady state (s.s.) titration. When using this method, H_2O_2 is added to cells at the beginning of the experiment, simultaneously with the appropriate units of the enzyme glucose oxidase (GO). With the glucose present in the media, GO will produce H_2O_2 at the same rate that it is consumed by the intracellular enzymes. Therefore, until the end of the incubation time, H_2O_2 concentration is kept constant. During the assay and at the end of it, the extracellular H_2O_2 concentration is measured with an oxygen electrode. The s.s. titration mimics the cell exposure to extracellular H_2O_2 *in vivo*, for example during inflammation.

As described in the General Introduction, the phagocytic oxidative burst process is characterized by an elevation of ROS, namely H_2O_2. Hence, H_2O_2 is able to escape the phagosome of leucocytes and exert signaling responses in endothelial and epithelial cells near the inflammatory local, where H_2O_2 concentrations can rise to 5-15 μM[122]. Thus, the

conditions used in this work, are a model of the effect that H_2O_2 produced during inflammation has on the NF-κB pathway of neighboring epithelial cells. The cytokine TNF-α is used as a positive control for NF-κB activation, since it is produced by activated macrophages and it is important at all stages of inflammation. Endothelium cells are really the first in contact with the leucocytes. However, epithelial cells are also responsive to inflammation, and so transmigration of the leucocytes across epithelial cells is also possible[197]. HeLa and MCF-7 cell lines are established cell lines from the cervical and mammary epithelium, respectively. Previous works reporting NF-κB activation by H_2O_2 justified the choice.

The results obtained are separated in Chapters II, III and IV of this thesis, which correspond to a published, in preparation and submitted manuscripts, respectively. An integrative discussion of the results is made in Chapter V.

In Chapter II, the different methods to expose culture cells to H_2O_2 are analyzed (s.s. titration, bolus addition and GO method). Working concentrations of H_2O_2 and TNF-α are stated for the study of NF-κB activation, which is monitored through the level of p65 subunit in the nuclear compartment. In order to validate the s.s. method, a comparative study of NF-κB activation between the s.s. titration and the bolus addition of H_2O_2 is investigated. To attribute biological significance to the changes of p65 level in the nucleus, gene expression is analyzed using microarrays with a set of 128 NF-κB-dependent genes.

Since H_2O_2 was able to stimulate NF-κB translocation to the nucleus in the presence of TNF-α, the events before translocation are studied in Chapter III, in order to find specific targets for H_2O_2 action. The levels of the three major IκBs (IκB-α, IκB-β and IκB-ε) and the proteasome activity are analyzed in the same conditions stipulated in Chapter II. Given that the three IκBs have different preferences for NF-κB dimers, apart from p65 the levels of nuclear c-Rel are also monitored.

The microarrays studies performed in Chapter II revealed a differential gene expression profile induced by H_2O_2. Therefore, NF-κB-dependent gene expression is explored in Chapter IV by using reporter plasmids coupled with the luciferase gene. Three different NF-κB-consensus sequences are used to make three plasmids with different luciferase expression, in order to evaluate the importance of the κB site for H_2O_2 role. Here, only HeLa cells are used because the transfection efficiency is better when compared with MCF-7 cells.

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Chapter II

A quantitative study of NF-κB activation by H_2O_2: relevance in inflammation and synergy with TNF-α*

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1. Abstract

While the germicide role of hydrogen peroxide (H_2O_2) released during inflammation is well established, a hypothetical regulatory function, either promoting or inhibiting inflammation, is still controversial. In particular, after 15 years of highly contradictory results it remains uncertain whether H_2O_2 by itself activates NF-κB or if it stimulates or inhibits the activation of NF-κB by pro-inflammatory mediators. We investigated the role of H_2O_2 in NF-κB activation using for the first time a calibrated and controlled method of H_2O_2 delivery – the steady-state titration – in which cells are exposed to constant, low and known concentrations of H_2O_2. This technique contrasts with previously applied techniques, which disrupt cellular redox homeostasis and/or introduce uncertainties in the actual H_2O_2 concentration to which cells are exposed. In both MCF-7 and HeLa cells H_2O_2, at extracellular concentrations up to 25 μM, did not induce significantly *per se* NF-κB translocation to the nucleus but it stimulated the translocation induced by TNF-α. For higher H_2O_2 doses this stimulatory role shifts to an inhibition, which may explain published contradictory results. The stimulatory role was confirmed by the observation that 12.5 μM H_2O_2, a concentration found during inflammation, increased the expression of several pro-inflammatory NF-κB-dependent genes induced by TNF-α (e.g. IL-8, MCP-1, TLR2, TNF-α). The same low H_2O_2 concentration also induced the anti-inflammatory gene coding for heme-oxygenase-1 (HO-1) and IL-6. We purpose that H_2O_2 has a fine-tuning regulatory role, comprising both a pro-inflammatory control loop that increases pathogen removal and an anti-inflammatory control loop, which avoids an exacerbated harmful inflammatory response.

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2. Introduction

Reactive oxygen species (ROS) and in particular hydrogen peroxide (H_2O_2) have a key role in the protection against invading pathogens during the innate immune response. Upon invasion by a pathogen, NADPH oxidase in neutrophils and monocytes is activated, liberating ROS, which combined with other factors attack the pathogen[1]. A deficient H_2O_2 production due to mutations in NADPH oxidase, as observed in patients with chronic granulomatous disease, increases the susceptibility to pathogens that do not release H_2O_2[2] and deregulates the inflammatory response[3].

NF-κB is a key regulator of the immune system since its activation induces several genes related to the innate immune and inflammatory responses. The recognition of pathogens by the TLR family of membrane receptors, mediation of neutrophil adhesion to the endothelium and transmigration from blood vessels to tissue interstitium, production of the pro-inflammatory cytokine TNF-α and its actions in target cells are all processes under NF-κB control[4]. Accordingly, bacteria that block NF-κB activation disrupt the innate immune response[5,6]. Originally, it was proposed that NF-κB activation induced by diverse stimuli shared a common secondary messenger, the oxidant H_2O_2[7]. For example, LPS a component of the outer membrane of Gram-negative bacteria, binds to TLR4, which activates NADPH oxidase, releasing H_2O_2 and activating NF-κB[8]. However, nowadays the role of H_2O_2 in NF-κB activation *in vivo* is highly controversial. In fact, H_2O_2 does not activate NF-κB in many cell lines[9,10]. Inhibition of TNF-α-induced NF-κB activation by the antioxidants *N*-acetyl-*L*-cysteine and pyrrolidine dithiocarbamate, previously interpreted as supporting a role played by H_2O_2[11,12], was recently shown to be independent of their antioxidant function[13]. H_2O_2 concentrations required to activate NF-κB are typically in the range 0.1 to 1 mM, which is much higher than the 5-15 μM range observed during inflammation[14,15], where H_2O_2 is produced at high rates. Because during inflammation cells are subjected simultaneously to both cytokines and H_2O_2, the combined actions of these species have been investigated in a number of studies. In two commonly used cell lines, Jurkat T-cells and HeLa, H_2O_2 was found to either stimulate[16,17] or inhibit[18,19] NF-κB activation by TNF-α. Contradictory results were also obtained in closely related alveolar epithelial cell lines [20-22]. The stimulatory effects have been explained by an independent pathway induced by H_2O_2 involving the small G protein Ras[20] or through phosphorylation of serine residues at the IκB kinase complex subunits[16], while the inhibitory effects were attributed either to direct inhibition of IκB kinase

complex activity through cysteine oxidation[21] or to inhibition of the proteasome thus blocking IκB-α degradation[22]. Thus, in spite of the wealth of data on the activation of NF-κB by H_2O_2, the fundamental question whether H_2O_2 will activate or inhibit NF-κB remains unanswered.

NF-κB activation has a dual and opposite dependence on oxidative events, because its translocation is favoured by oxidative events in the cytosol while binding to DNA requires a reductive environment in the nucleus[23-25]. So, higher levels of oxidative exposure can turn a potential positive stimulus by H_2O_2 into an inhibitory effect. We hypothesized that the contradictory results reported in the literature are due to the uncontrolled manner by which H_2O_2 was delivered to cells thus exposing cells to different experimental H_2O_2 concentrations. So, the aims of the present work are to address whether H_2O_2, at concentrations approaching those found *in vivo*, activates NF-κB, stimulates or inhibits the activation of NF-κB by TNF-α, and fulfils a regulatory role during inflammation. For the first time, NF-κB activation was investigated using a calibrated method that delivers H_2O_2 continuously, mimicking the endogenous production of H_2O_2. This method allowed a rigorous control of the extracellular H_2O_2 concentrations applied (in the range of 5-25 μM) as well as the extent of the exposure to H_2O_2 (from 15 min up to 6 h)[26]. The studies were carried out in two epithelial cell lines, MCF-7 and HeLa cells, by measuring both the translocation of the p65 subunit of the NF-κB complex into the nucleus and the IκB-α levels in the cytosol. To further address the relevance of the activation of NF-κB by H_2O_2, microarray analysis of a broad range of genes regulated by NF-κB was performed in the presence of H_2O_2.

3. Materials and Methods

Cell culture and reagents

MCF-7 (European Collection of Cell Cultures, Salisbury Wiltshire, UK) and HeLa cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 medium supplemented with 10% of fetal bovine serum, penicillin 100 U mL^{-1}, streptomycin 100 µg mL^{-1} and L-glutamine 2 mM, all from Cambrex, Verviers, Belgium. The MCF-7 cell line is immunologically active[27], undergoes NF-κB activation by H_2O_2[28], and has been used as a model of the mammary epithelium[29]. In spite of being a breast cancer cell line it belongs to a less aggressive and non-invasive/metastatic group[30]. HeLa cells are also immunologically active[31]. Dimethyl sulfoxide (DMSO), catalase (bovine liver), glucose oxidase (Aspergillus

Niger), IGEPAL CA-630, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), TNF-α (human recombinant), benzamidin, leupeptin, pepstatin, phenylmethylsulphonyl fluoride (PMSF) and dithilthreitol (DTT) were obtained from Sigma-Aldrich, Inc., Saint Louis, MO, USA. H_2O_2 was obtained from Merck & Co., Inc., Whitehouse Station, NJ, USA. All polyclonal antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, California, USA.

Cell incubations and viability

MCF-7 and HeLa cells were plated onto 100-mm dishes 48 h before the experiment to achieve approximately 1.8×10^6 cells and 1.5×10^6 cells per dish, unless otherwise referred. Fresh medium was added to cells 1 h before incubations. H_2O_2 concentration in the medium was determined on aliquots of the medium by measuring the oxygen released after catalase addition using an oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK). As described in[26], the H_2O_2 steady-state $[H_2O_2]_{ss}$ was achieved by adding an initial dose of H_2O_2 at the desired concentration; simultaneously, and to keep H_2O_2 concentration constant (steady-state) during the entire assay, an adequate amount of glucose oxidase was added in order to compensate for the rapid consumption of the initial H_2O_2 concentration by cells. The H_2O_2 concentration was checked periodically. Cell viability was assessed by the ability of cells to reduce MTT[32].

Estimation of intracellular H_2O_2 concentrations in MCF-7 cells

Intracellular concentrations of H_2O_2 were estimated from the determination of the H_2O_2 gradient across the plasma membrane as described in[33]. This gradient (1/R), i.e. the ratio between H_2O_2 concentration outside and inside (cytosol) the cell, may be inferred from H_2O_2 consumption by intact cells over the sum of enzyme activities (mainly catalase and glutathione peroxidase) that consume H_2O_2 in disrupted cells.

$$R = \frac{[H_2O_2]_{in}}{[H_2O_2]_{out}} = \frac{k_{intactcell}}{k_{catalase} + k_{GPx}} \qquad \text{(Equation 1)}$$

H_2O_2 consumption by intact cells was determined by adding an initial dose of 90 μM H_2O_2 and following the decay of H_2O_2 concentration along time using an oxygen electrode. H_2O_2 consumption is reported as a first-order rate constant ($k_{intactcell}$). Catalase activity (reported as

a first-order rate constant, $k_{catalase}$) was measured as previously described[34] using approximately 3×10^5 cells and in the presence of 1 μg mL^{-1} digitonin to permeabilize the plasma membrane. GPx activity (k_{GPx}) was measured as previously described[33,35] in a cell extract (corresponding to 8×10^5 cells) collected after centrifugation (5000 g for 5 min) of a cell lysate obtained with 0.1% (v/v) Triton X-100.

Preparation of cytosolic and nuclear extracts

Cells were washed twice with cold phosphate buffer saline (PBS) pH 7.2 (1.5 mM KH_2PO_4, 155 mM NaCl and 2.7 mM Na_2HPO_4) and lysed with 500 μL of cytosolic lysis buffer (50 mM HEPES pH 7.2, 2 mM EDTA, 10 mM NaCl and 250 mM sucrose plus freshly added 2 mM DTT, 0.1% IGEPAL CA-630 (v/v) and protease inhibitors: 1 mM PMSF, 1.5 µg mL^{-1} benzamidin, 10 µg mL^{-1} leupeptin and 1 µg mL^{-1} pepstatin). Cells were scrapped up and transferred to a microcentrifuge tube, and the remaining cells were collected from the dish with 100 µL of cytosolic lysis buffer. Cytosolic proteins were collected after centrifugation at 3000 g for 4 min at 4ºC. The pellet was washed once with the cytosolic lysis buffer and then ressuspended with the nuclear lysis buffer (same as the cytosolic buffer except that 400 mM NaCl and 20% glycerol (v/v) were used, but neither sucrose nor IGEPAL CA-630 were added to the buffer). Cells were put on ice during 20 min and mixed by vortexing three times during this incubation. Nuclear proteins were collected after centrifugation at 10 000g for 10 min at 4ºC. Protein concentration was assayed using the Bradford method[36].

Western Blot analysis

Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 8% polyacrylamide gel, followed by a semi-dry electroblotting of proteins onto a nitrocellulose membrane (Protan, Schleicher & Schuell). The membrane was blocked with 5% nonfat dry milk in PBS, followed by immunoblotting using primary antibodies to p65 (sc-109; 1:400) and IκB-α (sc-371; 1:800), and a secondary antibody conjugated to horseradish peroxidase (sc-2004; 1:1000). All incubations were performed at room temperature, during 2 h for the primary antibodies and 1 h for the secondary antibody. Signals were developed using the ECL chemiluminescence system (Amersham Biosciences, Little Chalfont Buckinghamshire, UK). Immunoblot films were digitalized and analysed with the ImageJ Software[37]. Control of protein loading was performed by analysis of the

membrane stained with Ponceau S red. To quantify the percentage of increase or decrease of the p65 levels in the presence of both TNF-α and H_2O_2 relative to the sum of the individual effects, equation 2 was used:

$$\text{Modulation} = \frac{(\text{nuclear p65})_{\text{TNF-}\alpha + H_2O_2}}{(\text{nuclear p65})_{\text{TNF-}\alpha} + (\text{nuclear p65})_{H_2O_2} - 1} \qquad \text{(Equation 2)}$$

When the effect observed is equal to the sum of the individual effects (addictive effect) there is no modulation and equation 2 equals 1. Positive modulations (synergism) and negative modulations (antagonism) will be above or under 1 respectively. The denominator was subtracted from 1 because nuclear translocation of p65 has relative units. For example, if both individual activations are the same as the control, the subtraction from 1 is needed in order for the sum to be also 1.

Microarray analysis

RNA from treated cells was extracted using the Array Grade Total RNA isolation kit (GA-013) and approximately 3 μg of RNA was used in the experiments. Labelling was performed over-night with biotin-16-uridine-5'-triphosphate (Roche, Mannheim, Germany) using the TrueLabeling-AMP 2.0 kit (GA-030). The probe was purified using the ArrayGrade cRNA cleanup kit (GA-012). Hybridization of 3 μg of probe to the Oligo GEarray Human NF-κB signalling pathway Microarray (OHS-025) was done over-night and detection was assayed by chemiluminescence, using the detection kit. Signal intensity was quantified using the ImageJ software[37]. All kits were from SuperArray, Bioscience Corporation (MD, USA), and the protocols were followed according to manufacturer instructions. Gene expression induced by both H_2O_2 and TNF-α – $(H+T)_i$ – was estimated as the average of the ratios between the expression caused by the simultaneous addition of the two agents divided by the expression caused by TNF-α (T_i) in each experiment, multiplied by the average expression caused by TNF-α stimulation in all experiments ($T_{average}$), equation 3:

$$\left((H+T)_i / T_i\right)_{average} \times T_{average},\ i = 1, \ldots, n \text{ (number of experiments)} \qquad \text{(Equation 3)}$$

Statistical analysis

Results are presented as the average ± SD. Results were analyzed by using the two-tailed one sample t-test comparing the average with 1, ***$p<0.001$; **$p<0.01$; * $p<0.05$; ·$p<0.10$.

4. Results

4.1 Calibrated exposure of MCF-7 cells to H_2O_2

In this work, delivery of H_2O_2 was performed using the steady-state titration method, in which cells are exposed to known and constant H_2O_2 concentrations ([H_2O_2]ss). To show the advantages of this method, we compared it with two other approaches: (a) the bolus addition, by far the most common method, in which H_2O_2 is added at the beginning of the experiment; and (b) the glucose oxidase method, in which glucose oxidase is added to continuously produce H_2O_2 from the oxidation of the glucose present in the medium (Figure 1).

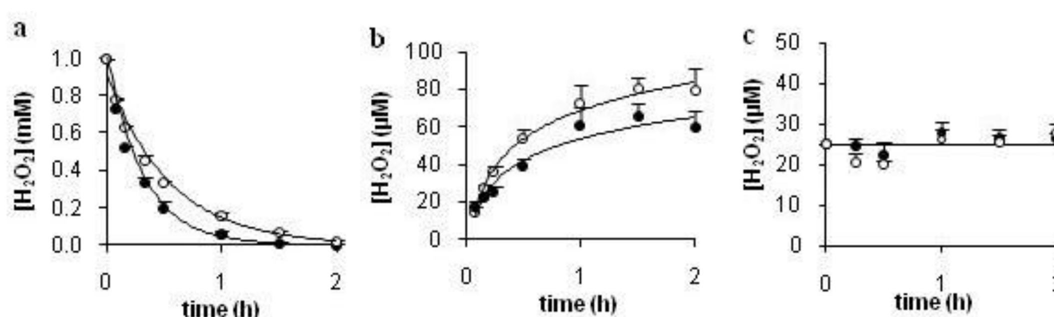

Figure 1 - Different methods of H_2O_2 delivery to cells. MCF-7 cells at a density of 1.8×10^6 cells (closed symbols) and 0.9×10^6 cells (open symbols) were exposed to H_2O_2 using different methods: a bolus addition of H_2O_2, an addition of glucose oxidase and a $[H_2O_2]_{ss}$. For the bolus additions **(a)** 1 mM H_2O_2 was added at the beginning of experiment, and for the glucose oxidase assay **(b)** 4 mU mL^{-1} of glucose oxidase were used **(c)** The $[H_2O_2]_{ss}$ was achieved with the initial addition of 25 µM H_2O_2 and the correspondent amount of glucose oxidase to maintain the 25 µM of H_2O_2. n=3 for all experiments.

As shown in Figure 1a, an initial bolus addition of 1 mM H_2O_2 was consumed by cells and after 2 h a very low concentration of H_2O_2 was measured. Therefore, the bolus addition is clearly unsuitable to investigate processes where H_2O_2 is present for more than a couple of hours, such as the inflammation process. Concerning shorter periods, this method is also inadequate because high initial doses of H_2O_2 are needed to observe the response under investigation due to the fast consumption of H_2O_2 by the cells. Thus, the high initial dose possibly leads to disruption of cellular homeostasis and the observed effects may be artifactual. When cells were exposed to glucose oxidase there was a gradual increase in H_2O_2 concentration that culminated in a near steady-state after approximately 2 h (Figure 1b). So,

when using this approach it is important to note that the desired H_2O_2 concentration is not obtained immediately, which becomes a problem when short exposures are required. Concerning longer periods of exposure, the glucose oxidase method could *a priori* be considered adequate. However, the simple exposure of cells with glucose oxidase (or to a bolus addition of H_2O_2) represents an uncalibrated and uncontrolled way of delivering H_2O_2 to cells. To illustrate this point, cells were exposed to H_2O_2 at two different cell densities (Figure 1a and Figure 1b). As can be seen the H_2O_2 concentration profile, including the near steady-state reached after 2 h with glucose oxidase, depends on the cell density used. Other factors, such as the cell type (different cells consume H_2O_2 with different rates), or the incubation medium (different media can consume or produce[38,39] H_2O_2 with different rates), can potentially affect the actual H_2O_2 concentration reached in the assay.

In the steady-state titration, the desired $[H_2O_2]_{ss}$ is given initially, together with the appropriate units of glucose oxidase that will produce H_2O_2 to the same extend of its consumption in the specific conditions of the assay, which include the cell density and the small day to day differences in cell behaviour. As shown in Figure 1c, it was possible to maintain the desired $[H_2O_2]_{ss}$ during the entire assay for the two cell densities. The key feature of this method is that it is calibrated on a daily basis because the amount of glucose oxidase added will be adjusted to match the consumption of H_2O_2 in the specific experimental conditions of the assay.

In conclusion, without measuring the H_2O_2 concentration achieved during the experiment it will not be possible to predict the concentration of H_2O_2 that cells are subjected to and the comparison of data obtained in different laboratories is not possible. The same initial dose of H_2O_2 or of glucose oxidase can represent very different levels of stress, even if the same line is used, and cause contradictory observations. In the steady-state titration, this problem is overcome by measuring the H_2O_2 concentrations in every assay and by adjusting the amount of glucose oxidase in order to reach the desired H_2O_2 concentration. This may be particularly relevant for biological processes subjected to a complex control by H_2O_2 levels, such as the dual dependence of NF-κB activation on H_2O_2[23-25].

4.2 Estimation of H_2O_2 cytosolic concentrations in MCF-7 cells

H_2O_2 diffusion across biomembranes is not "free" and gradients are formed when the source of H_2O_2 is separated from the sink by a biomembrane[33,40]. So, since H_2O_2 was added

extracellularly and NF-κB activation occurs in the cytosol it is important to estimate the actual cytosolic H_2O_2 concentrations achieved in our experiments. An H_2O_2 gradient across the plasma membrane of approximately 2 was obtained by applying equation 1 (materials and methods and Table 1), an equation that was previously applied by us in yeast and in Jurkat T-cells[33,40]. Therefore, for the extracellular H_2O_2 concentrations in the 5 to 25 μM range used in this work, the corresponding cytosolic concentrations in MCF-7 cells were in the 2.5 to 12.5 μM range.

Table 1 - Estimation of the gradient between extracellular and intracellular H_2O_2 concentrations in MCF-7 cells after exposure to extracellular H_2O_2[a].

Parameter	Value
$k_{intactcell}$	4.3 ± 0.15^b ($n = 3$)
$k_{catalase}$	4.2 ± 0.61^b ($n = 4$)
k_{GPx}	4.1 ± 0.63^b ($n = 3$)
$R = [H_2O_2]_{in}/[H_2O_2]_{out} = 1/\text{gradient}$	0.52

[a] Rate constants obtained from the consumption of H_2O_2 by intact MCF-7 cells ($k_{intactcell}$), from the catalase activity in disrupted cells with intact peroxisomes ($k_{catalase}$) and from GPx activity in disrupted cells (k_{GPx}) were measured as described in *Materials and Methods*. Gradient (1/*R*) was calculated according to equation 1; [b] units: $min^{-1} \times 10^{-7} cells \times mL$.

4.3 Titration of NF-κB activation with steady-state concentrations of H_2O_2 and with TNF-α in MCF-7 cells

To investigate the relevance of H_2O_2 as an NF-κB activator, p65 translocation into the nucleus was measured by western blot (Figure 2). We started by applying extracellular $[H_2O_2]_{ss}$ in the range between 5 and 25 μM, which are close to the H_2O_2 extracellular levels during inflammation[14,15]. In these conditions, cell viability was unaffected (not shown) and p65 translocation was induced significantly, up to 4-fold by H_2O_2 (Figures 2a and 2b), with a slow monotonous non-oscillatory kinetics. Removal of H_2O_2 reversed NF-κB activation, and the simultaneous addition of $[H_2O_2]_{ss}$ and catalase eliminated NF-κB activation after 4 h thus showing that the reversible NF-κB activation is caused by H_2O_2 production (Figure 2d). To assess if this level of activation is quantitatively important, we compared it with the activation of NF-κB induced by TNF-α, a well-known NF-κB inducer. TNF-α concentrations, both in the physiological range (0.2 to 1.5 ng mL^{-1} [41]) and in the supra-physiological range (higher than 1.5 ng mL^{-1}, but often used in experiments[16,23,42,43]) were used without affecting cell viability (not shown). For the physiological range, there was an up to 8-fold induction of p65 translocation, whereas for the supra-physiological range a 12-fold

induction was observed (Figure 2c). The recently discovered oscillatory dynamics of NF-κB activation (Figure 2e) were also observed[44].

