In Silico Analysis of Interactions Between NFkB and HSF Pathways

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Abstract: Motivation: Inhibition of NFkB pathway is known to promote apoptosis and therefore may constitute one of the goals in anticancer therapies. Experimental results show that heat shock induces such inhibition in cancer cells. However, the mechanisms of interactions between heat shock and NFkB pathways are not fully understood yet. Development of a combined mathematical model of these pathways and its subsequent computational analysis should help to uncover these mechanisms and determine the time window in which heat shock treatment preceding chemotherapy would be the most efficient. Results: An original mathematical model has been developed, allowing for computational testing of various hypotheses concerning main sources of interplay between HSF and NFkB pathways. Computational analysis strongly suggests that the competition for IKK, known from literature, cannot be the only mechanism. Two plausible hypotheses are that either a kinase activating IKK can misfold due to heat shock or that heat shock affects TNF receptors, blocking activation of NFkB pathway at the cell membrane.

1 INTRODUCTION

NFkB transcription factor regulates expression of various genes, including those important for cell survival. Therefore it has been the subject of research for many years now, and its role in promoting or blocking apoptotic pathways has been investigated as one of the key molecular players determining fate of cancer cells after radio- or chemotherapy. In particular, various ways of inhibition of NFkB pathway have been the focus of attention in many cancer studies (e.g. Yamamoto and Gaynor, 2001, Amman *et al*., 2009, Domingo-Domènech *et al*., 2008, Zanotto-Filho *et al*., 2011).

On the other hand, hyperthermia was suggested to have a potential of improving the efficacy of chemo- or radiotherapy by many authors (e.g. Neznanov *et al*., 2011) and be a promising treatment in itself (Lee Titsworth *et al*., 2014). Bringing cells under a heat stress initiates many biochemical processes, including, among others, an apparent inhibition of the NFkB pathway (Janus *et al*., 2011). While the precise mechanism of this inhibition is not clear, it has been observed that this inhibition remains in place for some time after the source of heat shock have been removed. Moreover, it is known that HSF1 is the primary transcription factor activated under stress conditions that is responsible for induction of genes encoding heat shock proteins (Fujimoto and Nakai, 2010, Morimoto 2011, Westerheide *et al*., 2012), hence the HSF1 pathway is a natural candidate for investigation. Uncovering the mechanisms behind this inhibition should yield two direct benefits: (i) finding the best (in terms of maximum reduction of cancer cell population) time lag between short thermal shock and subsequent irradiation or chemotherapeutic agent delivery, (ii) expanding knowledge about other possible molecular interactions that could be utilized to increase treatment efficacy even further.

Of the two components that are combined in this work, the NFkB pathway has been studied much more extensively and many models have been developed so far. It appears that one of the most often cited models is the one published in (Lipniacki *et al*., 2004), whose structure was later modified to some extent in later works (e.g., Wang *et al*., 2011, Wang *et al*., 2012, Zambrano *et al*., 2014). Much less attention was devoted to the HSF pathway, though one can find several papers dealing with

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mathematical modeling of the intracellular processes that are initiated by heat shock (Rieger *et al*., 2005, Szymanska and Zylicz, 2009, Petre *et al*., 2011, Rybinski *et al*., 2013). To our knowledge, there has been no published attempt to combine models of both pathways into a single one, which could bring some important conclusions about possible inhibition of NFkB pathway by heat shock, and its implications for clinical applications. While recently some work has been done on analysis of NFkB pathway dynamics under heat shock conditions, it was done only through manipulating the parameters of NFkB model (Sheppard *et al*., 2014). While such approach might be welcome as it does not necessitate increasing the complexity of the model, it is not useful if the question posed concerns the nature of interactions between the pathways, their dynamics and possible crosstalk with other pathways. In this work, we aim at building a model that might help answering these questions.

2 METHODS

The model presented in this work is clearly deterministic. While it has been proven that stochasticity plays a crucial role in intracellular processes described by signaling pathways models, deterministic approach is much more convenient when the initial model is developed. Stochastic modeling should inevitably follow, in particular when the analysis would be focused on determining the ultimate fate of cells. That, however, is beyond the scope of this paper.

