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# Heat Stress Induces Extended Plateau of Hsp70 Accumulation – A Possible Cytoprotection Mechanism in Hepatic Cells

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## ABSTRACT

The relevance of heat preconditioning resides in its ability to protect cells from different kinds of injury by induction of heat shock proteins, a process in which the intensity of heat stress (HS) and duration of subsequent recovery are vital. This study evaluates the effects of moderate HS (45 min/43°C) and the time-dependent changes during recovery period of HSP70, Bcl-2 and p53 gene and protein expression in HepG2 cells. We also evaluated the effects of 0.4 mM aspirin (ASA) as a potential pharmacological co-inducer of HSP, both alone and in a combination with HS (ASA + HS). HS alone and ASA + HS caused a major up-regulation of HSP70 mRNA in the first 2 h, while HSP70 protein increased gradually and was especially abundant from 2 h to 24 h. Regarding Bcl-2, all treatments rendered similar results: gene expression was down-regulated in the first 2 h, after which there was protein elevation (12–48 h after HS). mRNA expression of p53 in HS- and (ASA + HS)-cells was down-regulated in the first 12 h. The immediate decrease of p53 protein after HS was followed by a biphasic increase. In conclusion, 0.4 mM ASA + HS does not act as a co-inducer of HSP70 in HepG2 cells, but promotes Bcl-2 protein expression during prolonged treatment. Our suggestion is that hepatic cells are most vulnerable in the first 2–6 h, but may have a high capacity for combating stress 12–24 h after HS. Finally, short-term exposure HS might be a "physiological conditioner" for liver cells to accumulate HSP and Bcl-2 proteins and thus obtain cytoprotection against an additional stress. J. Cell. Biochem. 116: 2365–2374, 2015. 

**KEY WORDS:** HEAT PRECONDITIONING; HSP70; ASPIRIN; LIVER REGENERATION

A number of animal studies have shown that pre-exposure of the liver to transient ischemia, hyperthermia, or mild oxidative stress raises tolerance to additional injury, a phenomenon known as hepatic preconditioning [Carini and Albano, 2003]. Heat preconditioning has been proposed as a promising therapeutic strategy for ischemia/reperfusion injury in the liver [Terajima et al., 1999; Yamagami et al., 2003; Oba et al., 2010], as a mechanism for increasing the survival rate in a model of steatotic liver transplantation in the rat [Mokuno et al., 2004], and as an approach to preventing liver surgery injury [Carini and Albano, 2003].

Abbreviations: ASA, acetylsalicylic acid; Bcl-2, B-cell lymphoma 2; NSAIDS, non-steroidal anti-inflammatory drugs; HSP70, heat shock protein 70; HSR, heat shock response; STZ, streptozotocin.

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In particular, enhanced cell survival has been linked to the expression of specific molecular chaperones, such as heat shock proteins (HSPs). HSPs act not only as molecular chaperones, but also as housekeeping molecules [Zhu et al., 2009], and among them, HSP70 is a decisive negative regulator of the mitochondrial pathway of apoptosis that can block apoptosis at different levels [Schmitt et al., 2007]. Moreover, HSP70 seem to be intrinsically connected to the anti-apoptotic Bcl-2 and the proapoptotic stimulator p53 in regulating cell survival/cell death under stress conditions. In this sense, HSP70 has been shown to affect several transcription factors involved in the expression of Bcl-2 proteins, whereas Bcl-2 is among the transcriptional targets whose expression is repressed by the tumor suppressor protein p53. In addition, heat stress induces p53 protein by increasing p53 mRNA, a process that is involved in HSP70 regulation [Sharma, 2010].

More recently, beside the non-pharmacological induction of HSP through heat preconditioning, some pharmacological therapies involving chemical inducers of HSPs have also been applied, such as treatments with L-arginine [Chatterjee et al., 2005], poly(ADP-ribose) polymerase (PARP) inhibitors [Mota et al., 2008], glutamine [Ziegler et al., 2005], and aspirin [Jurivich et al., 1992]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as sodium salicylate, aspirin and indomethacin, originally known to inhibit cyclooxygenase activity, have been reported to modulate the heat shock response (HSR) in yeast [Giardina and Lis, 1995], Drosophila [Winegarden et al., 1996], rats [Fawcett et al., 1997] and different types of cells in culture [Jurivich et al., 1992; Lee et al., 1995; Koo et al., 2000]. However, salicylate triggers heat shock factor (HSF) differently to heat [Jurivich et al., 1995].

In general terms, the development of hepatic preconditioning has been differentiated into two stages: an immediate or early phase (minutes to hours) and a late stage, shown to offer protection for up to 2–3 days. The phenomenon of liver preconditioning by induction of HSPs is still not fully understood, but two factors seem to be determining: the right timing of the preconditioning and the size of the effect produced. Namely, it is very important to know the precise time-dependent changes, first of all those of HSP70 and also of other parameters, in order to estimate the sensitivity and resistance of the cells in additional application of other stress types or in the process of liver transplantation. In this study we have aimed to closely monitor the effect of non-pharmacological (moderate heat stress (HS) 45 min/ 43°C) and pharmacological (pre-treatment with aspirin) induction of HSP70 in cultured hepatic cells (HepG2) over a specific time course (0, 1, 2, 6, 12, 24, 36, and 48 h recovery period at 37°C). Dynamics of gene and protein expression of HSP70 were compared with those of two other relevant markers of cell damage: Bcl-2 and p-53. We have also addressed whether these cellular markers are affected by treatment with 0.4 mM aspirin (ASA- acetylsalicylic acid), alone and in a combination to HS.

## MATERIALS AND METHODS

### REAGENTS AND DRUGS

Unless stated otherwise, chemical reagents were purchased from Sigma-Aldrich (Steinheim, Germany), including aspirin (ASA-acetylsalycilic acid) (Cat No. A5376).

