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A proteasome inhibitor confers cardioprotection

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Abstract

Objective: In several cell types, proteasome inhibitors like carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) induce the 72 kDa heat shock protein (Hsp72) and exert cell protective effects. However, data in cardiomyocytes are currently lacking. **Methods and Results:** We investigated the effects of MG132 in cultured neonatal rat cardiomyocytes. MG132 time- and concentration-dependently induced Hsp72 and Hsp32 at mRNA and protein levels. Although Hsp60 mRNA was induced, Hsp60 protein levels were not altered. MG132 activated p38 MAP kinase already after 0.5 h. Hsp mRNA induction started after 2 h of MG132 treatment. Subsequently, Hsp72 and Hsp32 protein levels were increased after 4 h. SB202190, an inhibitor of p38 MAP kinase, concentration-dependently attenuated MG132-induced Hsp72-and Hsp32-elevations (by 59% and 41%, respectively, at 1 μM SB202190). In contrast, herbimycin A, a known inductor of Hsp72 in cardiomyocytes, enhanced the MG132-induced Hsp72 and Hsp32 expression even further: additionally applied 2 μM herbimycin A induced Hsp72 and Hsp32 about 2-fold higher than 1 μM MG132 alone. MG132 (1 μM) decreased the hyperthermia- or hydrogen peroxide-induced release of lactate dehydrogenase by 45% and by 35%, respectively ($P<0.05$, $n=5$). MG132 (1 μM) prolonged the spontaneous beating time of cardiomyocytes at 46 °C from 5±2 min (control hyperthermia) to 28±5 min ($P<0.05$, $n=4$). Thus, inhibition of the proteasome function by MG132 protects cardiomyocytes against hyperthermic or oxidative injury. This protective effect and Hsp induction were abolished by 1 μM SB202190. **Conclusion:** Proteasome inhibition results in p38 MAP kinase-dependent induction of Hsp72 and Hsp32 and might be a novel cardioprotective modality. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Gene expression; Myocytes; Protein kinases

1. Introduction

Hsp72, the archetypal heat shock protein, can protect the heart against stressors like ischemia, oxidative stress, or heat and prevent apoptosis and necrosis. This has been demonstrated in various model systems: hyperthermia increased myocardial Hsp72 levels and this protected the heart against the noxious effect of a second period of hyperthermia (reviewed in [1]). A causal link between Hsp72 expression and myocardial protection is further strengthened by experiments in transgenic mice: cardiac overexpression of Hsp72 in these genetically manipulated mice protected—in part—the heart against ischemic damage (reviewed in [1]).

Hsp60 is a major chaperone of mitochondria and is constitutively expressed in many tissues (reviewed in [2]). Ischemia released Hsp60 from the myocardium [3]. Hsp60 has been implicated in cardioprotection when co-expressed with Hsp10 [4].

Hsp32, too, exerted cardioprotective effects: enhanced expression of Hsp32 improved cardiac xenograft survival [5]. Likewise, upregulation of Hsp32 reduced myocardial infarct size and improved postischemic recovery [6]. Furthermore, hypoxia led to right ventricular dilatation and infarction in Hsp32-deficient mice [7].

Given the potential important cardioprotective effect of some Hsps it is of great interest to further examine potential non-toxic inducers of Hsps. Several drugs can induce Hsps in the heart. For instance, in cardiomyocytes, herbimycin A [8,9], L-glutamine [10], and dexamethasone [11] can induce Hsp72. However, proteasome inhibitors—either alone or in combination with other Hsp-inducing compounds—have not yet been studied in cardiomyocytes for Hsp induction. Why might they be cardioprotective,

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too? Proteasomes are intracellular multisubunit complexes that regulate the degradation of regulatory as well as damaged cellular proteins (reviewed in [12]). Proteasome function is important for the integrity of cells. Upon inhibition of proteasomes, certain regulatory as well as damaged proteins accumulate. These are intracellular signals for Hsp72 induction [13]. In non-myocardial cells such as canine kidney cells, the proteasome inhibitor MG132 induced Hsps including Hsp72 and protected these cells against lethal hyperthermic injury [14]. It has not yet been investigated whether MG132 can induce Hsp72, Hsp60, and Hsp32 in cardiomyocytes. Besides, proteasome inhibitors can activate the stress-responsive p38 mitogen-activated protein (MAP) kinase. Activating p38 MAP kinase can be cardioprotective (reviewed in [15]). This cardioprotective effect of p38 MAP kinase could be mediated by the induction of cardioprotective Hsps or by phosphorylation of other substrates that convey protection.

The present study was designed to answer the following questions: (1) Does a proteasome inhibitor (MG132) induce Hsp72 and/or other Hsps in cardiomyocytes? (2) What is/are the mechanism(s) of Hsp72 induction? (3) Does MG132 protect cardiomyocytes from injury? (4) Does herbimycin A (a known inducer of Hsp72) enhance MG132-mediated Hsp72 induction?

Here, we report for the first time: In cardiomyocytes inhibition of proteasome function leads to p38 MAP kinase-dependent induction of Hsp72 and Hsp32 that can protect cardiomyocytes from injury. Hence, MG132 might protect the heart against stress via the activation of the p38 MAP kinase pathway and concomitant Hsp induction.

2. Methods

2.1. Cell culture

The investigation conforms with the *Guide for the Care and the Use of Laboratory Animals* published by the US National Institutes of Health (publication No. 85-23, revised 1996). Whole hearts were isolated from 1- to 3-day-old Sprague–Dawley CD rats (Harlan Winkelmann, Borchen, Germany). Minced myocardial tissues were treated with phosphate-buffered saline (PBS) containing 0.25% trypsin for 10 min at 37 °C. Then, the tissue was digested with subsequent cycles of 0.05% Worthington collagenase CLS II (Biochrom, Berlin, Germany). The resulting cardiomyocytes were plated in culture dishes (Nunc, Wiesbaden, Germany) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Eggenstein, Germany) containing 10% horse serum and 2% fetal bovine serum (both from Gibco), 2 mmol/l glutamine (Sigma, Deisenhofen, Germany), and antibiotics (penicillin 100 IU/ml and streptomycin 100 μg/ml, both from Gibco). After 3–5 days during which cardiomyocytes formed a confluent spontaneously beating monolayer, the medium was changed to serum-free DMEM supplemented with 2 mmol/l glutamine, 25 μg/ml bovine serum albumin, 25 μg/ml bovine insulin, 25 μg/ml iron-saturated transferrin (all from Sigma), and antibiotics as described above. The experiments were performed after equilibrating the cells under serum-free conditions for 20–24 h.

