

## INDUCTION OF HEME OXYGENASE-1 AND HEAT SHOCK PROTEIN 70 IN RAT HEPATOCYTES: THE ROLE OF CALCIUM SIGNALING

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**Abstract:** Stress response genes including heat shock proteins are induced under a variety of conditions to confer cellular protection. This study investigated the role of calcium signaling in the induction of two stress response genes, heme oxygenase-1/hsp32 and hsp70, in isolated rat hepatocytes. Both genes were induced by cellular glutathione depletion. This induction could be inhibited by BAPTA-AM. Culturing in a calcium-free medium prevented the induction of hsp70 gene expression after glutathione depletion without affecting heme oxygenase-1 gene expression. Thapsigargin increased the gene expression of heme oxygenase-1 but not that of hsp70. Thapsigargin-induced heme oxygenase-1 induction was completely inhibited by BAPTA-AM. Incubation with the  $\text{Ca}^{2+}$ -ionophore A23187 augmented heme oxygenase-1 (two-fold) and hsp70 (5.2-fold) mRNA levels. Our data suggests a significant role of  $\text{Ca}^{2+}$ -dependent pathways in the induction of the two stress genes. An increase in the cytoplasmic  $\text{Ca}^{2+}$  activity seems to play a key role in the cascade of signaling leading to the induction of the two genes. However, the source of  $\text{Ca}^{2+}$  that fluxes into the cytoplasm seems to be different. Our data provides evidence for a compartmentalization of calcium fluxes, i.e. the  $\text{Ca}^{2+}$  flux from intracellular

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Abbreviations used: AP-1 – activator protein-1; BAPTA-AM – (1,2-bis(o-amino-phenoxy)ethane-*N,N,N',N'*-tetra (acetoxymethyl)ester; DMSO – dimethylsulfoxide; EDTA – ethylene-diamine-tetraacetic-acid; ER – endoplasmic reticulum; GSH – glutathione; HO-1 – heme oxygenase-1; hsp – heat shock protein; KHB – Krebs-Henseleit buffer; PBS – phosphate buffered saline; P/BSO – 2,6 dimethyl-2,5 heptadien-4-on (phorone)/buthionine sulfoximine; Tg – thapsigargin

stores (e.g. the endoplasmic reticulum) plays a major role in the induction of heme oxygenase-1. By contrast,  $\text{Ca}^{2+}$  flux from the extracellular medium seems to be a mechanism initiating the cellular signaling cascade leading to hsp70 gene induction.

**Key words:** Stress response genes, Hepatocytes, Calcium, Gene expression

## INTRODUCTION

Heat-shock proteins (HSP) are highly conserved proteins found in all prokaryotes and eukaryotes. Hsp90 is one of the most abundant cellular proteins. Heat shock protein 70 consists of a constitutively expressed isoform (Hsc70) and an isoform that is induced under various stress conditions (Hsp70). Under physiological conditions, there is no or only a low expression of hsp70 [1]. However, *de novo* synthesis of heat shock proteins is substantially induced by a wide variety of stress events. This so-called cellular stress response is initiated by different environmental (heat shock, exposure to heavy metals and ultraviolet radiation), pathological (infections, inflammation, malignancy, autoimmunity) or physiological stimuli (hormonal stimulation, growth factors, cell differentiation). The induction of heat shock proteins has been described as a protective mechanism against cellular injury following stress events in different cell types *in vivo* and *in vitro* [2-5]. Although the exact mechanisms by which HSPs confer protection are not clearly understood, their gene expression can be modulated by cell signal transducers, such as changes in intracellular pH, cyclic AMP,  $\text{Na}^+$ , inositol triphosphate, protein kinase C, and protein phosphatases [6, 7]. Free intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) is known to be a potent regulator of gene expression [8], and it appears to play a significant role in the induction of hsp gene expression [9]. However, the pathways leading to the induction of a given hsp seem to be cell-type specific. In cultured rat neuronal cells, Linden *et al.* [10] demonstrated an increase in hsp32/HO-1 gene expression by a decrease in endoplasmic reticulum (ER) calcium activity, but not by the corresponding increase in cytoplasmic calcium activity. By contrast, Choi *et al.* [9] showed an increase in hsp32/HO-1 gene expression dependent on cytoplasmic calcium activity in human diploid fibroblasts. Thus, the mechanisms of regulation of stress gene expression by changes in  $[\text{Ca}^{2+}]_i$  are not fully understood. However, there is evidence from different experimental studies to suggest that multiple pathways might be involved depending on the cell type, stimulus, and gene examined [9-13].