### CELL CULTURE AND TREATMENTS

Experiments were performed with the human hepatocellular carcinoma cell line HepG2 (ATCC® HB-8065™), a model routinely employed for a variety of functional studies, including liver toxicity testing. Cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate, penicillin (50 units/mL) and streptomycin (50 μg/mL). Unless stated otherwise, all the reagents employed in the cell culture were purchased from GIBCO (Invitrogen, Eugene, OR). Cell cultures were maintained in an incubator (IGO 150, Jouan, Saint-Herblain Cedes, France) at 37°C, with a humidified atmosphere of 5% $CO_2$/95% air (AirLiquide Medicinal, Valencia, Spain). Treatments were performed in complete cell culture medium.

Heat stress treatment (at 43°C) was administered for 45 min (T-25 flasks were placed in another incubator, with regulated temperature of 43°C, with the rest of the parameters kept constant), followed by varying periods of recovery at 37°C (0, 1, 2, 6, 12, 24, 36 and 48 h) before collection of the cells. Another set of T-25 flasks (both with and without HS-exposure) were treated with 0.4 mM ASA. This concentration of ASA was selected as it does not affect cell proliferation or modify heat resistance when applied alone [Amici et al., 1995]. According to Raza et al. (2011) concentrations higher than 1 mM ASA have been shown to cause cell apoptosis, oxidative stress, and cell cycle arrest in Hep2G cells. Importantly, we confirmed the lack of cytotoxicity when 0.4 mM ASA was used in our model. The stock solution (40 mM) was prepared by dissolving the drug in 1 M Tris–HCl pH 7.6 and adjusted to pH 7.45 with 4 N HCl, analogous to the dissolving protocol of Raza et al. (2011). Final dilution of ASA was prepared in DMEM and filtrated with 0.2 μm filter (VWR, Barcelona, Spain). Three different treatments were performed: 1) HS- groups: cells exposed only to HS and allowed to recover at 37°C over different periods of time; 2) HS + ASA- groups: cells treated with ASA just before the HS-exposure and allowed to recover at 37°C for different periods; 3) ASA-groups: normothermic cells treated with ASA alone, applied 45 min before initiation of the recovery period and maintained during the recovery period.

### PROLIFERATION AND VIABILITY (MTT ASSAY)

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diprenyl tetrazolium bromide) assay is a colorimetric assay based on the ability of cells to reduce a soluble yellow tetrazolium salt to blue formazan crystals [Mosmann, 1983]. This reduction takes place only when mitochondrial reductase enzymes are active, and is thus a marker of cell viability related to mitochondrial function. Treatments were performed in 96-well plates, and MTT reagent (Roche Diagnostics, Mannheim, Germany) was added for the last 4 h of treatment (20 μL/well). Cells were then solubilized with DMSO (100 μL/well, 5 min, 37°C) and absorbance (570 nm) was detected using a "Multiscan" plate-reader spectrophotometer (Thermo Labsystems, Thermoscientific, Rockford, IL).

### PROTEIN EXTRACTS AND WESTERN BLOTTING

Whole cell protein extracts (n = 5–10) were obtained using T-25 flask cell cultures. Whole-cell protein extracts were obtained by lysing cell pellets in 50–100 μL complete lysis buffer (20 mM

HEPES pH = 7.4, 400 mM NaCl, 20% (v/v) glycerol, 0.1 mM EDTA, 10 μM $Na_2MoO_4$, 1 mM DTT) supplemented with protease inhibitors ("Complete Mini" protease inhibitor cocktail, and "Pefabloc," both from Roche Diagnostics) and phosphatase inhibitors mixture (10 μM NaF, 10 mM $NaVO_3$, 10 mM p-nitrophenylphosphate (PNPP) and 10 mM β-glycerolphosphate). Samples were vortexed, incubated on ice for 15 min, vortexed again and centrifuged in a microcentrifuge at 16100*g* for 15 min at 4°C. Protein content was quantified employing the "BCA Protein Assay Kit" (Pierce, Thermo Scientific, Rockford, IL). SDS–PAGE and WB were performed using standard methods (BioRad, Hercules, CA), with 30 μg of the protein extract.

We used the following primary antibodies: Anti-HSP70 mouse mAb (C92F3A), Cat No. 386032, Calbiochem, applied at 1:1000; Anti-Bcl-2 mouse mAb (100): Cat No. sc-0509, Santa Cruz Biotechnologies, 1:500; Anti-p53, rabbit polyclonal N- Terminal, Cat No. SAB4503001, Sigma–Aldrich, 1:1000; Anti-PARP ½ (H-250), rabbit polyclonal, Cat No. sc-7150, Santa Cruz Biotechnologies, 1:500 and anti-actin rabbit polyclonal, Sigma–Aldrich 1:500. We used the following secondary antibodies: Peroxidase-labeled anti-rabbit IgG from Vector laboratories (Burlingame, CA) at 1:5000 and anti-mouse antibody from Dako (Glostrup, Denmark) at 1:2000. Immunolabelling was detected using the enhanced chemiluminescent reagent ECL (Amersham, GE Healthcare, Little Chalfont, UK) or SuperSignal WestFemto (Pierce, Thermo Scientific, Rockford, IL), and was visualized with a digital luminescent image analyzer (FUJIFILM LAS 3000, Fujifilm). ImageQuant software v. 4.0. was employed for densitometric analysis.