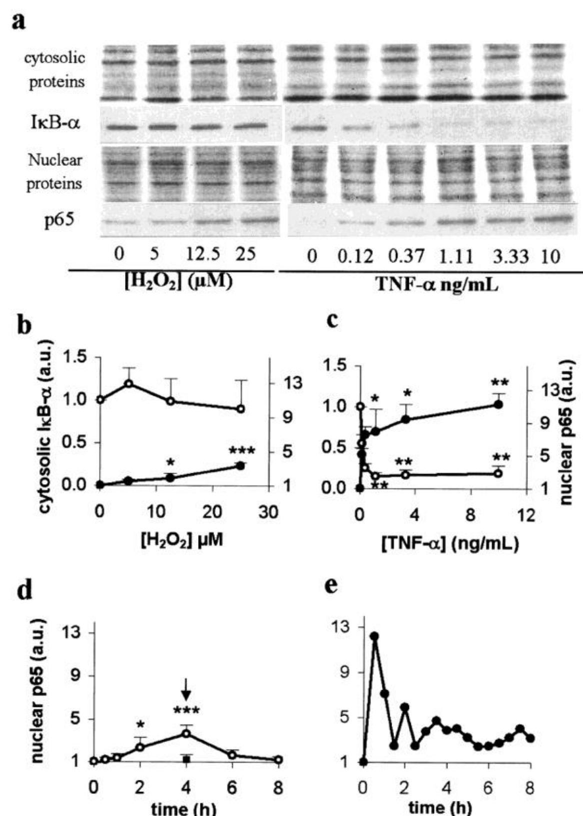

Figure 2 - NF-κB activation by H_2O_2 and TNF-α: titration and time course studies. MCF-7 cells were treated with $[H_2O_2]_{ss}$ during 4 h (n=4) and with TNF-α during 30 min (n=3): **(a)** protein loading and immunoblots of representative experiments; **(b)** and **(c)** quantification of p65 levels in the nucleus and of IκB-α in the cytosol by signal intensity analysis. Time courses for the translocation of p65 to the nucleus induced by **(d)** 25 μM of $[H_2O_2]_{ss}$, n=5, (exposure for periods longer than 4 h lead to cell death – data not shown) and **(e)** 0.37 ng mL^{-1} of TNF-α. In panel d, $[H_2O_2]_{ss}$ was removed after 4h (indicated by the arrow) by replacing with fresh medium; (■) simultaneous addition of catalase and $[H_2O_2]ss$ and analysis of p65 translocation after 4h (n=3). Protein levels in both H_2O_2- (○) and TNF-α-treated (●) samples are expressed as arbitrary units relative to control, showing statistical differences versus control values. In (c) the data with 0.12 and 0.37 ng mL^{-1} TNF-α is statistically different from control ($p<0.05$ for 0.12 ng mL^{-1} and $p<0.01$ for 0.37 ng mL^{-1}).

The differences observed on the levels of p65 translocation may reflect different mechanisms of NF-κB activation. Contrary to TNF-α, NF-κB activation by $[H_2O_2]_{ss}$ was not associated with a decrease in IκB-α levels (Figures 2a, 2b and 2c) and was not affected by proteasome inhibition (not shown). Thus, H_2O_2 may either induce the phosphorylation of tyrosine 42 of IκB-α, which releases IκB-α from the complex with NF-κB without the subsequent degradation, allowing NF-κB translocation[45], or inhibit NF-κB efflux from the nucleus, allowing NF-κB to slowly accumulate in the nucleus due to the low basal NF-κB activation[46].

From these results it can also be extrapolated that NF-κB activation by H_2O_2 does not occur during normal metabolism, where intracellular H_2O_2 levels are in the 0.01-0.1 μM range[47], and that H_2O_2 is at best a poor NF-κB activator when compared with TNF-α in MCF-7 cells (Figure 2b and 2c). This comparison has physiological relevance because the extracellular $[H_2O_2]_{ss}$ applied (5-25 μM) are probably only found *in vivo* when other inducers of NF-κB (e.g. TNF-α) are also present, such as during the inflammatory response.

4.4 Modulation by H_2O_2 of NF-κB activation by TNF-α

Due to the weak capacity of H_2O_2 to activate NF-κB, and taking into account that during inflammation both H_2O_2 and TNF-α are formed, we investigated whether H_2O_2 could modulate NF-κB activation induced by TNF-α. Cells were exposed to either a $[H_2O_2]_{ss}$ of 25 μM or TNF-α (0.37 ng mL^{-1}) alone, or to both agents simultaneously (Figure 3).

In both cell lines, the effect of the simultaneous addition of TNF-α and H_2O_2 on p65 levels in the nucleus was significantly higher than the sum of the individual effects of TNF-α and H_2O_2 (Figure 3a and 3c), i.e. H_2O_2 had a significant positive modulatory effect – a synergism – on the activation of NF-κB by TNF-α. In MCF-7 cells, a synergism of 20% was first observed at 30 min, a time corresponding to a peak of TNF-α-induced NF-κB activation but to no activation of NF-κB by H_2O_2, while a maximal synergism of 40% was observed at 2 h (Figure 3b). In HeLa cells a synergism on NF-κB activation of about 100% (Figure 3d) was observed at 2 h and 4 h. This high synergism is easily visualised because NF-κB activation by $[H_2O_2]_{ss}$ was almost negligible (Figure 3c).

Most published studies, e.g. [21,22,43], report that H_2O_2 inhibits the activation of NF-κB by TNF-α, although a few report a positive effect [16,20] in agreement with our results. We hypothesized that the inhibitory effects observed in most studies could be related to the delivery of H_2O_2 as a large bolus initial dose, which causes severe oxidation and thus inhibits NF-κB activation. As shown in Figure 3e and 3f for MCF-7 cells and in Figure 3g and 3h for HeLa cells, under the artifactual conditions of a bolus addition, TNF-α-dependent translocation of p65 is significantly inhibited by H_2O_2 (between 40% and 60%). This negative modulation decreases when H_2O_2 is consumed (Figure 3f and 3h). The kinetics of NF-κB activation by H_2O_2 was much faster with the bolus addition than with the steady-state exposure, reaching a maximum at 60 min, as observed by others in other cell lines[45,48]. This fast activation process is still slower than the achieved by TNF-α and, probably, not physiologically relevant. In fact, 1 mM H_2O_2 is an extremely high concentration that most likely is not even reached in the phagocytic vacuole of polymorphonuclear neutrophils[1]. While no cytotoxicity was observed for TNF-α alone or with $[H_2O_2]_{ss}$, there was a significant loss of cell viability (approximately 35% for MCF-7 cells and 40% for HeLa cells) for 1 mM H_2O_2 bolus addition after 1 h (data not shown).

We hypothesized that the high oxidation levels imposed by the H_2O_2 bolus addition to cells could also be mimicked by a longer incubation with a lower $[H_2O_2]_{ss}$. To test this hypothesis MCF-7 cells were pre-exposed to a $[H_2O_2]_{ss}$ of 25 μM for 3 h before the addition of TNF-α (Figure 3i). Under these conditions, H_2O_2 had an antagonistic effect on TNF-α-induced p65 translocation, with an inhibition of up to 60% (Figure 3j), but contrary to the bolus addition, there was a sustained antagonism and cell viability was maintained (not shown).

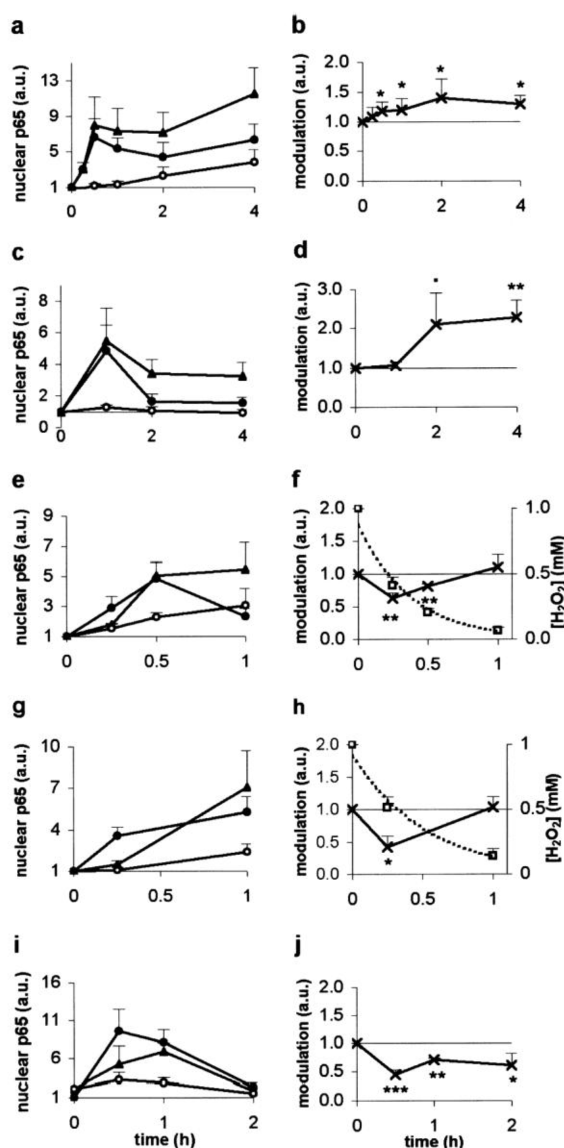

Figure 3 - H_2O_2 modulates NF-κB activation by TNF-α but this modulation is dependent on the method of H_2O_2 delivery. Three methods of H_2O_2 delivery were used: $[H_2O_2]_{ss}$ of 25 μM (**panels a to d**); bolus addition of 1 mM H_2O_2 (**panels e to h**); and pre-incubation with a $[H_2O_2]_{ss}$ of 25 μM for 3 h followed by the addition of TNF-α (**panels i and j**). MCF-7 (**panels a, b, e, f, i and j**; n=5) and HeLa cells (**panels c, d, g and h**; n=4) were subjected to H_2O_2 (○) and TNF-α (initial dose of 0.37 ng mL^{-1}) (●) individually, or to both agents simultaneously (▲). Translocation of p65 and modulation by H_2O_2 (×) calculated with equation 2 (see materials and methods) are plotted in the left and right panels, respectively. In **panel i**, H_2O_2 induced NF-κB activation at time zero because cells were pre-incubated for 3 h with H_2O_2. In addition, a representative time course of H_2O_2 consumption after the bolus addition is shown in **panels f** and **h** (□).

Overall, our results indicate that H_2O_2 has a modulatory effect on NF-κB activation by TNF-α. Under conditions of inflammation where TNF-α and H_2O_2 are simultaneously present, a positive modulatory role for H_2O_2 in TNF-α induced NF-κB activation is predicted. Nevertheless, this modulatory role is dependent on the cellular redox status and may switch from stimulatory to inhibitory thus explaining the contradictory results found in the literature.

4.5 Modulation by H_2O_2 of gene expression induced by TNF-α via NF-κB

To address the importance of the positive modulatory role of H_2O_2 on the activation of NF-κB by TNF-α, we analyzed with microarrays the expression of the NF-κB dependent genes listed in Table 1A of supplementary information (SI, see end of Chapter). The action of H_2O_2 was studied using both a short (1 h) and a long (6 h) time of exposure to mimic the beginning of an inflammatory situation, when residential macrophages in the tissue are activated and pro-inflammatory cytokines are liberated – immediate innate immunity – and the progression to an acute inflammatory response, which includes the additional recruitment of effector cells and induction of acute phase proteins[49].

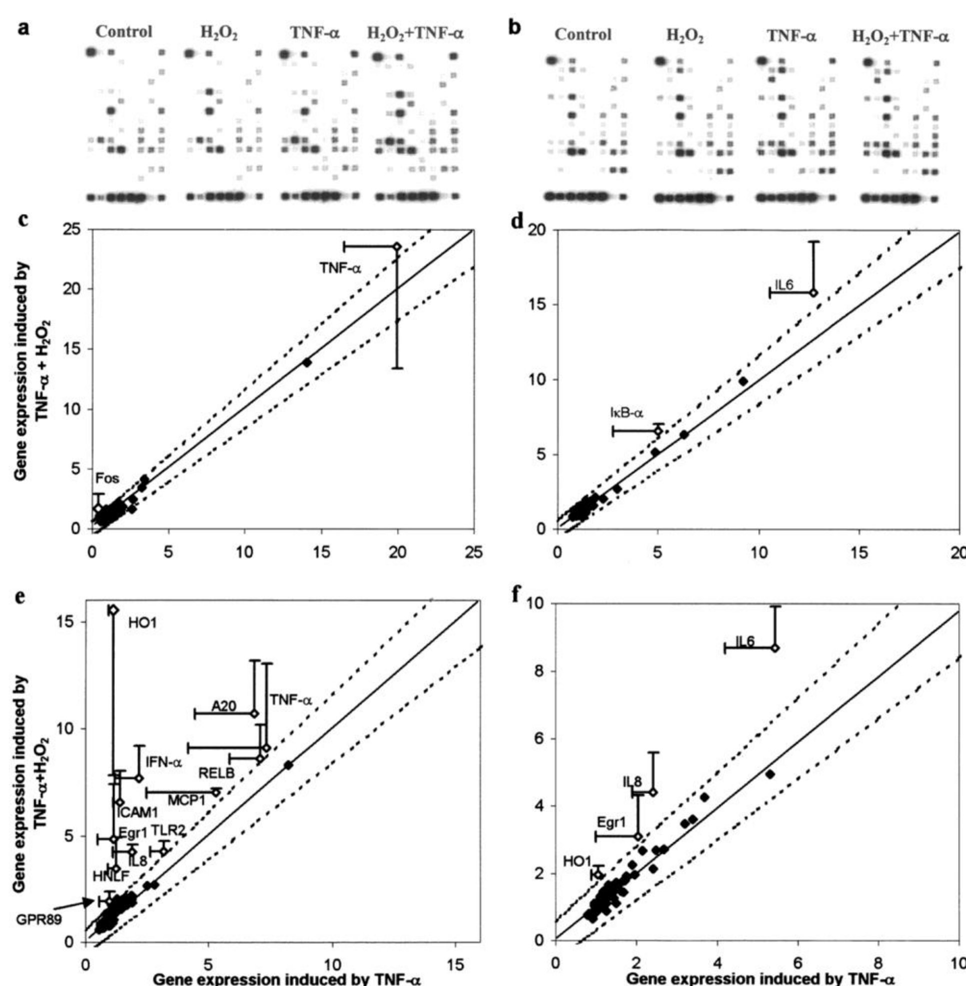

Figure 4. A low/moderate dose of H_2O_2 stimulates gene expression induced by TNF-α for a long time exposure. MCF-7 cells (left panels) and HeLa cells (right panels) were exposed to 0.37 ng mL^{-1} TNF-α and $[H_2O_2]_{ss}$ alone or simultaneously. **(a)** and **(b)** Microarray pattern of a representative experiment of MCF-7 cells treated for 6 h with a $[H_2O_2]_{ss}$ of 12.5 μM and HeLa cells treated with a $[H_2O_2]_{ss}$ of 25 μM for 1 h, respectively. The expression of individual genes induced by the simultaneous presence of H_2O_2 and TNF-α (equation 3, *materials and methods*) is plotted as a function of the expression regulated by TNF-α, for 1 h with a $[H_2O_2]_{ss}$ of 25 μM **(c, d)** and 6 h with a $[H_2O_2]_{ss}$ of 12.5 μM **(e, f)**. No effects by H_2O_2 are represented by the continuous line y = x. Based on the dispersion of results observed an error range was defined where modulation by H_2O_2 was not considered significant. The boundaries of this range are indicated by the dashed lines, which were defined to be $y = 1.1x + 0.6$ and $y = 0.9x – 0.6$.

After 1 h of exposure to TNF-α, many genes were induced in MCF-7 cells (Table 1B of SI) namely its own mRNA (TNF-α); the chemokines MCP-1 and IL-8 that attract leucocytes to site of inflammation; and genes of the NF-κB pathway, such as IκB-α and the zinc-finger protein A20, both responsible for the negative feedback of NF-κB activation. HeLa cells were also very responsive, with high expression of IL-8, TNF-α, A20 and IκB-α, as observed for MCF-7 cells. In addition, HeLa cells expressed the cytokine IL-6, which has an important role for resolution of the inflammation process and initiation of the immune response[50]. Thus, the MCF-7 and HeLa epithelial cell lines have a functional NF-κB pathway, and are able to switch on the production of cytokines and chemokines, as it would be expected at the beginning of an inflammatory response for an immunological active cell. This has been previously observed for other epithelial cell lines and is in agreement with an active role for epithelial cells during the innate immune response[51].

After 1h, a $[H_2O_2]_{ss}$ of 25 μM did not induce genes in HeLa cells and induced only a limited number of genes in MCF-7 cells, with more relevance for the cytokine TNF-α, the adhesion molecule ICAM-1 and the transcription factor Jun that belongs to the AP-1 family (Table 1B and 1C of SI). This is consistent both with the absent or low translocation of p65 into the nucleus induced by H_2O_2 at 1 h (Figure 3a and 3c) and the fact that the induction observed for the three genes could be due to activation of transcription factors other than NF-κB[52-54].

Concerning the modulation of the activation of NF-κB by TNF-α, Figure 4 shows the relationship between gene expression induced by the simultaneous addition of H_2O_2 plus TNF-α and gene expression induced by only TNF-α.

Only two genes in MCF-7 and in HeLa cells were modulated significantly by H_2O_2 and therefore it can be concluded that H_2O_2 does not have a regulatory role in the modulation of TNF-α action at 1h (Figure 4c and 4d and Table 2). After 6 h of incubation, both in MCF-7 and HeLa cells TNF-α still lead to an increased expression of genes related with the inflammatory/immune response and the NF-κB pathway (Table 1B and 1C of SI), as expected from the oscillatory prolonged p65 translocation caused by the continuous presence of TNF-α (Figure 2e). Concerning H_2O_2, and in contrast to what was observed at 1 h incubation, a $[H_2O_2]_{ss}$ of 12.5 μM induced several genes in MCF-7 cells (Table 1B of SI), which seems to indicate a late effect of H_2O_2 and is consistent with the slow kinetics of p65 nuclear translocation induced by H_2O_2 observed in Figure 2d. In particular, heme oxygenase-1 (HO-1), ICAM-1, IFN-α, the putative NF-κB activating protein HNLF and early growth

response-1 (Egr-1) expressions were higher in the presence of H_2O_2 than in the presence of TNF-α at 6 h. In HeLa cells, H_2O_2 also activated some genes after 6 h, especially Egr-1, IL-6, ICAM-1 and A20. However, some of these genes are probably being expressed under the control of other transcription factors (for example, HO-1 is probably activated by H_2O_2 via NF-E2-related factor-2[55]). More importantly, when comparing gene induction caused by TNF-α with gene induction caused by H_2O_2 plus TNF-α, it can be seen that an important sub-set of genes involved in the regulation of the inflammatory response (HO-1, G-protein coupled receptor 89 (GPR89), ICAM-1, IL-8, IFN-α, MCP-1, TLR2, TNF-α in MCF-7 cells and IL-6, IL-8, HO-1, and Egr-1 in HeLa cells) is positively modulated by H_2O_2 (Figure 4e and 4f). Remarkably, by itself H_2O_2 did not induce significantly some of these genes (GPR89, IL-8, MCP-1, TLR2), which emphasizes the synergistic action of H_2O_2 on TNF-α activation of NF-κB (Table 2).

Table 2 - H_2O_2 effect on gene expression induced by TNF-α after 1 or 6 h of exposure[a].

	Gene Name	H_2O_2	TNF-α	H_2O_2 + TNF-α
MCF-7				
1-h incubation				
Regulation of the inflammatory response	TNF-α	2.1 ± 1.0[b]	19.9 ± 3.5[b]	23.5 ± 10.2[b]
Transcription factors	Fos	1.7 ± 0.9	0.4 ± 0.0	1.7 ± 1.3
6-h incubation				
Regulation of the inflammatory response	GPR89	1.3 ± 0.1	1.0 ± 0.4	1.9 ± 0.5
	HO-1	11.0 ± 4.6	1.1 ± 0.2	15.5 ± 7.7
	ICAM-1	4.8 ± 1.3[b]	1.4 ± 0.3[b]	6.6 ± 1.5[b]
	IL-8	1.3 ± 0.2	1.9 ± 0.8	4.2 ± 0.4
	IFN-α	5.8 ± 2.6[b]	2.2 ± 1.0[b]	7.7 ± 1.5[b]
	MCP-1	n.d.	5.3 ± 2.8[b]	7.0 ± 0.2[b]
	TLR2	1.2 ± 0.1	3.2 ± 0.6	4.3 ± 0.5
	TNF-α	3.1 ± 0.8[b]	7.4 ± 3.2[b]	9.1 ± 3.9[b]
Regulation of the NF-κB pathway	A20	2.0 ± 0.5	6.9 ± 2.4	10.7 ± 2.5
	HNLF	2.5 ± 1.2	1.2 ± 0.3	3.5 ± 1.4
	RelB	1.8 ± 0.2	7.1 ± 1.2	8.6 ± 1.6
Transcription factors	Egr-1	3.0 ± 0.4	1.1 ± 0.6	4.8 ± 2.6
HeLa				
1-h incubation	IκB-α	1.4 ± 0.4	5.0 ± 1.7	6.6 ± 0.5
Regulation of the NF-κB pathway	IL-6	1.5 ± 0.3	12.7 ± 3.6	15.8 ± 3.4
6-h incubation				
Regulation of the inflammatory response	HO-1	1.5 ± 0.4	1.1 ± 0.2	2.0 ± 0.3
	IL-6	2.1 ± 0.2	5.4 ± 1.2	8.7 ± 1.2
	IL-8	1.9 ± 0.4	2.4 ± 0.5	4.4 ± 1.2
Transcription factors	Egr-1	2.5 ± 0.4	2.1 ± 1.1	3.1 ± 1.2

[a] MCF-7 and HeLa cells were exposed for 1 h with a $[H_2O_2]_{ss}$ of 25 µM and/or TNF-α 0.37 ng mL^{-1} and for 6 h with a $[H_2O_2]_{ss}$ of 12.5 µM and/or TNF-α 0.37 ng mL^{-1}. mRNA levels were quantified using microarray analysis. Expression values were normalized to non-treated cells. H_2O_2 + TNF-α effect was calculated as shown in equation 3. n.d. – not detected; [b] No expression was detected for non-treated cells, so the value shown is a lower limit.

In conclusion, in conditions mimicking inflammation and in the presence of TNF-α, H_2O_2 has a modest modulatory role after 1 h since only a few genes have an increased expression; however at 6 h H_2O_2 has an important modulatory role as shown by the increased expression of many genes, mainly those involved in the inflammatory response.