2.2. Experimental protocols

Spontaneously contracting cardiomyocytes were pretreated with solvent (dimethyl sulfoxide, DMSO) or the inhibitor of p38 MAP kinase SB202190 (Biomol, Hamburg, Germany) for 0.5 h. After this pretreatment cardiomyocytes were incubated with solvent (DMSO), or the proteasome inhibitor carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132, Biomol) at different concentrations. In separate experiments cardiomyocytes were treated with 2 μM herbimycin A (Sigma), or 1 μM MG132 alone, or a combination of both for 4 h.

Cardiomyocyte cultures were placed into a temperature-controlled sealed Plexiglas chamber equilibrated in humidified air containing 5% CO_2 and mounted onto the stage of an inverted phase-contrast microscope (Labovert FS, Leica, Bensheim, Germany). Contracting cardiomyocytes were observed at a magnification of 100-fold and the resulting image was sent to a video camera (FK7512-IQ, Pieper, Schwerte, Germany), viewed on a monitor (WV-5410, Pieper), and simultaneously stored on a video cassette recorder (AG7350, Panasonic, Japan) as reported earlier by our group [16]. Under continuous observation the cardiomyocytes were heat treated at 46 °C for 1 h. During this severe hyperthermic injury MG132 was still present. We measured the time until complete cessation of beating was observed (time to arrest). Alternatively, MG132 was removed from cardiomyocytes by decanting the media and washing the cells two times for 10 min with PBS. Then, cardiomyocytes were injured by exposure to 1 mM hydrogen peroxide (Sigma) for 2 h. Viability of cardiomyocytes was determined by measuring their release of lactate dehydrogenase (LDH) into the culture media using a commercial kit following the instructions of the manufacturer (Sigma) and were expressed as U/10^4 cells/h.

2.3. Papillary muscles

Studies in rat papillary muscle preparations were performed as described [17]. MG132 was added cumulatively to the organ bath for 10 min at each concentration (1.0, 10.0 and 100 μM).

2.4. Northern blotting

Northern blotting was performed as described [18]. Membranes were hybridized against a 745 kb Eco RI/Hind III fragment of human Hsp72 cDNA (Stressgen, Victoria,

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Canada) for Hsp72. Hybridizations for Hsp60 and Hsp32 were performed using radiolabeled cDNA probes generated by polymerase chain reaction (PCR) using primers by Clontech Laboratories, Palo Alto, USA.

2.5. Western blotting

Western blotting was performed as described recently [18]. Antibodies for Hsp72 and Hsp60 were from Stressgen and for Hsp32 from Affinity BioReagents (Golden, CO, USA). Radioactive bands were visualized and quantitated in a PhosphorImager™ as described above.

2.6. Immunological assay for phosphorylation state of p38 MAP kinase

The immunological assay to determine the phosphorylation state of p38 MAP kinase was performed as described recently [17].

2.7. Statistics

Data are means±S.E.M. Statistical analysis was performed using Student's t-test or analysis of variance (ANOVA) followed by Bonferroni's t-test for multiple comparisons. A P-value less than 0.05 was considered significant.

3. Results

3.1. Concentration-dependent induction of Hsps in response to MG132

First, we studied whether in cardiomyocytes MG132 induced Hsp72. We incubated the cells with MG132 (0.1 to 100 μM) for 3 h. Fig. 1A depicts a representative Northern blot showing two transcripts for inducible Hsp72 at 2.6 and 3.0 kb. Whereas untreated cells did not express notable amounts of Hsp72 mRNA, the induction started at 1 μM MG132 and further increased until 100 μM (the highest