### RT-PCR

Real time RT-PCR (from 3 to 6 repeats, in duplicate) was performed using mRNA of t-25 flask cell cultures. Total RNA was extracted (RNeasy Mini Kit, Qiagen, Hilden, Germany), eluted in 30–50 μL of water and quantified (NanoDrop - ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE). For first strand cDNA synthesis (Prime Script RT Reagent Kit, Takara, Otsu, Japan), 2 μg of total RNA was reverse-transcribed in a final volume of 20 μL (15 min/37°C and 5 s/37°C).

PCR reactions (Carousel-based LightCycler® 2.0 Real Time PCR System, Roche Applied Biosystems) were performed mixing 2 μL of cDNA with LightCycler® FastStart DNA MasterPLUS SYBR Green I master mix (Roche Applied Science, Mannheim, Germany). Primers 1 μM (TIB MOLBIOL, Berlin, Germany, Table I) were added in final reaction volume of 10 μL. The reactions were: denaturation- 30 s/95°C, amplification- 5 s/95°C, 20 s/60°C (60 cycles), melting- 15 s/65°C and cooling - 30 s /40°C. Normalized results (values for each sample divided by the corresponding value for actin, employed as a housekeeping gene) were expressed as fold expression of the specific gen/actin ratio vs. control (normothermic cells).

### LIVE CELL FLUORESCENCE MICROSCOPY COUPLED WITH STATIC CYTOMETRY

All treatments were performed in duplicate in 48-well plates, and 16-30 live cell images per well were immediately recorded with a fluorescence microscope (IX81, Olympus, Hamburg, Germany) coupled with "ScanR" version 2.03.2 static cytometry software (Olympus). Nuclei were stained with the fluorochrome Hoechst 33342 (2.5 μM) for the last 30 min of treatment. Specific fluorochromes indicative of several aspects of mitochondrial function were added for the last 30 min of treatment. TMRM (5 μM), MitoSox (2.5 μM) (both from Molecular Probes, Invitrogen) were used to assess $\Delta\Psi_m$ and superoxide production, respectively. Rotenone (mitochondrial Complex I inhibitor) and CCCP (uncoupler of oxidative phosphorylation) were employed as positive controls for assessment of superoxide generation and $\Delta\Psi_m$ respectively.

### PRESENTATION OF DATA AND STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism v.3 software with a Student's *t*-test. All values are expressed as a mean ± SEM (statistical significance $P < 0.05$). Data are represented as % of control, with untreated, normoxic cells being considered 100%.

## RESULTS

### OVER-TIME DYNAMICS OF HSP70 mRNA AND PROTEIN EXPRESSION

As shown in Figure 1A, HSP70 mRNA expression peaked (about 50–200 fold increase) just after HS, maintained until 2 h post-HS (0 h, 1 h, 2 h), and subsequently waned, reaching control values or even slightly lower. Interestingly, ASA alone caused a significant decrease of HSP70 mRNA from the very beginning of treatment, with normalization (control values) being detected by the end of the experimental period. The combined treatment of ASA + HS did not produce any significant changes in mRNA expression when compared to HS alone.

The increase in HSP70 protein levels (Fig. 1B,C) manifested a certain time delay and followed a more gradual dynamics than mRNA; it peaked from 6 h to 24 h (approximately a 6–14 fold increase) and a slow decrease took place thereafter without reaching control values (untreated cells), even 48 h after treatment. ASA treatment alone caused a small but significant increase (1.3-1.7 fold, $P < 0.05$) of HSP70 protein levels with respect to untreated control cells only in the first 6 h following treatment. The combined treatment (HS + ASA) manifested virtually the same dynamics as exposure to HS alone, but values for the HSP70 protein level (at 6 and 12 h) were non-significantly changed.

### OVER-TIME DYNAMICS OF BCL-2 mRNA AND PROTEIN EXPRESSION

All three treatments (ASA, HS and ASA + HS) manifested almost the same dynamics regarding expression of the Bcl-2 gene in the recovery period (Fig. 2A). Namely, a significant decrease was observed in the first 6 h of the recovery period, followed by a gradual recovery towards normalized levels at 24 h of HS recovery. Bcl-2 gene expression was also slightly decreased at the end of the experimental period (36 and 48 h). Interestingly, ASA alone caused a significant up-regulation of Bcl-2 protein levels (Fig. 2B,C), reaching statistical significance form 12 h to 48 h. Both HS-cells and ASA-treated HS-cells manifested almost the same dynamics of change, with maximum protein expression being recorded at 12, 24, and 36 h, and tendency towards normalization by the end of the experimental period.

TABLE I. Primer Sequences of Specific PCR Products for Each of the Genes Analyzed

| Gene Symbol | Sequence direction | Sequence |
|---|---|---|
| actin | s | 5′-GGACTTCGAGCAAGAGATGG-3′ |
| | as | 5′-AGCACTGTGTTGGCGTACAG-3′ |
| HSP70 | s | 5′-TAC AAG GGG GAG ACC AAG GCA-3′ |
| | as | 5′-TGG GAG TCG TTG AAG TAG GC-3′ |
| Bcl-2 | s | 5′-ATC GCC CTG TGG ATG ACT GAG-3′ |
| | as | 5′-CAG CCA GGA GAA ATC AAA CAG-3′ |
| p53 | s | 5′-TGA CAC GCT TCC CTG GAT TG-3′ |
| | as | 5′-GCT CGA CGC TAG GAT CTG AC-3′ |

### OVER-TIME DYNAMICS OF P53 mRNA AND PROTEIN EXPRESSION

Single ASA treatment produced a small but significant increase of p53 mRNA expression in the first 2 h, followed by significantly lower expression towards the end of the experimental period (Fig. 3A) compared to normothermic cells. The p53 mRNA expression in HS- and ASA-treated HS-cells was significantly lower than in control cells at all times, with varying intensity of down-regulation being recorded during the recovery period and control values almost being reached over time.