5. Discussion

The question of whether H_2O_2 stimulates or inhibits NF-κB has been under dispute because opposite results have been reported. A positive effect may exacerbate inflammation leading eventually to inflammation-related diseases[16,20] while an inhibitory effect is anti-inflammatory and may be viewed as protecting from chronic inflammation or sepsis[21,56,57]. This depiction may be simplistic because it may be advantageous to stimulate inflammation in order to have a strong defence response, while a putative anti-inflammatory action could be negative by blocking this defensive mechanism. In this work, a switch from a stimulatory to an inhibitory role was achieved by changing the way H_2O_2 was delivered to cells. While a bolus addition or a pre-treatment with $[H_2O_2]_{ss}$ of 25 μM for 3 h lead to a strong inhibitory effect, the simultaneous addition of the same $[H_2O_2]_{ss}$ with TNF-α caused a synergistic effect. A pre-treatment with H_2O_2 has been interpreted by some investigators as representative of chronic inflammation because cells have time to adapt to H_2O_2[17]. Cellular adaptation to H_2O_2, among other alterations, includes increasing the activity of H_2O_2 removal enzymes[40], leading to an increased consumption of H_2O_2 by cells and a consequent decrease in H_2O_2 levels during the experiments. During the incubations with H_2O_2, steady-states obtained were stable and did not require further additions of glucose oxidase, indicating that under our experimental conditions H_2O_2 removal enzymes were not inactivated and that cells were not adapted to H_2O_2. In fact, the time required for adaptation to H_2O_2 is around 18 h[58], while "permanent" long-term adaptations would require for treatments with H_2O_2 lasting weeks[59], which contrasts with our up to 6 h shorter exposures. Therefore we think that H_2O_2 role in TNF-α induced NF-κB activation switches from stimulatory to inhibitory due to an increase in the oxidative load, and not due to an adaptive response of the cells. This is consistent with the dual regulation of NF-κB by the redox state of the cell[23-25].

Acute inflammation starts approximately 4 hours after infection or trauma, peaks around 24 hours lasting up to 4 days and, in an animal model of LPS-induced cystitis, NF-κB-dependent gene induction peaks around 4 h[60]. Under our conditions, after a 6 h incubation with a

$[H_2O_2]_{ss}$ of 12.5 μM, a concentration which can be found *in vivo* under inflammatory conditions after neutrophil activation[14,15], H_2O_2 clearly stimulated the expression of many NF-κB dependent genes in MCF-7 cells, induced by TNF-α. Besides some regulatory genes of the NF-κB pathway, most genes stimulated by H_2O_2 have a pro-inflammatory role namely the adhesion molecule ICAM-1, the pro-inflammatory cytokine TNF-α, the chemokines MCP-1 and IL-8, and the chemokine receptor GPR89. TLR2 and IFN-α, which are important for antibacterial and antiviral protection, respectively, were also up-regulated by H_2O_2.

It is also highly relevant that the increased expression of these pro-inflammatory genes was accompanied by the activation of the HO-1 gene, which was observed in both cells lines studied, with a higher expression in MCF-7 cells. HO-1 controls the oxidative degradation of heme, forming as an end product carbon monoxide (CO), which has an anti-inflammatory function[61]. In LPS activated macrophages, CO mediates the inhibition of the production of pro-inflammatory cytokines, such as IL-1b, TNF-α, and chemokines, such as MIP-2[61]. HO-1 also inhibits ICAM-1 in endothelial cells[62]. The induction of HO-1 by H_2O_2 may not involve NF-κB, but rather the NF-E2-related factor-2[55]; nevertheless, the high expression of this gene after 6 h of exposure to H_2O_2 can potentially fulfil a negative feedback role to avoid an excess of pro-inflammatory conditions. In HeLa cells, this anti-inflammatory role of H_2O_2 may be mediated also through stimulation of IL-6. After the initiation of a local and systemic inflammation IL-$6^{-/-}$ mice showed a higher expression of pro-inflammatory cytokines, such as TNF-α and MIP-2, than IL-$6^{+/+}$ mice, which indicates an anti-inflammatory role for IL-6[63]. It is also interesting to note that in MCF-7 cells, where this cytokine was not induced, H_2O_2 fulfilled its putative anti-inflammatory role through a higher stimulation of HO-1.

Although the proposal that H_2O_2 has simultaneously a pro- and an anti-inflammatory action may seem contradictory, the biological design in which the activation of a pathway produces an inhibitor which blocks the activation process is a classic feed-back mechanism that is very common not only in metabolic pathways, but also in gene networks. For example, this design is found in the pairs NF-κB/(IκB,A20), p53/Mdm2, HSF-1/HSP70 and in many others[64]. Therefore, the coexistence of two regulatory loops, one pro-inflammatory in which H_2O_2 induces the production of pro-inflammatory cytokines, through a synergistic action with TNF-α, and another anti-inflammatory in which H_2O_2 either activates the production of CO through HO-1 or of IL-6, allows H_2O_2 to fine-tune the inflammatory process. Among other anti-inflammatory actions, HO-1 inhibits NF-κB activation[65]. Like IκB-α and A20, which act

as negative feed-back regulators of NF-κB activation, HO-1 can also act as a negative feed-back regulator of the H_2O_2-dependent synergistic activation of NF-κB. However CO, which is a highly diffusible gas, would not limit its action to the cell of origin having a potential important role in cell-cell communication at the site of inflammation. The anti-inflammatory role for H_2O_2 is supported by the increased IL-8 production found in neutrophils isolated from patients with chronic granulomatous disease[3] and by the deregulated excessive inflammatory responses (such as increased TNF-α and IL-1β levels) observed in animal knockout models of this disease when stimulated with sterile inflammatory stimulus[66,67]. On the other hand, the pro-inflammatory role for H_2O_2 is supported by the decreased formation of MCP-1 observed both in glucan-induced pulmonar granuloma *in vivo* when H_2O_2 is removed by catalase addition[68], and in a whole animal ischemia model when ROS are removed by antioxidants[69]. Furthermore, *in vitro*, many studies show that H_2O_2 stimulates the formation of chemokines[70-74].

Due to their localization, epithelial cells are the first to contact the pathogens thus constituting a first defensive physical barrier against them. Besides this passive role, there is recent evidence that epithelial cells participate actively in the inflammatory and innate immune responses, including pathogen recognition mediated by TLR receptors, production of pro-inflammatory cytokines, chemokines, adhesion molecules and growth factors[51,75,76]. The results obtained in this work with the two epithelial cell-lines MCF-7 and HeLa further support an active role for the epithelium (Figure 5). After phagocyte activation, epithelial cells will be subjected to pro-inflammatory cytokines, such as TNF-α and to exogenous H_2O_2 produced by phagocytes. H_2O_2 will stimulate the activation of NF-κB by cytokines in epithelial cells, producing cytokines and chemokines that will attract more phagocytes to the site of assault, thus amplifying the inflammation. This amplification is homeostatically controlled by the H_2O_2-dependent induction of the HO-1 or IL-6 genes in epithelial cells, which lead to the release of the anti-inflammatory mediators CO and IL-6.

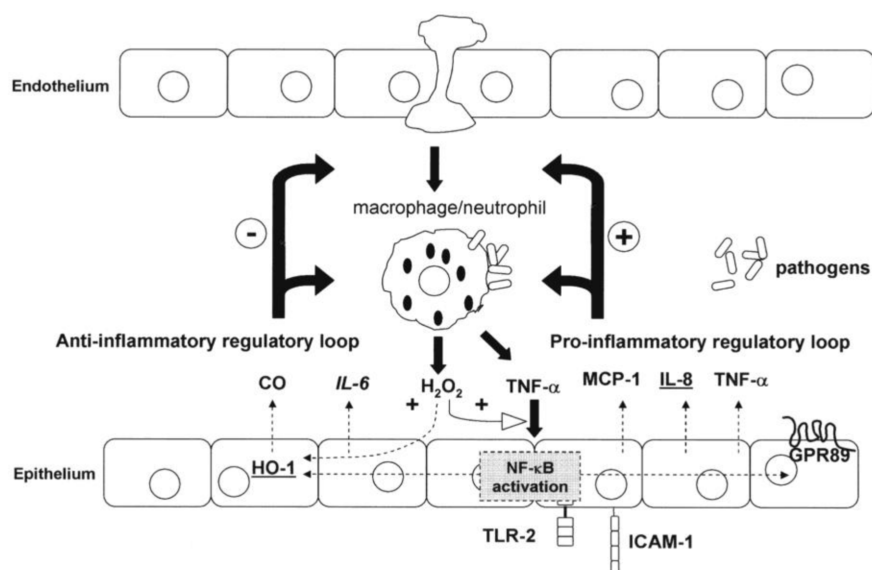

Figure 5 - H_2O_2 as a modulator of the inflammatory response. Scheme illustrating the regulatory role exerted by H_2O_2 on epithelial cells during the inflammatory response. H_2O_2 produced in phagocytes during the oxidative burst will diffuse quickly into epithelial cells where it modulates the action of TNF-α through the production of both pro- and anti-inflammatory mediators. Immune mediators whose expression was found in this work to be modulated by H_2O_2 are shown in the scheme: genes modulated only in MCF-7 cells, only in HeLa cells, and in both cell lines are indicated in bold, italic and underline typefaces, respectively.

While this work was carried out with two epithelial cell lines, there are implications for other cell types. The dependency of the regulation of NF-κB activation, either inhibitory or stimulatory, on the level of oxidative stress observed in MCF-7 and HeLa cells, may be a general phenomenon, taking in account the vast literature reporting contradictory findings for NF-κB regulation by H_2O_2. Also the weak capacity of H_2O_2 to activate by itself NF-κB is a result of general importance, because usually large doses of H_2O_2 are needed to observe NF-κB activation. Most probably for most cell types, *in vivo* H_2O_2 concentrations activate NF-κB weakly, and the main biological role of H_2O_2 is the stimulation of NF-κB activation by other species, such as TNF-α. The individual genes whose expression is increased by H_2O_2 are cell-type dependent, although this work suggests a general pattern in which H_2O_2 activates both pro- and anti-inflammatory genes. We propose that in addition to its well-established germicide function H_2O_2 has an important dual role as a fine-tuning regulator of the inflammatory process.

6. References

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7. Supplementary information

Table 1 – Activation of NF-κB-dependent genes by H_2O_2 and/or TNF-α. (**A**) Description of genes included in the Human NF-κB signaling pathway microarray used in this work; mRNA analysis of (**B**) MCF-7 and (**C**) HeLa cells after incubations with H_2O_2 (12.5 µM or 25 µM) and 0.37 ng mL^{-1} TNF-α for 1 h or 6 h[a]

A

	Description	Symbol
1	Ribosomal protein S27a[b]	RPS27A
2	Angiotensinogen	AGT
3	V-akt murine thymoma viral oncogene homolog 1	AKT1
4	Apolipoprotein L, 3	APOL3
5	Activating transcription factor 1	ATF1
6	Activating transcription factor 2	ATF2
7	B-cell CLL/lymphoma 10	BCL10
8	B-cell CLL/lymphoma 3	BCL3
9	B-factor, properdin	BF
10	Baculoviral IAP repeat-containing 2	BIRC2
11	Complement component 3	C3
12	Caspase recruitment domain family, member 10	CARD10
13	Caspase recruitment domain family, member 11	CARD11
14	Caspase recruitment domain family, member 14	CARD14
15	Caspase recruitment domain family, member 4	CARD4
16	Caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	CASP1
17	Caspase 8, apoptosis-related cysteine protease	CASP8
18	Chemokine (C-C motif) ligand 2	MCP1/CCL2
19	CASP8 and FADD-like apoptosis regulator	CFLAR
20	Conserved helix-loop-helix ubiquitous kinase	CHUK
21	Colony stimulating factor 2 (granulocyte-macrophage)	CSF2
22	Colony stimulating factor 3 (granulocyte)	CSF3
23	CTL2 gene	CTL2
24	CXXC finger 5	CXXC5
25	Extracellular matrix protein 1	ECM1
26	EDAR-associated death domain	EDARADD
27	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	EDG2
28	Early growth response 1	EGR1
29	ELK1, member of ETS oncogene family	ELK1
30	Coagulation factor II (thrombin) receptor	F2R
31	Fas (TNFRSF6)-associated via death domain	FADD
32	V-fos FBJ murine osteosarcoma viral oncogene homolog	FOS
33	Gap junction protein, alpha 1, 43kDa (connexin 43)	GJA1
34	G protein-coupled receptor 89	GPR89
35	Heme oxygenase (decycling) 1	HO1
36	Putative NF-κB activating protein HNLF	HNLF
37	5-hydroxytryptamine (serotonin) receptor 2B	HTR2B
38	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	ICAM-1
39	Interferon, alpha 1	IFNA1/INF-α
40	Interferon, beta 1, fibroblast	IFNB1
41	Interferon, gamma	IFNG
42	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	IKBKB
43	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	IKBKE
44	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	IKBKG

45	Interleukin 10	IL-10
46	Interleukin 1, alpha	IL-1A
47	Interleukin 1, beta	IL-1B
48	Interleukin 1 receptor, type I	IL-1R1
49	Interleukin 6 (interferon, beta 2)	IL6
50	Interleukin 8	IL-8
51	Interleukin-1 receptor-associated kinase 1	IRAK1
52	Interleukin-1 receptor-associated kinase 2	IRAK2
53	V-jun sarcoma virus 17 oncogene homolog (avian)	JUN
54	Lipopolysaccharide-induced TNF factor	LITAF
55	Lymphotoxin alpha (TNF superfamily, member 1)	LTA
56	Lymphotoxin beta receptor (TNFR superfamily, member 3)	LTBR
57	Mucosa associated lymphoid tissue lymphoma translocation gene 1	MALT1
58	Mitogen-activated protein kinase kinase 3	MAP2K3
59	Mitogen-activated protein kinase kinase 4	MAP2K4
60	Mitogen-activated protein kinase kinase 6	MAP2K6
61	Mitogen-activated protein kinase kinase kinase 1	MAP3K1
62	Mitogen-activated protein kinase kinase kinase 14	NIK/MAP3K14
63	Mitogen-activated protein kinase kinase kinase 3	MAP3K3
64	Mitogen-activated protein kinase kinase kinase 7	MAP3K7
65	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	MAP3K7IP1
66	Mitogen-activated protein kinase kinase kinase 7 interacting protein 2	MAP3K7IP2
67	Mitogen-activated protein kinase 14	MAPK14
68	Mitogen-activated protein kinase 3	ERK/MAPK3
69	Mitogen-activated protein kinase 8	JNK/MAPK8
70	Myeloid differentiation primary response gene (88)	MYD88
71	NACHT, leucine rich repeat and PYD containing 12	NALP12
72	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	p105/NFKB1
73	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	p100/NFKB2
74	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	IκB-α/NFKBIA
75	Polo-like kinase 2 (Drosophila)	PLK2
76	Protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform	PPM1A
77	Protein phosphatase 5, catalytic subunit	PPP5C
78	V-raf-1 murine leukemia viral oncogene homolog 1	RAF1
79	V-rel reticuloendotheliosis viral oncogene homolog (avian)	REL
80	V-rel reticuloendotheliosis viral oncogene homolog A. nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	RELA
81	V-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	RELB
82	Ret finger protein 2	RFP2
83	Ras homolog gene family, member A	RHOA
84	Ras homolog gene family, member C	RHOC
85	Receptor (TNFRSF)-interacting serine-threonine kinase 1	RIPK1
86	Receptor-interacting serine-threonine kinase 2	RIPK2
87	Solute carrier family 20 (phosphate transporter), member 1	SLC20A1
88	Signal transducer and activator of transcription 1, 91kDa	STAT1
89	TANK-binding kinase 1	TBK1
90	Toll-like receptor adaptor molecule 2	TICAM2
91	Toll-like receptor 1	TLR1
92	Toll-like receptor 10	TLR10
93	Toll-like receptor 2	TLR2
94	Toll-like receptor 3	TLR3
95	Toll-like receptor 4	TLR4

96	Toll-like receptor 6	TLR6
97	Toll-like receptor 7	TLR7
98	Toll-like receptor 8	TLR8
99	Toll-like receptor 9	TLR9
100	Tumor necrosis factor (TNF superfamily, member 2)	TNF-α
101	Tumor necrosis factor, alpha-induced protein 3	A20/TNFAIP3
102	Tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A
103	Tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B
104	Tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A
105	CD40 antigen (TNF receptor superfamily, member 5)	CD40
106	Tumor necrosis factor receptor superfamily, member 7	TNFRSF7
107	Tumor necrosis factor (ligand) superfamily, member 10	TNFSF10
108	Tumor necrosis factor (ligand) superfamily, member 14	TNFSF14
109	Fas ligand (TNF superfamily, member 6)	FASLG
110	TNFRSF1A-associated via death domain	TRADD
111	TNF receptor-associated factor 5	TRAF5
112	TNF receptor-associated factor 6	TRAF6
113	TIR domain containing adaptor inducing interferon-beta	TRIF
114	VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa	VAPA
115	PUC18 Plasmid DNA	PUC18
116		Blank
117		Blank
118	Artificial Sequence 1 Related 2 (80% identity)(48/60)	AS1R2
119	Artificial Sequence 1 Related 1 (90% identity)(54/60)	AS1R1
120	Artificial Sequence 1	AS1
121	Glyceraldehyde-3-phosphate dehydrogenase[b]	GAPDH
122	Beta-2-microglobulin[b]	B2M
123	Heat shock 90kDa protein 1, beta[b]	HSPCB
124	Heat shock 90kDa protein 1, beta[b]	HSPCB
125	Actin, beta[b]	ACTB
126	Actin, beta[b]	ACTB
127	Biotinylated Artificial Sequence 2 Complementary sequence[b]	BAS2C
128	Biotinylated Artificial Sequence 2 Complementary sequence[b]	BAS2C

B

		H_2O_2 25 µM 1h incubation			H_2O_2 12.5 µM 6h incubation		
	Symbol	**H_2O_2**	**TNF-α**	**H + T [c]**	**H_2O_2**	**TNF-α**	**H + T [c]**
3	AKT1	0.7 ± 0.1	0.8 ± 0.0	1.0 ± 0.3	0.9 ± 0.2	0.8 ± 0.3	0.9 ± 0.3
5	ATF1	0.6 ± 0.1	1.1 ± 1.0	1.5 ± 1.4	0.9 ± 0.1	1.0 ± 0.3	1.3 ± 0.0
8	BCL3	1.0 ± 0.2	1.7 ± 1.0	1.8 ± 0.9	1.1 ± 0.1	1.7 ± 0.4	1.8 ± 0.9
9	BF	n.d.	n.d.	n.d.	n.d.	8.2 ± 4.9 [d]	8.3 ± 2.4 [d]
10	BIRC2	0.7 ± 0.0	0.8 ± 0.2	1.1 ± 0.4	0.8 ± 0.1	1.2 ± 0.6	1.5 ± 0.1
11	C3	0.8 ± 0.3	1.3 ± 0.6	1.2 ± 0.3	1.1 ± 0.5	2.5 ± 1.3	2.7 ± 1.0
12	CARD10	0.8 ± 0.2	1.2 ± 0.6	1.3 ± 0.4	1.1 ± 0.2	0.8 ± 0.2	0.9 ± 0.5
18	MCP1/CCL2	n.d.	n.d.	n.d.	n.d.	5.3 ± 2.8 [d]	7.0 ± 0.2 [d]
20	CHUK	0.7 ± 0.3	0.5 ± 0.2	1.1 ± 0.7	0.9 ± 0.0	1.0 ± 0.4	1.4 ± 0.3
22	CSF3	0.8 ± 0.4	0.9 ± 0.6	0.9 ± 0.1	n.d.	n.d.	n.d.
23	CTL2	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	1.1 ± 0.3	1.3 ± 0.5
24	CXXC5	0.9 ± 0.2	0.6 ± 0.3	0.6 ± 0.2	0.7 ± 0.3	0.8 ± 0.3	0.7 ± 0.1
25	ECM1	0.6 ± 0.1	0.6 ± 0.0	0.9 ± 0.3	1.0 ± 0.2	1.1 ± 0.0	1.4 ± 0.5
28	EGR1	1.2 ± 0.2	0.9 ± 0.2	1.7 ± 0.4	3.0 ± 0.4	1.1 ± 0.6	4.8 ± 2.6
31	FADD	1.0 ± 0.1	1.5 ± 1.2	1.1 ± 0.5	1.0 ± 0.2	1.0 ± 0.2	0.8 ± 0.4

32	FOS	1.7 ± 0.9	0.4 ± 0.0	1.7 ± 1.3	1.4 ± 0.4	0.8 ± 0.2	1.3 ± 0.2
34	GPR89	1.0 ± 0.0	0.9 ± 0.1	1.1 ± 0.5	1.3 ± 0.1	1.0 ± 0.4	1.9 ± 0.5
35	HO1	1.2 ± 0.1	0.9 ± 0.2	1.2 ± 0.1	11.0 ± 4.6	1.1 ± 0.2	15.5 ± 7.7
36	HNLF	0.9 ± 0.1	0.9 ± 0.2	1.0 ± 0.2	2.5 ± 1.2	1.2 ± 0.3	3.5 ± 1.4
38	ICAM-1	1.6 ± 0.0	1.3 ± 0.3	1.6 ± 0.2	4.8 ± 1.3 [d]	1.4 ± 0.3 [d]	6.6 ± 1.5 [d]
39	IFNA1/INF-α	1.1 ± 0.1	0.6 ± 0.2	1.2 ± 0.2	5.8 ± 2.6 [d]	2.2 ± 1.0 [d]	7.7 ± 1.5 [d]
40	IFNB1	1.0 ± 0.2	1.1 ± 0.7	1.2 ± 0.6	1.4 ± 0.5	1.0 ± 0.2	1.5 ± 0.3
42	IKBKB	1.0 ± 0.2	1.4 ± 1.0	1.0 ± 0.8	0.8 ± 0.4	1.0 ± 0.0	1.3 ± 0.5
44	IKBKG	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.3	1.2 ± 0.2	0.9 ± 0.0	1.0 ± 0.6
45	IL-10	0.9 ± 0.2	1.7 ± 1.6	1.5 ± 0.8	0.8 ± 0.2	0.6 ± 0.1	0.8 ± 0.1
47	IL-1B	1.0 ± 0.6	0.8 ± 0.3	0.7 ± 0.1	0.8 ± 0.2	1.0 ± 0.4	0.8 ± 0.4
48	IL-1R1	1.0 ± 0.2	0.7 ± 0.1	0.9 ± 0.4	0.8 ± 0.2	0.8 ± 0.1	1.1 ± 0.4
50	IL-8	1.0 ± 0.1	2.7 ± 1.0	2.5 ± 0.9	1.3 ± 0.2	1.9 ± 0.8	4.2 ± 0.4
51	IRAK1	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.3
53	JUN	1.5 ± 0.1	1.7 ± 0.5	2.2 ± 0.4	1.7 ± 0.4	1.0 ± 0.1	1.8 ± 0.7
54	LITAF	1.0 ± 0.3	0.7 ± 0.1	1.0 ± 0.4	1.1 ± 0.4	1.4 ± 0.4	1.9 ± 0.7
56	LTBR	1.1 ± 0.2	1.1 ± 0.4	1.2 ± 0.5	0.9 ± 0.2	0.9 ± 0.1	1.1 ± 0.2
59	MAP2K4	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.5	1.0 ± 0.2	0.9 ± 0.2	1.2 ± 0.0
60	MAP2K6	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	0.6 ± 0.1	0.9 ± 0.2
61	MAP3K1	0.6 ± 0.0	0.6 ± 0.1	1.0 ± 0.3	n.d.	n.d.	n.d.
62	NIK/MAP3K14	1.3 ± 0.3	1.5 ± 0.2	1.3 ± 0.1	1.9 ± 0.3	1.3 ± 0.1	2.1 ± 0.4
64	MAP3K7	1.0 ± 0.2	1.4 ± 1.2	1.5 ± 1.0	1.1 ± 0.0	1.3 ± 0.3	1.8 ± 0.0
65	MAP3K7IP1	1.1 ± 0.1	1.6 ± 0.7	1.4 ± 0.7	1.1 ± 0.0	1.2 ± 0.1	1.6 ± 0.2
66	MAP3K7IP2	1.2 ± 0.2	1.8 ± 0.8	1.8 ± 0.1	1.2 ± 0.2	1.6 ± 0.5	2.1 ± 0.3
68	ERK/MAPK3	1.0 ± 0.2	1.2 ± 0.6	1.4 ± 0.6	1.2 ± 0.4	1.1 ± 0.3	1.2 ± 0.4
69	JNK/MAPK8	1.0 ± 0.1	0.8 ± 0.0	1.0 ± 0.4	1.3 ± 0.5	1.2 ± 0.6	1.4 ± 0.2
70	MYD88	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.4	0.9 ± 0.1	1.0 ± 0.2	0.8 ± 0.4
72	p105/NFKB1	0.8 ± 0.1	0.9 ± 0.2	1.0 ± 0.4	0.9 ± 0.1	1.9 ± 0.3	2.0 ± 0.1
73	p100/NFKB2	1.0 ± 0.1	2.0 ± 0.5	1.9 ± 0.6	1.1 ± 0.3	1.9 ± 0.6	1.9 ± 0.5
74	IκB-α/NFKBIA	1.1 ± 0.2	3.4 ± 1.4	4.2 ± 1.3	1.2 ± 0.2	2.8 ± 1.2	2.7 ± 1.1
75	PLK2	0.9 ± 0.1	1.3 ± 0.1	1.4 ± 0.3	1.2 ± 0.1	1.3 ± 0.2	1.8 ± 0.4
77	PPP5C	1.0 ± 0.4	1.6 ± 1.3	1.4 ± 0.8	1.1 ± 0.2	0.9 ± 0.2	1.1 ± 0.2
78	RAF1	0.9 ± 0.2	1.5 ± 0.7	1.2 ± 0.6	0.9 ± 0.2	0.8 ± 0.3	1.0 ± 0.2
80	RELA	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.4	1.0 ± 0.2	1.1 ± 0.2
81	RELB	1.0 ± 0.1	2.6 ± 1.1	1.6 ± 0.7	1.8 ± 0.2	7.1 ± 1.2	8.6 ± 1.6
82	RFP2	1.2 ± 0.2	1.8 ± 0.3	1.5 ± 0.5	1.4 ± 0.3	1.9 ± 0.4	2.2 ± 0.6
83	RHOA	1.0 ± 0.2	0.7 ± 0.2	1.0 ± 0.6	0.9 ± 0.1	1.0 ± 0.3	1.1 ± 0.0
84	RHOC	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
86	RIPK2	0.9 ± 0.1	0.9 ± 0.1	1.2 ± 0.4	0.9 ± 0.2	1.1 ± 0.3	0.9 ± 0.4
87	SLC20A1	0.9 ± 0.1	1.2 ± 0.2	0.9 ± 0.1	1.1 ± 0.3	0.9 ± 0.2	1.0 ± 0.1
88	STAT1	0.8 ± 0.1	1.2 ± 0.8	1.1 ± 0.5	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
93	TLR2	0.9 ± 0.1	1.4 ± 0.6	1.7 ± 0.7	1.2 ± 0.1	3.2 ± 0.6	4.3 ± 0.5
95	TLR4	0.7 ± 0.3	0.8 ± 0.2	0.6 ± 0.0	0.9 ± 0.4	0.8 ± 0.0	0.9 ± 0.3
96	TLR6	0.9 ± 0.1	1.1 ± 0.0	0.8 ± 0.0	1.2 ± 0.3	0.9 ± 0.3	1.1 ± 0.4
98	TLR8	1.0 ± 0.1	1.6 ± 0.3	1.4 ± 0.3	1.0 ± 0.2	1.5 ± 0.3	1.6 ± 0.7
100	TNF-α	2.1 ± 1.0 [d]	19.9 ± 3.5 [d]	23.5 ± 10.2 [d]	3.1 ± 0.8 [d]	7.4 ± 3.2 [d]	9.1 ± 3.9 [d]
101	A20/TNFAIP3	1.4 ± 0.4 [d]	14.0 ± 5.6 [d]	13.9 ± 2.2 [d]	2.0 ± 0.5	6.9 ± 2.4	10.7 ± 2.5
102	TNFRSF10A	0.8 ± 0.2	1.4 ± 0.3	1.5 ± 0.2	1.3 ± 0.4	1.2 ± 0.5	1.4 ± 0.8
103	TNFRSF10B	0.9 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	1.3 ± 0.5	1.7 ± 0.7	1.8 ± 0.5
104	TNFRSF1A	0.9 ± 0.1	1.3 ± 0.4	1.0 ± 0.2	1.3 ± 0.3	1.0 ± 0.3	1.3 ± 0.3
110	TRADD	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.3	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.1