In previous studies, we demonstrated an induction of hsp32/HO-1 and hsp70 in hepatocytes in a rat model of hemorrhagic shock and resuscitation through oxygen free radical-dependent AP-1 activation [2, 4, 14, 15]. In the same animal model, we observed a substantial disturbance of hepatocellular  $\text{Ca}^{2+}$  regulation [16]. At present, no data is available assessing the role of the intracellular  $\text{Ca}^{2+}$  messenger system in the activation of hsps in liver parenchymal cells. Therefore, in this study, we used an *in vitro* oxidative stress model with cellular glutathione

depletion by incubating the cells with a combination of phorone and buthionine sulfoximine (P/BSO). To evaluate the role of cellular calcium regulation for gene expression in this model, extracellular and cytosolic  $[Ca^{2+}]$  were respectively depleted by preincubation of the hepatocytes with the  $Ca^{2+}$  chelators EDTA and BAPTA-AM. Furthermore, we studied the effect of increasing cytoplasmic  $Ca^{2+}$  concentrations on hsp gene expression by using the calcium ionophore A23187 and an experimental depletion of ER calcium stores by thapsigargin, with and without depletion of the cytoplasmic  $Ca^{2+}$  pool by BAPTA-AM.

## MATERIALS AND METHODS

### Chemicals

All the chemicals were purchased from Sigma chemicals (Deisenhofen, Germany) or Roth (Karlsruhe, Germany) if not specified otherwise.

### Animals

The experiments were performed using male Sprague-Dawley rats (250-350 g body weight; Charles River, Sulzfeld, Germany) which had free access to tap water and standard chow food prior to the experiment. The study had the approval of the Regional Review Board for the Care of Animal Subjects, and was run in accordance with the National Institute of Health guidelines for animal care. The rats were anesthetized with pentobarbital (50 mg/kg body weight intraperitoneally) and placed in the supine position for surgical preparation.

### Hepatocyte isolation

The rats were heparinized (1 U/g body wt), and after a midline laparotomy, the portal vein was cannulated. The livers were perfused for 10 min *in situ* with oxygenated (95%  $O_2$ /5%  $CO_2$ )  $Ca^{2+}$ - and  $Mg^{2+}$ -free Krebs-Henseleit buffer (KHB) (37°C, pH 7.4). The livers were then perfused *ex situ* in a recirculating system for an additional 20 min with an oxygenated KHB-solution (0.1 mM  $Ca^{2+}$ ) containing 0.05% collagenase (collagenase-hepatocyte-qualified, 212 U/mg, prepared from clostridium histolyticum, Life Technologies, Grand Island, NY). After mincing the digested livers, hepatocytes were separated from the cell suspension in two centrifugation steps (50 x g, 2 min, 4°C). The cells were finally resuspended in Williams E-medium supplemented with 5% fetal calf serum and gentamycine 50  $\mu$ g/ml (Gibco BRL, Life Technologies, Scotland) in a final concentration of  $1 \times 10^6$  cells per mL. Only hepatocyte suspensions exhibiting a viability higher than 90% as assessed by the trypan blue exclusion technique were used for further experiments.

### Cell culture

The collagen coating of the culture dishes was performed as described elsewhere [17], with minor modifications, using 40  $\mu$ g collagen from rat tails/25cm<sup>2</sup> plate. Three x  $10^6$  cells/plate were seeded. After incubation for 6 h in a humidified

atmosphere (37°C, 19:1 air:CO<sub>2</sub>) adherent cells were washed with phosphate buffered saline (PBS) and cultured for an additional 12 h in serum-free Williams-E medium containing gentamycine.

### **Experimental procedures**

The experiments were started 20 to 24 hours after the isolation of the cells according to the different experimental protocols (see below). At the beginning of each experiment, the cells were washed once with PBS (37°C) and supplied with fresh serum-free medium.

### **Glutathione depletion**

Cellular glutathione stores were depleted by incubation with phorone (2,6 dimethyl-2,5 heptadien-4-one; Fluka, Neu Ulm, Germany, 0.1 mg/ml), which decreases the intracellular glutathione concentration by increasing glutathione transferase activity. Phorone was used in combination with buthionine sulfoximine (BSO, 2 mM), which inhibits  $\gamma$ -glutamylcysteine synthetase, an enzyme essential for glutathione (GSH) synthesis. After 75 min of incubation, the cells were washed with PBS and incubated for a further 6 h with fresh serum-free medium.