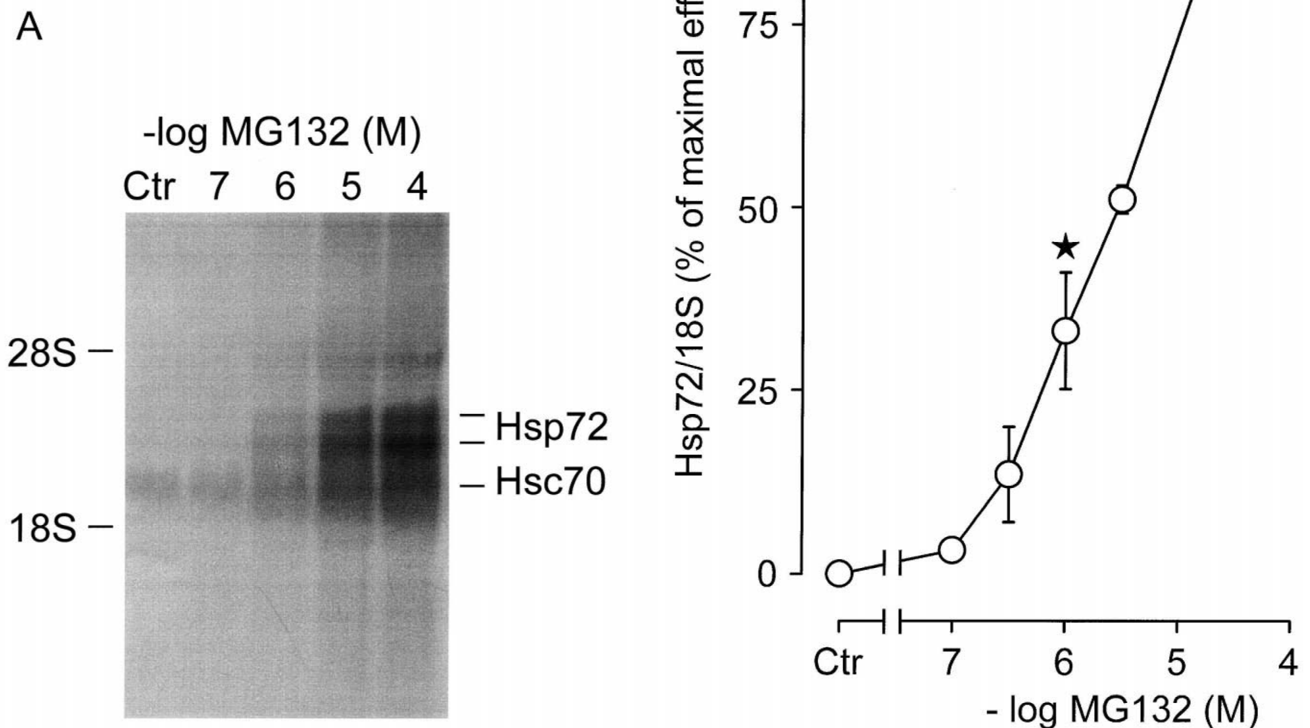

Fig. 1. Concentration-dependent effects of MG132 on the expression of heat shock proteins (Hsp). Contracting cardiomyocytes were treated with MG132 for 3 h and then Northern blot analysis was performed as described in the Methods. Representative autoradiograms are shown and the locations of 28S and 18S ribosomal RNAs are indicated on the left. (A) Representative autoradiogram for Hsp72. (B) Quantification of Hsp72 mRNA levels normalized to 18S. Abscissa: concentration of MG132 in mol/l (M). Ordinate: ratio of Hsp72 mRNA to 18S. (C) Representative autoradiogram for Hsp60. (D) Quantification of Hsp60 mRNA levels. (E) Representative autoradiogram for Hsp32. (F) Quantification of Hsp32 mRNA levels. (*$P<0.05$ vs. Ctr, $n=3$).

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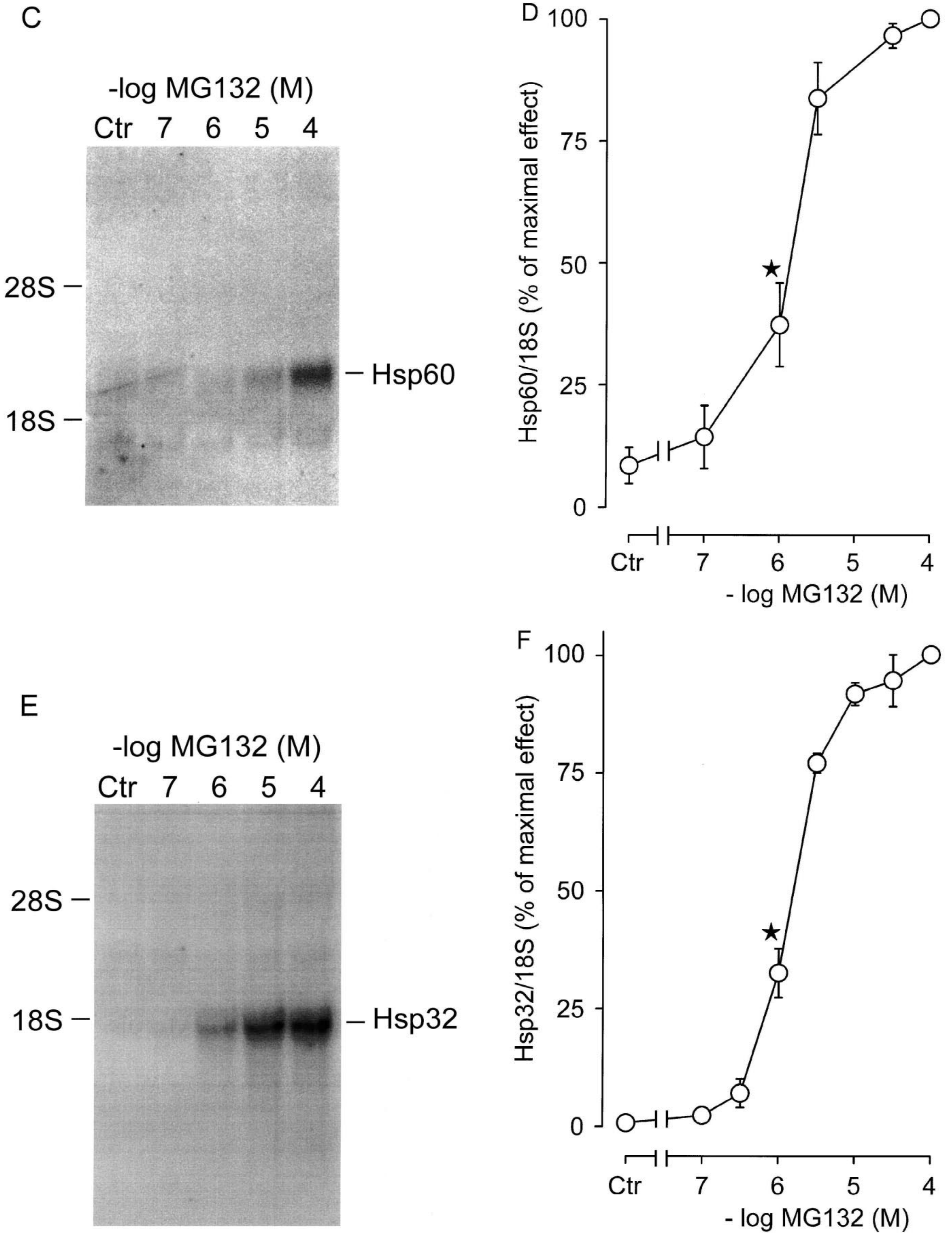

Fig. 1. (*continued*)

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concentration studied). Blots were then hybridized against 18S ribosomal RNA. Because MG132 treatment did not alter 18S expression, we normalized Hsp72 mRNA levels to 18S levels. These experiments are summarized in Fig. 1B. The half-maximal effective concentration of MG132 for Hsp72 mRNA induction was approximately 3 μM.

Fig. 1C, E shows representative Northern blots with transcripts of Hsp60 at 2.4 kb and Hsp32 at 1.8 kb. Several experiments are summarized in Fig. 1D, F. MG132 concentration-dependently induced Hsp60 and Hsp32 mRNAs with half-maximal effective concentrations of 1.5 and 2 μM, respectively.