C

		H_2O_2 25 µM 1h incubation			H_2O_2 12.5 µM 6h incubation		
	Symbol	**H_2O_2**	**TNF-α**	**H + T [c]**	**H_2O_2**	**TNF-α**	**H + T [c]**
3	AKT1	0.8 ± 0.1	1.0 ± 0.4	1.1 ± 0.3	0.8 ± 0.0	1.1 ± 0.1	1.0 ± 0.2
5	ATF1	0.7 ± 0.2	0.9 ± 0.4	0.9 ± 0.2	1.1 ± 0.4	1.3 ± 0.5	0.9 ± 0.1
6	ATF2	1.4 ± 0.8	1.3 ± 0.9	0.9 ± 0.1	0.8 ± 0.5	1.1 ± 0.7	1.0 ± 0.7
7	BCL10	1.0 ± 0.3	1.2 ± 0.6	1.0 ± 0.0	0.8 ± 0.1	1.0 ± 0.4	0.8 ± 0.2
8	BCL3	1.0 ± 0.5	2.3 ± 1.7	2.0 ± 0.4	0.8 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
9	BF	1.2 ± 0.1	1.2 ± 0.2	1.6 ± 0.4	0.9 ± 0.3	1.4 ± 0.2	1.6 ± 0.5
10	BIRC2	0.8 ± 0.1	1.2 ± 0.4	1.4 ± 0.5	1.1 ± 0.1	2.2 ± 0.3	2.7 ± 0.6
11	C3	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	2.4 ± 0.7	2.2 ± 0.2
12	CARD10	0.7 ± 0.1	0.9 ± 0.3	1.2 ± 0.3	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.2
13	CARD11	0.9 ± 0.2	1.2 ± 0.5	1.2 ± 0.1	1.1 ± 0.5	1.4 ± 0.8	1.4 ± 0.4
16	CASP1	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	1.5 ± 0.7	1.3 ± 0.2
18	MCP1/CCL2	0.7 ± 0.1	1.9 ± 0.5	2.1 ± 0.6	1.2 ± 0.2	3.7 ± 1.3	4.3 ± 0.3
20	CHUK	0.7 ± 0.0	1.1 ± 0.3	1.0 ± 0.2	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.2
22	CSF3	1.1 ± 0.2	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.4	1.1 ± 0.7	1.1 ± 0.4
23	CTL2	0.9 ± 0.1	1.0 ± 0.3	1.1 ± 0.4	0.8 ± 0.1	1.0 ± 0.2	0.9 ± 0.1
24	CXXC5	0.7 ± 0.2	0.8 ± 0.3	1.0 ± 0.4	0.6 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
25	ECM1	1.0 ± 0.2	1.2 ± 0.3	1.3 ± 0.2	0.9 ± 0.3	1.1 ± 0.3	1.3 ± 0.0
27	EDG2	1.1 ± 0.1	1.2 ± 0.3	1.0 ± 0.2	n.d.	n.d.	n.d.
28	EGR1	1.1 ± 0.1	1.4 ± 0.2	1.9 ± 0.7	2.5 ± 0.4	2.1 ± 1.1	3.1 ± 1.2
29	ELK1	0.8 ± 0.3	1.0 ± 0.5	0.9 ± 0.0	1.2 ± 0.4	1.5 ± 0.5	1.1 ± 0.2
30	F2R	0.8 ± 0.3	0.9 ± 0.4	1.0 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	0.7 ± 0.6
31	FADD	0.8 ± 0.1	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.0	1.1 ± 0.2	1.2 ± 0.1
32	FOS	0.9 ± 0.3	0.9 ± 0.4	1.3 ± 0.2	0.8 ± 0.1	0.9 ± 0.4	0.8 ± 0.2
33	GJA1	0.8 ± 0.1	0.9 ± 0.4	1.1 ± 0.4	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
34	GPR89	0.9 ± 0.0	1.0 ± 0.2	1.2 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	1.5 ± 0.2
35	HO1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.5 ± 0.4	1.1 ± 0.2	2.0 ± 0.3
36	TMED4	0.8 ± 0.1	1.1 ± 0.4	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.4 ± 0.1
38	ICAM-1	0.9 ± 0.3	1.6 ± 1.0	1.7 ± 0.4	1.5 ± 0.2	2.7 ± 0.9	2.7 ± 0.1
39	IFNA1/INF-α	0.8 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	1.2 ± 0.3	1.7 ± 0.7	1.8 ± 0.2
40	IFNB1	0.8 ± 0.2	0.9 ± 0.3	0.9 ± 0.2	1.1 ± 0.3	1.7 ± 0.6	1.8 ± 0.4
42	IKBKB	0.9 ± 0.2	1.6 ± 0.5	1.8 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	1.5 ± 0.1
44	IKBKG	0.9 ± 0.1	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.1	1.3 ± 0.4	1.3 ± 0.1
45	IL-10	1.2 ± 0.3	1.0 ± 0.2	1.3 ± 0.4	1.2 ± 0.6	1.2 ± 0.8	1.4 ± 0.3
46	IL-1A	1.2 ± 0.1	3.0 ± 0.9	2.7 ± 0.5	1.3 ± 0.4	2.0 ± 0.5	2.0 ± 0.0
47	IL-1B	1.3 ± 0.4	1.2 ± 0.4	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.1	1.0 ± 0.2
48	IL-1R1	0.9 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	1.1 ± 0.3	1.4 ± 0.3	1.4 ± 0.3
49	IL6	1.5 ± 0.3	12.7 ± 3.6	15.8 ± 3.4	2.1 ± 0.2	5.4 ± 1.2	8.7 ± 1.2
50	IL-8	1.2 ± 0.2	9.2 ± 3.2	9.9 ± 1.5	1.9 ± 0.4	2.4 ± 0.5	4.4 ± 1.2
51	IRAK1	1.0 ± 0.3	1.0 ± 0.3	1.2 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2
53	JUN	1.2 ± 0.3	1.6 ± 0.7	1.9 ± 0.2	1.4 ± 0.2	1.4 ± 0.1	1.6 ± 0.2
54	LITAF	0.9 ± 0.3	1.0 ± 0.4	1.0 ± 0.1	0.9 ± 0.2	1.1 ± 0.2	1.0 ± 0.1
56	LTBR	0.9 ± 0.3	1.0 ± 0.2	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	1.0 ± 0.1
57	MALT1	0.9 ± 0.1	1.5 ± 0.4	1.4 ± 0.1	1.4 ± 0.3	1.6 ± 0.3	1.7 ± 0.1
58	MAP2K3	0.8 ± 0.1	1.8 ± 0.6	1.6 ± 0.2	1.1 ± 0.1	1.5 ± 0.2	1.7 ± 0.0
59	MAP2K4	0.9 ± 0.1	1.2 ± 0.2	1.3 ± 0.4	1.2 ± 0.3	1.3 ± 0.1	1.5 ± 0.3
60	MAP2K6	0.9 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.5 ± 0.1
61	MAP3K1	1.1 ± 0.2	1.4 ± 0.3	1.0 ± 0.1	n.d.	n.d.	n.d.
62	NIK/MAP3K14	1.0 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.2 ± 0.2
64	MAP3K7	0.8 ± 0.1	1.1 ± 0.4	1.2 ± 0.4	0.9 ± 0.1	1.1 ± 0.1	1.2 ± 0.2

65	MAP3K7IP1	0.9 ± 0.1	1.3 ± 0.4	1.3 ± 0.3	1.0 ± 0.1	1.2 ± 0.2	1.3 ± 0.2
66	MAP3K7IP2	0.9 ± 0.0	1.8 ± 0.5	1.9 ± 0.2	1.3 ± 0.0	1.8 ± 0.1	1.9 ± 0.4
67	MAPK14	1.0 ± 0.1	1.4 ± 0.1	1.1 ± 0.2	1.4 ± 0.0	1.2 ± 0.3	1.5 ± 0.1
68	ERK/MAPK3	0.9 ± 0.1	1.0 ± 0.2	1.1 ± 0.2	0.9 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
69	JNK/MAPK8	0.9 ± 0.2	1.0 ± 0.4	1.0 ± 0.1	1.1 ± 0.3	1.1 ± 0.1	1.2 ± 0.1
70	MYD88	0.8 ± 0.2	1.0 ± 0.2	1.1 ± 0.3	1.1 ± 0.2	1.2 ± 0.3	1.3 ± 0.2
72	p105/NFKB1	0.8 ± 0.1	1.3 ± 0.3	1.1 ± 0.2	1.1 ± 0.0	1.9 ± 0.3	2.3 ± 0.4
73	p100/NFKB2	0.8 ± 0.1	1.5 ± 0.6	1.7 ± 0.4	1.3 ± 0.2	2.5 ± 0.6	2.7 ± 0.3
74	IκB-α/NFKBIA	1.4 ± 0.4	5.0 ± 1.7	6.6 ± 0.5	1.3 ± 0.2	3.4 ± 0.7	3.6 ± 0.5
75	PLK2	0.8 ± 0.2	1.3 ± 0.1	1.7 ± 0.2	1.2 ± 0.0	1.3 ± 0.1	1.7 ± 0.2
77	PPP5C	0.8 ± 0.0	1.0 ± 0.3	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.3 ± 0.3
78	RAF1	0.9 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
79	REL	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.2	1.0 ± 0.3	1.1 ± 0.3	1.2 ± 0.2
80	RELA	0.8 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.0	1.1 ± 0.1	1.2 ± 0.1
81	RELB	0.7 ± 0.1	1.2 ± 0.5	1.2 ± 0.3	1.4 ± 0.3	3.2 ± 1.2	3.5 ± 0.6
82	RFP2	0.8 ± 0.2	1.2 ± 0.6	1.3 ± 0.4	1.0 ± 0.2	1.4 ± 0.4	1.6 ± 0.3
83	RHOA	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.2	1.1 ± 0.1
84	RHOC	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.0	1.1 ± 0.2	1.1 ± 0.1
86	RIPK2	0.7 ± 0.3	1.3 ± 0.4	1.3 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	1.3 ± 0.2
87	SLC20A1	0.9 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.0
88	STAT1	0.8 ± 0.2	1.0 ± 0.4	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	1.1 ± 0.1
89	TBK1	1.0 ± 0.2	1.3 ± 0.3	1.1 ± 0.1	1.3 ± 0.3	1.7 ± 0.7	1.4 ± 0.3
95	TLR4	1.1 ± 0.2	1.1 ± 0.3	1.0 ± 0.1	0.9 ± 0.3	1.0 ± 0.1	1.0 ± 0.2
96	TLR6	1.1 ± 0.2	1.0 ± 0.4	1.0 ± 0.1	0.8 ± 0.3	1.1 ± 0.2	1.0 ± 0.3
98	TLR8	0.7 ± 0.2	0.9 ± 0.5	1.2 ± 0.3	1.0 ± 0.1	1.4 ± 0.0	1.5 ± 0.3
100	TNF-α	n.d.	4.9 ± 1.3 [d]	5.2 ± 0.1 [d]	n.d.	n.d.	n.d.
101	A20/TNFAIP3	0.8 ± 0.1	6.3 ± 1.9	6.3 ± 0.4	1.5 ± 0.2	5.3 ± 1.4	5.0 ± 0.9
102	TNFRSF10A	0.8 ± 0.2	1.1 ± 0.4	1.2 ± 0.4	1.0 ± 0.2	1.3 ± 0.2	1.3 ± 0.3
103	TNFRSF10B	0.7 ± 0.1	0.8 ± 0.2	1.0 ± 0.5	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.2
104	TNFRSF1A	0.7 ± 0.3	0.7 ± 0.2	0.8 ± 0.2	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.1
110	TRADD	0.9 ± 0.2	1.2 ± 0.4	1.1 ± 0.3	1.2 ± 0.0	1.3 ± 0.1	1.1 ± 0.2
111	TRAF5	0.8 ± 0.2	1.0 ± 0.3	1.0 ± 0.0	1.0 ± 0.3	1.0 ± 0.4	0.9 ± 0.1
112	TRAF6	1.0 ± 0.3	1.0 ± 0.4	1.1 ± 0.2	1.0 ± 0.3	1.2 ± 0.6	1.1 ± 0.2

[a] Results are from 4 independent experiments for MCF-7 cells and 3 independent experiments for HeLa cells and are presented as average ± SD. Individual expressions were normalized to non-treated cells. n.d. – not detected.

[b] Housekeeping genes used as internal control

[c] Calculated with equation 3 in *Materials and Methods*.

[d] No expression was detected for non-treated cells so the value presented is a lower limit.

Chapter III

Cooperative effect of H_2O_2 and TNF-α on NF-κB activation: modulation of IκB-α, IκB-β and IκB-ε†

Virgínia Oliveira-Marques, Teresa Silva, H. Susana Marinho, Fernando Antunes, Luísa Cyrne

1. Abstract

NF-κB is the key regulator of the inflammatory response. Hydrogen peroxide (H_2O_2) is an atypical modulator of NF-κB, with contradictory data pointing to both positive and negative effects. We have recently shown that H_2O_2 at moderate and steady-state doses is able to synergize with TNF-α by increasing nuclear p65 subunit of NF-κB and thereby up-regulating several NF-κB-dependent genes. Degradation of the inhibitory proteins IκBs precedes NF-κB translocation to the nucleus and so the outcome of these proteins was studied in this work. IκB-α and IκB-β responded to TNF-α induction in MCF-7 and HeLa cells, while small changes occurred in IκB-ε. The addition of H_2O_2 simultaneously with TNF-α decreased drastically IκB-α levels in MCF-7 cells, and concomitantly the nuclear levels of p65 and c-Rel proteins increased, whereas IκB-β was almost unresponsive to the addition of H_2O_2. The stimulatory effect of H_2O_2 is a result, at least in part, of the increased activity of the 26S proteasome that was observed when H_2O_2 and TNF-α were added simultaneously. This regulation was cell type-dependent and is in agreement with the estimated intracellular concentration of H_2O_2, which was lower in HeLa than in MCF-7 cells. In HeLa cells, H_2O_2 stimulated IκB-α degradation and p65 translocation but the proteasome activity was not increased. Also, in opposition to what was observed for MCF-7 cells, c-Rel translocation was inhibited by H_2O_2, probably because it was sequestered in the cytosol bound to IκB-ε. During an inflammatory process, H_2O_2 and TNF-α are expected to cooperate for an increased activation of NF-κB by modulating IκB levels, especially IκB-α, in order to induce a

† Manuscript in preparation. Experiments of IκB-β and IκB-ε proteins were performed by Teresa Silva.

selective gene expression profile. The particular responses for different cell types are probably a result from the different sensibilities to H_2O_2.

2. Introduction

An inflammatory environment is rich in cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1); chemokines, such as IL-8 and monocyte chemotactic protein-1 (MCP-1) and also leucocytes, such as neutrophils and macrophages. The latter produce reactive oxygen species (ROS) for a germicide action; however some ROS, especially hydrogen peroxide (H_2O_2), may leak the phagosome and participate in other cellular processes[1,2]. H_2O_2 is a small molecule, relatively stable and able to cross membrane barriers. Although H_2O_2 has been linked to oxidative stress, nowadays the mild oxidative properties of H_2O_2 and the tight control of intracellular H_2O_2 levels support its role in signal transduction[3,4]. We have recently shown that H_2O_2, at concentrations close to the ones existing during an inflammatory situation, 5-15 μM[2,5], is able to synergize with TNF-α by increasing the translocation levels of the p65 protein from the cytosol to the nucleus. p65 belongs to the NF-κB/Rel family of transcription factors, which have key regulatory roles in inflammation, innate and adaptive immune response, proliferation and apoptosis[6,7]. Apart from p65, the NF-κB/Rel family comprises c-Rel and RelB, which bear transactivation domains and also the regulatory subunits p50 and p52[6,8,9]. NF-κB/Rel proteins can form homo or heterodimers and the prototypical NF-κB is a heterodimer composed by the p50 and p65 subunits (p50/p65). In the classical activation pathway, NF-κB dimers remain inactive in the cytosol bound to its inhibitory proteins, the IκBs, which possess the typical ankyrin repeats that mask the nuclear localization signal (NLS) of NF-κB thus preventing its translocation to the nucleus[6,7]. The three most common members of the IκBs family are IκB-α, IκB-β and IκB-ε. After a specific signal, such as the binding of TNF-α to its cell receptors, the IκB kinase complex (IKK) is activated and phosphorylates the different IκBs at specific conserved serine residues, which are subsequently marked by ubiquitination leading to recognition and degradation by the 26S proteasome[10,11]. Then, free NF-κB migrates to the nucleus in order to activate the target genes. Contradictory data from the literature indicate either a synergism between H_2O_2 and TNF-α, as a result of IKK stimulation by H_2O_2[12] or an antagonism by inhibition of IKK[13,14] or the proteasome[15] by H_2O_2. As little is known about the effect of H_2O_2 in the other two IκBs, IκB-β and IκB-ε, here we propose to elucidate part

of the mechanism involving the synergistic activation by H_2O_2 and TNF-α by monitoring the levels of the three major IκBs. In addition to p65, c-Rel levels were monitored since the three IκBs also bind c-Rel. IκB-α and IκB-β bind preferentially to heterodimers p50/p65 and p50/c-Rel[7], while IκB-ε binds preferentially to p65 homodimers and c-Rel/p65 heterodimers[16]. Because the contradictory data about the role H_2O_2 has on the NF-κB pathway is probably a result of the method of cell exposure to H_2O_2, a controlled and calibrated method – the steady-state (s.s.) titration – was used to overcome this issue[3,17].

3. Materials and methods

Cell culture and reagents

HeLa (American Type Culture Collection, Manassas, VA, USA) and MCF-7 (European Collection of Cell Cultures, Salisbury Wiltshire, UK) cells were grown in RPMI 1640 medium supplemented with 10% of fetal bovine serum, penicillin 100 UmL^{-1}, streptomycin 100 µg mL^{-1} and L-glutamine 2 mM, all from Cambrex, Verviers, Belgium. Glucose oxidase (*Aspergillus Niger*), TNF-α (human recombinant), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the chymotrypsin proteasome substrate N-Succinyl-leucine-leucine-valine-tyrosine-7-amido-4-methylcoumarin (SucLLVY-AMC) were obtained from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). H_2O_2 was obtained from Merck & Co., Inc. (Whitehouse Station, NJ, USA). Proteasome inhibitors MG132 and Lactacystin were acquired from Calbiochem (Darmstadt, Germany).

Cells incubations

Cells were counted and plated approximately 46 h before the experiment. Fresh medium was added to cells 1 h before the incubations. Exposure to H_2O_2 was performed using the steady-state titration (s.s.)[17], the method is extensively described in[3]. Briefly, a steady-state level of H_2O_2 is maintained during the entire assay by adding simultaneously with an initial dose of H_2O_2, a quantity of glucose oxidase enough to counteract H_2O_2 consumption by cells. All experiments were performed with 25 µM H_2O_2 s.s. and 0.37 ng mL^{-1} of TNF-α. The proteasome inhibitor MG132 was used at a concentration of 10 µM.

Estimation of intracellular H_2O_2 concentrations in HeLa cells

Intracellular H_2O_2 concentrations were estimated from the H_2O_2 gradient across the plasma membrane as described in[3,18]. The gradient, i.e. the ratio between H_2O_2 concentration outside and inside (cytosol) the cell, may be inferred from H_2O_2 consumption by intact cells ($k_{\text{intactcell}}$) over the sum of enzyme activities of catalase (k_{catalase}) and glutathione peroxidase (k_{GPx}) that consume H_2O_2 in disrupted cells (equation 1).

$$\text{Gradient} = \frac{[\mathrm{H_2O_2}]_{out}}{[\mathrm{H_2O_2}]_{\text{in}}} = \frac{k_{\text{catalase}} + k_{\text{GPx}}}{k_{\text{intactcell}}} \quad \text{(Equation 1)}$$

Protein extraction and immnunoblot analysis

HeLa and MCF-7 cells were plated onto 100-mm dishes to achieve respectively 1.5×10^6 and 1.8×10^6 cells per dish at the day of experiment. Preparation of cytosolic and nuclear extracts and immunoblot assays were performed as described previously[3]. All proteins were analyzed onto a 8% polyacrylamide gel, except for IκB-β where a 12.5% gel was used. To analyze the phosphorylated IκB-α (P-IκB-α), the phosphatase inhibitor cocktail (5 mM NaF, 1 mM $NaVO_3$, 10 mM β-glycerophosphate and 10 mM *p*-nitrophenyl phosphate) was added to the extraction buffer. The antibody #9246 from Cell Signaling Technology (Danvers, MA, USA) was used to identify phosphorylated Ser32 and Ser36 of IκB-α (1:1000). Antibodies sc-372 (1:1000), sc-70 (1:300), sc-371 (1:800), sc-945 (1:400) and sc-7156 (1:800) were used to identify p65, c-Rel, IκB-α, IκB-β and IκB-ε, all from Santa Cruz Biotechnology, Santa Cruz, California, USA. The corresponding bands for each proteins were quantified by signal intensity analysis, normalized to the protein loading (membrane stained with Ponceau S), using the ImageJ software[19].

