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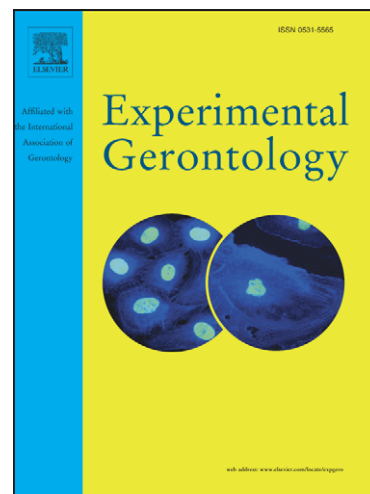
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**Short Term Hyperthermia Prevents the Activation of Mitogen-
Activated Protein Kinase p38**

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Abstract

We demonstrated earlier that hyperthermia (HT), a form of balneotherapy, suppresses transcription and translation of a number of proinflammatory genes. Here we show that short term HT not only acts by preventing the activation of NF- κ B, but also by blocking the activation of the MAPK p38. Data are presented that show that the effects of HT on p38 are clearly independent of HT effects on NF- κ B. This is demonstrated by the ability of short term HT to prevent IL-1 β induced activation of MAPK p38 dependent, but NF- κ B independent genes. In general it is assumed that the protective effects of HT are mediated by de novo synthesis of a number of heat shock proteins. However, data presented herein imply the need to distinguish early and late acting mechanisms of short term HT. Neither a recovery period nor de novo protein synthesis is essential for the early protective effects of HT. The effectiveness of short term HT in preventing the activation of two important signaling pathways and the relative ease by which the temperature in joints of arthritis patients might be modulated, seemingly offer an appealing opportunity to prevent or diminish inflammation by balneological means.

Introduction

A number of in vitro and in vivo models have been employed to study heat shock proteins (HSPs) and the modi operandii that account for the protective effects of stress proteins. Out of such studies, a pattern emerged that linked HSPs, newly synthesized by hyperthermia, fever, or chemical means, to the reduction of a number of pro-inflammatory genes that are characterized by their dependence on the activation of the transcription factor nuclear factor kappaB (NF- κ B) (1). Interactions between HSP70 and

members of the NF- κ B family have been associated with heat shock mediated NF- κ B inhibition. It has, for example, been shown that blocking the degradation of the inhibitor kappaB (I κ B), a protein that sequesters NF- κ B in the cytoplasm, is one of the key events that account for the anti-inflammatory effects of HT (2, 3).

In general it is rather difficult to change the body core temperature by artificial means without risking undesired effects. One disease that affects many might offer the unique opportunity to utilize HT as a mean to ameliorate inflammation without potentially harmful effects to vital organs in the body core. It seems, that due to their location, the temperature in joints of patients suffering from certain forms of arthritis could be modulated with relative ease. It is for this reason that we initiated studies to evaluate HT in an in-vitro model for its effectiveness in dampening the inflammation associated with rheumatic diseases (4).

Fibroblast-like synoviocytes (FLS) play an important role in creation and propagation of rheumatoid arthritis and are characterized by their ability to up-regulate numerous genes that take part in inflammatory processes (5). As reported earlier from our laboratory, exposing FLS to HT resulted in significantly reduced mRNA and protein levels of many pro-inflammatory genes that would otherwise be highly upregulated in response to IL-1 β (4). We further demonstrated by electrophoretic mobility shift assays (EMSA) and western blot experiments that HT prevented I κ B degradation and nuclear translocation of NF- κ B in this cell type (4).

It is generally thought that it is the newly synthesized HSPs that account for the majority of protective effects of the stress response induced by HT. However, like us, others also demonstrated discrepancies in the time course of HSP70 and/or HO-1 expression and the inhibition of NF- κ B mediated by HT (4, 6). Similar to our results

using FLS, it has been reported that I κ B degradation in heat shocked macrophages is blocked for up to four hours (7). However, HSP70 protein expression induced by HT in the aforementioned publication (as well as in our experiments) peaked after 7 hours. In addition, in FLS, significantly elevated levels of HSP70 protein can be observed for up to 24 hours, a time at which the effect of HT on I κ B/NF- κ B has long since subsided (4, 6). Such data, together with relative high HSP70 levels in resting, non-stressed FLS, prompted us to further investigate mechanisms and the involvement of HSPs in anti-inflammatory effects of short term HT.

As reported, mammalian cells are able to withstand temperatures of up to 45°C without significant effects on their viability (8). By and large, HT effects are thought to be due to the activation of a number of heat shock proteins (9, 10). The extent of the heat shock response is thought to be a function of the duration and magnitude of the temperature increase. Furthermore, it is widely assumed that a recovery period at 37°C is essential for the protective effects of HT. Such a period is thought to allow for maximal expression of a number of protective HSPs. However, as mentioned, the heat shock proteins HSP70 and HO-1 are maximally expressed at time points at which the protective effect of HT is seemingly past its peak. In the following, data are presented resulting from an investigation into the very early events mediated by short term hyperthermia.

Materials and Methods

Reagents. If not stated otherwise, reagents e.g. Interleukin-1 β (human recombinant), phorbol myristate acetate (PMA) etc. were from Sigma (Sigma, Vienna, Austria). Anti-I κ B α and the phosphorylation specific antibodies directed against p-I κ B,

p-p38, p-JNK/SAPK and p-ERK were from CellSignaling, (CellSignaling, Bedford, MA, USA). The MAPK inhibitor set I and II as well as the MAPK inhibitors SP600125, SB 203580, PD 98059 were from Calbiochem, (Merck, Darmstadt, Germany). Anti- α -tubulin was from LabVision (Lab Vision Corporation, Fremont, CA, USA). The anti HSP70 and HO-1 antibodies were from Santa Cruz Biotechnology, (Santa Cruz, CA, USA).

Cell culture. A series of human CD90 positive fibroblast-like synoviocytes (FLS), isolated from rheumatoid arthritis patients, were used in these experiments. Cells were either purchased from Dominion Pharmakine (Dominion Pharmakine, Derio, Bizkaia, Spain), or were a gift from Dr. G. Steiner, Medical University Vienna, Austria. FLS were cultured and propagated essentially as described (4). The temperature of the medium was measured and monitored in cell culture dishes that contained the same amount of medium as dishes containing FLS used in experiments.

Western blot experiments. SDS-PAGE and Western blot experiments were carried out as described (4). In short, cells were washed twice in ice-cold PBS and subsequently dissolved in SDS sample buffer. Proteins were quantitated, separated, blotted, and stained with indicated antibodies. Proteins were made visible using RenaissancePlus (Perkin Elmer Life Science, Boston, USA) and Kodak BioMax MR films or the chemiluminescence detection device GeneGnome (Syngene, Cambridge, UK). In western blot experiments, lower concentrations of proteins were loaded on separate gels that served as controls for loading and protein transfer (LC). Such blots were stained with an antibody recognizing tubulin, or, like other blots, were stained with Ponceau red.

Real time RT-PCR, data analysis and quality controls. Gene expression in FLS was measured by real-time RT-PCR on a Mx3000P (Stratagene, Amsterdam, The Netherlands), using SYBR green as reporter fluorophore for quantitating mRNA levels (4). To normalize the amount of total RNA present in each reaction, mRNA levels of HPRT and/or actin were used. Results are expressed as relative threshold cycle (ΔC_t -values) (Ct values of mRNA levels in stimulated minus Ct values of a given gene in resting cells). Primer sequences used but not reported previously are as follows: IL-1 α (forward) ATC AGT ACC TCA CGG CTG CT, IL-1 α (reverse) CTT CAT CTT GGG CAG TCA CA, HAS1 (forward) CAA GCC GGA GAG AAG AGA GA, HAS1 (reverse) GTA GGC CCA GGT CAT GAG G. Primers, amplification curves, and equations for the quantitation of mRNA for other genes have been reported elsewhere (4, 11, 12).

Basal mRNA expression levels in unstimulated FLS were chosen to represent 1X expression of a given gene. Data shown in figure 7B resulted from two independent experiments done in duplicates; all other experiments were done at least three times. Where statistical analysis was done a P-value ≤ 0.05 was considered significant.