To investigate the role of extracellular calcium in the induction of hsp gene expression by phorone/buthionine sulfoximine (P/BSO), cells were incubated with the extracellular Ca<sup>2+</sup>-chelator EDTA (ethylene-diamine-tetraacetic-acid; 10 mM) in the absence or presence of P/BSO. To evaluate the potential role of cytoplasmic calcium activity in the induction of hsp gene expression by P/BSO, cells were incubated with the cytoplasmic Ca<sup>2+</sup>-chelator BAPTA-AM (1,2-bis-(o-Aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester; 33.3  $\mu$ M) in the absence or presence of P/BSO. The used concentration of BAPTA-AM was also sufficient to completely block the thapsigargin-induced (Tg-induced) rise in the cytoplasmic calcium activity in cultured cells [10].

### **Modulation of the cellular calcium homeostasis**

Endoplasmic reticulum stores were depleted by exposing cells to Tg, a specific, irreversible inhibitor of ER Ca<sup>2+</sup>-ATPase, leading to an increased cytosolic Ca<sup>2+</sup> concentration. Tg stock solution (100  $\mu$ M) was prepared immediately before use, by dissolving Tg in DMSO. Tg was used at different concentrations (0.1, 0.25 and 1  $\mu$ M) [18] in the absence or at 1  $\mu$ M in the presence of P/BSO. To study whether the Tg-induced hsp gene expression was caused by a decrease in the ER calcium concentration or by a corresponding increase in the cytoplasmic calcium activity, cells were preloaded (30 min) with BAPTA-AM before exposure to Tg. Incubation with the highest concentration of Tg (1  $\mu$ M) yielded a final concentration of 1% DMSO in the culture medium. The control cells were either left untreated (the negative control group) or supplemented with 1% DMSO (the DMSO control group).

The calcium ionophore A23187 permeabilizes the plasma membrane and induces a leakage of Ca<sup>2+</sup>, which increases cytoplasmic Ca<sup>2+</sup>-concentrations.

A23187 was prepared as a stock solution (500  $\mu$ M in DMSO) and was used at different concentrations (0.2, 1 and 5  $\mu$ M). Different time points were studied using the highest concentration (1, 2, 4 and 6 h). Incubation with the highest concentration of A23187 (5  $\mu$ M) yielded a final concentration of 1% DMSO in the culture medium. The control cells were either left untreated (control) or supplemented with 1% DMSO (DMSO).

Six hours after the start of each experiment, the cells were washed once with PBS, and RNA was collected by adding 500  $\mu$ L of guanidine isothiocyanate solution (4 M guanidine isothiocyanate, 20 mM sodium citrate, 0.5% *N*-lauroyl-sarcosine, 0.1 M 2-mercaptoethanol) to the cells, scraping the cells off with a rubber policeman, and aspirating the obtained suspension twice through a 22-G needle. All the experiments were performed in triplicate. Since incubation with calcium chelators causes the cells to progressively detach from the culture plates during the experiments, six independent experiments were performed with BAPTA-AM and EDTA, used to compensate for cell loss during washing procedures and to harvest significant amounts of cells for RNA isolation.

#### **Isolation of RNA and Northern blot analysis**

Total RNA was extracted from hepatocytes according to the acid guanidinium-phenol-chloroform extraction method [2]. Similar amounts of RNA from the corresponding 3 or 6 experiments were pooled. Aliquots (10  $\mu$ g) of pooled RNA were dried under a vacuum, dissolved in a 15- $\mu$ l gel loading buffer (50% formamide, 2.2 M formaldehyde, 50 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.0, 2.72 mg sucrose, 34  $\mu$ g bromphenol blue, 0.55  $\mu$ g ethidium bromide) at 65°C, separated by electrophoresis on 1.2% agarose formaldehyde gels, and blotted to charged modified nylon membranes (GeneScreen Plus, Du Pont, Brussels, Belgium). The accuracy of RNA loading was confirmed by staining the membrane with methylene blue (0.03% in 3 M sodium acetate, pH 5.2). An EcoR I/Hind III restriction fragment (0.9 kb, kindly provided by Dr. S. Shibahara, Sendai, Japan) from rat HO-1 complementary DNA and a commercially available human hsp70 cDNA (ATCC # 57494) were used as hybridization probes for Northern blot analysis. Hybridization was performed at 65°C in hybridization buffer (RapidHyb, Amersham Biosciences, Freiburg, Germany) using the hsp32/HO-1 and hsp70 cDNA probe radiolabeled with  $\alpha$ -[<sup>32</sup>P]dCTP (50  $\mu$ Ci) (Amersham Biosciences, Freiburg, Germany) according to the random primer method, followed by spin column centrifugation. Autoradiographs of the blots were obtained after stringency washes and exposure to diagnostic film at -70°C (X-Omat XAR-5, Eastman Kodak Co., Rochester, NY). Autoradiograms were analyzed densitometrically using a scanning densitometer (Bio-Rad GS-700 Imaging Densitometer, BioRad, Hercules, CA). Signals were normalized to the 18S ribosomal RNA band to correct for unequal loading.