26S and 20S proteasome activity

Proteasome activity was measured as reported[20,21] with some modifications. Cells were washed 3 times with cold PBS and collected with a scrapper in the hypotonic buffer (50 mM Tris-HCl, 0.5 mM EDTA, 5 mM $MgCl_2$ and 250 mM sucrose) daily supplemented with 2 mM ATP and, when indicated 2 mM DTT. Cell lysis was performed with three freeze/thaw cycles using liquid nitrogen and a water bath at 37ºC and then the soluble fraction was

collected after centrifugation at 13000 g for 15 minutes at 4ºC. Proteasome assay was performed using a microplate reader (SpectraMax GeminiEM, Molecular Devices) and by adding sequentially to each well (final volume of 200 µL): 26S (50 mM Tris-HCl, 5 mM $MgCl_2$ and 0.5 ng mL^{-1} BSA) or 20S (50 mM Tris-HCl, 0.5 mM EDTA, 0.035% SDS and 0.5 ng mL^{-1} BSA) buffer warmed at 37ºC, 2 mM ATP (only for 26S) and 75 µM of the fluorogenic substrate SucLLVY-AMC (chymotrypsin-like) dissolved in DMSO and incubated for 5 min. The reaction was started by the addition of 10 µg of the protein extract. Proteolytic activity was measured by monitoring the release of the fluorescent group AMC, with excitation at 380 nm and emission at 460 nm. Less than 5% of fluorescence intensity was measured after the treatment of the extract with 20 µM Lactacystin, a specific proteasome inhibitor, indicating the correct measurement of the proteasome activity.

Statistical analysis

All data is presented as the average ± standard deviation of at least four independent experiments. In Figure 1, independent time points were compared with control levels (1 r.u.) by using the two-tailed t-test and the relevant results are in the text description. Otherwise, multiple comparisons where performed using analysis of variance (ANOVA) with the Student-Newman-Keuls post-hoc test.

4. Results

4.1 Estimation of H_2O_2 cytosolic concentrations in HeLa cells

The exogenously added H_2O_2 is able to cross the plasma membrane, but it is not a completely “free” process[22]. Since H_2O_2 passage through the plasma membrane is rate-limiting, the formation of a gradient across the membrane occurs when an external source is added[22]. In Table 1 we present the H_2O_2 consumption activities of catalase and GPx in both cell lines and the resulting gradient by the application of equation 1 for HeLa cells and compared them with the results previously obtained for MCF-7 cells[3]. Although for both cell lines the H_2O_2 consumption rate is approximately the same in intact cells, the intracellular concentration of H_2O_2 estimated for HeLa cells is lower than the one for MCF-7 cells because of the higher (about seven times) GPx activity. The higher catalase activity found in MCF-7 cells is not enough to compensate the GPx activity and, consequently, these cells have a lower H_2O_2 gradient. This indicates that for an extracellular concentration of 25 µM H_2O_2, the

intracellular concentration for HeLa cells is expected to be about 3.7 μM, whereas for the MCF-7 cells this value increases to approximately 12.5 μM.

Table 1 - Estimation of the gradient across the plasma membrane in HeLa cells after exposure to extracellular H_2O_2.

Parameter	HeLa	MCF-7[b]
$k_{intactcell}$	0.50 ± 0.017[a]	0.43 ± 0.015[a]
$k_{catalase}$	0.21 ± 0.042[a]	0.42 ± 0.061[a]
k_{GPx}	3.18 ± 0.450[a]	0.41 ± 0.063[a]
$[H_2O_2]_{out}/[H_2O_2]_{in}$	6.8±2.3	1.9±0.6

[a] units: $min^{-1} \times 10^{-6}$cells × mL; [b] Adapted from[3]

4.2 Characterization of IκB-α, IκB-β and IκB-ε responses to H_2O_2

To characterize NF-κB activation by a moderate dose of H_2O_2 s.s. the cytosolic levels of the three more abundant IκBs: IκB-α, IκB-β and IκB-ε were analyzed in MCF-7 (Figure 1a, 1b) and HeLa cells (Figure 1a, 1c). As the degradation of IκBs precedes NF-κB translocation to the nucleus, IκB responses were correlated with the nuclear levels of p65 and c-Rel, which are able to form different NF-κB dimers and activate gene expression through their TAD. In MCF-7 cells (Figure 1b left panel), H_2O_2 did not induce significantly degradation of IκB-α or IκB-β. Note the significant increase above control levels of IκB-α at 0.5 h ($P<0.05$), which may indicate a relocalization of IκB-α in the cytosol, as it has the capacity to shuttle between the nucleus and the cytosol[23]. IκB-ε, which also makes nucleo-cytosolic shuttling[24], followed an oscillatory pattern that does not match the nuclear response, in which H_2O_2 induced a slight translocation of p65 and c-Rel. This indicates that probably the small pool of p65 and c-Rel eventually complexed with IκB-ε is not enough to affect the global response of these proteins.

With the classical NF-κB-activator TNF-α (Figure 1b, middle panels), IκB-α responded promptly, with maximal degradation after 30 min of treatment followed by a recover, as expected for an NF-κB-dependent gene[25,26]. IκB-β also showed a fast response, although the degradation was incomplete and more sustained over time. This was expected from the data in the literature because IκB-β expression is not dependent on NF-κB[27,28]. As observed for H_2O_2, IκB-ε presented an oscillatory response to TNF-α. IκB-ε has been pointed as a regulator by dampening oscillations provoked by IκB-α in TNF-α-treated-murine fibroblast cells, through an antiphase IκB-ε expression over IκB-α expression[29], but IκB-ε responses are highly dependent on the cell type and treatment[30]. In fact, the nuclear levels of p65 are in

accordance with a combined effect of both IκB-α and IκB-β degradation, with the maximal translocation corresponding to the maximal IκB-α degradation, while the sustained activation of p65 at longer timer seems to be due to IκB-β. Moreover, TNF-α induced a sustained increase of nuclear c-Rel, which is also in accordance with IκB-β behavior.

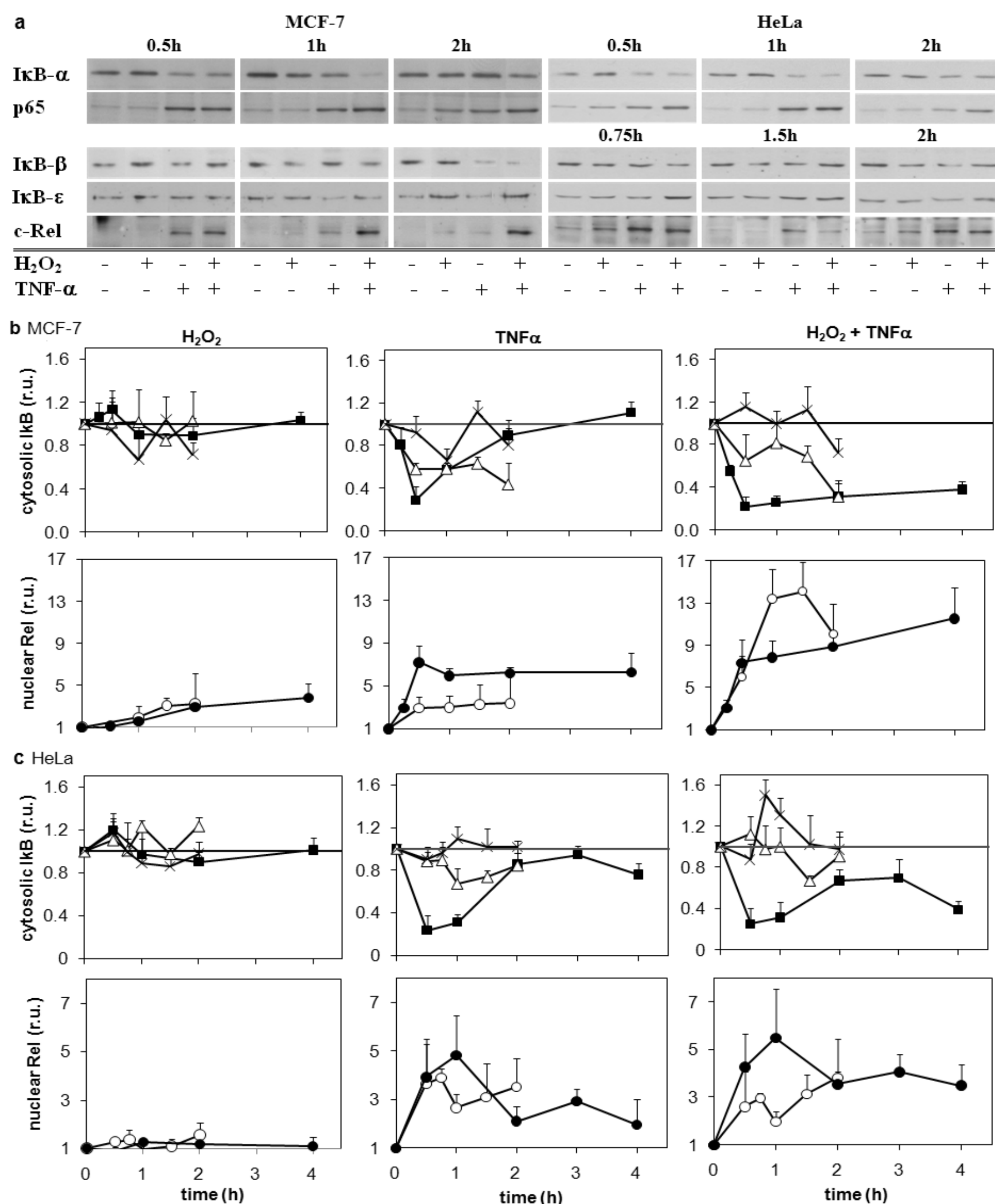

Figure 1 – Differential modulation of IκB and Rel proteins by H_2O_2 and TNF-α. (a) Representative immunoblot showing the effect of H_2O_2 and TNF-α on IκB-α, IκB-β, IκB-ε, p65 and c-Rel. MCF-7 **(b)** and HeLa **(c)** cells were exposed to either 25 μM H_2O_2 s.s. (left panels) or 0.37 ng mL^{-1} TNF-α (middle panels) or both agents simultaneously (right panels). The levels of cytosolic IκB-α (■), IκB-ε (×) and IκB-β (Δ) and nuclear p65 (●) and c-Rel (○) were followed by western blot. In MCF-7 cells, the values for c-Rel are a minimal value because no band was observed in non-treated-cells and the normalization was made with the lighter band visualized in the immunoblot.

We have recently showed that H_2O_2 is able to positively modulate NF-κB activation by TNF-α, through an increase of nuclear p65 levels[3]. Because H_2O_2 by itself did not induce degradation of the IκBs, next we investigated if the increased translocation of NF-κB induced by H_2O_2 in TNF-α-treated cells was due to an increased degradation of the IκBs. The right panels of Figure 1b show the result of the simultaneous exposure of MCF-7 cells to H_2O_2 and TNF-α. Since the response of IκB-β was approximately the same observed with TNF-α alone, a modulation role for H_2O_2 was ruled out for IκB-β. IκB-ε showed again an oscillatory behavior, although with no degradation at 1 h incubation. When H_2O_2 and TNF-α were simultaneously present, important differences were observed for IκB-α levels, when compared with TNF-α alone. After 1 h, the recovery of IκB-α was mostly prevented, and this effect was in agreement with the increased nuclear levels of p65 and c-Rel, indicating that NF-κB dimers containing p65 and c-Rel are for the most part bound to IκB-α. It should be noted that for c-Rel there is a high synergistic behavior in the presence of H_2O_2 and TNF-α.

In HeLa cells, IκB-α response to H_2O_2 itself (Figure 1c left panels) was similar to the one observed in MCF-7 cells. However, no degradation was observed for IκB-ε and in the time interval studied IκB-β levels sometimes increased above control levels. In HeLa cells, p65 and c-Rel were mostly unresponsive to H_2O_2. Thus, the changes in IκB-β levels probably are not high enough as to implicate changes in nuclear p65 and c-Rel levels. The addition of TNF-α (middle panels), induced a response similar to the one obtained for MCF-7 cells, although the degradation of IκB-β occurred later in time and was shorter. In opposition to MCF-7 cells, c-Rel had an oscillatory response to TNF-α.

The simultaneous addition of H_2O_2 and TNF-α (Figure 1c, right panels) also decreased the recovery of IκB-α after maximal degradation, but less dramatically than in MCF-7 cells. Again, the presence of H_2O_2 did not change the pattern of IκB-β but it induced a significant increase of 50% and 30% of IκB-ε levels after 45 min and 1h, respectively (P=0.002 for 45 min and P=0.035 for 1 h). While for p65 once again there was a synergism between H_2O_2 and TNF-α, for c-Rel no effects were observed except at short incubation times where an antagonism was observed (compare with TNF-α, middle panel). Interestingly, the raise of IκB-ε above control levels is related with the inhibition of c-Rel translocation, which is in opposition with results obtained for MCF-7 cells, but consistent with other reports. In macrophages, a pre-incubation with 250 μM H_2O_2 was also shown to inhibit c-Rel

translocation in LPS plus interferon γ-treated cells[31] and in murine lungs IκB-ε was shown to increase after LPS instillation[32]. This indicates that both IκB-ε and c-Rel regulation is cell-type dependent.

Taken together, in both MCF-7 and HeLa cells, p65 translocation results from both IκB-α and IκB-β degradation when cells are treated with TNF-α; however, the synergism observed in the presence of H_2O_2 results only from a modulation by IκB-α. IκB-ε had a different behavior in both cell lines. It is known that IκB-ε is degraded with slower kinetics than IκB-α and IκB-β[33], that it actively shuttles between the nucleus and cytosol[24] and that its expression is regulated by NF-κB[16]. Therefore, the increased IκB-ε levels observed might be a result of newly synthesized IκB-ε, due to NF-κB activation via degradation of both IκB-α and IκB-β, or an accumulation of IκB-ε in the cytosol, which in unstimulated cells may be distributed between the nucleus and cytosol. In HeLa cells, c-Rel dimers are probably bound to, IκB-ε whereas in MCF-7 cells c-Rel dimers, including c-Rel/p65 dimers are bound chiefly to IκB-α.

4.3 H_2O_2 does not affect IκB-α synthesis in MCF-7 cells

The ability of H_2O_2 to maintain IκB-α at low levels when compared with TNF-α might be a result of an impairment of IκB-α synthesis or an accelerated degradation of IκB-α, due to either increased activities of IKK and/or the 26S proteasome. We have shown before that IκB-α mRNA was not decreased by H_2O_2 in both HeLa and MCF-7 cells[3]. To further elucidate about IκB-α synthesis issue, the proteasome inhibitor MG132, which has also been described as an NF-κB inhibitor[15,34,35] was used (Figure 2).

Cells were exposed to TNF-α with or without H_2O_2 for 30 min, to allow the almost complete degradation of IκB-α (Figure 1, middle and right panels) and then MG132 was added. If H_2O_2 is responsible for an inhibition of IκB-α synthesis, it would be expected that blocking IκB-α degradation would not result in any significant accumulation of IκB-α, since the recovery of IκB-α is guaranteed by its *de novo* synthesis.

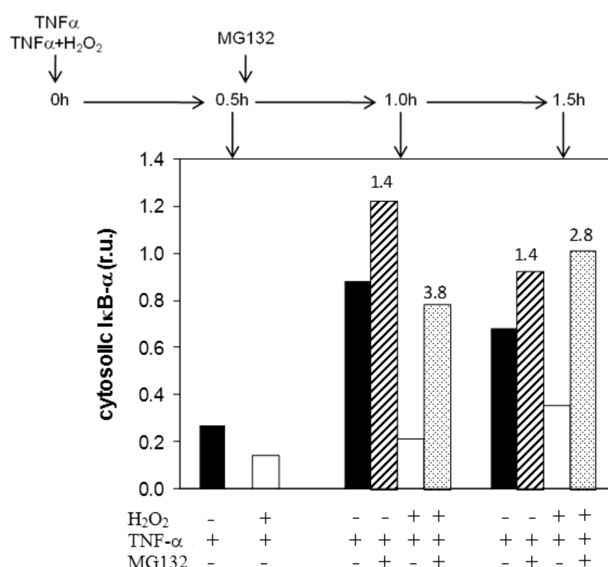

Figure 2 – H_2O_2 is not affecting IκB-α *de novo* synthesis. MCF-7 cells were pre-exposed to 0.37 ng mL^{-1} TNF-α alone (■) or simultaneously with 25 μM H_2O_2 s.s. (□) for 30 min, and then the proteasome inhibitor MG132 was added for a further 30 min and 1 h exposure (▨ TNF-α+MG132, ⬚ H_2O_2+TNF-α+MG132). Cytosolic IκB-α levels were measured by western blot. The ratio between IκB-α levels in the presence and in the absence of MG132 is presented in the top of the respective bar. Representative experiment of a total of three.

The representative experiment in Figure 2 shows that the presence of MG132 lead to an accumulation of the newly synthesized IκB-α in MCF-7 cells exposed to TNF-α and to both H_2O_2 and TNF-α at the two time points studied: 1 h and 1.5 h. Thus, the results obtained are in agreement with the mRNA data[3] and allowed the rejection of the hypothesis that H_2O_2 was inhibiting IκB-α synthesis. Unfortunately, it was not possible to reproduce the experiment on HeLa cells, because MG132 induced the degradation of IκB-α and thus activation NF-κB (data not shown). This result is in agreement with other reports using HeLa[36] and also other cell lines[37].

4.4 H_2O_2 and TNF-α together stimulate the 26S proteasome activity in MCF-7 cells

Subsequently to its phosphorylation at specific serines, IκB-α is modified by ubiquitination and, consequently, recognized by the 26S proteasome, which is responsible for its degradation. A recent report showed that, depending on its concentration, H_2O_2 could either activate or inhibit proteasome activity[38]. Therefore, the low levels of IκB-α found in cells exposed to H_2O_2 and TNF-α simultaneously might be a result of an increased proteasomal activity. The chymotrypsin-like activity of the 26S and 20S proteasomes in both MCF-7 (Figure 3a) and HeLa cells (Figure 3b) was assayed.

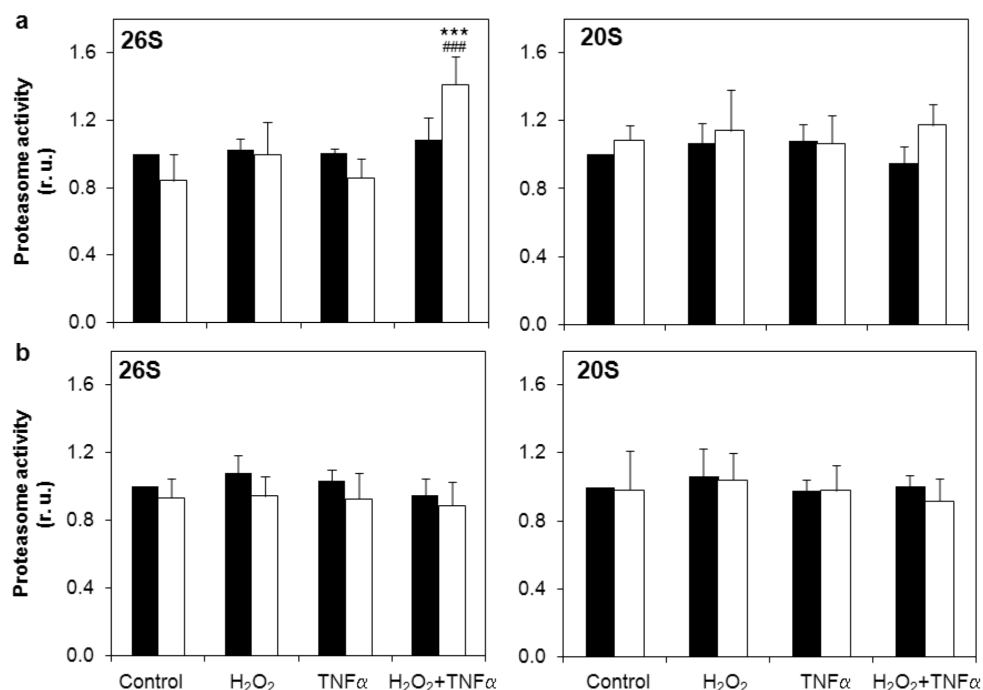

Figure 3 – H_2O_2 stimulates proteasome activity in MCF-7 cells. (a) MCF-7 and **(b)** HeLa cells were treated with 25 μM H_2O_2 s.s., 0.37 ng mL^{-1} TNF-α and H_2O_2 s.s.+ TNF-α and the chymotryptic activity of the 26S (left panels) and 20S (right panels) proteasome particles was measured. Protein extraction was performed in the presence (■) and in the absence (□) of DTT. Proteasome activity is normalized to control cells extracted with DTT. ****P*<0.001 vs. respective control, $^{###}P$<0.001 vs. same treatment with DTT.

For these activity assays two different extraction buffers were used, one containing the reducing agent dithiothreitol (DTT) and the other without DTT, because a reversible oxidative modification of the proteasome by H_2O_2 could be masked by the action of DTT. A slight decrease in the 26S proteasome activity was observed in control cells extracted without DTT, which is more evident in MCF-7 cells (Figure 3a). Importantly, there was a significant enhancement of the 26S proteasome activity in MCF-7 cells treated simultaneously with H_2O_2 and TNF-α ($P<0.001$, vs. control without DTT). H_2O_2 by itself induced a slight increase in proteasomal activity, but no effect was induced by TNF-α alone which indicate that H_2O_2 is only able to oxidatively modify a component of the 26S proteasome in the presence of TNF-α. This modification is reversible, since the addition of DTT in the extraction buffer abolished this effect. The 26S proteasome is composed by the catalytic core 20S plus other regulatory subunits, such as the 19S complex, the 22S regulator and the PA700 proteasome activator[39]. The 20S proteasome is not active by itself in the living cells and it is not able to degrade polyubiquitinylated proteins, such as IκB-α[20], which means that studying the activity of the 20S proteasome is not a physiologic approach. Even though, the results obtained also indicate that the effects of H_2O_2 rely on a regulatory component of the

26S particle, rather than on the catalytic core 20S, because the activity of the 20S particle was not changed by the H_2O_2 plus TNF-α treatment. In HeLa cells no differences were observed in proteasomal activity for the different treatments (Figure 3b).

Together, these results indicate that the proteasome activity partially explains the effect of H_2O_2 on TNF-α-treated cells. In fact, in MCF-7 cells the reason why IκB-α levels remain lower in the presence of H_2O_2 when compared to HeLa cells, is probably because of the higher proteasome activity. The outcome of this higher activity is to enable a higher translocation of p65 and c-Rel dimers to the nucleus.

4.5 The proteasome inhibitor MG132 decreases NF-κB activation in MCF-7 cells

To confirm the results obtained for the proteasome activity in MCF-7 cells, the proteasome was inhibited by a short pre-incubation with MG132 followed by exposure to the usual agents. If the stimulatory action of H_2O_2 in TNF-α-induced NF-κB activation is dependent on a higher proteasome activity, then it should be expected that stimulation by H_2O_2 would be abolished in the presence of the proteasome inhibitor MG132 (Figure 4).

As it can be seen in Figure 4a and 4b, MG132 completely abolished the increase in p65 and c-Rel levels, respectively, caused by H_2O_2. Concerning IκB-α (Figure 4c) and P-IκB-α (Figure 4d) MG132 abolished almost completely the stimulatory action of H_2O_2. The ratio between the levels of IκB-α in TNF-α-treated cells and TNF-α + H_2O_2-treated cells decreased from 2.7 to 1.3, which difference was no longer significant. For P-IκB-α levels, this ratio decreased from 3.4 to 1.2.

Overall, from these results a major role for the proteasome activation as the mechanism by which H_2O_2 stimulates NF-κB activation becomes apparent. It is not clear why there is still a residual synergism on IκB-α and P-IκB-α. It might be due to a slight increase of constitutive degradation of free IκB-α, which is not only dependent on the proteasome. In fact, the turnover IκB-α is dependent on phosphorylation by casein kinase II (CKII), which was shown to be up-regulated by 300 μM H_2O_2 in a mouse lymphoma cell line[40,41].