Viability of cells was neither affected by the stimuli used, by exposure to hyperthermia, nor by a combination of hyperthermia and stimuli.

Results

Effects of short term HT on IL-1 β induced NF- κ B signaling pathways in FLS. As shown by data presented in figure 1A and 1B, in which FLS were exposed to short term HT (39, 40, 41, and 43°C for 30 min.) I κ B α degradation, induced by IL-1 β (5 ng/ml for 20 minutes), can be affected by HT for at least 20 hours. However, the

effectiveness of HT to block the NF- κ B activation pathway is more pronounced at earlier time points i.e. when cells are challenged immediately after short term HT rather than after 20 hours at which time HSPs are maximally expressed. If cells are stimulated immediately after HT, 41°C is sufficient to completely block IL-1 β induced I κ B α degradation (figure 1A). However, prior exposure to 41°C is no longer sufficient to block I κ B α degradation in cells that were given time (20 hours) to maximally express HSPs (figure 1B). Also shown in figure 1A and B is that the temperature had to be increasing to 43°C in order to prevent I κ B α degradation even after 20 hours of recovery. This is in accordance with observations made by others, namely, that exposing cells to higher temperatures will result in a more pronounced/prolonged protective effects of HT (8).

Next to HO-1, HSP70 is one of the best studied HSPs and also one that very quickly responds to temperature changes (13-15). HSP70 has many times been shown to be involved in, and is thought to account for the majority of protective effects of, the heat shock response (10). Nevertheless, we found that in FLS HSP protein levels remain unchanged for at least 40 minutes after HT (4), (data not shown). This led to the assumption that HT might also initiate events that are independent of newly synthesized HSPs. Shown in figure 1C is an experiment that demonstrates that in FLS, IL-1 β induced I κ B α phosphorylation is an event that starts at 30 seconds and peaks at around 5 minutes. Experiments like the one shown in figure 1D were done to investigate the time requirements for HT mediated inhibition of the NF- κ B pathway. FLS were kept at 37°C or were exposed to 41°C for 5, 10, and 15 minutes. Afterwards, IL-1 β was added for 5 minutes. I κ B α phosphorylation was therefore chosen to monitor the minimal duration of HT necessary to prevent NF- κ B activation. Figure 1D shows that 15 minutes of HT are

sufficient to completely block IL-1 β induced I κ B α phosphorylation. Shown in figure 1C is a representative Western blot experiment depicting IL-1 β induced I κ B phosphorylation in FLS.

Protein synthesis is not essential for HT effects on NF- κ B activation. In the following, we show data that further support the hypothesis that short term HT exerts effects that do not depend on a recovery period that allows for the generation of HSPs. FLS were left untreated or were pretreated either with Cycloheximide (figure 2A) and Emetine (figure 2B) - two protein synthesis inhibitors. After 60 minutes pretreatment with these substances, parts of the cells were exposed to 41°C for 30 min. Where indicated, cells were subsequently stimulated with IL-1 β for 5 minutes at 37°C. Phosphorylation of I κ B α was monitored by Western blots. As shown in figure 2, short term HT prevents IL-1 β induced I κ B phosphorylation even in FLS that were prevented from synthesizing protective heat shock proteins in response to being exposed to 41°C. That the NF- κ B signaling cascade in Cycloheximide and Emetine treated cells is intact is demonstrated by data showing phosphorylation of I κ B α in protein synthesis inhibitor treated cells exposed to IL-1 β (Figure 2, lane “CYC + IL-1 β “, and lane “EME + IL-1 β “). Such data point at early, HT mediated mechanisms that do not require de novo protein synthesis.

Is protein denaturation involved in HT mediated prevention of I κ B phosphorylation? Data shown in figure 2 point to mechanisms other than de novo synthesis of HSPs by which HT is able to block NF- κ B activation. One mechanism that might be at play is based on the assumption that some proteins could be more sensitive to a moderate increase in temperature than others. For example, a method that is frequently

used to selectively inactivate proteins of the complement cascade consists of heating serum to 56°C for 30 minutes. Whether exposing cells to 41°C for 15 minutes is sufficient to denature proteins is less clear. However, a recently published article seems to imply heat induced denaturation/insolubilization as one mechanism by which HT mediates its effects (16). Shown in figure 3 are data that implicate that a method used in A459 cells to demonstrate HT induced protein insolubilization is not suited to prove or disprove such a mechanism in FLS. The reason is that in FLS glycerol (used to prevent protein aggregation) itself exerts profound effects on the IL-1 β induced NF- κ B pathway.

FLS were incubated with 1M of glycerol for 1 hour and subsequently treated with IL-1 β (5 ng/ml for 5 min). Unlike the demonstration that 1M of glycerol will restore the NF- κ B pathway in HT treated A459 cells, we found that in FLS, glycerol itself prevents I κ B phosphorylation. Shown in figure 3A is a dose curve experiment demonstrating that IL-1 β induced I κ B phosphorylation is blocked at high concentrations of glycerol. FLS were incubated with glycerol (1 to 1000 mM) for 1 hour. Afterwards, cells were treated with IL-1 β for 5 more minutes. As in other Western blot experiments, staining blots with tubulin served as control for protein loading and transfer in addition to staining membranes with Ponceau red.

As shown in figure 3B, preincubating FLS with 1M glycerol for one hour, will not restore IL-1 β induced I κ B phosphorylation in cells that were heat treated. If glycerol indeed prevents protein insolubilization, then data presented herein would exclude protein denaturation as the modus operandi of HT in FLS. However, as also demonstrated in figure 3, glycerol itself is able to prevent IL-1 β induced I κ B phosphorylation. As

shown in figure 3B, lane “GLY + IL-1 β “, treating FLS with 1M glycerol for one hour will prevent IL-1 β induced I κ B phosphorylation rather than restore it.

Differential effect of short term HT on PMA induced gene activation. To get a better sense of early events affected by HT, PMA induced activation of proinflammatory genes was studied next. One reason for using PMA was that it reportedly does not rely on cell surface receptors for its action(s). Cell surface proteins could be among the first molecules affected by a temperature increase to 41°C. Data presented in figure 4 show that HT effects on PMA induced activation are selective and gene dependent. FLS were left untreated or exposed to HT for 30 minutes. Immediately after HT, PMA (2 ng/ml) was added where indicated. After 4 hours stimulation at 37°C, experiments were terminated by removing medium and dissolving FLS in TRIzol for subsequent mRNA isolation. As shown in figure 4, HT was able to completely prevent PMA induced accumulation of IL-1 β mRNA. The effects on IL-1 α and COX-2 mRNA levels were, however, less pronounced. Steady state levels of IL-1 α mRNA in hyperthermia treated cells are slightly less than 50% of controls and COX-2 mRNA levels were only reduced by approximately 50%.

Short term HT has a profound impact on mitogen activated protein kinases (MAPK). The MAPKs p38, JNK/SAPK and ERK take part in the initiation and progression of many inflammatory events. Due to their central position at the intersection of many signaling pathways, MAPK have increasingly become the target of drug interventions aimed at blocking activation of proinflammatory genes (17).

To investigate whether short term HT affects MAPK activation was the aim of the following experiments. FLS were left untreated, exposed to HT (41°C, 30 minutes), treated with IL-1 β (5 ng/ml) for 5 minutes, or first exposed to HT and subsequently

treated with IL-1 β . Shown in figure 5A are the somewhat surprising results of a series of such experiments. HT blocked IL-1 β induced phosphorylation of the MAPK p38 in FLS that were stimulated with IL-1 β . However, in FLS, HT alone resulted in the activation of ERK as well as of JNK/SAPK. For data shown in figure 5A, phosphorylation of p38 was measured at 5 minutes. ERK and JNK phosphorylation patterns were measured 60 minutes after simultaneously treating cells with IL-1 β and transferring cells back to 37°C.

Time course experiments show that HT induced ERK and JNK/SAPK phosphorylation is detectable within minutes but peaks at about 60 minutes in FLS that were exposed to HT for 30 minutes followed by incubation at 37°C (data not shown).