Taking into account that the separate models of each of the two pathways under consideration have already been published, we decided to arbitrarily chose their representatives and introduce the necessary modifications concerning both the proposed crosstalk mechanisms and parameter values that would compensate the crosstalk in control (i.e. not excited) cells. Additionally, the HSF pathway has been further modified, to take into account both assumptions needed to apply the law of mass action that is behind the most part of the deterministic modeling and the implications of experimental results that are available.

The detailed description of canonical NFkB and HSF pathways can be found in many papers, including those cited in the Introduction section. Here they will be only briefly summarized in the following two paragraphs.

In unstimulated cells, NFkB forms a cytoplasmic complex with its inhibitor IκB proteins. The pathway can be activated, among others, by Tumor Necrosis Factor (TNF) stimulation. Then, the kinase IKK is activated and it subsequently phosphorylates the inhibitors, targeting them for degradation. Freed NFkB is imported into the nucleus, where it serves as a transcription factor for many genes. These genes include, among others, the genes coding IκB inhibitors. Thus a negative feedback is formed. Another feedback loop involves the A20 protein, whose gene is also activated in the pathway.

In the heat shock activated pathway, in turn, the main players are HSP and HSF proteins. Under normal conditions, they mostly reside in complexes with each other. Following heat shock, some of the proteins in a cell denature and become misfolded. These denaturated proteins cause HSP|HSF complex to dissociate, following HSP binding to the misfolded proteins. Free HSF molecules undergo phosphorylation and subsequently form trimers, which are transported into nucleus. Once there, the trimers serve as transcription factors of heat-shock inducible genes, including the family of HSP proteins. Newly produced HSP proteins can bind HSF, thus creating a negative feedback loop.

In all simulations aimed at checking the possible inhibition of the NFkB pathway, the excitation protocol comprised of a short, 30 minute heat shock of 42 degrees, followed by TNF stimulation. The time lag between the end of heat shock and beginning of TNF treatment was varied, to check when the NFkB pathway regains its functionality. Cytoplasmic IKK and nuclear NFkB proteins were chosen to represent the pathway response. In order to check if the pathway was inhibited, the results were compared to the ones obtained when only TNF was used (see Figure 1).

It was assumed that the model should satisfy the following assumptions, indicated by experimental data (Ciocca and Calderwood 2005; Daugaard *et al*., 2007, Morimoto, 2011, Janus *et al*., 2011 and our own, unpublished results):

- there are two types of HSP: constitutive, present at high levels all the time, and inducible; though they are represented by separate variables in the model, they perform the same actions; therefore the model should be applicable both in the case when they are the same and when they are different species of HSP.
- Most of the constitutive HSP is located in the cytoplasm in a normal physiological state.
- Following the beginning of a heat shock, most of the HSP is transported to the nucleus so that in less than 1 hour most of it appears in the nucleus.

- 3 to 4 hours after the heat shock begun, inducible HSP is observed in cytoplasm.
- Of all possible complexes formed by HSF1 taken into account in the model, only the trimer can shuttle between nucleus and cytoplasm.
- If the TNF is given earlier than 6 hours after heat shock has ended, a partial inhibition of the NFkB pathway is observed. If this time lag is greater than 6 hours, inhibition does not take place.

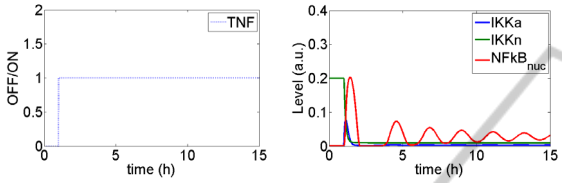

Figure 1: The reference plots for not inhibited NFkB pathway.

3 THE MATHEMATICAL MODEL

One of the most frequently mentioned mechanisms of possible crosstalk between the two pathways under consideration is the competition for IKK proteins through creation of IKK|HSP complexes (e.g. Ran *et al.*, 2004). Therefore, this process has been incorporated into our model and it involves both constitutive and inducible HSPs (Figure 2a). Another possible mechanism might involve a kinase responsible for IKK activation. Heat shock causes, among others, misfolding of some proteins. If that kinase was misfolded (Figure 2b), it would be unable to activate IKK (however, this could be repaired by the constitutive HSP). Yet another possibility is the effect of heat shock on the TNF receptor. Receptor-dependent sensitivity of NF-κB pathway has already been reported in another context (Yang and Zhou, 2013). If TNF receptors are inactivated by heat shock, then obviously the NFkB pathway would be inhibited. In that case, one could always choose model parameters to adjust the time of inactivation and since the results of such simulation are obvious, they are not presented in the paper.