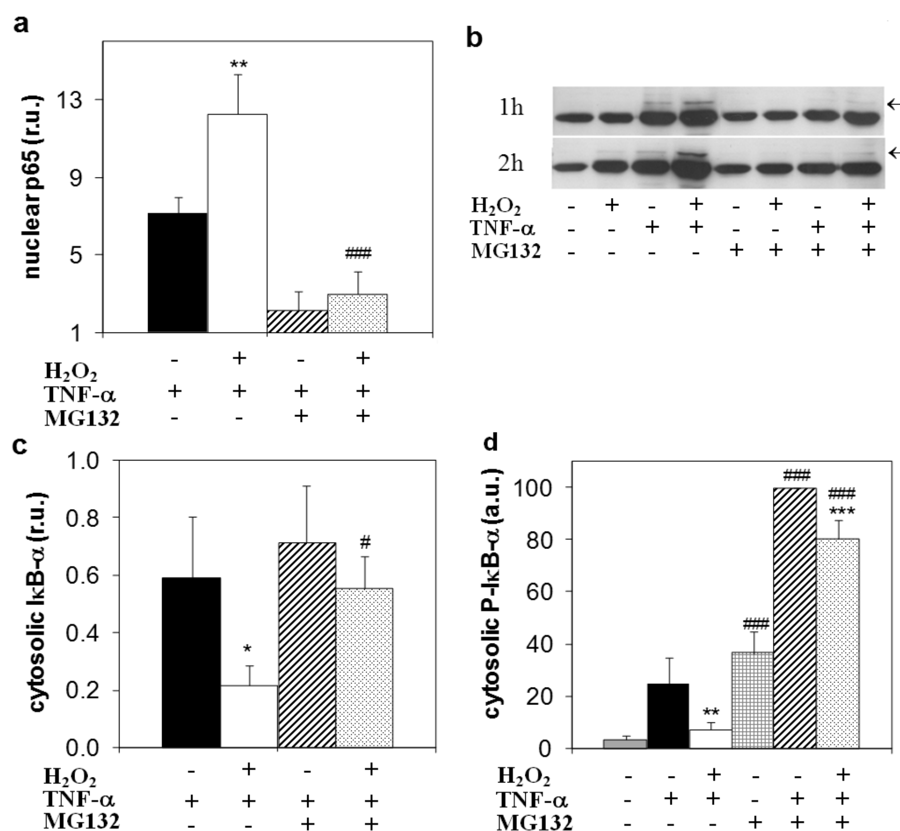

Figure 4 – IκB-α degradation and Rel proteins translocation to the nucleus stimulated by H_2O_2 is impaired by proteasome inhibition. (**a**) Nuclear p65 and (**b**) c-Rel, (**c**) Cytosolic IκB-α and (**d**) P-IκB-α levels were determined in TNF-α (■) and H_2O_2 + TNF-α (□) exposed-MCF-7 cells for 2 h and compared with the simultaneous addition of MG132 (◪ and ⊡, respectively). (**b**) Representative immunoblot for c-Rel variations indicated with an arrow. It was not possible to quantify c-Rel signal because of the contamination of p65 signal (lower and higher intensity band) and the low intensity for samples treated with MG132. In (**d**) control levels of P-IκB-α (▣) are also presented because the inhibitor MG132 significantly increased the basal levels of IκB-α phosphorylation (⊞). Therefore, data is presented as arbitrary units relative to the higher signal intensity band. $^{**}P<0.01$and $^{***}P<0.001$ for H_2O_2 +TNF-α vs. TNF-α; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ for treatment in the absence of MG132 vs. respective treatment in the presence of MG132.

5. Discussion

The role of H_2O_2 in the NF-κB pathway has been widely studied. It was first described as the common messenger of any NF-κB-inducer, but nowadays a modulatory role is the most accepted paradigm. During inflammation H_2O_2 is produced and, together with pro-inflammatory cytokines, may reach neighboring cells where it exerts signaling roles. Using a moderate dose of H_2O_2 s.s. (25 μM), delivered as to mimic *in vivo* H_2O_2 formation, we previously described an increased translocation of the subunit p65, which consequently lead to a specific up-regulation of NF-κB-dependent genes[3]. Here we aimed to elucidate which targets of H_2O_2 lead to this NF-κB stimulation. By treating cells with TNF-α it was found that the levels of both IκB-α and IκB-β decreased, although the latter at a lower extent, and that the simultaneous addition of TNF-α and H_2O_2 leads to a higher degradation of only IκB-

α. Also, it was found that IκB-α reaches higher degradation levels than IκB-β. Chen *et al.* identified a pool of IκB-β bound to NF-κB dimers and another one bound to a κB-Ras protein, which masks the signal-induced phosphorylation serines, avoiding an exacerbated degradation of IκB-β[42]. So, it should be expected that only specific inducers can liberate the IκB-β/NF-κB heterodimer from the κB-Ras and consequently lead to a total IκB-β degradation. Therefore, H_2O_2 is probably not involved in this specific degradation of IκB-β.

Besides forming complexes with p65, IκB proteins can also form complexes with c-Rel dimers and thus c-Rel nuclear levels were also monitored. A significant synergism between H_2O_2 and TNF-α in MCF-7 cells, and an antagonism at short incubation times in HeLa cells, was obtained. These results may suggest the existence of different proportions of NF-κB/IκB complexes in different cell types, indicating also that c-Rel dimers may bind chiefly to IκB-α in MCF-7 cells, and to IκB-ε in HeLa cells. This differential regulation of c-Rel may have consequences in the c-Rel-dependent gene expression. c-Rel can complex with p65, p50 and with itself, and binds preferentially to different κB sites than the ones that p65 binds to. For example, the canonical κB consensus sequence for p50/p65 is the following: GGGRNNYYCC (R for purine, Y for pyrimidine and N for any base), while the sequence for the heterodimer c-Rel/p65 is HGGARNYYCC (H for not G)[43]. The human chromosome 22 contains 35% of p65 canonical κB sites, but only 6% c-Rel/p65 κB sequences[44]. In our previous work[3], it was found that H_2O_2 up-regulated the level of several NF-κB-dependent genes such as the intracellular adhesion molecule-1 (ICAM-1)[45], IL-8[46], MCP-1[47]. These genes are known to be regulated by both p65 and c-Rel dimers, including c-Rel/p65 heterodimers. So, this suggests that the higher levels of expression for these genes reached in MCF-7 cells when compared with HeLa cells, occur because in MCF-7 cells both p65 and c-Rel levels are increased.

The sustained low levels of IκB-α that occur in the presence of H_2O_2 plus TNF-α are responsible for the stimulatory effect on p65 and c-Rel translocation observed in MCF-7 cells. In these cells, it was possible to show that H_2O_2 is not affecting IκB-α *de novo* synthesis and that the stimulation of NF-κB activation was a consequence of an increased activity of the 26S proteasome, only significant for the simultaneous addition of H_2O_2 plus TNF-α. This response was observed for the first time and clearly indicates a cooperation between H_2O_2 and TNF-α in the activation of NF-κB. The proteasome degrades the majority of the proteins, especially abnormal proteins, and thus this increased activity can also

contribute for cell survival and for the anti-apoptotic role attributed to NF-κB[9]. Interestingly, Thomas *et al.* described a bell-shaped response of the proteasome activity with increasing concentrations of H_2O_2[38]. They concluded that H_2O_2 could lead the endothelial cells into a pro-survival pathway through an increase in the proteasome activity caused by low H_2O_2 concentrations, or into a pro-apoptotic pathway, induced by a decrease of proteasomal activity for higher H_2O_2 concentrations[38].

Other mechanisms may also contribute for the synergism between H_2O_2 and TNF-α on NF-κB activation. The IKK complex is composed by the proteins IKKα, IKKβ and IKKγ, the latter with regulatory properties. It was recently reported that IKKγ forms dimers through the formation of disulfide bonds, dependent on Cys54 and Cys347 residues, and that these dimers are required for the correct activation of NF-κB. H_2O_2 was able to induce IKKγ dimerization, but a pre-treatment with 200 μM H_2O_2 before the addition of TNF-α prevented this activation of IKK, probably by interfering with the IKKβ subunit[48]. Thus, an increased activity of the IKK might explain the increased translocation of p65. Enesa *et al.*[49]recently reported that H_2O_2 stimulation of NF-κB transcription activation in IL-1-treated cells was modulated, in part, by suppression of p65 exportation. This suppression was caused both by the inhibition of the NF-κB-pathway down-regulator cezanne, which is a new inhibitor similar to A20, and by an enhanced degradation of the newly synthesized IκB-α. These mechanisms of NF-κB regulation may account for the H_2O_2 synergistic effect in TNF-α-treated HeLa cells, given that IκB-α is probably responsible for the increased translocation of p65 and that in these cells the proteasome activity was not increased by H_2O_2.

In this study, we were able to clarify some important issues for H_2O_2 modulation of the NF-κB pathway. H_2O_2 is a controversial regulator of the NF-κB pathway because the results are highly dependent on the concentration and time of exposure used, even for the same cell line. H_2O_2 is a mild oxidant, with highly controlled intracellular levels, but the effective H_2O_2 concentration may vary from cell to cell. The addition of an exogenous dose of H_2O_2 results in a H_2O_2 gradient across the plasma membrane of about two in MCF-7 cells[3] and seven in HeLa cells, in great part because of the high GPx activity in HeLa cells. Therefore, when adding an extracellular concentration of 25 μM H_2O_2 s.s, MCF-7 cells are estimated to have a higher intracellular concentration of H_2O_2 (approximately 12.5 μM) than HeLa cells (approximately 3.7 μM). Exposing HeLa cells to a higher extracellular concentration of H_2O_2 in an attempt to obtain a similar intracellular concentration in both cell types used and then

test if the proteasome activity increases is not feasible because it will damage HeLa cells. In fact, an increase in the rate of H_2O_2 production of approximately 3.4 would be needed, which will exert an increase of 3.4 times in the intracellular consumption of H_2O_2. A concomitant formation of oxidized glutathione (GSSG) would increase because in HeLa cells H_2O_2 consumption is mostly done by GPx, affecting the intracellular redox balance and thus inducing oxidative stress.

In conclusion, our data clearly show that H_2O_2 is an important physiological modulator of the NF-κB pathway, with specific cell-type actions that potentially cause a more effective inflammatory response. However, at high doses, H_2O_2 can lead to oxidative stress, which is linked with abnormal NF-κB activation and, therefore, with cancer and inflammatory diseases. A better knowledge of H_2O_2 metabolism in each cell type present in different organs is probably a good approach for specific therapies involving NF-κB-targeting.

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Chapter IV

Modulation of NF-κB-dependent gene expression by H_2O_2: a major role for a simple chemical process in a complex biological response‡

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1. Abstract

We have recently observed that H_2O_2 regulates inflammation via up-expression of a few NF-κB-dependent genes, while leaving expression of most NF-κB-dependent genes unaltered. Here we test the hypothesis that this differential gene expression is dependent on the affinity of κB sites in the gene promoter regions towards NF-κB. Accordingly, in cells transfected with three reporter plasmids containing κB sequences with different affinities for NF-κB, it was shown that the lower the affinity the higher the range of TNF-α concentrations where H_2O_2 up-regulated gene expression. Mathematical models reproduced the key experimental observations indicating that H_2O_2-up-regulation ceased when NF-κB fully occupied the κB sites. *In vivo*, it is predicted that genes with high-affinity sites remain insensitive to H_2O_2, while genes with lower affinity sites are up-regulated by H_2O_2. In conclusion, a simple chemical mechanism is at the root of a complex biological process such as differential gene expression caused by H_2O_2.

2. Introduction

Hydrogen peroxide (H_2O_2), the most abundant reactive oxygen species *in vivo*, has been given a central role in cell signaling[1,2]. The tight control of intracellular H_2O_2 levels and mild oxidative properties of H_2O_2 support such a role. H_2O_2 shows specificity to its redox-sensitive targets, such as cysteine residues in proteins, which upon oxidation activate transcription factors such as OxyR in bacteria[3] and Yap1 in yeast[4]; it also modulates signaling enzymes

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such as tyrosine phosphatases, which are inhibited[5] and tyrosine kinases, which are activated[6,7]. Other transcription factors and signaling molecules that are known to be modulated by H_2O_2 include activator protein-1 (AP-1), hypoxia-inducible factor-1 (HIF-1), NF-E2-related factor-2 (NRF-2) and nuclear factor κB (NF-κB)[8], but the precise molecular oxidative events are not known.

In addition to these global actions on the cellular signaling network, H_2O_2 is able to selectively modulate the expression of specific individual genes, leaving the expression of other genes unchanged, even when they share common transcription factors. For example, we have recently observed that a low dose of H_2O_2 only modulates the activation of an handful set of genes among 100 genes whose expression is dependent on NF-κB[9]. The molecular basis for this selectivity constitutes the topic of this work and we will use the modulation of NF-κB-dependent genes activated by the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) as a test model. NF-κB is a transcription factor with a key regulatory role in inflammation, adaptive immune response and apoptosis. The prototypical NF-κB is a heterodimer composed by two proteins, the p65 and p50 subunits, which remains inactive in the cytosol bound to its inhibitory proteins, the IκBs. After a specific signal, such as the binding of TNF-α to its cell receptors, IκBs are phosphorylated by the IκB kinase (IKK) complex and subsequently degraded by the 26S proteasome. Free NF-κB migrates to the nucleus in order to activate the target genes. The biological role of H_2O_2 as a regulator of NF-κB activation is biphasic since, in HeLa and MCF-7 cells, NF-κB translocation into the nucleus is inhibited by high levels of H_2O_2 (mM range), while low doses of H_2O_2 (μM range) similar to those found *in vivo*, stimulate this translocation[9]. Once inside the nucleus, NF-κB binds to the promoter/enhancer regions of the target genes[10], the κB sites, which have a general consensus sequence of GGGRNNYYCC (R is purine, Y is pyrimidine and N is any base). The modulation of specific genes leaving others unchanged, even upon an increase in the levels of nuclear NF-κB, can be achieved in a number of ways. The transactivation potential of NF-κB depends on modifications in the NF-κB subunits and DNA, such as acetylations[11] and phosphorylations[12,13] and also on the efficient recruitment of all the transcriptional machinery to form the enhanceosome, which was shown recently to be a dynamic binding-release process[14]. All these processes depend on co-activators, such as histone acetyltransferases (HATs) and co-repressors, such as histone deacetylases (HDACs), whose recruitment is essential to coordinate transcription[15]. Leung *et al.* showed that even the

conformation of the complex (NF-κB|κB), which can be changed by only one nucleotide in the κB site, determines the recruitment of the essential co-activators, controlling the initiation of transcription[16]. All together, transcription is a tightly regulated and complex event and each of these processes could be selectively targeted by H_2O_2 causing the up-regulation of some genes but not others. However, ultimately, the interaction of NF-κB with κB binding sites is determined by the equilibrium between free NF-κB, free κB sites and the complex (NF-κB|κB), and can be regarded as a simple chemical process. We hypothesized that for each κB binding site (a) H_2O_2 is able to shift this equilibrium towards a higher degree of κB site occupancy, by increasing NF-κB translocation into the cell nucleus, and (b) the magnitude of this shift is dependent on the affinity of the κB binding site to NF-κB. It is important to note that a single modification of a nucleotide within a κB site can change abruptly its affinity towards NF-κB[17]. According to our hypothesis, genes containing low or medium affinity κB sites should be positively modulated by H_2O_2, while genes with high affinity κB sites should be mostly insensitive to H_2O_2, since occupancy of their κB regions is already maximal in the presence of low TNF-α levels. To test these predictions, HeLa cells were transfected with plasmids coupled to a luciferase reporter gene containing κB sequences with three different affinities. After transfection, cells were exposed to TNF-α in the absence or presence of a controlled steady-state (s.s.) concentration of H_2O_2 to check whether the profile of luciferase expression in the presence of H_2O_2 was dependent on the affinity of the κB sequence towards NF-κB. Results obtained were successfully simulated by two kinetic mathematic models based on simple chemical equilibriums reactions.

3. Materials and Methods

Cell culture and reagents

HeLa cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 medium supplemented with 10% of fetal bovine serum, penicillin 100 UmL^{-1}, streptomycin 100 μg mL^{-1} and L-glutamine 2 mM, all from Lonza, Basel, Switzerland. Glucose oxidase (*Aspergillus Niger*), tumor necrosis factor-α (TNF-α) (human recombinant) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). H_2O_2 was obtained from Merck & Co.,

Inc. (Whitehouse Station, NJ, USA). All plasmid reporter vectors were kindly provided by Dr. Helena Soares (Instituto Gulbenkian de Ciência, Portugal).

Cells incubations

HeLa cells were counted and plated approximately 46 h before the experiment. Fresh medium was added to the cells 1 h before the incubations. H_2O_2 exposure was performed using the steady-state titration[18], the method is extensively explained in[9]. Briefly, a steady-state level of H_2O_2 is maintained during the entire assay by adding, simultaneously with an initial dose of H_2O_2, a quantity of glucose oxidase enough to counteract H_2O_2 consumption by cells. All experiments were performed with an initial concentration of 25 μM, ending at approximately 21 μM. TNF-α was used at a range of 0.036 – 75 ng mL^{-1}.

Protein extraction and immnunoblot analysis

HeLa cells were plated onto 100-mm dishes to achieve 1.5×10^6 cells per dish at the day of experiment. Preparation of nuclear extracts and immunoblot assays were performed as described previously [9]. p65 analysis was done with antibodies sc-372 and sc-109 (Santa Cruz Biotechnology, Santa Cruz, California, USA).

Plasmid constructs

The κB reporter plasmids were generated by inserting a minimal promoter in the pGL3-basic vector (Promega, Madison, WI, USA) with BglII (5'end) and HindIII (3'end) restriction enzymes (New England Biolabs, Ipswich, England): 5'– GATCTGGGTATATAATGGATCCCCGGGTACGCAGCTCA – 3'. Three different κB sequences, which were chosen based on the work of Udalova *et.al.*[17], were inserted upstream the minimal promoter, between the KpnI/SacI restriction site, with the following general sequence: 5' – GCT-**κB**-CTGGCTCCT-**κB**-CTCAGCT –3' (Table 1).

Table 1 – κB sequences inserted in the plasmid construct

Plasmid	Affinity	κB sequence (5'-3')
κBl	Low	GGGGACTTCC
κBm	Medium	GGGGATTCCC
κBh	high	GGGAATTTCC

Reporter gene assays

Cells were distributed onto 24-well plates at a density of 4.5×10^4 cells/well. After 24 h, the medium was replaced to RPMI 1640 without antibiotics and the cells were transfected with 18 μL of Opti-MEM containing a total of 360 ng of DNA (180 ng of κB experimental plasmid, 9 ng of pRL-SV40 control plasmid and 171 ng of pGL3-basic plasmid) and 0.9 μL of fugeneHD (Roche, Mannheim, Germany). Cells recovered for 24 h and then were exposed to different conditions for 4 h. Lysis and luciferase analysis was assayed using the Dual-Luciferase Reporter Assay Sytem (Promega, Madison, WI, USA), accordingly to manufacturer instructions. Luminescence was read with the luminometer Zenyth 3100 with 1 s of integration time, one sample at a time because of the rapid decreased of the renilla signal. Each sample was read in triplicate.

Viability assays

Cells were distributed in 96-well plates at a density of 7500 cells/well. The procedure used for transfection was the same as described before, adding 3 μL of the complex prepared. After exposure to TNF-α and/or H_2O_2 cell viability was assessed by the ability of cells to reduce MTT reagent[19]. Briefly, the medium was changed and cells were incubated for 2 h with MTT (0.5 mg mL^{-1} in PBS). Reduced MTT was dissolved using dimethyl sulfoxide (DMSO) and the absorbance was read at 570 nm with reference at 630 nm in a microplate reader (Tecan Sunrise).

Mathematical modeling

A simple model formed only by the association-dissociation between free NF-κB and κB regions was set up. Equation 1, which describes this chemical equilibrium, was solved in order to (NF-κB|κB) yielding Equation 2.

$$K_{eq} = \frac{[(NF-\kappa B \mid \kappa B)]}{[NF-\kappa B_{free}][\kappa B_{free}]}$$

Equation (1)

$$[(NF-\kappa B \mid \kappa B)] = \frac{1 + K_{eq}[\kappa B_{tot}] + K_{eq}[NF-\kappa B_{tot}] - \sqrt{-4[\kappa B_{tot}][NF-\kappa B_{tot}]K_{eq}^{2} + (1 + K_{eq}[\kappa B_{tot}] + K_{eq}[NF-\kappa B_{tot}])^{2}}}{2K_{eq}}$$

Equation (2)

(NF-κB|κB) represents the complex formed and ultimately is proportional to the activation of the reporter gene (Equation 2); $[NF\text{-}\kappa B_{free}] = [NF\text{-}\kappa B_{tot}] - [(NF\text{-}\kappa B|\kappa B)]$, $[\kappa B_{free}] = [\kappa B_{tot}] - [(NF\text{-}\kappa B|\kappa B)]$ and K_{eq} is the equilibrium constant. We assumed a κB_{tot} concentration of 10^{-3} μM and three K_{eq} of 20, 200 and 2000 μM^{-1} to represent the low, medium and high affinity sequences, respectively. The total nuclear NF-κB concentration was assumed in the range of 10^{-4} to approximately 4 μM.

The extended model was developed to introduce competition between the endogenous κB and plasmidic κB sites for a co-activator, cooperativity in the binding of NF-κB to κB sites, and endogenous luciferase expression in non-treated cells. Therefore, to represent the endogenous DNA, 100 hypothetical κB sites were set up in the model with the same rate constant for the first binding of NF-κB to the promoter region - 24 $\mu M^{-1}s^{-1}$ - and a ten-times increase for the second binding to the promoter region, to simulate cooperative binding. The dissociation rate constant used for the higher affinity κB sequence was $2.0\times10^{-2}\ s^{-1}$. The other 99 κB sites were set up with a dissociation rate constant (k_d) according to the following geometric sequence: k_d for $\kappa Bsite_{n+1}$= (k_d for $\kappa Bsite_n$)$\times10^{(1/25)}$ (with n=1,…, 100 and for n=1, $k_d=2.0\times10^{-2}\ s^{-1}$). The competition was restricted to those endogenous κB sites that have a similar order of magnitude affinity to the plasmidic κB region: for the κBh, κBm, κBl regions, the endogenous regions considered were regions 11-36, 30-55, and 50-75, respectively. The dissociation constants for the low, medium and high affinity sites in the plasmids were 1.8, 0.3 and 0.05 s^{-1}, respectively. The co-activator had a higher affinity to endogenous κB sites, with a association constant of $2.4\times10^{4}\ \mu M^{-1}s^{-1}$, while the constant for plasmid DNA was 24 $\mu M^{-1}s^{-1}$, with the same dissociation constant (2 s^{-1}). It was assumed that each of the plasmidic κB concentrations was 5×10^{-5} μM, the co-activator concentration was 5×10^{-4} μM and the nuclear NF-κB concentration was 1×10^{-4} μM. Endogenous luciferase expression was modeled assuming (NF-κB|κB|co-activator) concentrations of 1.0×10^{-11} μM, 4.8×10^{-10} μM, and 1.2×10^{-9} μM for the κBl, κBm, κBh regions, respectively. This model could not be solved analytically, instead we applied numerical simulation using the PLAS software[20]. The description of the mathematical model can be found in Supplementary Tables 1, 2 and 3.

Statistical analysis

All data is presented as the average ± standard deviation.