That short term HT can have a long lasting effect on IL-1 β induced p38 activation is shown in figure 5B. Where indicated, FLS were exposed to 41°C for 30 minutes and subsequently challenged with IL-1 β for 5 minutes, either immediately after HT or after the indicated periods at 37°C. Such Western blot experiments demonstrate that HT not only blocks p38 phosphorylation immediately after HT, but does so even 4 hours after HT. Additional data (not shown) imply that even 7 to 10 hours after HT, IL-1 β induced p38 activation is still significantly diminished.

Since, as shown in figure 5C, IL-1 β induced phosphorylation of p38 peaks roughly 5 minutes after addition of IL-1 β , the effect of HT on the activation of this kinase was analyzed at 5 minutes. Non-specific bands (marked with asterisks) and tubulin serve as loading and protein transfer controls.

Effects of HT on p38 and the NF- κ B signaling pathway are two independent events. From the above data the question arises whether inhibition of p38 by HT ultimately also accounts for the inhibition of the many ways HT is reportedly able to

prevent the activation of NF- κ B-dependent proinflammatory genes. Although unlikely, we tested whether blocking activation of MAPKs plays any role in the activation of NF- κ B in FLS. Cells were preincubated with known inhibitors of p38, JNK/SAPK and ERK. For comparison, FLS were left untreated or exposed to hyperthermia for 30 minutes prior to being stimulated with IL-1 β . As shown in figure 6, blocking p38 activation had no effect on IL-1 β induced I κ B α degradation. As in many experiments before, HT (30 minutes, 41°C) completely blocked IL-1 β mediated I κ B α degradation. Even more remote is the possibility that other MAPKs play a role in the NF- κ B pathway upstream of IL-1 β induced I κ B degradation. This possibility has, however, also been refuted by experiments demonstrating that in FLS blocking JNK/SAPK and ERK were without any effect on IL-1 β mediated I κ B α degradation (data not shown).

HT prevents transcriptional activation of genes that are regulated by the MAPK p38. Two observations made in this laboratory were used to further test the hypothesis that inhibition of p38 by HT is entirely independent of effects of HT on the NF- κ B pathway. We demonstrated earlier that TGF- β activates hyaluronansynthase1 (HAS1) via a NF- κ B independent pathway (12). However, TGF- β induced activation of HAS1 in FLS relies on an intact p38 MAPK signaling pathway (18). We made use of this phenomenon and show in figure 7 that blocking p38 by short term HT results in highly significant inhibition of TGF- β induced HAS1 transcription. Where indicated, cells were exposed to 41°C for 30 minutes prior to stimulation with TGF- β (2 ng/ml) for either 8 or 14 hours. At both time points analyzed, HAS1 mRNA levels in cells exposed to short term HT were significantly lower than in cells that did not undergo such treatment.

Discussion

Despite many novel treatment options available today, rheumatoid arthritis is still a debilitating disease whose progression can only be slowed but not cured. Inflammation plays an important role in ongoing joint destruction. For this reason most drugs used today aim at interrupting cell to cell communication that is contributing to inflammatory processes. While immunocompetent white blood cells clearly play an important role in arthritis, evidence has been presented that FLS could be of significance in the etiology of rheumatoid arthritis (5).

We and others have demonstrated that FLS are not only able to respond to but also express and/or release a number of proinflammatory cytokines, chemokines and adhesion molecules known to be potent mediators of inflammation (4). We demonstrated earlier that short term hyperthermia might offer the appealing opportunity to prevent or reduce inflammation associated, for example, with rheumatoid arthritis by blocking the activation of NF- κ B (4). Rheumatoid arthritis seems a disease that offers the rather unique opportunity to make use of short term HT to disrupt or at least diminish inflammatory processes. Due to tight control mechanisms, it is nearly impossible to raise the body core temperature significantly without harming the organism. However, normally the temperature in joints is not only considerably lower than the body core temperature, it also seems much easier to increase the temperature in joints to the degree needed for the initiation of a sustained, protective heat shock response. The temperature in joints is about 32°C and the reportedly needed increase in temperature by 6°C for prolonged protective effects (8) might not pose a serious hurdle for an in vivo implementation of HT as a means to ameliorate inflammation in joints.

While, as previously shown, unwanted gene activation in FLS could indeed be diminished by short term HT, the mechanisms involved seem manifold and still largely unknown. By and large, it is thought that the protective effects of HT are due to the transcription and translation of heat shock proteins that in turn interact with the activation of proinflammatory genes by a multitude of mechanisms (19). However, time course experiments done in this laboratory pointed at yet another mode of action that accounts for the very early anti-inflammatory effects of HT.

As shown in this manuscript, two very important signaling pathways are blocked by short term HT. Also noteworthy is that, at least in FLS, HT clearly blocks these signaling pathways independently of the need for de novo synthesis of heat shock proteins, as protective effects of HT are detectable long before the appearance of newly synthesized HSPs. Over the years, substantial effort has been put into finding ways to specifically block the NF- κ B pathway. As demonstrated again herein, short term HT is a very effective method to prevent events in FLS that would otherwise result in the activation of NF- κ B dependent genes.

Of similar importance is the demonstration that short term HT is able to block the activation of p38, a serine kinase that plays a central role in numerous proinflammatory responses (17). Similar to the effect on NF- κ B, HT effects on MAPK p38 do not require de novo protein synthesis. As demonstrated, a mere 10 to 15 minutes exposure to 41°C is sufficient to block IL-1 β induced p38 activation for several hours.

The MAPK p38 has increasingly become the focus of researchers aiming at interrupting inflammatory processes. As outlined by Schieven (17) the main reasons are that the MAPK p38 regulates the production of key inflammatory mediators such as TNF α , IL-1 β and COX-2 and, in addition, also acts downstream of cytokines such as

TNF α . The potential efficacy of p38 inhibition may thus be greater than would be expected from the inhibition of the mediators alone (17).

It was surprising to note the differential effect of short term HT on MAPKs. As shown, IL-1 β induced activation of p38 is blocked for several hours. However, HT acts as a potent inducer of ERK and JNK/SAPK phosphorylation in FLS. While the consequences of ERK and JNK/SAPK activation by HT are currently unclear, the relevance of MAPK p38 inhibition for the benefit of arthritis patients seems obvious. Inhibition of p38 has been hailed as a new approach for treating inflammatory disorders such as rheumatoid arthritis (20). It has also been shown, that blocking p38 activation will prevent bone destruction (21). In addition, several studies confirmed the central role of p38 in the progression of rheumatic disorders (21-25). Because of their promise as a potent treatment option, several of the p38 inhibitors are currently under development or are already in phase I or II clinical trials (21).

There are only a limited number of reports analyzing early effects of HT on MAPK. Among these, one group reported activation of p38 by HT (26). Whether such differences are due to the cell type used, CCL39 and HeLa versus FLS, or to the fact that cells were exposed to 44°C in their experiments, is currently unclear (26). Yet, preliminary data from our laboratory imply that under the conditions used to treat FLS (41°C for 30 minutes), HT seemingly also blocks p38 in cells other than FLS (data not shown). However, other mechanisms could be at work as well, since cell type specific variations in the activation pattern of HSPs by HT have indeed been demonstrated (27). Then again, the early events leading to the prevention of p38 as well as NF- κ B activation in FLS are independent of the need for de novo synthesis of HSPs.

Taken together, data are presented which demonstrate that short term HT is able to prevent independently the activation of two important signaling pathways that are essential for the upregulation of many proinflammatory genes. If these in vitro data could be reproduced in in-vivo experiments and/or extended to other cell types, it would be appealing to speculate that eventually patients could greatly benefit from this simple and inexpensive method to ameliorate inflammation associated with arthritis.

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Figure Legends

Figure 1. Short term HT prevents NF- κ B activation. FLS were exposed to temperatures of up to 43°C and subsequently stimulated with IL-1 β (5 ng/ml) for 20 minutes. Figure 1A shows that exposing FLS to 41°C for 30 minutes is sufficient to completely prevent IL-1 β induced I κ B α degradation. As shown in figure 1B, FLS exposed to 41°C for 30 minutes regain the ability to utilize the NF- κ B pathway within 20 hours. As also shown in figure 1B, increasing the temperature to 43°C for 30 minutes blocks IL-1 β induced I κ B α degradation even after 20 hours. Figure 1C shows a representative time course experiment of IL-1 β induced I κ B phosphorylation. Figure 1D demonstrates that exposing FLS to 41°C for 5 or 10 minutes is not sufficient to block IL-1 β (5 ng/ml for 5 min) induced I κ B phosphorylation. However, after 15 minutes at this temperature cells are no longer able to respond to IL-1 β with the phosphorylation of I κ B α if challenged with IL-1 β for 5 minutes at 37°C. Next to staining with Ponceau red, tubulin (Tub) as well as non-specific bands (ns) serve as controls for protein transfer. In the gel in figure 1B, two medium and IL-1 controls were run. In Fig. 1C, the time of expose to IL-1 β is indicated in seconds and minutes.