## RESULTS

### The induction of heme oxygenase-1 gene expression

#### *The effect of glutathione depletion and its modulation by intra- and extracellular $Ca^{2+}$ chelation*

The *in vitro* oxidative stress model of glutathione depletion with P/BSO significantly increased hsp32/HO-1 mRNA levels relative to the controls. Co-incubation with the intracellular  $Ca^{2+}$ -chelator BAPTA-AM prevented P/BSO-induced hsp32/HO-1 mRNA induction. Incubation with BAPTA-AM alone did not influence hsp32/HO-1 expression (Fig. 1A). Co-incubation of hepatocytes with the extracellular acting  $Ca^{2+}$ -chelator EDTA further increased hsp32/HO-1 induction by P/BSO. Incubation of the cells with EDTA alone slightly increased hsp32/HO-1 mRNA induction relative to the controls (Fig. 1B).

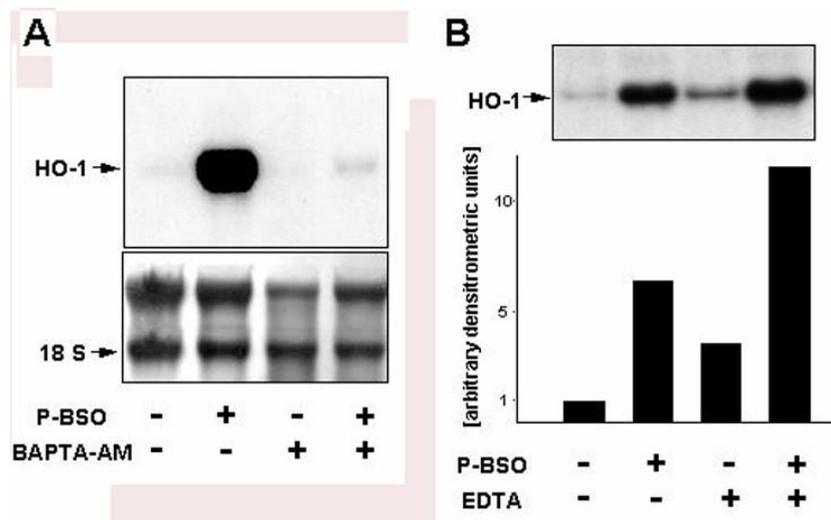


Fig. 1. The effect of oxidative stress on hsp32/HO-1 mRNA levels and its modulation by  $Ca^{2+}$ -chelation. The role of the cytosolic  $Ca^{2+}$  in P/BSO-induced hsp32/HO-1 expression was evaluated by intracellular  $Ca^{2+}$ -chelation with BAPTA-AM 33.3  $\mu$ M (A), and the role of extracellular  $Ca^{2+}$  was investigated by  $Ca^{2+}$ -chelation with EDTA 10 mM (B). Total RNA was prepared from time-matched unmanipulated control cells and 6 h after the start of incubation with Phorone/BSO. Independent experiments were performed in triplicate, and experiments involving BAPTA-AM and EDTA were performed six times with hepatocytes isolated from different animals. RNA was pooled from equal amounts of RNA from each experiment and further analyzed for hsp32/HO-1 by Northern blot hybridization. Thus, the shown blots reflect the mean expression of these independent experiments. The results are shown as blots from pooled RNA (10  $\mu$ g), blots after staining with methylene blue (1A), and densitometrical analysis of hsp32/HO-1 after normalization to the methylene blue signal (1B).

### The effect of the modulation of cellular calcium homeostasis

Incubation of hepatocytes in the presence of thapsigargin significantly increased the expression of hsp32/HO-1 transcripts in a dose-dependent manner (Fig. 2A). This effect was completely blocked by simultaneous incubation with BAPTA-AM (Fig. 2B). Co-incubation with P/BSO further augmented the effect of the Tg-induced hsp32/HO-1 mRNA-expression (Fig. 2A). Incubation with the  $\text{Ca}^{2+}$ -ionophore A23187 induced a two-fold increase of hsp32/HO-1 mRNA expression relative to the untreated controls (Fig. 3).