MG132 concentration-dependently induced Hsp72 and Hsp32 proteins with half-maximal effective concentrations similar to mRNA data. In contrast, despite low mRNA levels, Hsp60 protein was highly expressed under control conditions. Although Hsp60 mRNA was clearly induced by MG132, Hsp60 protein levels did not further increase at MG132 concentrations up to 100 μM.

3.2. Time-dependent induction of Hsps in response to MG132

In order to investigate the time-course of Hsp induction, we exposed cardiomyocytes to 10 μM MG132 for different periods of time. As shown in representative autoradiograms (Fig. 2A, C, E) the Hsp72, Hsp60, and Hsp32 mRNAs were clearly elevated after 2 h of treatment. They increased until 5 h (the longest time studied). These experiments are summarized in Fig. 2B (Hsp72), D (Hsp60), and F (Hsp32). Whereas Hsp72 seemed to reach a plateau after 4 h, Hsp60 and Hsp32 increased throughout the time frame studied here.

Representative autoradiograms of Western blots for Hsp72, Hsp60, and Hsp32 are depicted in Fig. 3A. Data are summarized in Fig. 3B (Hsp72), C (Hsp60), and D (Hsp32) showing increased protein expression of Hsp72 and Hsp32, but not Hsp60 at 3 h. For comparison, time-courses of respective mRNA levels are plotted again in these figures. Hsp72 and Hsp32 protein induction started about 1 h later than the induction of respective mRNAs indicative of transcriptional regulation. At 1 μM, MG132 induced Hsp72 and Hsp32 protein levels with a similar time-course but to a lesser extent (data not shown).

To determine whether MG132 acts via the p38 MAP kinase, we determined the levels of dually phosphorylated (activated, P-p38) as well as total levels of p38 MAP kinase. The total levels (sum of phosphorylated and non-phosphorylated forms, T-p38) of p38 MAP kinase remained unchanged (see Fig. 3A) and hence were used as a reference point for calculating the ratio of phospho-p38 and total p38 MAP kinase levels. Typical autoradiograms are shown in Fig. 3A. In unstimulated cardiomyocytes very low levels of activated (e.g., phosphorylated) p38 MAP

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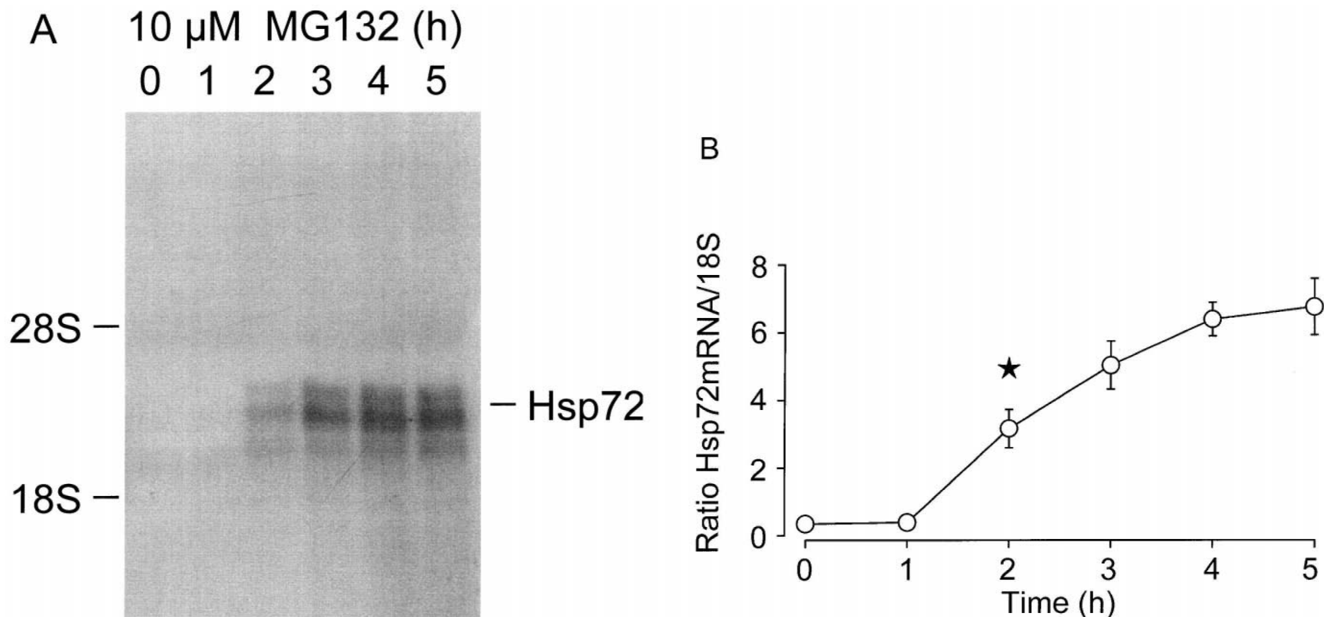

Fig. 2. Time-dependent effects of MG132 on expression of Hsps in rat cardiomyocytes. Contracting cardiomyocytes were treated with 10 μM MG132 for various periods of time as indicated and then Northern blotting was performed as described in the Methods. Representative autoradiograms are shown. The locations of 28S and 18S ribosomal RNAs are indicated on the left. (A) Representative autoradiogram for Hsp72. (B) Quantification of the time-dependent effects of MG132 on Hsp72 mRNA levels. Abscissa: duration of treatment with MG132 in hours (h). Ordinate: ratio of Hsp72 mRNA to 18S. (C) Representative autoradiogram for Hsp60. (D) Quantification of time-dependent effects of MG132 on Hsp60 mRNA levels. (E) Representative autoradiogram for Hsp32. (F) Quantification of time-dependent effects of MG132 on Hsp32 mRNA levels. *$P<0.05$ vs. 0 h, $n=5$–6.