Figure 2. De novo synthesis of HSPs is not essential for HT mediated blockage of NF- κ B activation. FLS were pretreated either with Cycloheximide (figure 2A) or Emetine (figure 2B) to prevent de novo protein synthesis in response to the 30 minutes of HT. As shown here and in figure 1, HT blocked IL-1 β induced I κ B phosphorylation. More importantly, HT prevents also I κ B phosphorylation in FLS whose protein synthesis machinery was blocked.

Figure 3. Glycerol prevents IL-1 β induced I κ B phosphorylation. As shown in figure 3A, at high concentrations, glycerol itself prevents IL-1 β induced I κ B phosphorylation in FLS. Figure 3B shows that glycerol does not restore IL-1 β induced activation of the NF- κ B pathway in cells exposed to short term HT. Cells were pretreated with glycerol (1 – 1000 mM) for 1 hour and stimulated with IL-1 β (5 ng/ml) for 5 minutes. Where indicated, cells were exposed to HT for 30 minutes prior to stimulation with IL-1 β .

Figure 4 HT affects steady state mRNA levels of proinflammatory genes in PMA treated cells. In FLS, HT (41°C, 30 min.) completely prevents PMA (2 ng/ml for 4 hours) induced IL-1 β transcription. Short term HT, however, is less effective in reducing IL-1 α and COX-2 steady state mRNA levels in PMA treated FLS.

Figure 5. HT blocks IL-1 β induced phosphorylation of p38 but activates ERK and JNK/SAPK. IL-1 β induced phosphorylation is blocked in FLS that were exposed to short term HT (41°C, 30 min). Also shown in figure 5A is that HT alone results in phosphorylation of ERK and JNK/SAPK. The effect of short term HT on p38 lasts for several hours as shown in figure 5B. Shown in figure 5C is a time course experiment analyzing IL-1 β induced p38 phosphorylation.

Figure 6. The MAPK p38 plays no role in IL-1 β induced I κ B α degradation. Cells were preincubated (45 minutes) with known inhibitors of p38, JNK/SAPK and ERK

activation and subsequently stimulated with IL-1 β (5 ng/ml) for 20 minutes. The concentration of the p38 inhibitor SB203580 (SB) used is sufficient to completely block IL-1 β induced p38 phosphorylation (data not shown). As shown in figure 6, IL-1 β induced I κ B α degradation was not affected by the presence of SB203580. Blocking JNK/SAPK and ERK were without any effect on IL-1 β mediated I κ B α degradation (data not shown). Also shown are a number of controls showing that neither SB nor HT (alone or in combination) had any effect on I κ B α degradation.

Figure 7. HT prevents transcriptional activation of p38 dependent genes. Steady state mRNA levels of HAS1 are significantly lower in FLS that were exposed to short term HT (30 minutes, 41°C) prior to stimulation with TGF- β for 8 (left panel) and 14 hours (right panel) respectively. The labels “MED” and “TGF” indicate data resulting from positive and negative controls.