### Induction of hsp70 gene expression

*The effect of glutathione depletion and its modulation by intra- and extracellular  $\text{Ca}^{2+}$  chelation*

P/BSO significantly increased hsp70-gene induction. This induction was prevented by loading the cells with the cytoplasmic  $\text{Ca}^{2+}$ -chelator BAPTA-AM.

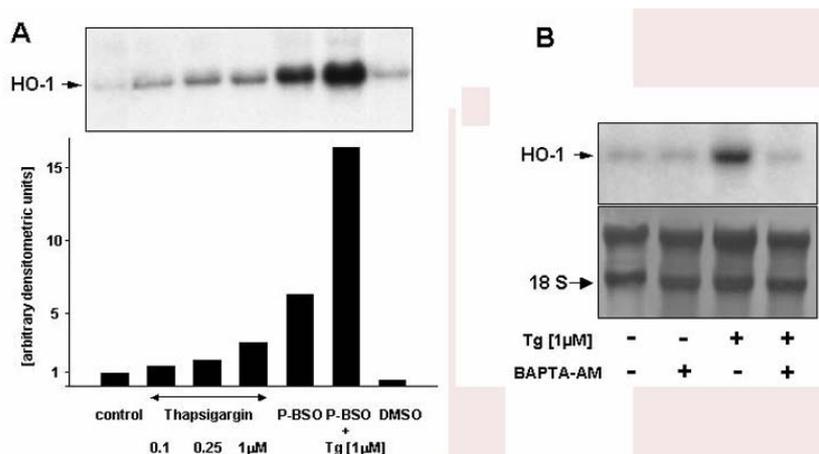


Fig. 2. Analysis of expression of hsp32/HO-1 mRNA by modulating the cellular  $\text{Ca}^{2+}$ -homeostasis with thapsigargin. Endoplasmic reticulum (ER) stores were depleted by exposing cells to thapsigargin (Tg), a specific, irreversible inhibitor of ER  $\text{Ca}^{2+}$ -ATPase, at different concentrations as indicated. Tg incubation was continued in combination with phorone and buthionine sulfoxime (P/BSO). Untreated cells (control) or those incubated with 1% DMSO (DMSO) served as controls (A). To study whether the Tg-induced rise in gene expression was caused by a decrease in ER calcium concentration or by the corresponding increase in cytoplasmic calcium activity, cells were preloaded (30 min) with BAPTA-AM before exposure to Tg (B). Independent experiments were performed in triplicate, and experiments involving BAPTA-AM and EDTA were performed six times with hepatocytes isolated from different animals. RNA was pooled from equal amounts of RNA from each experiment and further analyzed for hsp32/HO-1 by Northern blot hybridization. Thus, the shown blots reflect the mean expression of these independent experiments. The results are shown as blots from pooled RNA (10  $\mu\text{g}$ ) and densitometrical analysis of hsp32/HO-1 after normalization to the methylene blue signal.

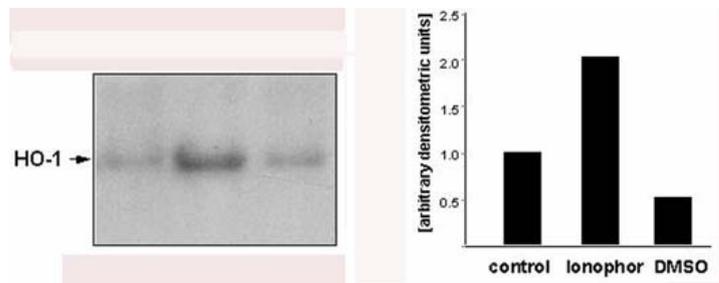


Fig. 3. Analysis of the expression of hsp32/HO-1 mRNA by modulating the cellular  $\text{Ca}^{2+}$ -homeostasis with ionophore A23187. The calcium ionophore A23187 permeabilizes the plasma membrane and induces a leakage of  $\text{Ca}^{2+}$  which increases cytoplasmic  $\text{Ca}^{2+}$ -concentration. A23187 was used at a concentration of  $1 \mu\text{M}$ . Untreated cells (control) or those incubated with 1% DMSO (DMSO) served as controls. Independent experiments were performed in triplicate. RNA was pooled from equal amounts of RNA from each experiment and further analyzed for hsp32/HO-1 by Northern blot hybridization. The results are shown as blots from pooled RNA ( $10 \mu\text{g}$ ) and densitometrical analysis of hsp32/HO-1 after normalization to the methylene blue signal.