Treatment with ASA alone had no significant effects on p53 protein levels during the course of the experimental period (Fig. 3B, C). On the contrary, HS alone and in combination with ASA significantly affected said levels with biphasic dynamics during the recovery period: after a dramatic decrease immediately following HS (0′), there was a gradual increase until 2 h later, after which point levels declined again. The second phase consisted of a gradual increase between 6 and 24 h of recovery, and a decline towards control values in the 48 h- recovery groups. Moreover, ASA + HS produced lower values than HS (at 12, 24, and 36 h of recovery), although differences were not significant.

### PARP PROTEIN EXPRESSION

Western blot analysis showed no cleavage of PARP during heat exposure and recovery, or under ASA treatment (Fig. 4), which indicated lack of DNA-damage in the treated cells. As a positive control for PARP cleavage, we used streptozotocin (STZ), a DNA damaging agent provided in a 20 mM solution and freshly dissolved in 0.1 M citrate buffer (pH 4.5). As expected, STZ-treatment produced cleavage of PARP in two fragments (119 kDa and 89 kDa).

### ASSESSMENT OF CELLULAR VIABILITY AND CELL FLUORESCENCE MICROSCOPY

We also set out to evaluate whether these treatments compromised cellular viability, and to do this we employed several approaches. We performed an MTT test in the first 12 h after HS or ASA + HS treatment, which did not reveal any significant differences in cellular viability after heat exposure or ASA treatment (Supplementary Figure 1A). Moreover, heat shock preconditioning of the liver is believed to specifically protect mitochondria. This has been shown in the case of warm ischemia, which is characterized by loss of mitochondrial membrane integrity [Ishikawa et al., 1999], and protection against this deleterious effect seems to be vital in providing the liver with ischemic tolerance. With this in mind, we also evaluated mitochondrial function following HS, ASA, or HS + ASA treatment. Two parameters - mitochondrial superoxide production and membrane potential - were assessed in the first 12 h after HS or ASA + HS treatment. The results obtained showed no significant changes in either of the two parameters (Supplementary Figure 1B).

## DISCUSSION

Transient sublethal hyperthermia followed by a recovery period (heat preconditioning) provides a cytoprotective countereffect to different kinds of insults through the induction of a specific intracellular protective response, denominated as HSR (heat shock response) [Terajima et al., 1999]. A large body of evidence shows that the process of liver regeneration can benefit from hepatic preconditioning, which seems to involve a genetic response - triggering of HSP expression [Pirkkala et al., 2001]. Recent evidence obtained in $Hsp70^{-/-}$ mice undergoing partial hepatectomy points to a fundamental role of HSP70 in the early stages of liver regeneration [Wolf et al., 2014], and supports earlier findings of low Hsp70 expression levels as a marker of acute rejection in human liver transplantation [Flohe et al., 1998].

Moreover, it is important to note that the induction of HSP in response to stress and the subsequent cytoprotective effect are transient: when the stress element is removed, cells continue to function normally and HSP returns to basal levels with time [Samali and Orrenius, 1998]. Several studies have examined the specific dynamics of HSP70 gene expression and protein level [King et al., 1996; Salminen et al., 1996] in different kinds of cells, temperature, and the duration of heat exposure, bur generally, higher intensity of HSP70 induciotn was observed 24 h after the given stress. Thus, it is of considerable relevance to establish the correct timing of preconditioning, the rate of HSPs produced and the optimum time-frame of the protection of the tissues/cells. In this sense, we evaluated the dynamics of the expression of HSP and other stress markers (Bcl-2 and p53), as well as the duration of this protective mechanism in the cells after single moderate HS (45 min/ 43°C). Our results reveal that HSP70 expression in hepatic (HepG2) cells is induced by HS at both mRNA and protein levels. HSP70 mRNA increased considerably in the first 2h following HS (200-250 fold), after which it returned to basal levels, or even lower. Importantly, HSP70 protein expression followed a time delay pattern and a varying dynamics; the time frame during which hepatic cells exhibited enhanced protein levels of HSP70 after HS insult was extensive (2 h to 24 h and even 36 h). This interesting finding could point to HSP70 as a crucial factor in cell survival against second stress stimulus.