As indicated in the preceding section, the model proposed in this work is built on the previously published ones, which described either NFkB or HSF1 pathways separately. Of these, the NFkB model comes from (Lipniacki *et al.*, 2004) and was changed only in the parts that are responsible for the crosstalk between the pathways, shown in the Figure 2 and summarized in the Table 1.

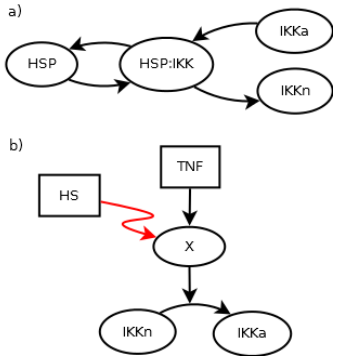

Figure 2: Possible crosstalk mechanisms between HSF and NFkB pathways: (a) creation of IKK|HSP complexes, (b) misfolding of kinase activating IKK.

The existing HSF1 pathway model (Szymanska and Zylicz, 2009) had to be modified to a much greater extent and these changes are summarized below:

- First, both this and other models (e.g. Rieger *et al.*, 2005, Petri *et al.*, 2011) treat the cell as a single compartment. Here, in order to satisfy the assumptions presented in the preceding section, it was necessary to distinguish cytoplasmic and nuclear levels of proteins and complexes.
- Constitutive and inducible HSPs are described by separate variables.
- The complexes of the HSF1 trimer and its corresponding Heat Shock Element (HSE) in the promoter region of the HSP gene are not modeled explicitly. It seems that here law of mass action is not applicable due to a small number of HSEs and to take into account these complexes properly one should use a stochastic approach. Instead, transcription rate of the HSP gene is assumed to be proportional to the nuclear level of the trimer.
- The function describing temperature-dependent protein degradation rate has been modified from its original version (Peper *et al.*, 1997) which was subsequently used in (Rybinski et al., 2013) to be zero for the temperature of 37 degrees.
- In addition to forming complexes with HSF1 and misfolded proteins, HSP can also form complexes with IKK.

While the introduction of nuclear and cytoplasmic compartments, as well as constitutive and inducible forms of HSP have not changed the main reasoning behind the previously published HSF pathway models, the number of changes might be confusing without a proper presentation. Therefore, the list of reactions in the model is presented in the Table 1.

Table 1: Reaction list for the HSF pathway.

Reactions both in cytoplasm and nucleus:
Prot → mfProt
mfProt + HSP_{cons} → HSP_{cons}:mfProt
mfProt + HSP_{ind} → HSP_{ind}:Prot
HSP_{cons}:mfProt → HSP_{cons} + Prot
HSP_{ind}:Prot → HSP_{ind} + Prot
HSP_{cons} + HSF → HSP_{cons}:HSF
HSP_{ind} + HSF ↔ HSP_{ind}:HSF
HSP_{cons}:HSF + mfProt → HSP_{cons}:mfProt + HSF
HSP_{ind}:HSF + mfProt → HSP_{ind}:mfProt + HSF
3HSF → HSF_3
HSF3 + HSP_{ind} → HSP_{ind}:HSF + 2 HSF
HSP_{ind} →
mRNA →
Reactions taking place only in the nucleus:
HSF_3 → HSF_3 + mRNA
Reactions taking place only in cytoplasm:
mRNA → mRNA + $HSPF_{ind}$
HSF_{cons} + IKKa → HSP_{cons}:IKK
HSF_{ind} + IKKa → HSP_{ind}:IKK
HSP_{cons}:IKK → HSP_{cons} + IKKn
HSP_{ind}:IKK → HSP_{ind} + IKKn
Transport:
$HSP_{cons,cyt}$ ↔ $HSP_{cons,nuc}$
$HSP_{ind,cyt}$ ↔ $HSP_{ind,nuc}$
$HSF_{3\ cyt}$ ↔ $HSF_{3\ nuc}$
Additional reactions in the model B
IKKn $\xrightarrow{TNF,X}$ IKKa
X $\xrightarrow{T}$ mfX
mfX + HSP_{kons} → HSP_{kons}:mfX
mfX + HSP_{ind} → HSP_{ind}:mfX
HSP_{kons}:mfX → HSP_{kons} + X
HSP_{ind}:mfX → HSP_{ind} + X