4. Results

4.1 H_2O_2 modulates NF-κB translocation by TNF-α

Previously, we have observed that H_2O_2 by itself is a very weak NF-κB activator, when compared with the classical NF-κB activator TNF-α. Nevertheless, H_2O_2 is able to modulate NF-κB by stimulating the activation caused by TNF-α, provided H_2O_2 delivery to cells mimics *in vivo* conditions. This observation was obtained for a single dose of TNF-α. Here these studies were extended by characterizing the action of H_2O_2 for a wide range of TNF-α concentrations, including physiological levels of TNF-α (0.2-1.5 ng mL^{-1})[21] and also doses that are much higher, but that are typically used in experimental settings (e.g. 10 ng mL^{-1})[22-25]. Figure 1B shows that NF-κB activation by TNF-α had an hyperbolic dependence on TNF-α concentration with an half-maximal response obtained with a TNF-α concentration of 0.324±0.126 ng mL^{-1} and of 0.206±0.092 ng mL^{-1} in the presence of 25 μM H_2O_2. The maximal NF-κB translocation (NF-$κB_{max}$) induced by TNF-α (6.59±0.475 a.u) was increased by H_2O_2 (7.49±0.582 a.u, P=0.054, n=4). The stimulatory effect of H_2O_2 on TNF-α-dependent NF-κB activation was more pronounced for low TNF-α concentrations (Figure 1C). Altogether, these data indicate that H_2O_2, at concentrations that can be found *in vivo* in an inflammatory environment[26,27], is a positive modulator of NF-κB activation by TNF-α, particularly for physiological TNF-α concentrations. Therefore, H_2O_2 is expected to have a regulatory role in NF-κB activation during the inflammatory response, *in vivo*.

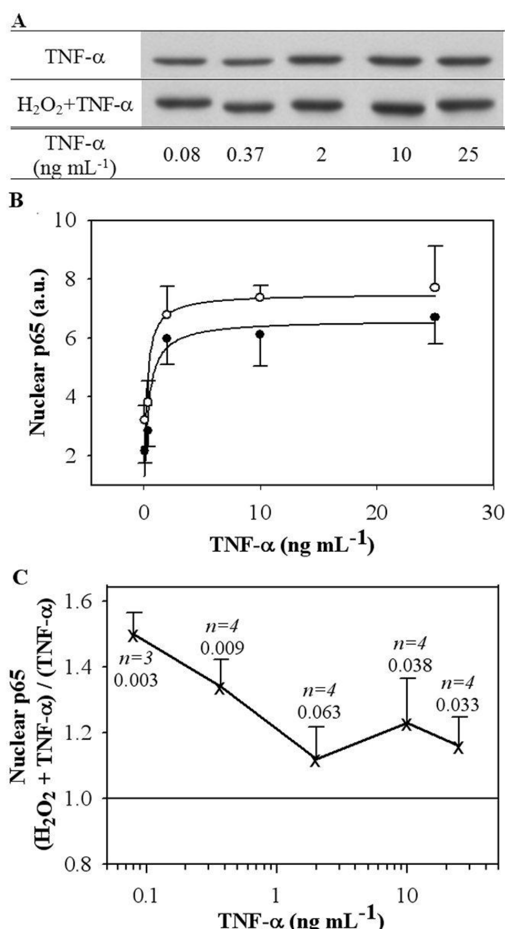

Figure 1 – H_2O_2 increases the translocation of NF-κB to the nucleus, in the presence of TNF-α. Hela cells were treated with the indicated TNF-α concentrations alone or simultaneously with 25 μM H_2O_2 delivery as a steady-state **(A)** Representative western blot against nuclear p65. **(B)** Quantification of western blots of cells exposed to TNF-α (●) or 25 μM H_2O_{2ss} simultaneously with TNF-α (○). **(C)** Ratio of the p65 measured in cells incubated with both TNF-α and H_2O_2 over the p65 measured in cells incubated only with TNF-α. Data passed the Kolmogorov-Smirnov normality test and was compared with 1 (no modulation by H_2O_2) using the one-tailed student's t-test, except for the TNF-α concentration of 2 ng mL^{-1}. In this latter case the non-parametric sign test was used instead. *P* and n values are indicated.

4.2 Effects of transfection: viability and NF-κB activation

The doses of H_2O_2 and TNF-α used in this work did not damage HeLa cells (not shown). Conditions used for transfection in subsequent studies were also analyzed to check for possible interferences. As shown in Figure 2, about 50% of the cells died after transfection, but the addition of 25 μM H_2O_2 in s.s., alone or simultaneously with either 10 or 50 ng mL^{-1} of TNF-α, did not lead to a loss of viability when compared with non-treated cells (control). It was also checked whether transfection could alter NF-κB activation. Transfection had a slight effect on the basal levels of NF-κB activation, but did not change NF-κB activation in the presence of H_2O_2 and/or TNF-α (not shown).

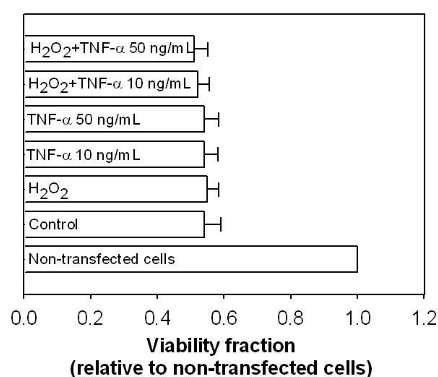

Figure 2 – Viability of transfected cells is not affected by H_2O_2 and TNF-α treatments. Viability was measured by the MTT assay both in non-transfected cells and transfected cells. Viability of transfected cells exposed to 25 μM H_2O_{2ss} with or without 10 and 50 ng mL^{-1} TNF-α is also shown.

4.3 The transcriptional activity of the reporter gene is dependent on its κB sites

To test our working hypothesis that the modulatory effect of H_2O_2 on the activation of NF-κB-dependent genes by TNF-α is dependent on the affinity of NF-κB towards the κB sites in the promoter region, HeLa cells were transfected with three plasmids coupled to a luciferase reporter gene with different κB sequences. The choice for the κB sequences was based on the data provided by Udalova *et al.* who showed that minor changes in the κB region can have a large impact on the affinity of NF-κB towards the κB region[17]. In spite of just two nucleotide changes in the κB region of the plasmids used (Table 1), the levels of luciferase activity measured for each plasmid showed large differences (Figure 3). Taking in account that all other factors were maintained constant, and that the plasmids had similar transfection efficiencies, the differences obtained in luciferase activity were probably a consequence of the apparent affinity of the NF-κB toward that particular κB site.

The three reporter plasmids were named according to their apparent affinity: κBh, κBm, and κBl for the highest, medium and lowest affinity plasmids, respectively (Table 1 and Figure 3). Since by enhancing TNF-α concentration, there was an increase in the nuclear levels of NF-κB (Figure 1B), the binding of the free κB sites is also expected to increase with TNF-α concentration. The final expected outcome should be a higher luciferase expression, until saturation. The luciferase expression of the constructs κBm and κBh fitted a hyperbolic curve, with similar half-maximal responses (0.390±0.112 ng mL^{-1} and 0.349±0.035 ng mL^{-1}, respectively), which suggests that the saturation observed was not due to binding to a κB region, but to a process occurring at an earlier stage. Moreover, these half-maximal responses were close to those obtained for the p65 nuclear translocation in Figure 1B, indicating that there is a limiting step before translocation, possibly at the level of either the TNF-α receptor, IKK activation or proteasome activity. The maximal level of luciferase was 0.0653±0.004

a.u. for the κBm construct and 0.476±0.011 a.u. for the κBh construct, and so the apparent affinity of κBh was seven times higher than that of κBm. Concerning the κBl sequence, a similar hyperbolic behavior was observed, but the error associated with the measurement of low levels of luciferase was too high to allow a meaningful fitting. Even so, by using the maximal value of luciferase activity it could be inferred that the apparent affinity of this κB sequence towards NF-κB was approximately six times lower than that of the κBm sequence. These results confirm that changing just two nucleotides within the κB site can originate different transcriptional activities.

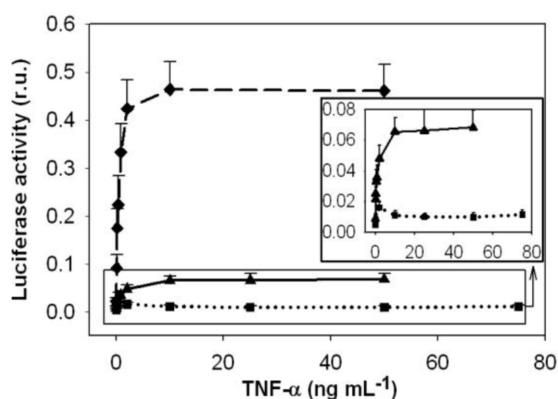

Figure 3 – Luciferase activity is dependent on the κB reporter plasmid. Cells transfected with κBl (■), κBm (▲) and κBh (◆) reporter plasmids were exposed to increasing concentrations of TNF-α in the range of 0.016 - 75 ng mL^{-1} for 4 h.

4.4 H_2O_2 modulates the transcriptional activity of the reporter genes

By assuming that the binding of NF-κB to the free κB sites follows a dynamic equilibrium, it should be expected that an increase of nuclear p65 stimulated by the presence of H_2O_2, in TNF-α-treated cells (Figure 1), would favor complex formation between free NF-κB and free κB sites, therefore increasing luciferase expression. H_2O_2 alone did not induce luciferase expression (not shown), which is in agreement with the negligible NF-κB translocation observed in the presence of H_2O_2. As expected, when added simultaneously with TNF-α, H_2O_2 induced synergistic effects on luciferase expression but, surprisingly, it also induced antagonistic effects (Figure 4). These opposing effects were dependent on the TNF-α concentration used, with synergistic effects being observed in a wide range of low TNF-α concentrations and antagonistic effects observed in a narrow range of high TNF-α concentrations. For the low-affinity κBl construct and a TNF-α concentration up to 25 ng mL^{-1}, H_2O_2 stimulated significantly luciferase expression. This upper limit in the range of TNF-α concentrations where synergistic effects by H_2O_2 occur, decreased to 0.83 ng mL^{-1} for the medium-affinity κBm construct, and to 0.08 ng mL^{-1} for the high-affinity κBh construct.

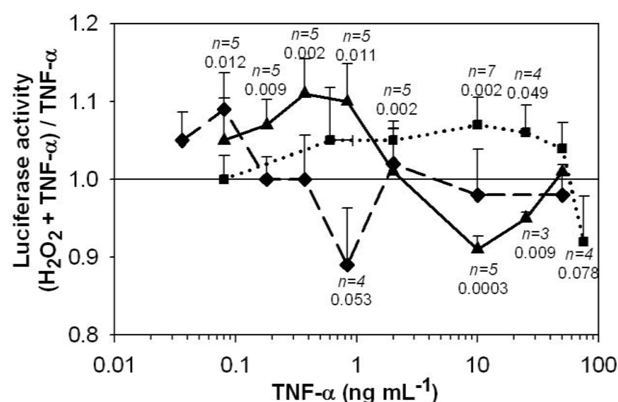

Figure 4 – H_2O_2 modulation of reporter plasmid expression is dependent both on the TNF-α concentration and the κB sequence on the gene. Cells transfected with κBl (■), κBm (▲) and κBh (◆) constructs were exposed to TNF-α concentrations in the range of 0.016 - 75 ng mL^{-1} with or without of 25 μM H_2O_{2ss} for 4 h. The ratio of the luciferase activity measured in presence and absence of H_2O_2 is shown. Experimental data passed the Kolmogorov-Smirnov normality test and was compared with 1 (no modulation by H_2O_2) using the two-tailed student's t-test. n and *P* values are indicated for data at a significance level below 10%.

An unexpected observation was that for all concentrations of TNF-α studied, maximum synergistic effects in luciferase expression was around 10 %, while H_2O_2 synergistic stimulation of translocation was around 50 % for physiological doses of TNF-α (Figure 1C). Since H_2O_2 is involved in the activation of other transcription factors, such as AP-1[28], that are dependent on the same co-activators, one possible explanation may rely on a competition with other transcription factors for the same co-activators and co-repressors. Another possible explanation is based on the activation by H_2O_2 of the early growth response protein-1 (Egr-1). Egr-1 has a zinc finger domain which interacts with the p65 subunit and, consequently, by inhibiting its transcriptional activity decreases NF-κB-dependent gene expression[29].

To understand these results, a minimal model of NF-κB binding to κB sites based on a simple equilibrium between free NF-κB and the κB sites in the promoter region of the target genes was used to simulate the experimental observations. With this simple model, it was possible to reproduce in part the experimental results, since a positive modulation by H_2O_2 for (NF-κB|κB) formation was obtained in the range of NF-κB concentrations considered in the model. This positive modulation, occurred within a wider interval of NF-κB concentrations for the low-affinity κBl sequence than for the high-affinity κBh sequence (Figure 5A). These results obtained with the model can be understood considering that when low concentrations of NF-κB are present in the nucleus (i.e. when low concentrations of TNF-α are applied to the cell), the rise of nuclear NF-κB induced by H_2O_2 causes an increase in the complex formation. Since κB sites are still available for binding, H_2O_2 has a synergistic effect. When the nuclear NF-κB concentration increases, the occupancy of κB sites also increases until they are fully occupied by NF-κB. At this point, even though there is a higher nuclear NF-κB level induced by H_2O_2, H_2O_2 does not have an effect on the occupancy level of κB sites,

because the occupancy is already maximal. For higher affinity κB sites, the formation of the complex is favored, which means that κB sites become totally occupied at a lower NF-κB concentration. Thus, the higher the affinity of NF-κB towards a κB site, the lower the concentration of NF-κB (or TNF-α) at which H_2O_2 stops having a synergistic action.

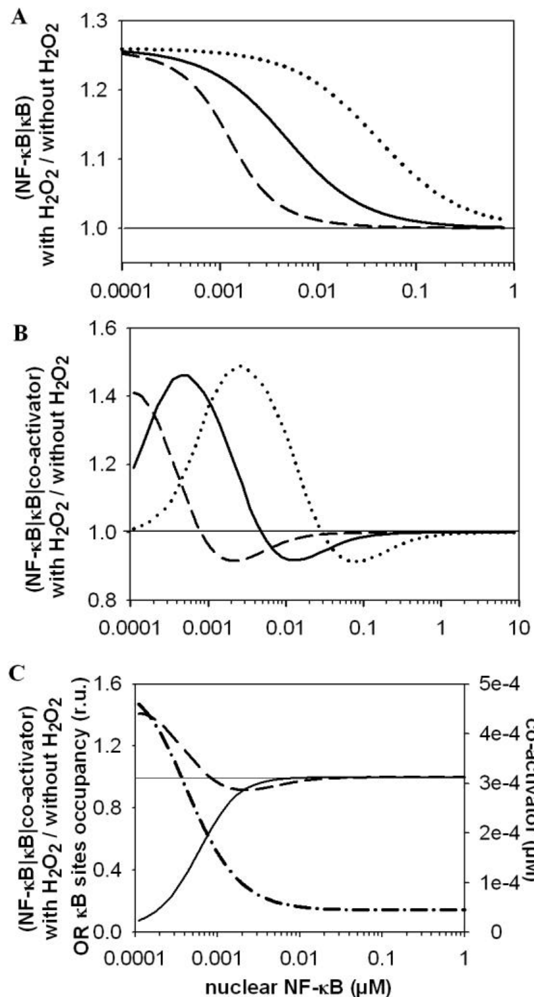

Figure 5 – Mathematical modeling predicts a differential modulation of gene expression by H_2O_2 based on the affinity constants of the κB regions towards NF-κB. **(A)** The simple model in which the concentration of the complex (NF-κB|κB) is given by Equation 2 was used; the ratio of this complex, with and without H_2O_2, is plotted as a function of the NF-κB concentration, for three different κB sites (κBl - dotted line; κBm - solid line; κBh - dashed line). The effect of H_2O_2 was modeled by assuming a 26 % increase of the nuclear NF-κB concentration. **(B)** As in (A) but the extended model with cooperativity in the binding of NF-κB between two κB sites within the same gene, competition for a co-activator and basal luciferase expression was used. The ratio between the (NF-κB|κB|co-activator) complex with and without H_2O_2 is plotted. **(C)** In addition to this ratio (dashed line), the concentration of the co-activator (dashed-dotted line) and the occupancy of the κB sites (solid line) for the κBh case are represented. For details see materials and methods.

The minimal model was extended since it was not able to reproduce three important experimental features, (1) the antagonistic effect of H_2O_2 on the transcriptional activity of the reporter genes observed experimentally at higher TNF-α concentrations (Figure 4). *A priori*, such behavior could be explained by a competition between different κB sites of the endogenous genes and the reporter genes bound to NF-κB for a transcriptional co-activator. An example of such co-activator is the cyclic AMP response element binding proteins (CREB)-binding protein/adenoviral protein E1A (CBP/p300), indispensable for the activation of transcription by NF-κB[30]. CBP/p300 exists in limiting quantities and the competition between different promoters can result in some down-regulation of gene transactivation[31]. Therefore, κB regions simulating the endogenous genes that compete with the luciferase

reporter genes for a hypothetical co-activator were introduced in the model. (2) In the minimal model, a large change in nuclear NF-κB concentration is needed in order for the synergistic effect of H_2O_2 to disappear, as opposed to the experimental results where the transition from synergism to no effect is observed in a narrow range of TNF-α concentrations (Figure 4). Such steep transitions are often an indication of cooperativity, and it has been shown that NF-κB binding to DNA involves cooperativity, e.g. between two or more κB regions within the same gene, as observed in the IL-1β and iNOS[32,33] promoters, or by the cooperative binding of the NF-κB dimer to the κB region in the promoters. Therefore, cooperativity for NF-κB binding to κB sites was included in the model. (3) While the experimental results show that synergism induced by H_2O_2 increases for low TNF-α concentrations before decreasing, in the minimal model the synergism is constant in the low range of NF-κB concentrations. This discrepancy probably occurs both due to the lack of experimental sensitivity when measuring the very low luciferase expression obtained at very low TNF-α levels and also because in the model the existence of an endogenous luciferase expression in the absence of TNF-α was not considered. Therefore, this endogenous luciferase expression was introduced in the extended model.

With this improved model, the main discrepancies between results obtained experimentally and by the model were eliminated (Figure 5B). In addition, the model provided a possible interpretation for some of the less intuitive experimental observations. Concerning the antagonism, at a certain critical NF-κB level when almost all the κB sites of the plasmids become occupied, additional NF-κB will bind to other κB regions in the endogenous DNA with lower affinity sequences. The outcome is an increased competition for the transcriptional co-activator and consequently its displacement to the endogenous κB sites, decreasing luciferase expression – antagonism (Figure 5C). If NF-κB concentrations are further increased, the antagonism disappears, and at high NF-κB concentrations luciferase activity is not induced by H_2O_2. According to the model, this recovery is caused by the increased saturation of the endogenous κB regions, which makes the competition with the transcriptional co-activator less important. Taken together, the modulation by H_2O_2 of NF-κB-dependent genes activated by TNF-α is dependent on both the affinity of the κB region towards NF-κB and the level of TNF-α, thus providing a rationale for the selective modulation of a sub-set of genes.

5. Discussion

We provided evidence that a simple chemical equilibrium principle based on the association-dissociation between NF-κB and κB sites in the promoter region of genes may explain the selective modulation of some genes by H_2O_2, leaving other genes unchanged. This explanation has predictive power because it is based on the affinity of the promoter regions towards the transcription factor.

Based on our results several general predictions can be made concerning the role of H_2O_2 as a regulator of inflammation. During inflammation, some of the H_2O_2 generated during phagocytosis leaks from the phagosome. Therefore, cells present at inflammation sites are simultaneously subjected to H_2O_2 and other pro-inflammatory cytokines, such as TNF-α. In the present work, three NF-κB-dependent reporter genes were used to simulate three hypothetical genes with different affinities towards NF-κB to investigate the differential activation of genes. Although a broad range of TNF-α concentrations was used to study the full pattern of response, in most *in vivo* situations TNF-α levels are within the range 0.2 and 1.5 ng mL^{-1}. We predict that genes bearing high-affinity sites are probably not modulated by H_2O_2 *in vivo*, because at a 0.2 ng mL^{-1} level TNF-α will probably trigger NF-κB translocation to the nucleus at high enough levels to fully occupy high-affinity κB sites. Thus, these genes will be insensitive to further increases in NF-κB translocation caused by H_2O_2. On the other hand, genes containing medium affinity κB sites and, particularly, genes containing low affinity κB sites, are predicted to have their expression up-regulated by H_2O_2 during inflammation.

Making more concrete predictions by identifying specific genes that are expected to be up-modulated or unchanged by H_2O_2 is difficult. First, the affinity of κB regions is not uniquely defined by the nucleotide sequence in the κB region. Instead, the context of the neighboring sequence, either in the linear DNA sequence or in the tridimensional spatial organization of DNA, affects the affinity of the sequence towards NF-κB[34,35]. Thus, the κB sequence affinities obtained *in vitro* with the isolated sequence cannot be straightforwardly extrapolated to the cellular environment. Secondly, promoter or enhancer regions of most genes can have more than one κB site, including the presence of both high and low affinity κB sites in the same gene. For example, Taylor *et al.* showed that the different κB regions within the inducible nitric oxide synthase (iNOS) promoter have variable importance for the

gene expression in response to TNF-α. There is a vital κB site for iNOS expression, other three with regulatory effects and another one with no apparent importance for the overall expression. In our context, this latter κB site might be a good candidate for regulation by H_2O_2, which would probably cause an increased expression of iNOS[33].

Analysis of the affinities of κB sites towards NF-κB could also be very useful when investigating the NF-κB mis-regulations that have been implicated in the development of human diseases, especially chronic inflammatory and autoimmune diseases and cancer. Genetically inherited or somatic mutations in the NF-κB family members that can affect NF-κB activation have been identified and are at the origin of several disorders[36]. Other mutations associated with the NF-κB signaling, such as polymorphisms within κB sites of NF-κB-target genes, have been correlated with human disease. Rossouw *et al.* identified a significant association between a polymorphism within the NF-κB binding site of the interferon γ (IFN-γ) promoter and susceptibility to tuberculosis, probably because of the decreased levels of IFN-γ expression[37]. Similarly, Kang *et al.* identified one polymorphism within the NF-κB binding site of the cyclooxygenase-2 (COX-2) gene which leads to changes in COX-2 expression and have been associated with increased risks of bladder cancer[38]. Understanding the regulation by H_2O_2 of NF-κB-dependent gene expression is also important in this context because H_2O_2 is present in the inflammatory environment of NF-κB related-diseases and the resulting gene expression will be a function of both the polymorphism itself and the subsequent regulation by H_2O_2. According to our hypothesis, if a polymorphism decreases the affinity of a normally high-affinity κB site towards NF-κB, the stimulatory role of H_2O_2 will be potentiated. This could help maintain the expression level of the gene, i.e. to maintain a normal situation and to avoid the development of diseases, but it would also make gene expression dependent on oxidative stress. If the polymorphism increases the affinity of κB sites towards NF-κB, the H_2O_2 regulatory role will be attenuated or even lost, disrupting the fine-regulation of the gene, potentially triggering the pathology.

To account for the H_2O_2 regulatory role other effects caused by H_2O_2 should not be ruled out. In general there is a vast array of co-repressors and co-activators that regulate transcription[15]. Concerning H_2O_2, extensive work on the role of oxidative stress in chronic obstructive pulmonary disease and asthma[39] has implicated oxidants in the activation of HAT and in the inhibition of HDAC. A putative protective role, helping with the resolution of inflammation

can become harmful for the organism if the oxidants doses are higher and/or the exposure is longer.

In summary, while it has long been assumed that the affinity of transcription factors to the promoter region of the gene can be one of the elements that control the intensity of the expression, the consequences of this affinity in the differential gene expression induced by an agent have remained unnoticed. Here we provided for the first time conclusive proof of principle that a simple chemical mechanism like the equilibrium between bound and unbound transcription factor to the promoter region of a gene can explain differential gene expression. Application of this principle to the regulation of NF-κB dependent genes by H_2O_2 predicts that H_2O_2 will up-regulate the expression of low to medium affinity NF-κB-target genes leaving high affinity genes unchanged.

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7. Supplementary Information

Table 1. Description of the abbreviations used in the model.

Abbreviation	Description
kBsite	DNA endogenous κB sites (1 to 100)
kBluc	Plasmidic κB sites with gene reporter luciferase (κBh, κBm and κBl)
NFkBnuc	Nuclear NF-κB that binds to kBsite and kBluc
NFkBnuc2	Species bound to two NF-κB
kbind	Association constant
kbind2	Association constant for the second binding of NFkBnuc
kdiss	Dissociation constant (k_d)
Coact	Co-activator

Table 2. Set of reactions considered in the mathematical model for the activation of NF-κB.