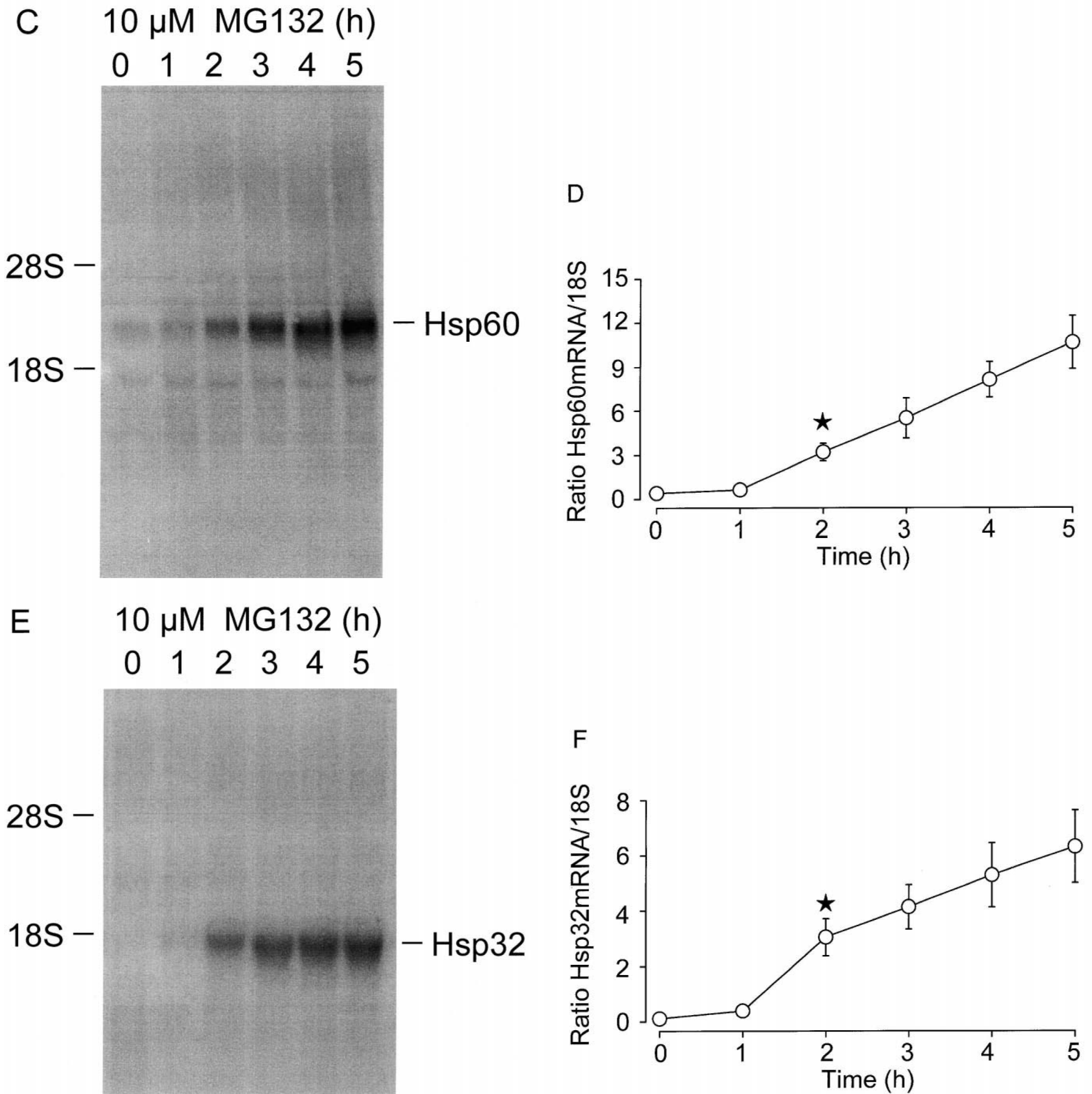

Fig. 2. (*continued*)

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kinase were detected. After 0.5 h of treatment p38 MAP kinase was activated. This pattern is exemplified in Fig. 3A. The summarized data are given in Fig. 3B–D. MG132 (1 μM) exhibited a similar time-course, albeit the maximum p38 MAP kinase activation was lower (data not shown). MG132 at 1 μM for 6 h induced Hsp72 15-fold compared to control levels in DMSO-treated cardiomyocytes. Hsp72 protein induction was 25-fold by heat shock at 42 °C for 30 min followed by 6 h recovery.

3.3. Effect of herbimycin A on MG132-induced Hsp expression

We investigated whether herbimycin A can enhance MG132-induced Hsp expression further. Cardiomyocytes were treated with 2 μM herbimycin A alone (HA), 1 μM MG132 alone (MG), or a combination of both (HA+MG) for 4 h. Hsp72 and Hsp32 protein levels were quantitated and are depicted in Fig. 4A, B, respectively. Thus, while