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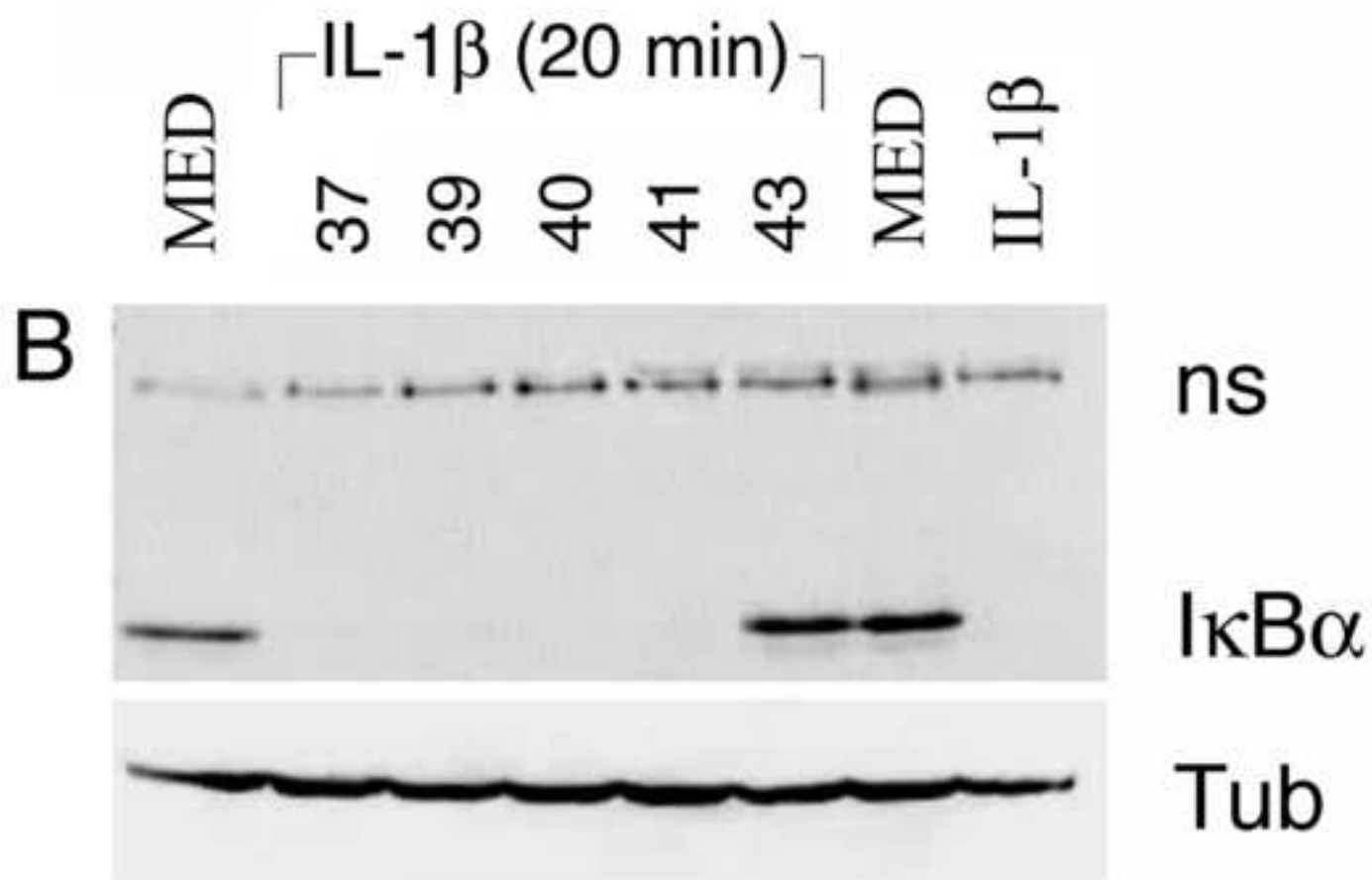
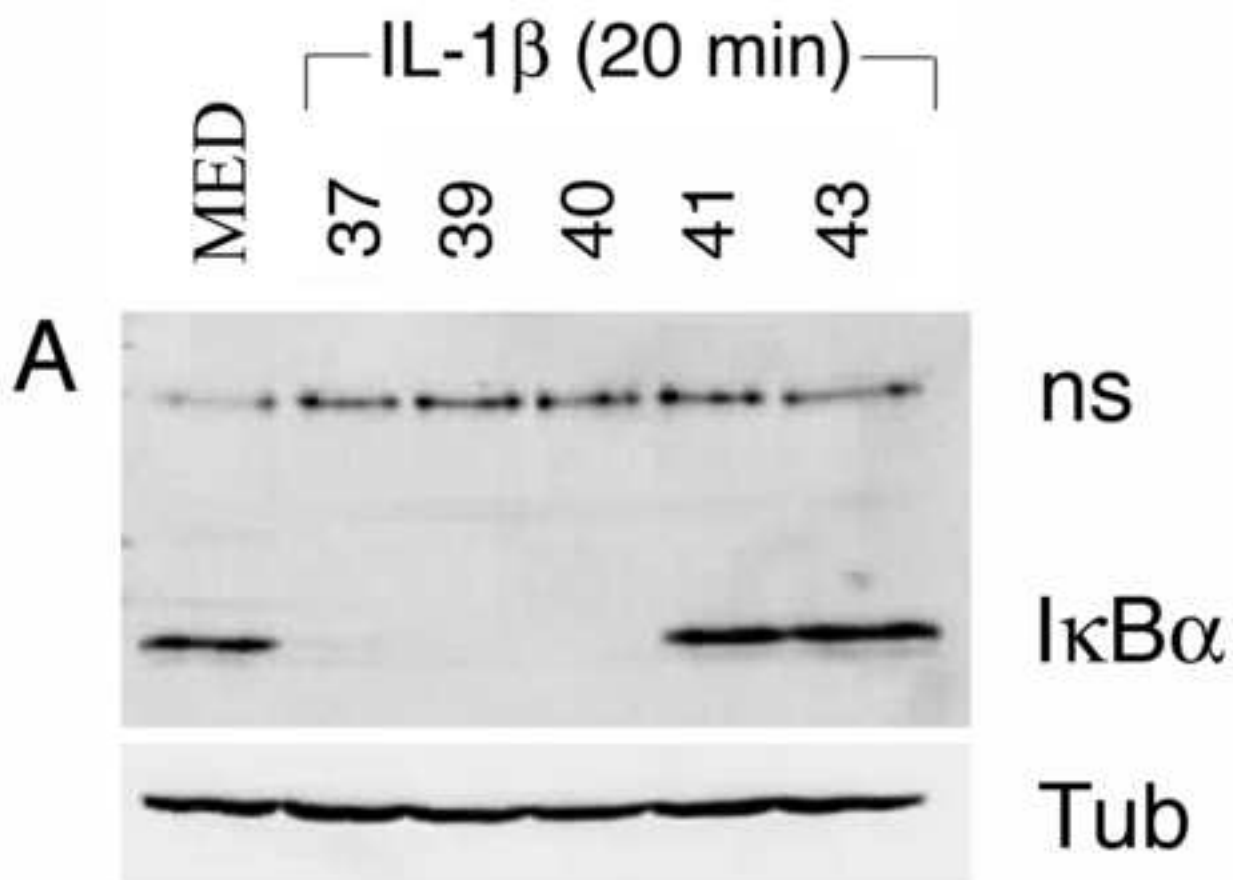
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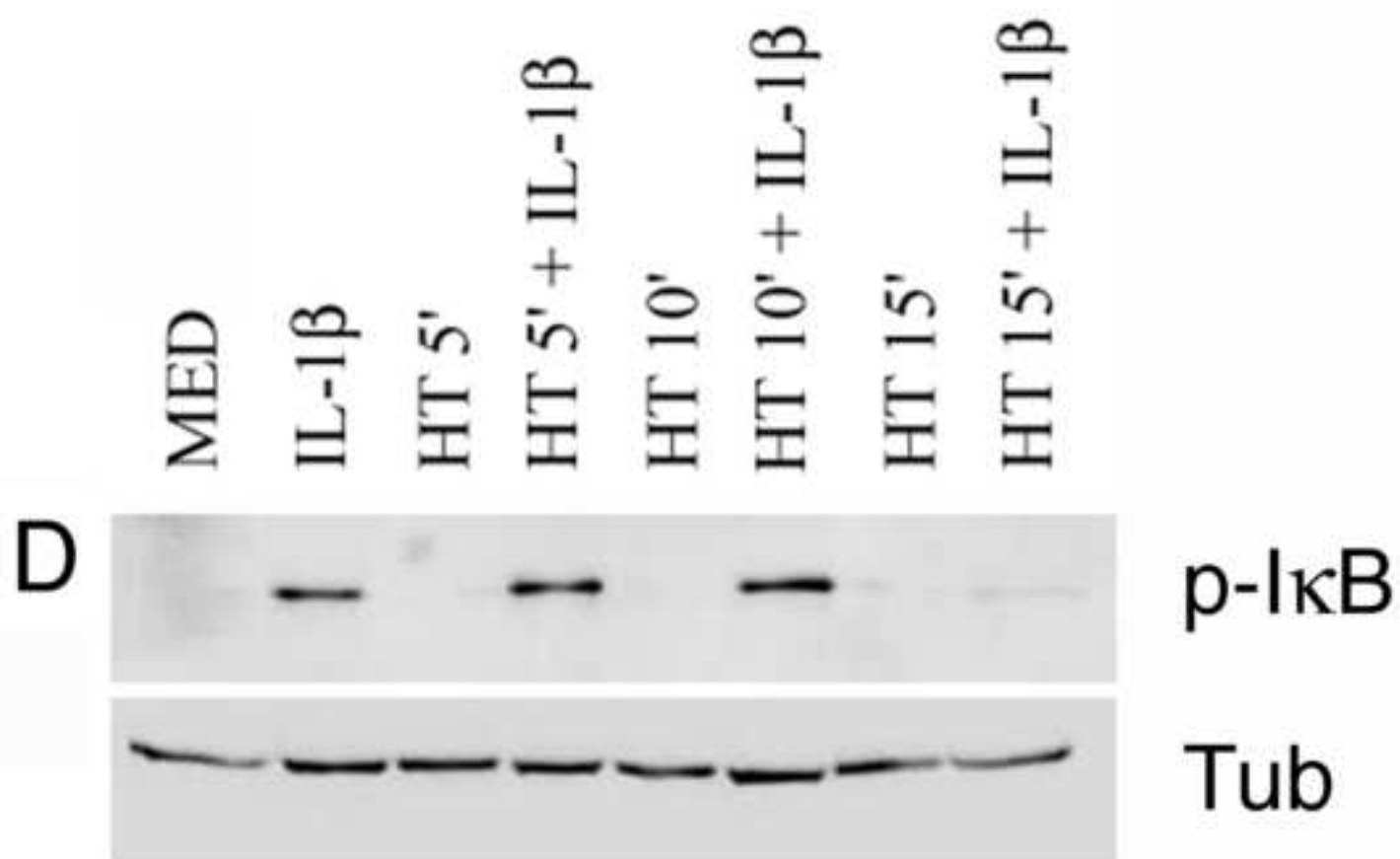