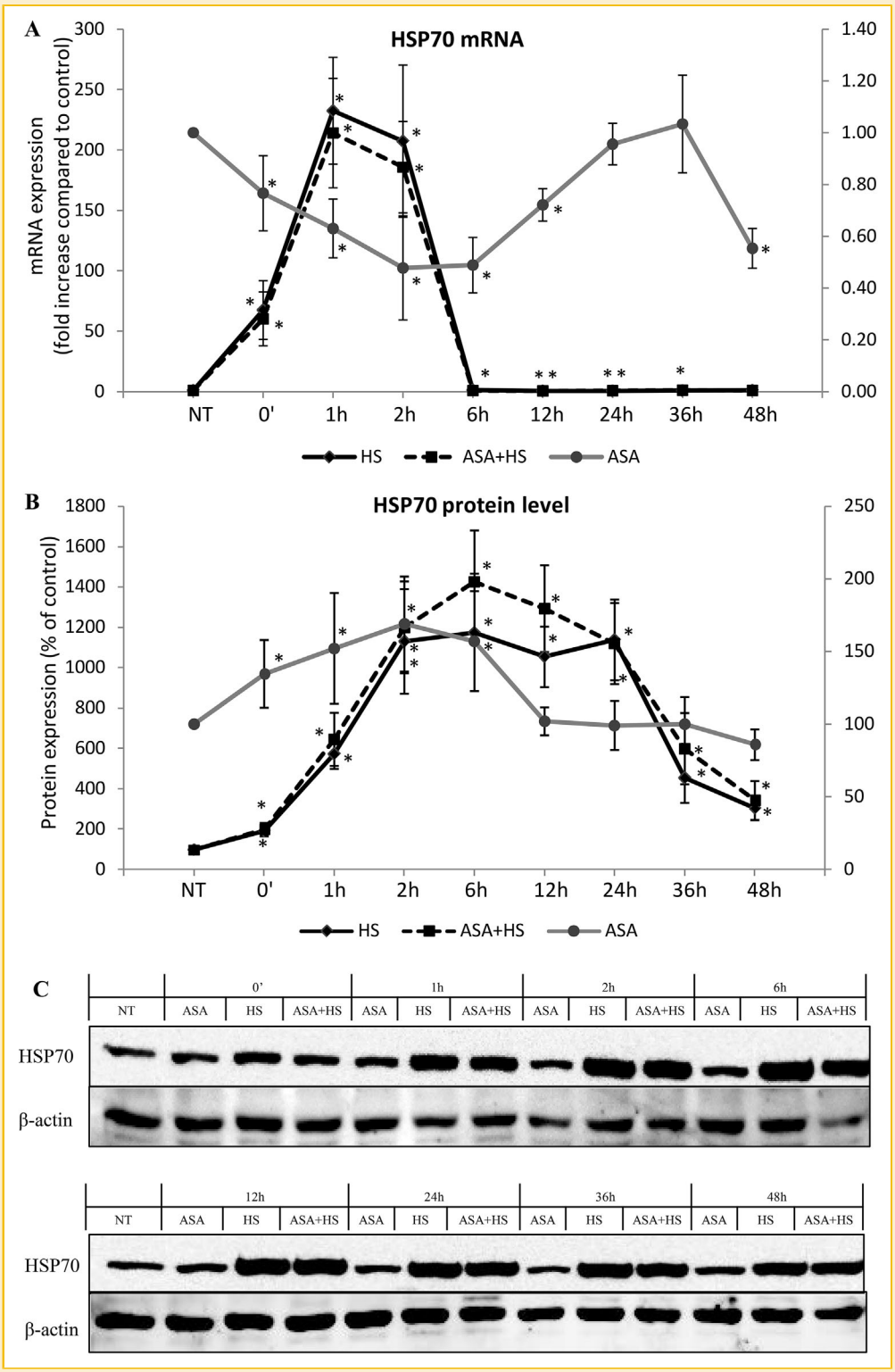


**Fig. 1.** Over-time dynamics of HSP70 mRNA and protein expression. Cells were exposed to heat stress (45 min/43°C) and allowed to recover (0, 1, 2, 6, 12, 24, 36, and 48 h at 37°C) and/or treated with 0.4 mM ASA which was added to the medium 45 min before the recovery period (ASA) or at the same time of starting HS (ASA + HS). (A) Quantification of mRNA levels. Line-plots for ASA-treatments are presented on a secondary axis; (B) Quantification of the densitometry data. Line-plots for ASA-treatments are presented on a secondary axis; (C) Representative Western blots. Results express relative expression (%) compared to that recorded in normothermic cells (100%) and are shown as mean ± SEM; n = 5–10. *Significant difference ($P < 0.050$) compared to NT-cells. Legend: NT: normothermia (untreated cells, 37°C); HS: heat stress; ASA: single 0.4 mM acetylsalicylic acid treatment, 45 min before and during the recovery period; HS + ASA: single HS in a combination with single ASA treatment (at the same time of HS exposure).