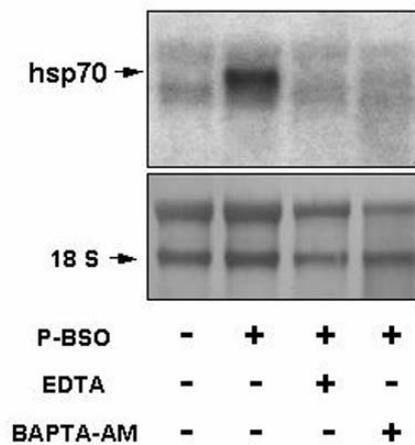


Fig. 4. The effect of oxidative stress on hsp70 mRNA levels and its modulation by the  $\text{Ca}^{2+}$ -chelators EDTA and BAPTA-AM. Hepatocyte cultures were incubated with P/BSO in the absence or presence of the intracellular  $\text{Ca}^{2+}$ -chelator BAPTA-AM ( $33.3 \mu\text{M}$ ) or the extracellular  $\text{Ca}^{2+}$ -chelator EDTA ( $10 \text{mM}$ ). RNA was pooled from equal amounts of RNA from each experiment and further analyzed for hsp70 by Northern blot hybridization. The results are shown as blots from pooled RNA ( $10 \mu\text{g}$ ), and corresponding methylene blue staining is shown.

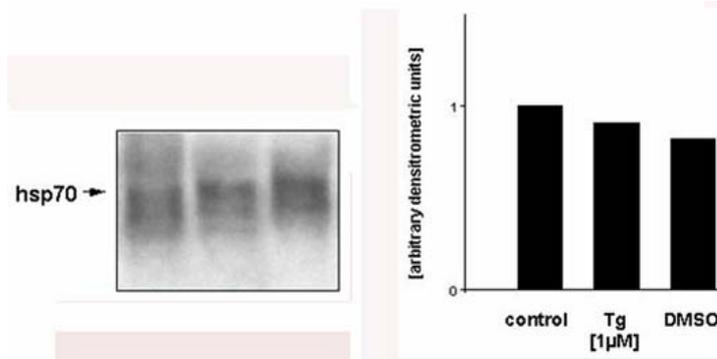


Fig. 5. The effect of thapsigargin on hsp70 mRNA levels. Hepatocytes were incubated with thapsigargin (Tg, 1 μM), an inhibitor of endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase. Unmanipulated cells (control) and those incubated with 1% DMSO (DMSO) served as controls. RNA was pooled from equal amounts of RNA from each experiment and further analyzed for hsp70 by Northern blot hybridization. The results are shown as densitometrical analyses of hsp70 after normalization to the methylene blue signal.

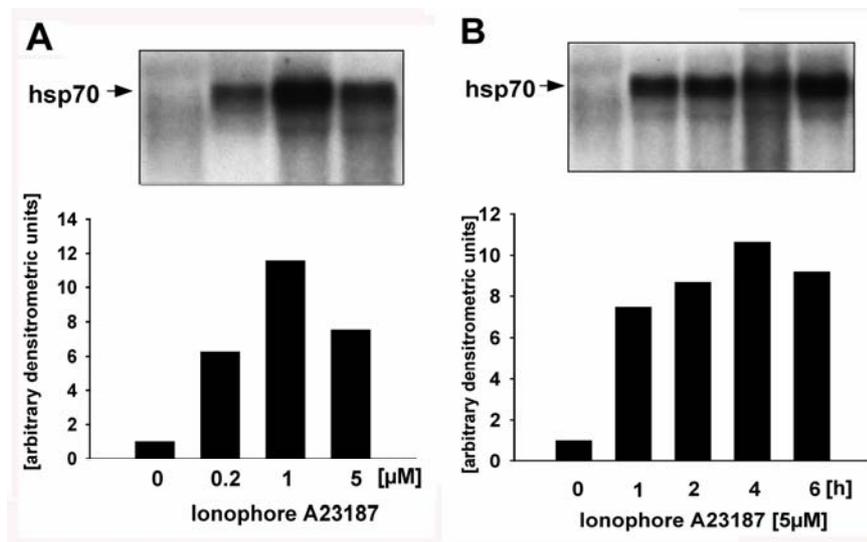


Fig. 6. The effect of Ionophore A23187 on hsp70 mRNA levels. A - Hepatocytes were incubated with different concentrations of Ionophore A23187 (0, 0.2, 1, and 5 μM). B - Hepatocytes were incubated with 5 μM of Ionophore A23187 for 1, 2, 4, or 6 h. The control cells were exposed to the DMSO vehicle. RNA was pooled from equal amounts of RNA from each experiment and further analyzed for hsp70 by Northern blot hybridization. The results are shown as densitometrical analyses of hsp70 normalization to the methylene blue signal.