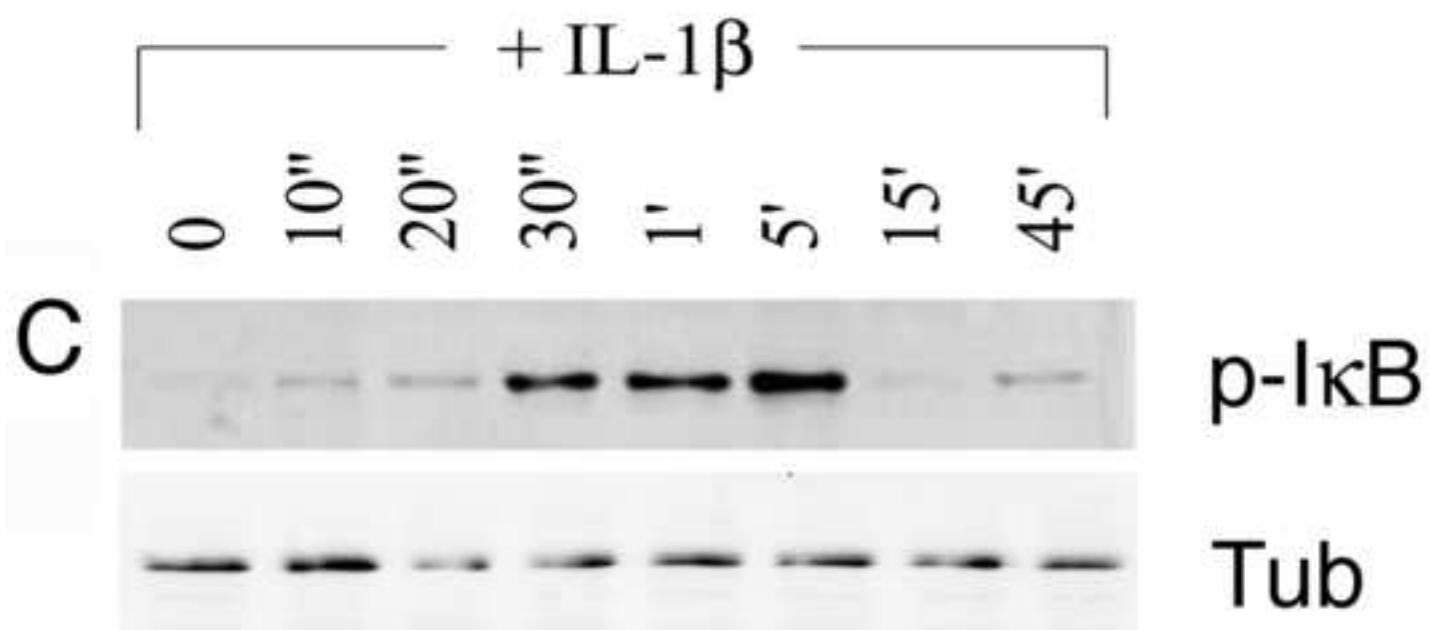
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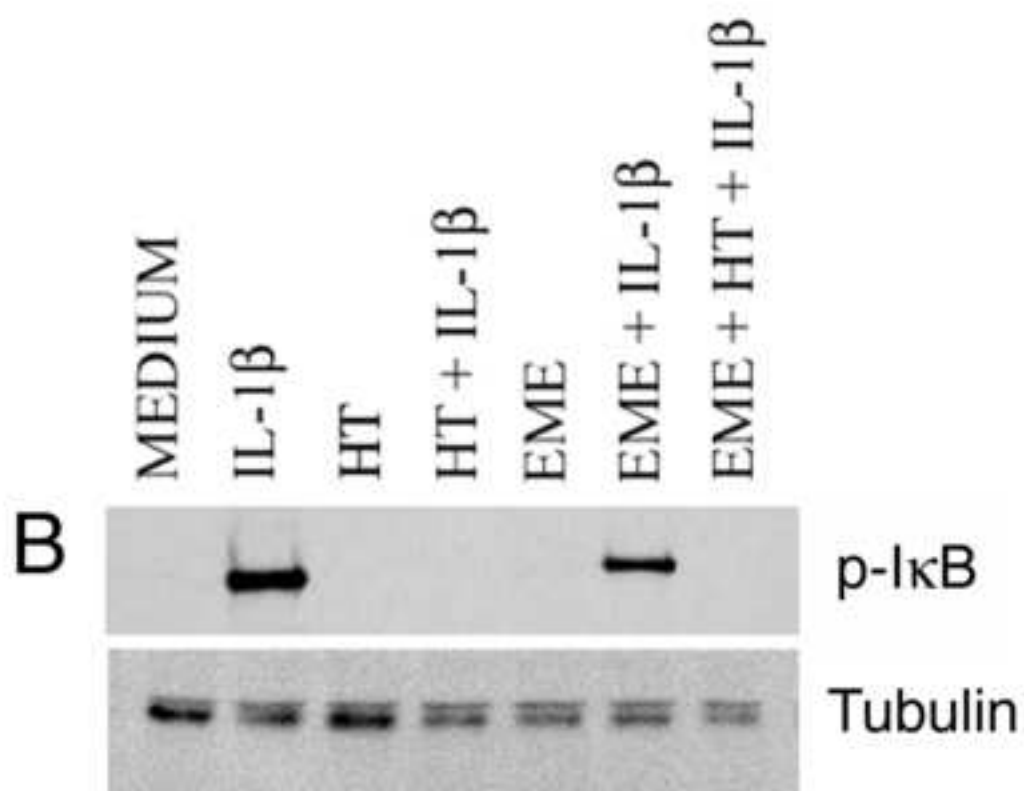
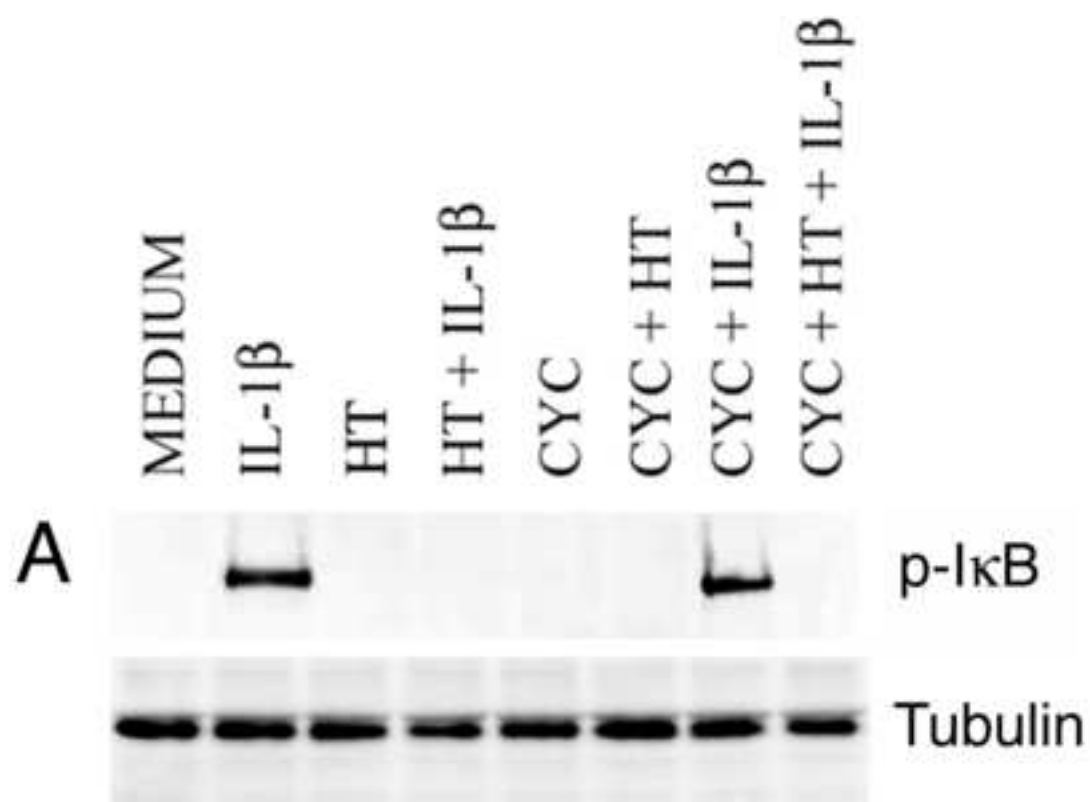