In all, three models have been tested. The first one took into account IKK|HSP formation as the only mechanism behind the inhibition of NFkB pathway. Since it has yield no positive results, its simulations are not included in the subsequent section. The second model (Model A) additionally incorporated nuclear import of HSP proteins as a temperature-dependent process to reflect nuclear accumulation of constitutive HSP following the heat shock. In the third model (Model B), concentration of a kinase activating IKK is explicitly modeled, with the kinase being prone to misfolding in a temperature-dependent manner.

4 RESULTS AND DISCUSSION

Due to abundance of constitutive HSP, despite formation of HSP|IKK complexes the NFkB pathway does not affect dynamics of the HSF1 pathway. Therefore, only exemplary results, showing the main molecules in the HSF1 pathway are shown in Figure 3. The remaining plots concentrate on possible inhibition of the NFkB pathway.

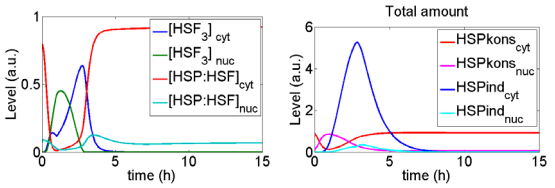

Figure 3: HSF and HSP levels following 30-minute heat shock.

The initial assumption that the IKK|HSP complexes formation alone might be responsible for the NFkB pathway inhibition have failed. Although one might choose the kinetic parameters to completely block all IKK in the complexes, this would subsequently result in a prolonged inhibition, not observed experimentally. Therefore, in addition to this process, other mechanisms had to be proposed.

First, the temperature-dpendent mechanism of nuclear import had to be introduced. Without it, one could not capture nuclear accumulation of constitutive HSP, when in a normal state most of it should be located in cytoplasm. Since the precise mechanism behind this transport is not known, instead of introducing new, unknown molecules that might mediate this ttansport, we decided to describe it in the simplest way, introducing the temperature-dependent transport ration into the equations:

$$HSP_{cyt} = k_{exp} \cdot HSP_{nuc} - k_{imp} \cdot HSP_{cyt} \cdot \cdot e^{1,4\cdot(T-37)} \quad (1)$$

$$HSP_{nuc} = -k_{exp} \cdot k_V \cdot HSP_{nuc} + k_{imp} \cdot k_V \cdot \cdot HSP_{cyt} \cdot e^{1,4\cdot(T-37)} \quad (2)$$

where k_{imp} and k_{exp} are nuclear import and nuclear export rates in normal conditions, k_v is the ratio of nuclear and cytoplasmic volumes. Both constitutive and inducible HSP is assumed to follow that kind of transport, though the normal rates might differ between them.

Other than in the model developed by Szymanska and Zylicz (2009) we wanted to distinguish between two different outcomes of HSP acting on the misfolded proteins: through creation of complexes with them, HSP can either repair them or target for degradation. Therefore we have created a pool of generic proteins, both in the nucleus and in cytoplasm, that are negatively affected by the temperature and transformed into misfolded proteins:

$$\frac{d\,Prot}{dt} = -k_5 \cdot \phi_T \cdot [Prot] + \alpha \cdot k_{-1} \cdot \quad (3)$$
$$\cdot [HSP_{kons}{:}mfProt] + \alpha \cdot k_{-1} \cdot [HSP_{ind}{:}mfProt]$$
$$\frac{d\,mfProt}{dt} = k_5 \cdot \phi_T \cdot [Prot] - k_1 \cdot \quad (4)$$
$$\cdot [HSP_{kons}] \cdot [mfProt] - k_1 \cdot [HSP_{ind}] \cdot [mfProt]$$