Thus, these effects were similar to the observations for the hsp32/HO-1 gene. However, by contrast to hsp32/HO-1, the incubation of the hepatocytes with P/BSO in presence of the extracellular  $\text{Ca}^{2+}$ -chelator EDTA also prevented hsp70 gene induction (Fig. 4).

#### **The effect of the modulation of cellular calcium homeostasis**

Administration of thapsigargin to the cell cultures did not increase hsp70 gene induction over the levels of the untreated controls or the controls incubated with the vehicle DMSO (Fig. 5). Ionophore A23187 strongly increased hsp70 induction, with the highest induction obtained using 1  $\mu\text{M}$  A23187 (Fig. 6A). Assessment of the kinetics of  $\text{Ca}^{2+}$  ionophore A23187 on hsp70 steady state transcript levels revealed increased mRNA concentrations as early as 1 h after incubation (Fig. 6B).

#### **DISCUSSION**

Oxidative stress events may lead to the upregulation of the stress genes hsp32/HO-1 and hsp70. The results of this study show that cellular glutathione depletion by P/BSO is a potent inducer of hsp32/HO-1 and hsp70 mRNA expression in primary cultures of rat hepatocytes. This data concurs with our recent observations on the human hepatoma cell line HepG2 [19]. Although there are numerous reports of a variety of stimuli that upregulate these two stress genes, less is known about the intracellular signaling intermediaries that regulate their expression. Furthermore, data from different studies suggests a differential induction pattern with respect to cell type and species.

Intracellular  $\text{Ca}^{2+}$  is a universal second messenger. Changes in free intracellular  $\text{Ca}^{2+}$  are quickly transformed into changes in the activity of several kinases including protein kinase A, PKC, and mitogen-activated protein kinases. Phosphorylation of the transcription factor cAMP response element-binding protein or Elk by these kinases may lead to the transactivation of the immediate early gene c-fos or Egr-1 in the nucleus [20]. Choi *et al.* showed a calcium-mediated expression of the stress response genes heme oxygenase, c-fos, Egr-1, gadd153, and hsp70 after treatment of human diploid fibroblasts with prostaglandin  $\text{A}_2$  [9]. Our study is to our knowledge the first examining the induction of the two hsp-genes hsp32/HO-1 and hsp70 in rat hepatocytes with a focus on intracellular calcium signaling.

Hsp32/HO-1 gene induction by P/BSO was attenuated in the presence of intracellular  $\text{Ca}^{2+}$ -chelation with BAPTA-AM. Incubation of hepatocytes in a calcium-free medium using EDTA did not attenuate hsp32/HO-1 induction. This suggests a mechanism of gene induction by an increased cytoplasmic  $[\text{Ca}^{2+}]_i$  independent from the extracellular  $\text{Ca}^{2+}$  pool. Incubation of hepatocytes with EDTA led to an approximately 3.5-fold induction in HO-1 mRNA expression compared to the controls. EDTA-mediated HO-1 induction was potentiated by glutathione depletion with phorone and buthionine sulfoximine (P/BSO). These results suggest a differential regulation of HO-1 by EDTA and

glutathione depletion. The level of HO-1 induction by co-incubation with EDTA and P/BSO was comparable to the level obtained by co-incubation with thapsigargin and P/BSO. One potential mechanism could be a  $\text{Ca}^{2+}$  shift along the calcium concentration gradient from the intracellular to the extracellular space initiated by EDTA-induced extracellular  $\text{Ca}^{2+}$  chelation. Compensatory release of  $\text{Ca}^{2+}$  from intracellular sources such as the mitochondria or endoplasmic reticulum might mimic an effect similar to the one seen after incubation with thapsigargin. The hypothesis of a mechanism of gene induction by an increased cytoplasmic  $[\text{Ca}^{2+}]_i$  independent from the extracellular  $\text{Ca}^{2+}$  pool is further supported by our data showing an increased hsp32/HO-1 induction in hepatocytes in the presence of thapsigargin. Thapsigargin depletes ER calcium stores with a corresponding increase in cytoplasmic calcium activity [21]. Since BAPTA-AM incubation prior to thapsigargin treatment prevented the expression of hsp32/HO-1 mRNA, the subsequent increased cytoplasmic calcium activity by thapsigargin rather than the corresponding depletion of ER calcium stores seems to be responsible for the induction. By contrast, pretreatment of cells with BAPTA-AM was not able to prevent thapsigargin-induced HO-1 expression in cultured rat neuronal cells [10]. Choi *et al.* demonstrated an induction of hsp32/HO-1 in human diploid fibroblasts by incubation with prostaglandin  $\text{A}_2$  which was attenuated by BAPTA-AM [9]. Furthermore, Terry *et al.* presented an induction of hsp32/HO-1 transcripts in human vascular endothelial cells by exposure to tumor necrosis factor- $\alpha$  and interleukin-1 $\alpha$ ; this was also prevented by incubation with BAPTA-AM [6]. Thus, the results of these two studies are in line with our results supporting the concept of a significant role of cytoplasmic calcium activity for the induction of hsp32/HO-1 following stress events.