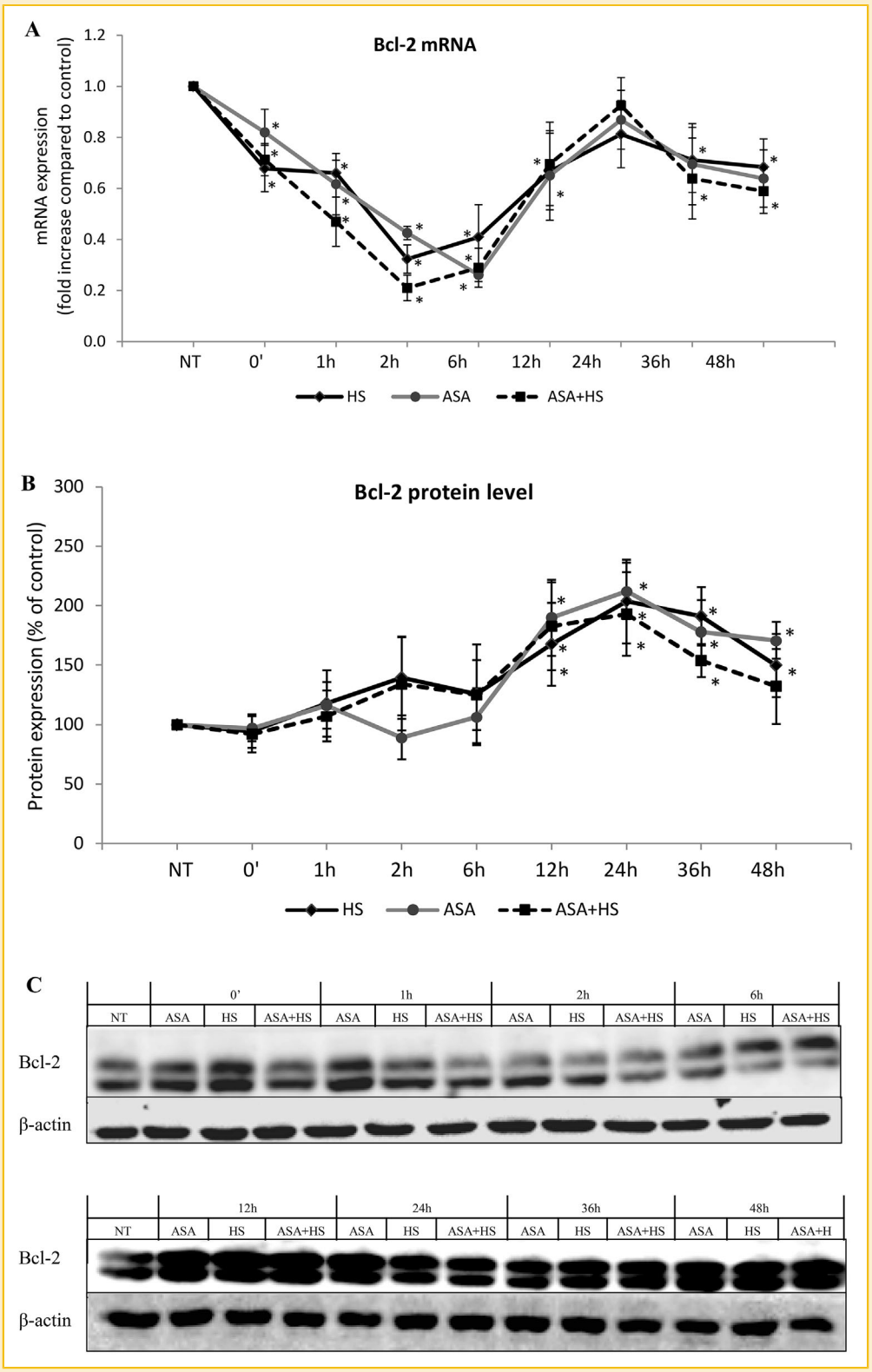


**Fig. 2.** Over-time dynamics of Bcl-2 mRNA and protein expression. Cells were exposed to heat stress (45 min/43°C) and allowed to recover (0, 1, 2, 6, 12, 24, 36 ,and 48 h at 37°C) and/or treated with 0.4 mM ASA which was added to the medium 45 min before the recovery period (ASA) or at the same time of starting HS (ASA + HS). (A) Quantification of mRNA levels; (B) Quantification of the densitometry data; (C) Representative Western blots. Results express relative expression (%) compared to that recorded in normothermic cells (100%) and are shown as mean ± SEM, n = 5–10. *Significant difference ($P < 0.050$) compared to NT-cells. Legend: NT: normothermia (untreated cells, 37°C); HS: heat stress; ASA: single, 0.4 mM acetylsalicylic acid treatment, 45 min before and during the recovery period; HS + ASA: single HS in a combination with single ASA treatment (at the same time of HS exposure).