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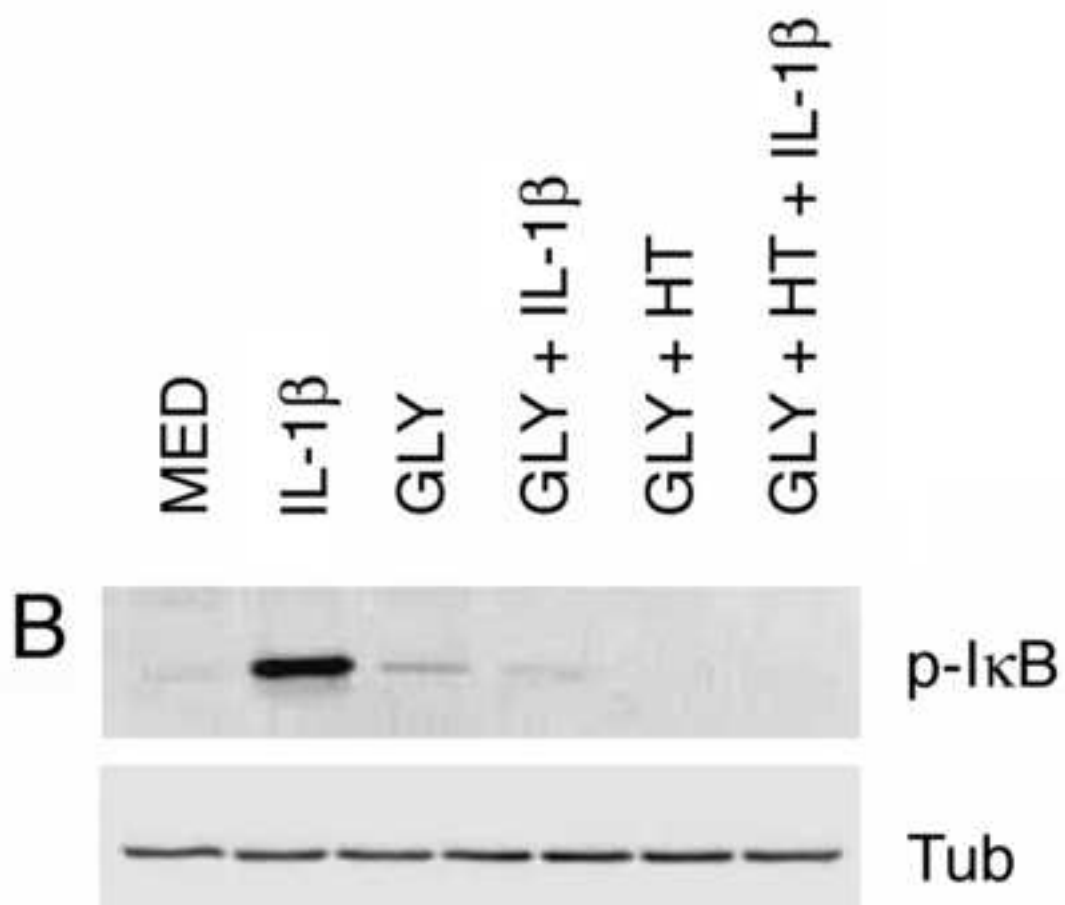
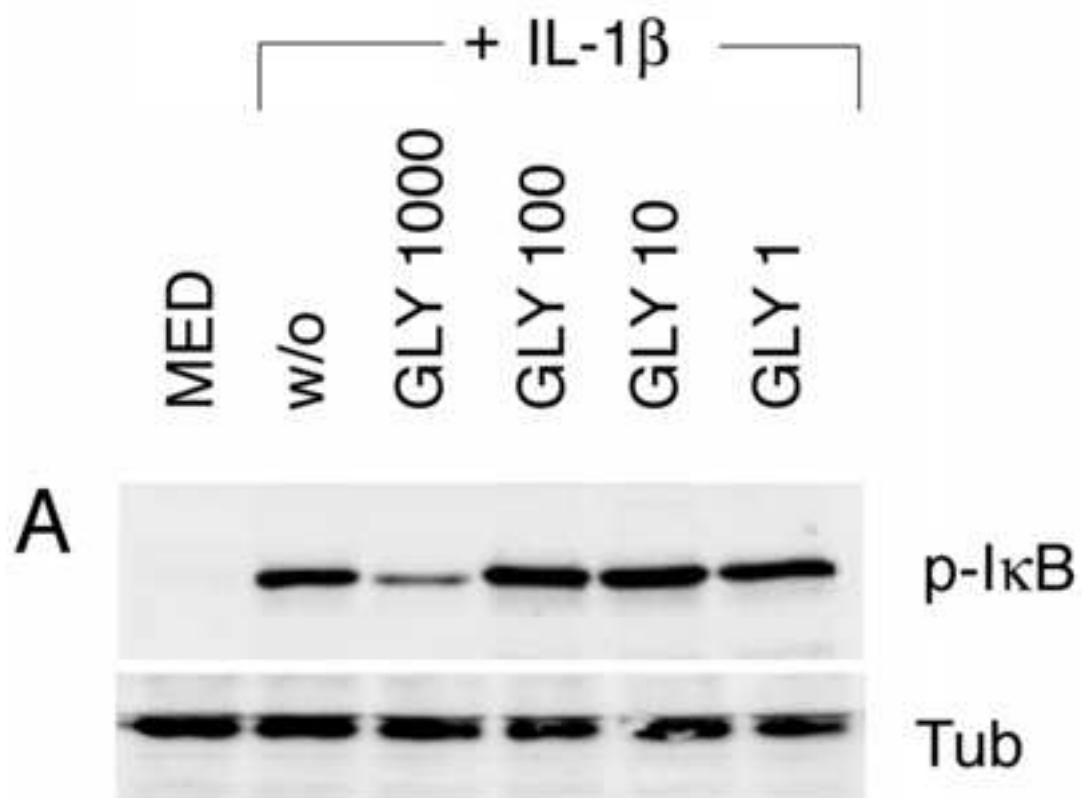
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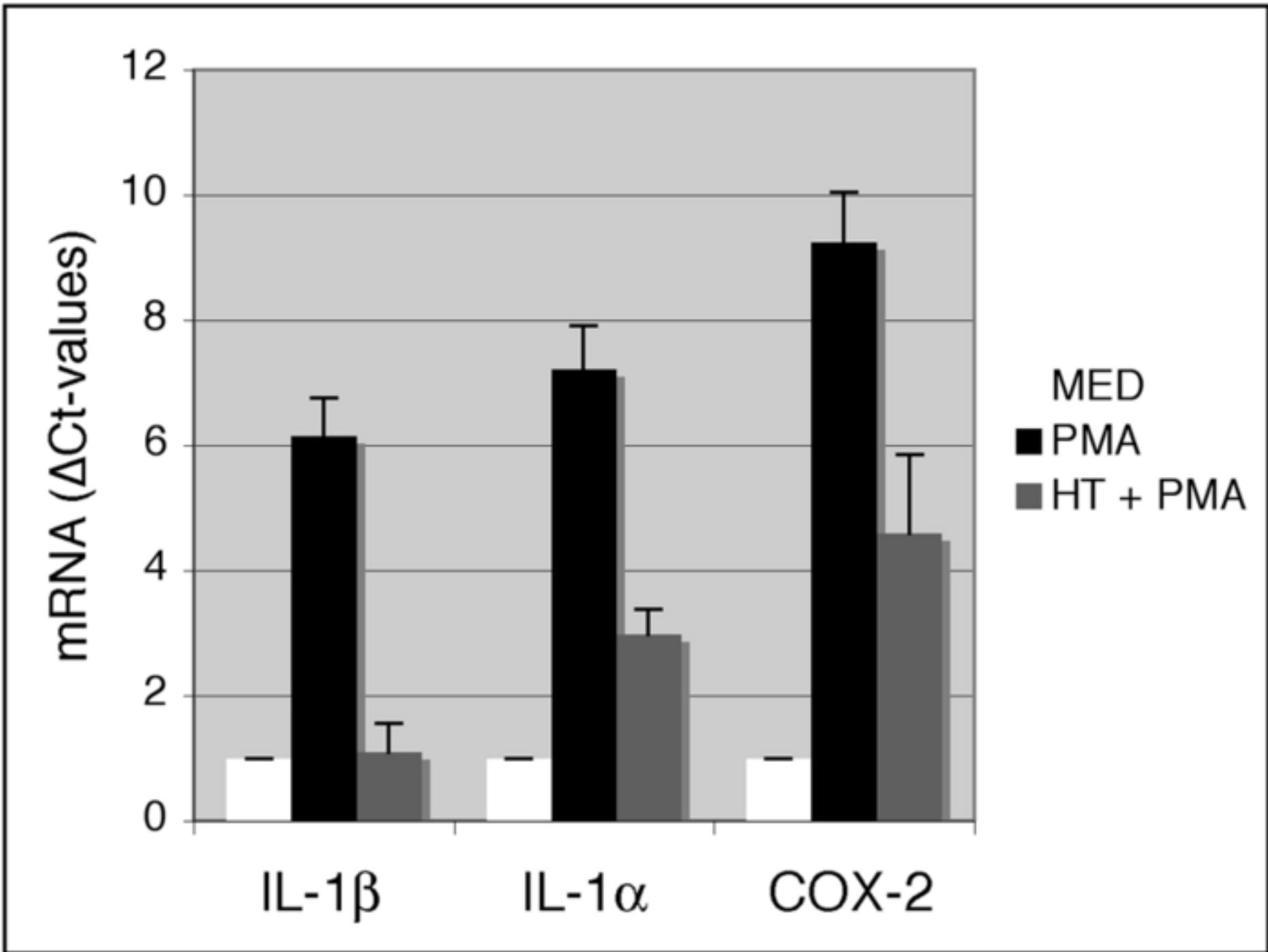