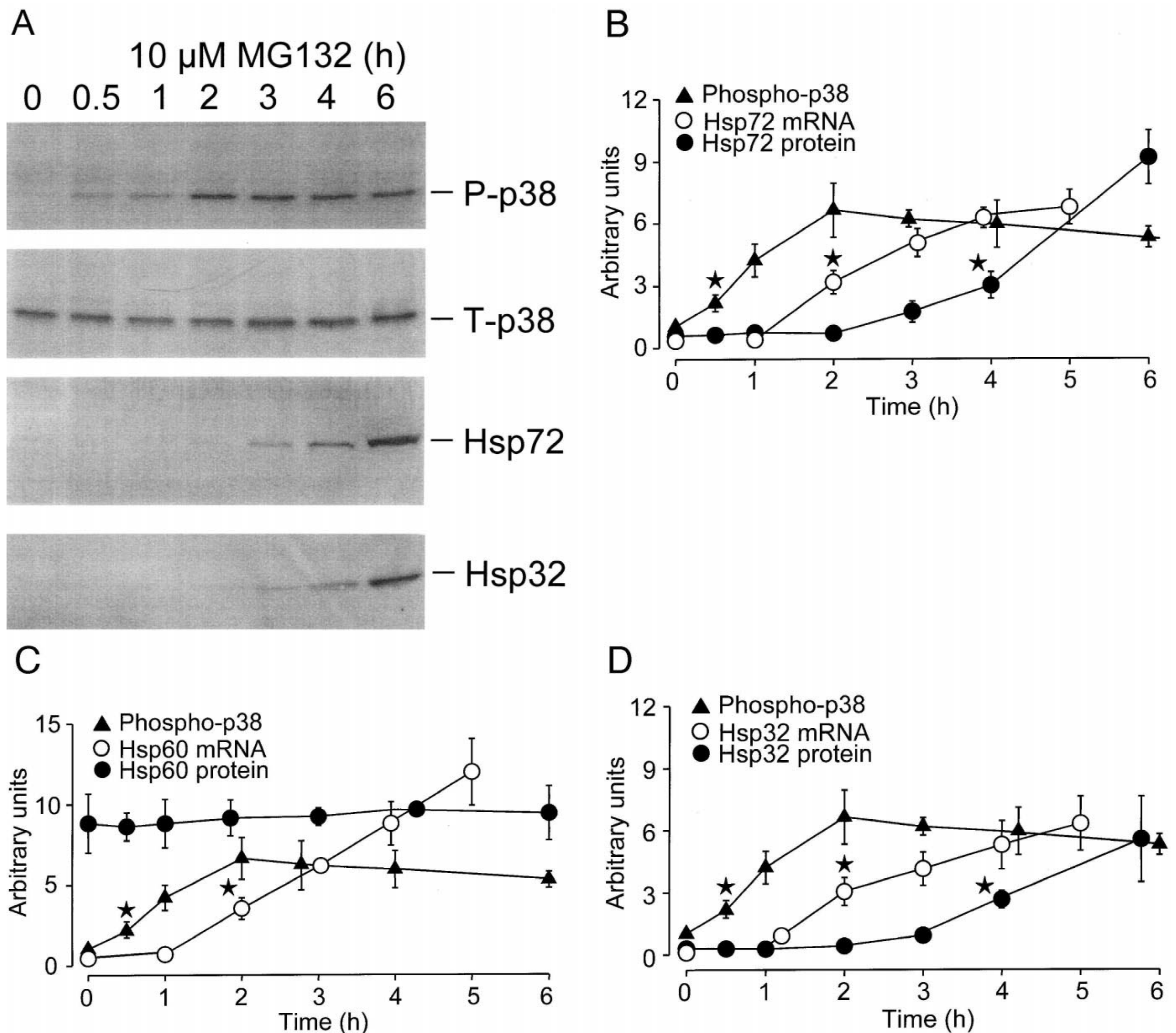

Fig. 3. Time-dependent effect of MG132 on p38 MAP kinase phosphorylation in cardiomyocytes. Contracting cardiomyocytes were treated with 10 μM MG132 and then Western blot analysis was performed as described in Methods. (A) Representative autoradiograms for activated (P-p38), total p38 MAP kinase (T-p38), Hsp72, and Hsp32 proteins. The duration of treatment in h (h) with MG132 is indicated. (B) Quantification of phospho-p38 levels normalized to total p38 MAP kinase as well as Hsp72 mRNA and protein levels. Abscissa: duration of treatment with MG132 in h (h). Ordinate: ratio of Phospho-p38/total p38, ratio of Hsp72 mRNA/18S, and Hsp72 protein levels in PhosphorImager units (* $P<0.05$ vs. 0 h, $n=5$–6). (C) Quantification of phospho-p38 levels normalized to total p38 MAP kinase as well as Hsp60 mRNA and protein levels. (D) Quantification of Hsp32 mRNA and protein levels. Abscissa: duration of treatment with MG132 in h (h). Ordinate: ratio of Phospho-p38/total p38, ratio of Hsp32 mRNA/18S, and Hsp32 protein levels in PhosphorImager units (* $P<0.05$ vs. 0 h, $n=5$–6).

herbimycin A alone did neither affect Hsp72 nor Hsp32 levels, the combination of MG132 and herbimycin A was more effective than MG132 alone. This synergism might indicate a common final pathway for both Hsps.

3.4. Effect of p38 MAP kinase inhibition on MG132-induced Hsp expression

MG132 time-dependently enhanced p38 MAP kinase activation. This activation preceded the induction of Hsp72 and Hsp32 mRNAs (Fig. 3B, D). Therefore, we hypothesized that p38 MAP kinase activation might be a prerequisite for Hsp72 and Hsp32 induction. To study a possible causal relationship between increased p38 activation and Hsp induction, we inhibited p38 MAP kinase activity by pre-treating cardiomyocytes with SB202190 at concentrations of 0.03 to 5 μM for 30 min prior to as well as during MG132-treatment. Fig. 5A depicts such an experiment. It shows a representative autoradiogram of a Western blot for Hsp72. SB202190 alone had no effect on

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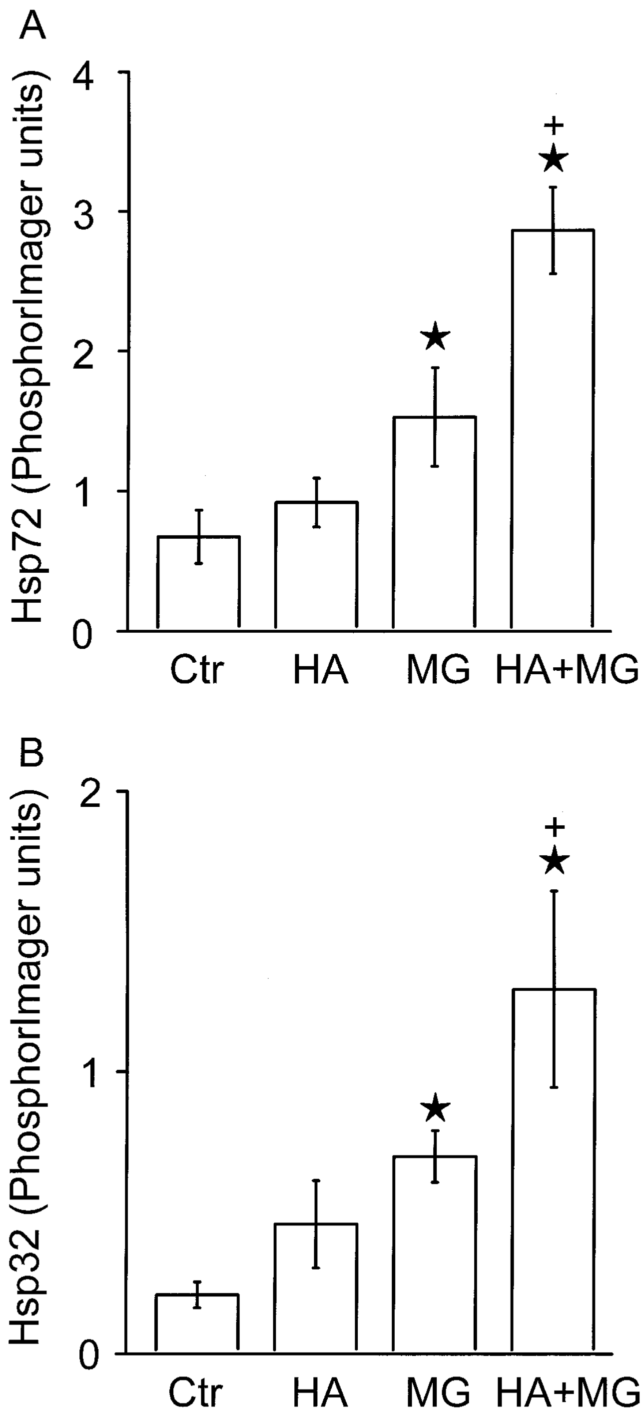