In our study, the incubation of hepatocytes with the calcium ionophore A23187, which permeabilizes the cell membranes for  $\text{Ca}^{2+}$  leading to an increase in intracellular  $\text{Ca}^{2+}$  concentrations [22], produced only a slight increase in hsp32/HO-1 induction when compared to thapsigargin incubation. This effect corresponds to the observations of Terry *et al.*, who also demonstrated only a slight expression of hsp32/HO-1 in human vascular endothelial cells following incubation with A23187 [6]. Moreover, since we could not demonstrate a decrease in hsp32/HO-1 induction by P/BSO in the presence of the extracellular  $\text{Ca}^{2+}$ -chelator EDTA, we conclude a subordinate role of the extracellular  $\text{Ca}^{2+}$ -pool in the pathway of hsp32/HO-1 gene induction. In summary, our observations demonstrate that a major signaling mechanism for the hsp32/HO-1 induction in isolated hepatocytes is increased cytoplasmic calcium activity, with the main source of calcium ions being from intracellular rather than from extracellular  $\text{Ca}^{2+}$  pools.

The hsp70 gene was also shown to be induced by the cellular stress model using P/BSO [19]. As demonstrated here for the hsp32/HO-1 gene, this P/BSO-triggered induction was suppressed after loading the cells with the intracellular  $\text{Ca}^{2+}$ -chelator BAPTA-AM. However, by contrast to hsp32/HO-1, the induction of hsp70 in this stress model was also prevented by incubation of the cells in

a  $\text{Ca}^{2+}$ -free medium containing EDTA. This suggests a significant role of the cytoplasmic  $\text{Ca}^{2+}$ -activity in the induction of the hsp70 gene. Furthermore, the role of the calcium-ions from the extracellular pool seems to be evident in the underlying signaling mechanism. Our data is in accordance with observations of Yamamoto *et al.* [23] in cultured rat proximal tubulus cells. They reported that hsp70 induction was reduced by lowering the extracellular  $\text{Ca}^{2+}$ -concentration and abolished by buffering the cytoplasmic  $\text{Ca}^{2+}$ -activity. In our study, incubating the hepatocytes with ionophore A23187, which produces an increased cytoplasmic calcium activity by calcium influx from the extracellular medium, increased the hsp70 gene expression. Likewise, in a rat hepatoma cell line, the  $\text{Ca}^{2+}$  ionophore ionomycin also induced hsp70 synthesis [13]. Thus, this data supports the involvement of extracellular and cytoplasmic  $\text{Ca}^{2+}$  in the regulation of the hsp70 gene. Our data further demonstrates that incubation with Tg did not significantly induce the hsp70 gene, by contrast to HO-1 gene induction. In a chondrocytic cell line, Elo *et al.* [12] did not observe any hsp70 gene induction in response to increased cytoplasmic  $[\text{Ca}^{2+}]_i$ , neither by incubation with Tg, nor by Ionophore A23187. From their data, those authors concluded that calcium-mediated responses are unlikely to cause the stress response in this cell line. However, from our study, it can be concluded that increased cytosolic  $\text{Ca}^{2+}$  activity with a role of calcium ions from the extracellular  $\text{Ca}^{2+}$  pool seems to be a major mechanism in the induction of the hsp70 gene in isolated rat hepatocytes.

In summary, we could identify different  $\text{Ca}^{2+}$ -dependent signaling mechanisms in the induction of the two stress genes, hsp32/HO-1 and hsp70, in primary cultures of rat hepatocytes. An increase in the cytoplasmic calcium activity seems to play a key role in the signaling cascade leading to induction of the two genes. However, the sources of the calcium ions which flux into the cytoplasm are different. Our data provides evidence that for the hsp32/HO-1 gene, a  $\text{Ca}^{2+}$  flux from intracellular stores (e.g. the endoplasmic reticulum), and for the hsp70 gene, a  $\text{Ca}^{2+}$  flux from the extracellular medium is a major mechanism in a complex cascade of cellular signal events.

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