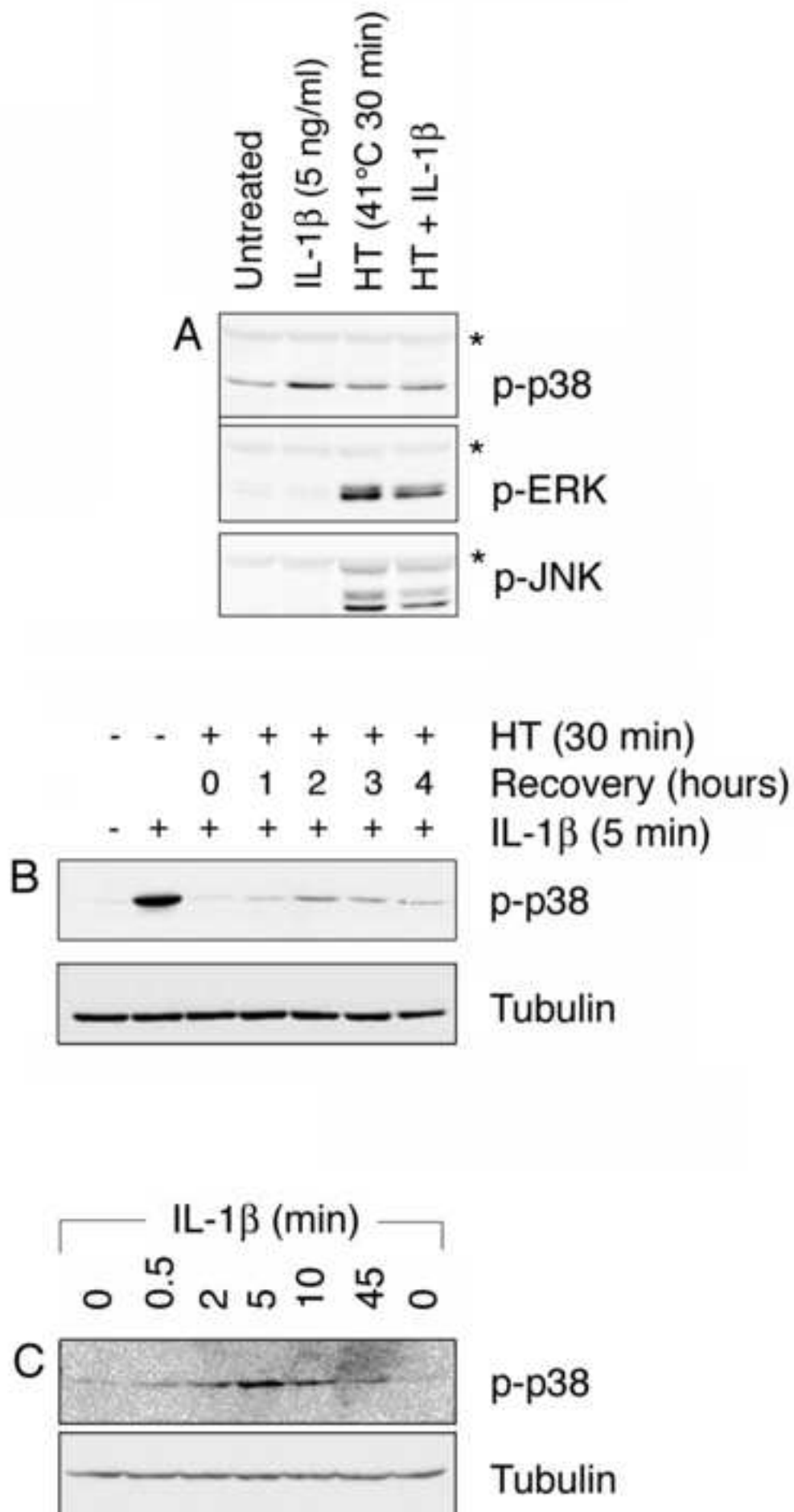


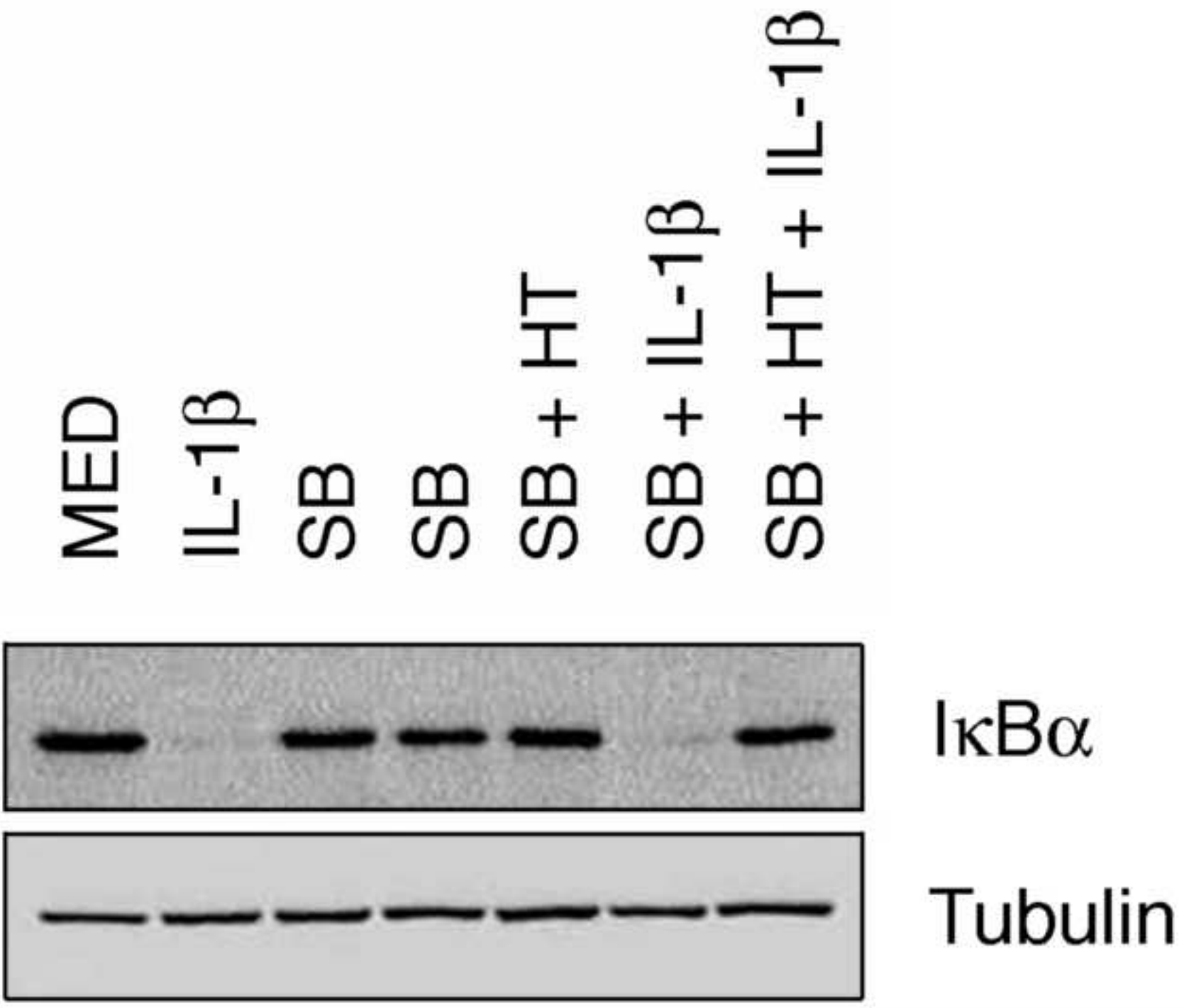




Figure(s) 4







Figure(s) 7