Fig. 4. Effect of herbimycin A on MG132-induced protein expression of Hsps in rat cardiomyocytes. Contracting cardiomyocytes were treated for 4 h with solvent (Ctr), 2 μM herbimycin A (HA), 1 μM MG132 (MG), or both (HA+MG) and then Western blotting was performed as described in Methods. (A) Hsp72 levels. Abscissa: treatments. Ordinate: Hsp72 protein levels in PhosphorImager units. (B) Hsp32 levels. Abscissa: treatments. Ordinate: Hsp32 protein levels in PhosphorImager units (* $P<0.05$ vs. Ctr, $^{+}$ $P<0.05$ vs. MG, $n=3$).

Hsp72 and Hsp32 levels, but it inhibited the MG132-induced Hsp protein induction starting at a concentration of 0.1 μM. Fig. 5B, C summarizes the data. At 1 μM,

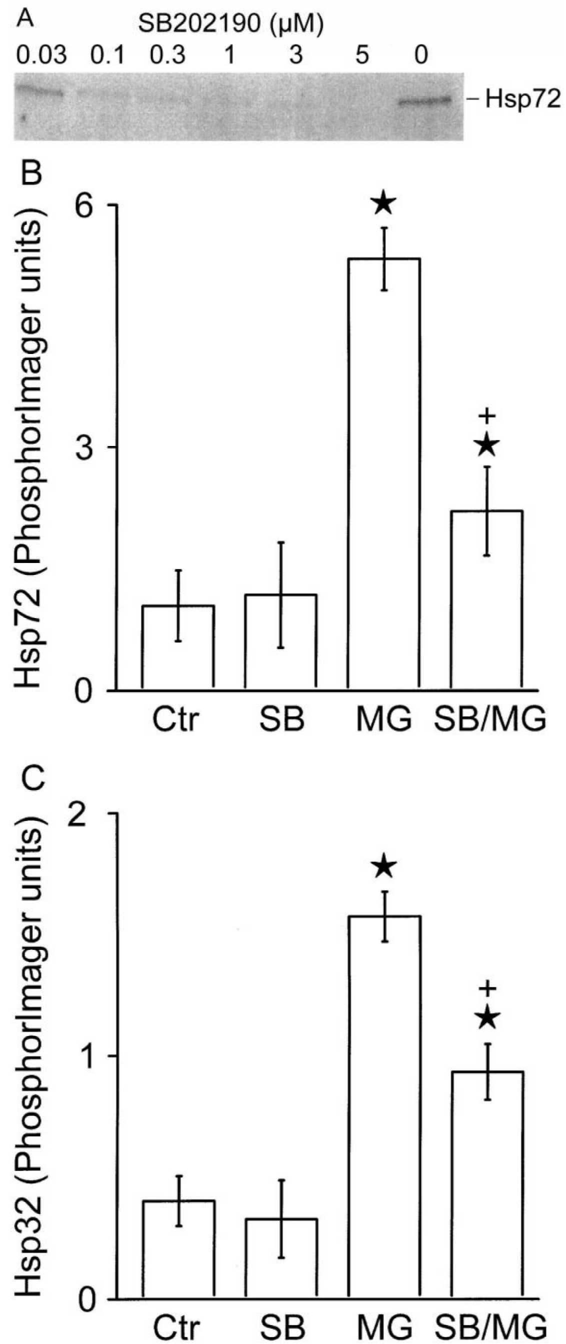

Fig. 5. Concentration-dependent effects of SB202190 on the induction of Hsps by MG132 in cardiomyocytes. Contracting cardiomyocytes were pretreated with different concentrations of the p38 MAP kinase inhibitor SB20219 for 0.5 h. Then, cardiomyocytes were treated with 10 μM MG132 for 4 h and Western blot analysis was performed as described in Methods. (A) Representative autoradiogram for Hsp72. (B) Quantification of Hsp72 protein levels in cardiomyocytes. Abscissa: control (Ctr), 1 μM SB202190 (SB), 10 μM MG132 (MG), pretreatment with 1 μM SB202190 for 0.5 h and then treatment with 10 μM MG132 for 4 h (SB/MG). Ordinate: Hsp72 protein levels in PhosphorImager units (* $P<0.05$ vs. Ctr; $^{+}P<0.05$ vs. MG, $n=5$). (C) Quantification of Hsp32 protein levels in cardiomyocytes. Abscissa: control (Ctr), 1 μM SB202190 (SB), 10 μM MG132 (MG), pretreatment with 1 μM SB202190 for 0.5 h and then treatment with 10 μM MG132 for 4 h (SB/MG). Ordinate: Hsp32 protein levels in PhosphorImager units (* $P<0.05$ vs. Ctr; $^{+}P<0.05$ vs. MG, $n=5$).