where α represents the efficacy of the repair process and ϕ_T is a function dependent on the temperature as in (Petre *et al.*, 2011):

$$\phi_T = \left(1 - \frac{0{,}4}{e^{T-37}}\right) \cdot 1{,}4^{T-37} \cdot 1{,}45 \cdot \quad (5)$$
$$\cdot\, 10^{-5} - 8{,}7 \cdot 10^{-6} \quad [s^{-1}]$$

with the last term introduced by us to have

$$\phi_{T=37} = 0 \quad (6)$$

Figure 4 illustrates partial inhibition of the NFkB pathway in the Model A (Figure 4a) which ceases to exist if the period between the end of heat shock and NFkB activation with TNF is too long (Figure 4b). Despite these promising results, if the TNF stimulation follows directly heat shock, no such inhibition is observed (Figure 5a). This is a direct result of the changes introduced to obtain nuclear accumulation of constitutive HSP following heat shock. Since HSP proteins, following their release from the complexes with HSF1 are transported to nucleus, too little of them remain in cytoplasm to effectively bind IKK. Only after inducible form of HSP is produced as a result of HSF1 pathway, these newly translated proteins are in place to block NFkB pathway through IKK inhibition.

As a result, the inhibition of the NFkB pathway stimulated shortly after the heat shock must be mediated otherwise than through IKK|HSP complexes formation. It seems that the inhibition should be located upstream of IKK activation. One of the possible explanations is temperature-dependent misfolding of a kinase activating IKK, which renders it incapable of such activation. This assumption is behind the modification introduced in the model B. Then, inhibition of the NFkB pathway is visible also in the case when TNF stimulations follows directly the heat shock (Figure 5b). It is worth noticing that the frequency of oscillations, regarded as a specific feature of the NFkB pathway, remains unchanged even when models of both pathways are coupled.

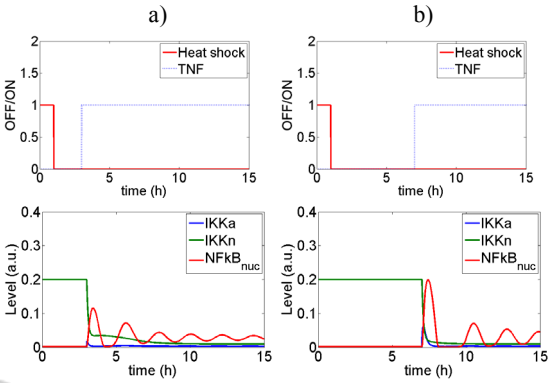

Figure 4: NFkB pathway responses for two different time lags between heat shock and TNF stimulation.

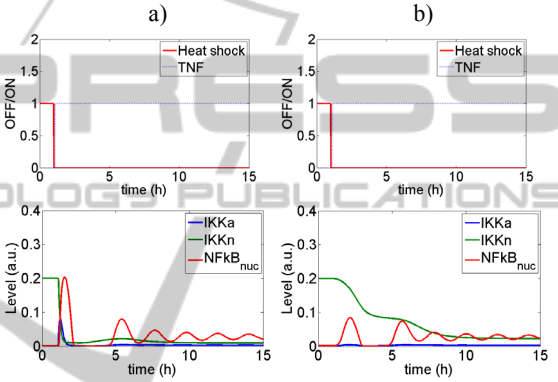

Figure 5: NFkB pathway response for (a) Model A and (b) Model B.

5 CONCLUSIONS

This paper presents results of a computational analysis of a combined HSF1-NFkB pathway. The model that has been developed captures the phenomenon of NFkB pathway inhibition through a heat shock that can last several hours after the heat shock has ended. The mechanisms behind this inhibition that have been proposed, i.e. (a) IKK|HSP complexes creation; (b) temperature-dependent nuclear import of HSP proteins; (c) temperature-dependent activation of IKK proteins do not involve purely theoretical new proteins and it seems that they are biologically viable. Nevertheless, another possible explanation could be that the inhibition takes place at the cell membrane and involves inactivation of the TNF receptor - a hypothesis that is currently the subject of experimental investigation.

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