	Reaction	Kinetic law
1	kBsite1 + NFkBnuc → kBsite1NFkBnuc	kbind×kBsite1×NFkBnuc
	⋮	⋮
100	kBsite100 + NFkBnuc → kBsite100NFkBnuc	kbind×kBsite100×NFkBnuc
101	kBsite1NFkBnuc → kBsite1 + NFkBnuc	kdiss1×kBsite1NFkBnuc
	⋮	⋮
200	kBsite100NFkBnuc → kBsite100 + NFkBnuc	kdiss100×kBsite100NFkBnuc
201	kBsite1NFkBnuc + NFkBnuc → kBsite1NFkBnuc2	kbind2×kBsite1NFkBnuc×NFkBnuc
	⋮	⋮
300	kBsite100NFkBnuc + NFkBnuc → kBsite100NFkBnuc2	kbind2×kBsite100NFkBnuc×NFkBnuc
301	kBsite1NFkBnuc2 → kBsite1NFkBnuc + NFkBnuc	kdiss1×kBsite1NFkBnuc2
	⋮	⋮
400	kBsite100NFkBnuc2 → kBsite100NFkBnuc + NFkBnuc	kdiss100×kBsite100NFkBnuc2
401	kBsite1NFkBnuc2 + Coact → kBsite1NFkBnuc2Coact	kbindCoact×kBsite1NFkBnuc2×Coact
	⋮	⋮
500	kBsite100NFkBnuc2 + Coact → kBsite100NFkBnuc2Coact	kbindCoact×kBsite100NFkBnuc2×Coact
501	kBsite1NFkBnuc2Coact → kBsite1NFkBnuc2 + Coact	kdissCoact×kBsite1NFkBnuc2Coact
	⋮	⋮
600	kBsite100NFkBnuc2Coact → kBsite100NFkBnuc2 + Coact	kdissCoact×kBsite100NFkBnuc2Coact
601	kBluc + NFkBnuc → kBlucNFkBnuc	kbindluc×kBluc×NFkBnuc
602	kBlucNFkBnuc → kBluc + NFkBnuc	kdissluc×kBlucNFkBnuc
603	kBlucNFkBnuc + NFkBnuc → kBlucNFkBnuc2	kbind2luc×kBlucNFkBnuc×NFkBnuc
604	kBlucNFkBnuc2 → kBlucNFkBnuc + NFkBnuc	kdissluc×kBlucNFkBnuc2
605	kBlucNFkBnuc2 + Coact → kBlucNFkBnuc2Coact	kbindCoactluc×kBlucNFkBnuc2×Coact
606	kBlucNFkBnuc2Coact → kBlucNFkBnuc2 + Coact	kdissCoactluc×kBlucNFkBnuc2Coact

Table 3. Parameter values used in the model.

Parameter	Value	Units
kBsite	5.0×10^{-5}	μM
kBluc	5.0×10^{-5}	μM
NFkBnuc	1.0×10^{-4}	μM
Coact	5.0×10^{-4}	μM
kdiss1	2.0×10^{-2}	s^{-1}
kdiss2	$Kdiss1\times10^{(1/25)}$	s^{-1}
⋮	⋮	
kdiss100	$Kdiss99\times10^{(1/25)}$	s^{-1}
kbind	2.4×10	μMs^{-1}
kbind2	kbind×10	μMs^{-1}
kbindCoact	$2.4E\times10^{4}$	μMs^{-1}
kdissCoact	2.0	s^{-1}
kbindluc	2.4×10	μMs^{-1}
kbind2luc	kbindluc×10	μMs^{-1}
kdissluc	kBh:0.05; kBm:1.8; kBl:0.3	s^{-1}
kbindCoactluc	2.4×10	μMs^{-1}
kdissCoactluc	2.0	s^{-1}

Chapter V – General discussion

This work elucidated some contradictions in the literature about the regulation of NF-κB by H_2O_2 and contributed with some advances in the field. Nevertheless, it is only a small piece of the vast NF-κB puzzle. Here, by using the controlled H_2O_2 delivery and a small TNF-α dose, three major points were addressed in the NF-κB activation pathway: the level of the three major IκB proteins (Chapter III), the translocation of p65 and c-Rel as a representation of the major Rel proteins in the canonical NF-κB pathway (Chapters II and III) and the regulation of NF-κB-dependent gene expression (Chapters II and IV). Although nowadays an enormous importance is given to the mechanistic and molecular modification events, it is crucial to first underlie rigorously the global events, i.e. the observational descriptions.

Three major keywords define this work: H_2O_2 s.s., NF-κB and inflammation.

The experimental model

ROS have undoubtedly an important germicide role during the inflammatory response. The presence of myeloperoxidase in leucocytes allows the formation of an extremely toxic product, hypochlorous acid, by using H_2O_2 originating from the dismutation of $O_2^{\bullet-}$ [1]. Therefore, this protective role constitutes a good justification for H_2O_2 production in cells. Still, H_2O_2 has unique chemical properties, especially its low reactivity and ability to cross membrane barriers. H_2O_2 is involved in specific oxidative modifications and its concentration is tightly controlled by the action of several peroxidases. So, H_2O_2 is a potential signaling molecule, but this role is often masked by the high concentrations employed in experiments, which highlight the damaging effects of H_2O_2 and the concept of oxidative stress. NF-κB is a ubiquitous transcription factor having as a primary function the control of inflammation, but it is also involved in proliferation and apoptosis. Since the discovery that NF-κB is redox-regulated, innumerous studies, in different cell lines, were performed to elucidate mechanistically the potential role of H_2O_2 as a regulator of NF-κB. Unfortunately, taken all the data together there are more contradictory results than consistent ones, thus preventing a clear view of what role H_2O_2 plays in NF-κB activation.

To understand the true importance of H_2O_2 in biological systems it is fundamental to study its role within a range of concentrations where there is an oxidative balance that allows signaling responses and not use doses where only its harmful role can be observed. The correct oxidative balance to obtain signaling responses without entering in a harmful role is the key to understand the true importance of H_2O_2 in biological systems. In addition, it is important to take in account that different cell types are expected to have particular response patterns due to their distinct protein expression levels. For example, the dimer p50/p65 is the most abundant in non-hematopoietic cells, while c-Rel dimers are in majority in hematopoietic cells, B and T cells, and consequently, for the same stimuli, the gene expression profile will be different[2]. Therefore, to be certain that the different results reported are a consequence of a particular cell type and not a result of inconsistencies due to H_2O_2 treatment, it is necessary to create an "universal" way to deliver H_2O_2 to cell cultures. This issue constituted the basis of this work. It was first established which was the most correct way to deliver H_2O_2 to cells, i.e. the method that more closely matched the *in vivo* production in an inflammatory situation. The s.s. titration, which is explained in detail in Chapter II, is a calibrated and controlled method in opposition to the traditional bolus addition, where only elevated H_2O_2 initial doses are used and the real concentration exerting the effects is not known.

Moreover, in this work an attempt was made to use conditions close to the physiological ones, present in an inflammatory situation. At an inflammatory site, extracellular concentrations of H_2O_2 can increase to 5-15 μM[3,4] and therefore H_2O_2 was used at 12.5 μM s.s. (longer exposures) and 25 μM s.s. (shorter exposures). TNF-α is one of the first cytokines produced by activated macrophages at the beginning of inflammation, its role is first to spread the inflammatory response followed by resolution of inflammation. In experiments found in the literature, TNF-α is usually used at concentrations above 10 ng mL^{-1}, which is far higher than the inflammatory concentrations (less than 1.5 ng mL^{-1} [5]). In fact, only in acute episodes of septic shock TNF-α serum levels attain a maximum level of 3 ng mL^{-1} [6]. *In vitro*, stimulated monocytes (10^6 cells) with LPS are able to produce and secrete a maximal concentration of 1.2 ng mL^{-1} of TNF-α after 8 h of incubation[7]. So, the TNF-α concentration chosen for the assays, 0.37 ng mL^{-1}, was low but still able to activate NF-κB significantly.

Overall, in this work by using epithelial cell cultures, the NF-κB pathway was revisited in conditions of H_2O_2 and TNF-α close to the ones occurring during an inflammatory response.

Integrative appreciation of the results

In chapters II, III and IV consistent results were obtained about the global effect of H_2O_2 on the NF-κB pathway (Figure 1).

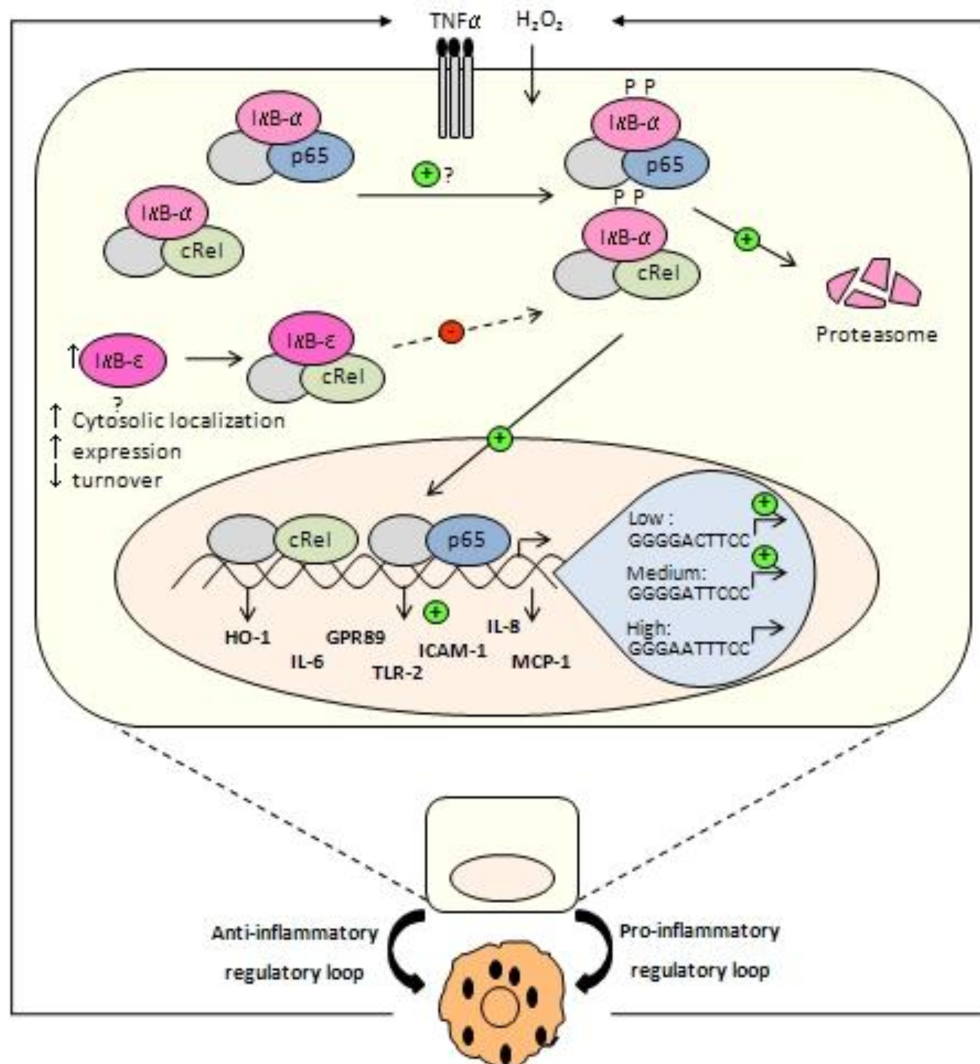

Figure 1 – Redox regulation of NF-κB activation by H_2O_2 and effects on gene expression. Schematic representation of the global events of NF-κB regulation described in this work in the simultaneous presence of H_2O_2 at moderate and s.s. concentrations and a low concentration of TNF-α, conditions which are hypothesized to be representative of an inflammatory situation. NF-κB/Rel proteins are represented by the major subunits p65 and c-Rel, which can homodimerize or heterodimerize with other elements of the family (in grey) and form complexes with the inhibitory subunits IκBs. During inflammation, activated macrophages produce H_2O_2 and TNF-α, which can affect epithelial cells. Dependently on the cell type, different proportions of NF-κB/IκB complexes are formed thereby affecting the global response to the inducers. Degradation of IκB-α is the major effect of H_2O_2 on TNF-α-induced cells, in part because of the increased proteasome activity, although it is probable that other mechanisms are also involved. Afterwords, higher levels of p65 and c-Rel dimers that were complexed with IκB-α become free, by the effect of H_2O_2, and translocate to the nucleus. Differential regulation of the other IκBs is probable, including temporary increases of IκB-ε levels, which impose an inhibition of NF-κB activation dependent on c-Rel dimers. The higher nuclear levels of p65 and, in some cases c-Rel, are predicted to contribute for the increased expression of genes bearing low/medium-affinity κB sequences, although the global transactivation of DNA is also dependent on other factors. Some NF-κB-dependent genes are stimulated by H_2O_2, including pro-inflammatory and anti-inflammatory genes, while others remain unresponsive. Protein expression and secretion of these mediators are both able to affect the macrophages and neutrophils, thereby helping in the control of inflammation.

H_2O_2 was found to be a poor activator of NF-κB, especially in HeLa cells. It had practically no effects on the degradation of the three major IκBs: IκB-α, IκB-β and IκB-ε and consequently, on NF-κB translocation and on the expression of NF-κB-dependent genes. The oxidative modifications provoked by H_2O_2 are probably not enough to induce NF-κB activation. Importantly, other pathways may be targets of H_2O_2 action, since we reported mRNA increases of HO-1 and ICAM-1, whose gene expression is probably dependent on the Nrf2 and AP-1 *cis*-elements, respectively[8,9]. Therefore, in a physiological context, it is not expected that transient increases of H_2O_2 concentration will affect NF-κB activation *per se*.

TNF-α is a classic inducer of NF-κB and a mediator of all stages of inflammation. After the first wave of pro-inflammatory gene expression, a decrease of TNF-α production and secretion is crucial for the resolution of inflammation[10]. In both HeLa and MCF-7 cell lines, treatment with 0.37 ng mL^{-1} TNF-α greatly increased the expression of its own mRNA after 1 h incubation, which was lowered after 6 h incubation. This observation suggests a correct balance of an initial pro-inflammatory response followed by its attenuation. Apart from the activation of NF-κB, binding of TNF-α to its receptors also activates a pro-apoptotic pathway by the recruitment of DD-containing mediators and activation of caspases, probably via JNK activation[11,12]. It is not well understood how cells keep the balance of this pro-apoptotic pathway with the simultaneous activation of NF-κB, which is known to have anti-apoptotic properties trough induction of expression of anti-apoptotic and proliferative proteins[13]. Although apoptosis was not monitored, viability assays indicated that the cells were not injured by exposure to TNF-α. The level of TNF-α is important for the appropriate balance of inflammation.

The combination of H_2O_2 s.s. and TNF-α had effects on NF-κB, that were useful for a better understanding of the contradictory data in the literature. First, it was possible to validate the s.s. titration method of delivering H_2O_2 to the cells. An inhibition of NF-κB activation was obtained with a bolus of 1 mM H_2O_2, while the 25 μM s.s. lead to a stimulation of NF-κB activation. Cells have potent antioxidant enzymes and thiols that are responsible for the maintenance of a reduced intracellular state, but high H_2O_2 concentrations may overload the total capacity of the cell response leading to an excessive oxidative damage, and thus necrosis and/or apoptosis or as, in this work, to NF-κB inhibition (bolus addition).

In MCF-7 cells, H_2O_2 s.s. raised the degradation of IκB-α induced by TNF-α, which lead to an increased concentration of nuclear p65 and c-Rel. These synergisms were a result of the

increased activity, approximately 65%, of the 26S proteasome induced by H_2O_2 and TNF-α, which indicates a faster degradation of the phosphorylated IκB-α. The proteasome is not only responsible for the degradation of signaling proteins, such as IκB-α, but also for the degradation of abnormal or oxidized proteins. Since H_2O_2 by itself did not alter proteasome activity, the changes observed in proteasome activity are not due to a massive oxidation of proteins, but to a specific role for H_2O_2 in conjunction with TNF-α. This observation lead to speculate that induction of the NF-κB pathway by TNF-α is necessary for the H_2O_2 action.

In HeLa cells, the NF-κB pathway also responded to H_2O_2 and TNF-α, with an increase of IκB-α degradation and translocation of p65 to the nucleus. The effect on IκB-α was not as pronounced as the one observed in MCF-7 cells, probably because no modulation of the proteasome was observed. This suggests that another stimulatory mechanism upstream IκB-α degradation is probably involved. Interestingly, for short incubation times, the presence of H_2O_2 in TNF-α-treated cells induced IκB-ε levels above control. Again, it seems that induction of the NF-κB pathway by TNF-α is important, perhaps for *de novo* synthesis of IκB-ε or its re-localization back to the cytosol, which is followed by an inhibitory mechanism induced by H_2O_2. In murine lungs, IκB-ε levels increased with LPS instillation, while IκB-α and IκB-β were degraded as expected[14]. This apparent contradictory regulation of the IκBs in some cell lines is not well understood. Here, increase of IκB-ε levels corresponded to a decrease of c-Rel levels in the nucleus. This short and particular effect reinforces the paradigm of H_2O_2 as being able to exert specific regulatory actions and indicates a tendency for its modulation of early-response genes, by favoring the ones only regulated by p65.

Once in the nucleus, NF-κB binds to its consensus κB sites to activate the target genes. A study of the expression levels of approximately 100 NF-κB-dependent genes confirmed the specific regulation by H_2O_2 in cells treated with TNF-α: a group of pro-inflammatory and anti-inflammatory genes was modulated by 12.5 μM H_2O_2 s.s. after 6 h incubation, while most of the genes remained insensitive to H_2O_2. Importantly, the study was performed for a maximal of 6 h, which still corresponds to the duration of a typical acute inflammatory response. In MCF-7 cells, H_2O_2 up-regulated more NF-κB-dependent genes than in HeLa cells, which is in accordance with the synergism in c-Rel translocation to the nucleus observed in MCF-7 cells, because c-Rel is also involved in the expression of other p65-regulated genes, e.g. IL-8 and ICAM-1.

Using artificial DNA sequences and mathematical modeling, it was concluded that the higher NF-κB concentration in the nucleus induced by H_2O_2 favors the modulation of genes bearing low- to medium-affinity κB sites towards NF-κB. Within the promoter/enhancer region of a gene a mixture of low, medium and/or high κB sites might be present and, in that case, the saturation of the low-affinity site induced by H_2O_2 may contribute either to a higher and/or longer expression level of the gene. Based on the results obtained with the plasmids, it is difficult to predict what is the affinity of the κB sites of the specific genes up-regulated by the simultaneous presence of H_2O_2 and TNF-α, because the overall modulation is dependent on the specific context of a particular κB site. The medium affinity κB site studied in this work belongs to the MHC class I gene, which was not studied in the microarrays, and the low affinity κB site is an artificial κB site. However, since the κB sequence of the high-affinity plasmid used in this work is one of the two κB sites existing in the MCP-1 enhancer, it is possible to give some speculative explanations. In transfected HeLa cells with the high-affinity plasmid and using 0.37 ng mL^{-1} of TNF-α, H_2O_2 did not induce any positive or negative effect on luciferase expression, probably because the κB site was already totally occupied with NF-κB dimers, induced by TNF-α itself. Interestingly, in the microarrays of HeLa cells, the mRNA of MCP-1 was not up-regulated by H_2O_2, which supports the results of the transfection assays. Nevertheless, in MCF-7 cells the simultaneous addition of H_2O_2 and TNF-α up-regulated MCP-1 mRNA. This allows speculating that the increase of nuclear c-Rel levels by H_2O_2 is important for the other κB site of MCP-1 to become also occupied, thereby increasing global MCP-1 expression. Moreover, it indicates that probably this κB site has more affinity towards c-Rel than p65, since c-Rel translocation is inhibited by H_2O_2 in HeLa cells.

In vivo, H_2O_2 produced during the inflammatory response is probably involved in the activation of epithelial cells, which may contribute for the overall inflammatory process. A set of pro-inflammatory genes, such as IL-8, ICAM-1, MCP-1 and anti-inflammatory/protective genes, such as HO-1 and IL-6, are important to balance the response. Cells where c-Rel, besides p65, is stimulated by H_2O_2 are expected to be more responsive during inflammation. Therefore, down-regulation of c-Rel might be a good therapeutic approach in these cells if resolution of inflammation is impaired. Apart from the crucial germicide role, ROS produced by NOX_{ph} are usually related with pro-inflammatory conditions, such as chronic inflammation[15]. However, the anti-inflammatory role of H_2O_2 is

supported by chronic granulomatous disease (CGD) models, a condition resulting from mutations in the components of NOX_{ph}, which originates a non-functional NOX_{ph} defective in ROS production. When compared with healthy monocytes, CGD-deriving monocytes respond to infection with LPS with higher levels of pro-inflammatory mediators, such as IL-8 and TNF-α, and a higher expression of NF-κB proteins, such as p65, c-Rel and p50[16]. Accordingly, in this disease model IκB-α, IκB-β and IκB-ε are not up-regulated, and thus an inflammatory condition with an aberrant NF-κB activation prevails[16].

The resolution of inflammation is also characterized by the triggering of apoptosis in neutrophils, in order to down-regulate pro-inflammatory signals. After apoptosis, the macrophages help with the clearance of the neutrophils. The anti-inflammatory role for H_2O_2 is also supported by the use of SOD for therapies against chronic conditions[15,17]. Artificial SOD mimetics are able to decrease neutrophil infiltrations to the tissues and inhibit the production of pro-inflammatory mediators, partially by inducing apoptosis of neutrophils, which was shown to be mediated by the production of H_2O_2[15,17].

From all the information available about the inflammatory environment, it is expected that TNF-α will be present at the same time with H_2O_2, simultaneously with other inflammatory mediators. NF-κB, as the principal regulator of inflammation, is targeted for both pro- and anti-inflammatory modulation by H_2O_2, which contributes for a more effective and controlled response.

Perspectives

Although the overall conclusions of this work were similar for HeLa and MCF-7 cells, there were still some particular and different responses dependent on the cell type. These dissimilarities arise probably from the differences in GPx activity which are reflected in the cellular consumption rates of H_2O_2. MCF-7 cells have a lower GPx activity than HeLa cells, resulting in higher intracellular H_2O_2 steady-state concentration and consequently leading to different changes in the intracellular redox state. As the couple GSH/GSSG constitutes the major redox buffer of cells, it will be important to measure the concentrations of GSH and GSSG in both cell types to analyze the oxidative environment provoked by H_2O_2 exposure. Moreover, monitoring formation of mixed disulfides, especially the levels of S-thiolated proteins, is also a good approach to elucidate the extent of modifications caused by H_2O_2. In fact, the concept of protein glutathionylation as a post-translational regulative modification,

in opposition to a biomarker of oxidative stress, has gained importance and may be involved in the signaling events observed in MCF-7 cells[18].

By maintaining the same conditions (cell types and exposure conditions) it is also important to address other factors that are responsible for the increased degradation of IκB-α. H_2O_2 may lead to a more favorable binding of TNF-α to its receptor, as it was demonstrated that H_2O_2 activates JNK through the TNF-R1 with no effect of the NF-κB pathway[19] or it can increase the activity of the IKK, as reported as well in some works[20,21]. The transactivation potential of Rel proteins is determined by its phosphorylation and acetylation status[22]. Although the major H_2O_2 effect is to increase the level of nuclear NF-κB, phosphorylations and acetylations in NF-κB itself or even in the histones are also possible modifications that might affect DNA transactivation. For example, some studies report that oxidative stress is implicated in inflammation by the down-regulation of the HDACs[23,24].

The next step should be to work with co-cultures of macrophages and epithelial cells and instead of adding H_2O_2 directly to medium, activate macrophages with PAMPS, e.g. LPS, and follow the subsequent activation of NF-κB in epithelial cells, while simultaneously monitoring of H_2O_2 concentration in the medium. Besides activating the endogenous production of H_2O_2, this procedure will also be important to address the anti-inflammatory role of H_2O_2 in the NF-κB pathway, i.e. the down-regulation of macrophages activation.

HeLa and MCF-7 are established cell lines obtained from tumors. They were an important starting point for our controlled studies because they are easily maintained, grow fast and can be preserved for periods of months. All these characteristics were fundamental to obtain reproducibility in the number of cells needed to obtain the same rates of consumption of H_2O_2 for the s.s. in each experiment. With all the information obtained in this study, it is now possible to try to reproduce the results in primary cultures, and so, address the H_2O_2 role in inflammation in a more physiological context.

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