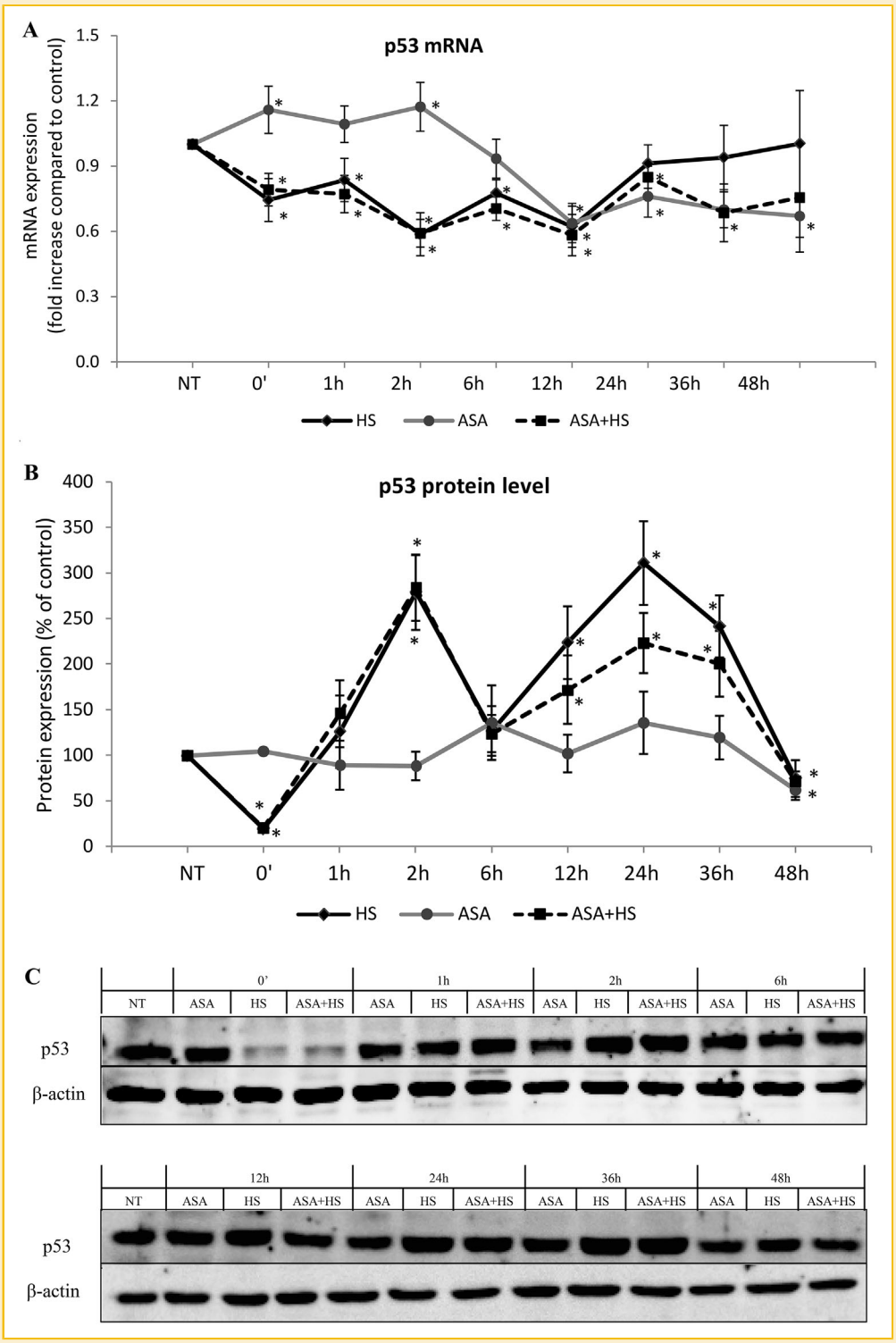


**Fig. 3.** Over-time dynamics of p53 mRNA and protein expression. Cells were exposed to heat stress (45 min/43°C) and allowed to recover (0, 1, 2, 6, 12, 24, 36, and 48 h at 37°C) and/or treated with 0.4 mM ASA which was added to the medium 45 min before the recovery period (ASA) or at the same time of starting HS (ASA+HS). (A) Quantification of mRNA levels; (B) Quantification of the densitometry data; (C) Representative Western blots. Results express relative expression (%) compared to that recorded in normothermic cells (100%) and are shown as mean ± SEM, n = 5–10. *Significant difference ($P < 0.050$) compared to NT-cells. Legend: NT: normothermia (untreated cells, 37°C); HS: heat stress; ASA: single, 0.4 mM acetylsalicylic acid treatment, 45 min before and during the recovery period; HS + ASA: single HS in a combination with single ASA treatment (at the same time of HS exposure).

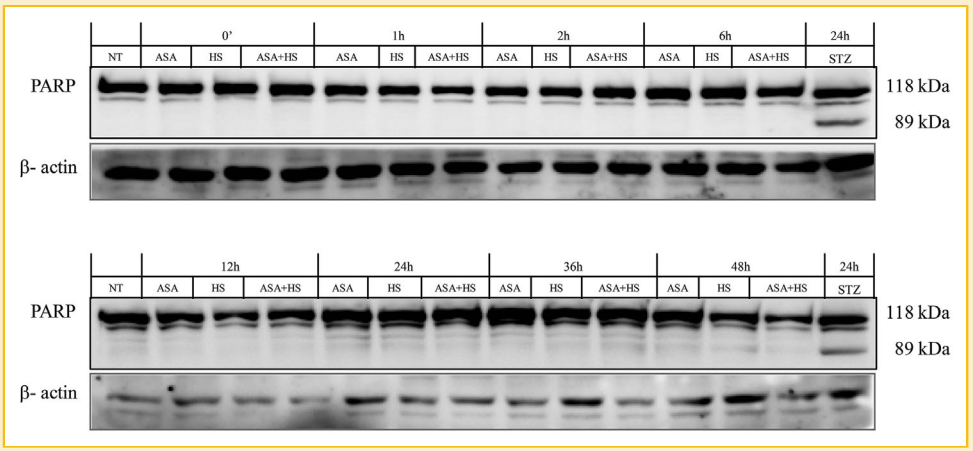


**Fig. 4.** Over-time dynamics of PARP protein expression. Cells were exposed to heat stress (45 min/43°C) and allowed to recover (0, 1, 2, 6, 12, 24, 36, and 48 h at 37°C) and/or treated with 0.4 mM ASA which was added to the medium 45 min before the recovery period (ASA) or at the same time of starting HS (ASA + HS). As a positive control, cells were exposed to the cytotoxic compound streptozotocin (20 mM solution, fresly dissolved in 0.1 M citrate buffer, pH 4.5). A representative image of Western blot is shown. Legend: NT: normothermia (untreated cells, 37°C); HS: heat stress; ASA: single, 0.4 mM acetylsalicylic acid treatment, 45 min before and during the the recovery period; HS + ASA: single HS in a combination with single ASA treatment (at the same time of HS exposure); STZ: streptozotocin.

Aspirin therapy has been reported to reduce hepatic artery thrombosis, one of the leading causes of early liver graft loss [Shay et al., 2013]. In addition, this NSAID was the first pharmacological agent shown to induce HSR in cells exposed at various time points at 42°C [Jurivich et al., 1992,1995]. In the study in question, short term-exposure of HeLa cells to sodium salicylate signaled the first step of the HSR cascade, although the transcription rate of the HSP70 gene was not up-regulated. Another in vitro study [Amici et al., 2005] suggested that, aspirin (400 μM) administered during or immediately after a hyperthermic treatment (20 min at 45°C) in human erythroleukemic cells, caused an increase in the amount of HSP70 synthesized and prolonged HSP70 synthesis for a period of several hours, an effect not attributed to increased HSP70 mRNA stability, but rather to an enhanced and prolonged HSP70 mRNA transcription. We observed that ASA, when administered alone, provoked only a small increase in HSP70 protein levels during the first 6 h of recovery, which, strangely, was paralleled by a decrease in mRNA levels (Fig. 1). However, we did not observe any cumulative effect of ASA + HS on HSP70 mRNA or protein expression, which may be due to cell type specificity or the intensity of heat stress.

Concerning in vivo studies, Fawcett et al. (1997) reported that treatment of rats with aspirin (100 mg/kg) elevated HSP70 mRNA and protein expression but only in the presence of heat (30 min at 37°C). Another in vivo investigation [Locke and Atance, 2000] found that salicylate in combination with a mild heat stress (40°C) induced heat shock factor activation, but not HSP synthesis and only the hearts from severely heat-stressed animals (42°C) demonstrated a significantly elevated myocardial Hsp72 content. We consider that both the in vivo and in vitro studies suggested dose- and temperature-dependent effect of aspirin/salicylate over HSR cascade.

Accumulation of HSPs has long been considered a marker of cell and tissue damage, which would seem to contradict their previously described anti-apoptotic properties. There is an evident overlap of the signals that induce a protective stress response and those that initiate apoptosis [Samali and Orrenius, 1998]. In our experiments, the Bcl-2 profile showed a significant down-regulation of mRNA, with protein levels remaining unchanged a few hours after heat stress. Contrary to our results, Basile et al. (2008) found that Bcl-2 protein level decreased by hyperthermia (42°C/1 h) and remained at low levels when cells returned to 37°C in the following 8 h. Since HepG2 cells constitutively express high levels of HSP70, we believe that this stress protein protects cells against heat-induced apoptosis. We suspect that increased Bcl-2 protein levels and maximum HSP70 protein expression are important determinants of the health of liver cells during the 24-hour post-stress period.

It is important to note that the effects of HS alone and of ASA + HS on the transcriptional and translational changes of Bcl-2 were very similar. Interestingly, ASA alone also produced a significant elevation of Bcl-2 protein levels in the long-term recovery period. Thus, it could be hypothesized that long-term ASA-treatment promotes anti-apopototic reactions in the cell, a possibility that deserves further study. This finding, together with the fact that ASA produced only a small increase in HSP70 with respect to HS, points to the presence of different regulatory mechanisms of Bcl-2 expression under the influence of these two stimuli.

HSP70 is a decisive negative regulator of the mitochondrial (intrinsic) pathway of apoptosis that can block apoptosis at different levels: at a premitochondrial stage, by inhibiting stress-inducing signaling; at the mitochondrial stage, by preventing mitochondrial membrane permeabilization and the release of several pro-apoptotic factors into the cytosol [Beere, 2004]; and also at the postmitochondrial level, by interacting with apoptosis-inducing factor (AIF) [Schmitt et al., 2007]. Concerning mitochondrial membrane potential, we found no changes immediately following HS (0 h–12 h post HS), which could be another indicator of the absence of apoptosis in the HepG2 cells. We also assessed the protein expression

of poly(ADP-ribose) polymerase (PARP), a DNA-damaging enzyme which undergoes protein cleavage while producing apoptotic changes in the cells. These findings, together with those observed by the MTT-test for unchanged viability of cell, confirmed that both, the intensity of HS, as well as the applied dose of ASA, did not cause cell or DNA damage, which was of great importance for the subsequent acquired condition of the cells.

According to Buzzard et al. (1998); constitutive expression of HSP72 inhibits PARP cleavage in cells after heat shock and halts the process of apoptotic cell death [Mosser et al., 1997]. We did not observe PARP cleavage, regardless of how long the recovery period lasted. Our observations are in accordance with those of Robertson et al., (1997) and suggest that HSP70 and Bcl-2 proteins act separately in preventing apoptosis, since each protein effectively restricts apoptosis, but can have synergistic effects when present together.

The tumor suppressor p53 is a universal sensor of genotoxic stress and is considered "the guardian of the genome" [Burns and El Deiry, 1999]. In our study, expression of p53 seemed to be modified through several phases during the course of the recovery period. Importantly, there was down-regulation of p53 mRNA immediately after HS, followed by a dramatic drop in the expression of this protein at the same time (HS + 0′). This is in line with published evidence obtained in the same cell line (HepG2) in which p53 protein levels were reported to be reduced during hyperthermia, but returned to high (control) levels when the cells were kept at 37°C for 4 h [Basile et al., 2008]. Moreover, in our experiments, while mRNA levels remained lower than basal values throughout the whole recovery period, p53 protein levels gradually and significantly increased during the first 2 h of recovery. This result is in accordance with recently published data obtained, once again, in the same cell line and showing an increase in p53 protein levels in the first hour of recovery after HS (43°C). In fact, in the report in question [Basile et al., 2008], as in ours, HS diminished p53 mRNA expression; this is highly relevant, as other stress stimuli, such as classic genotoxic stressors (gamma irradiation, UV, hypoxia, and virus infection), activate p53 by up-regulating its transcription. The abovementioned short-term changes were followed with normalization at 6 h and a "second wave" of increased p53 protein levels until 36 h of heat recovery, with normalization finally occurring at 48 h. The close connection between p53 and HSP70 has been previously reported in HepG2 cells, in which HS induced p53 and its inhibition down-regulated heat-induced Hsp70 [Sharma et al., 2010]. On the one hand, activation of p53 by HS has been reported to occur largely by increasing its DNA-binding activity [Zilfou and Lowe, 2009], while on the other, HS has been shown to modulate the p53 protein mainly at the post-transcriptional level by prolonging its half-life in hepatoma cells [Han et al., 2013]. We observed that combined HS+ASA treatment did not result in significant differences in p53 with respect to HS alone. Notably, ASA alone did not produce major changes in p53 expression at any point of the recovery period, except for a short-term increase in p53 mRNA (up to 2 h).

In conclusion, our findings suggest that there is a complex time-dependent dynamic in the expression of several proteins crucial for the survival of HS-stressed hepatic cells. Depending on HSP70 levels, cells would be most vulnerable in the first 2–6 h after HS and would have a greater capacity to combat additional stress stimuli 12–2 4h after moderate HS. In general in our experiments, 0.4 mM ASA failed to act as a co-inducer of HSP70, but enhanced Bcl-2 protein accumulation when treatment was prolonged. Our study suggests for extended plateau of HSP70 accumulation which would have cytoprotective effect against an injurious agent in HepG2 cells. Finally, short-term exposure to moderate heat may be a "physiological conditioner" of the capacity of liver cells to accumulate HSP and Bcl-2 proteins, a phenomenon that may be of relevance for ensuring cell survival during the process of liver regeneration.

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