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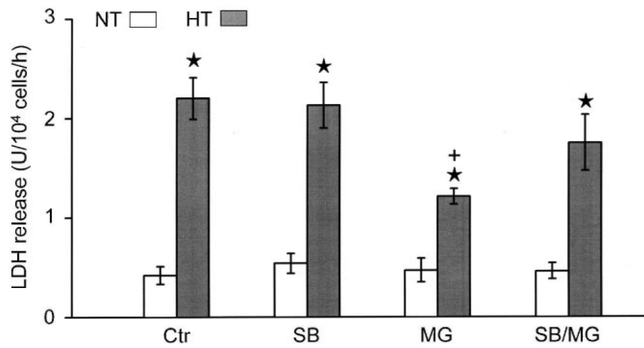

Fig. 6. Effects of MG132 and SB202190 on release of lactate dehydrogenase (LDH) from contracting rat cardiomyocytes during severe hyperthermic injury into the cell culture media. Cardiomyocytes were pre-incubated with solvent (Ctr) or 1 μM SB202190 for 0.5 h (SB). After this pre-incubation period, cardiomyocytes were treated with solvent (Ctr) or 1 μM MG132 (MG, SB/MG) for 6 h. Then, cardiomyocytes were either incubated at 37 °C (NT), or heat treated at 46 °C (HT) for 1 h. LDH release from cardiomyocytes into the cell culture media was determined as described in Methods. Abscissa: treatments. Ordinate: LDH release in U/10^4 cells/h. *$P<0.05$ vs. corresponding NT, $^+P<0.05$ vs. Ctr, ($n=3$–5).

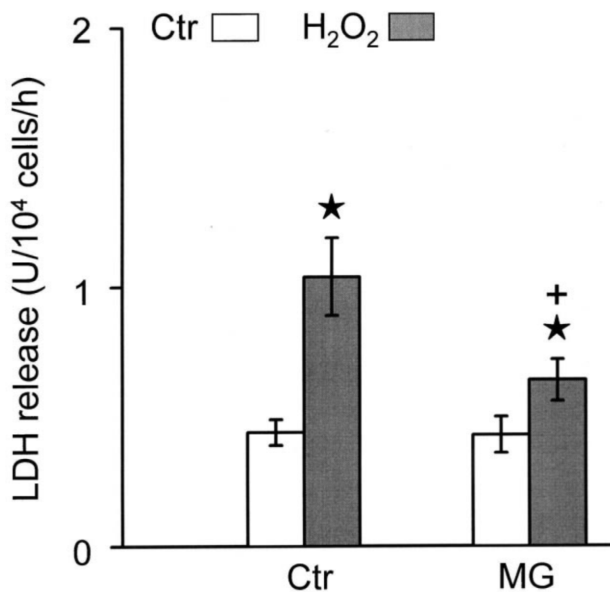

Fig. 7. Effects of MG132 and SB202190 on release of lactate dehydrogenase (LDH) from contracting rat cardiomyocytes during oxidative stress injury into the cell culture media. Cardiomyocytes were pre-incubated with solvent (DMSO) or 1 μM MG132 (MG) for 6 h. Then, MG132 was removed by two PBS washes and fresh medium was added. Then, cardiomyocytes were either incubated with solvent (Ctr), or with 1 mM hydrogen peroxide (H_2O_2) for 2 h. LDH release from cardiomyocytes into the cell culture media was determined as described in Methods. Abscissa: treatments. Ordinate: LDH release in U/10^4 cells/h. *$P<0.05$ vs. Ctr, $^+P<0.05$ vs. Ctr, ($n=3$–5).

SB202190 inhibited Hsp72 and Hsp32 protein induction by 59% and 41%, respectively. Thus, p38 MAP kinase might interfere with the pathway of induction of both Hsp72 and Hsp32.

3.5. Effect of MG132 on cardiomyocyte viability and contractility

Finally, we studied the effect of MG132 on the function of cardiomyocytes. We hypothesized that induction of Hsp72 or other Hsps may lead to protection of cardiomyocytes against stress. Here we applied heat as the classical stressor. As parameters for cardioprotection we used spontaneous contractions and LDH release. By inspection, cardiomyocytes continued to beat at MG132 concentrations up to 30 μM for 6 h. In isolated electrically-driven rat papillary muscles MG132 up to 100 μM (10 min incubation for each concentration) did not affect force of contraction (1.10±0.46 mN at 100 μM MG132 vs. 1.15±0.50 mN (predrug values), $n=3$, $P>0.05$).

As depicted in Fig. 6, the viability of cardiomyocytes (determined as the release of LDH) exposed to 1 μM MG132 for 6 h (MG), or 1 μM SB202190 for 6.5 h (SB), or both (SB/MG) at 37 °C (NT) was the same as in normothermic controls (Fig. 6). Since 1 μM MG132 induced Hsp72 (Fig. 4), we next examined whether treating cardiomyocytes with 1 μM MG132 confers thermotolerance. Heat treatment at 46 °C for 1 h (HT) led to a 5-fold increase of LDH release into the cell culture media compared to normothermic cultures (NT). 1 μM SB202190 did not alter normothermic and hyperthermic LDH release (SB, Fig. 6). However, treatment of cardiomyocytes with 1 μM MG132 resulted in a 45% decrease of hyperthermic LDH release (MG, Fig. 6). This effect was attenuated when cardiomyocytes were pretreated with SB202190 prior to treatment with MG132 (SB/MG, Fig. 6).

Contractions of the cardiomyocytes from the experiments shown in Fig. 6 were observed through a microscope as described in Methods. Under normothermic conditions (NT) cardiomyocytes with or without 1 μM MG132 contracted throughout 1 h (the longest time studied). However, at 46 °C (HT) in MG132-treated cardiomyocytes, time to arrest amounted to 28±5 min compared to 5±2 min without MG132 treatment ($P<0.05$, $n=4$). Thus, time to arrest was prolonged almost 6-fold. In order to study if this protective effect of MG132 is accompanied by Hsp induction, after the experiments described above, cardiomyocytes were lysed and analysed by Western blotting: whereas Hsp60 protein was not elevated by MG132 treatment, both Hsp72 and Hsp32 proteins were induced in MG132 treated cardiomyocytes (data not shown).

We also exposed cardiomyocytes to oxidative stress, namely to hydrogen peroxide. The results are given in Fig. 7. They show a cardioprotective effect of MG132 against

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oxidative stress. Hence, MG132 could confer cardioprotection in the assays used here.

4. Discussion

In cardiomyocytes the proteasome inhibitor MG132 induced Hsp72 and Hsp32 in a p38 MAP kinase-dependent fashion and protected them against severe injury. This is the main new finding of the present work.

In cardiomyocytes (this paper), MG132 concentration- and time-dependently induced Hsp72, and Hsp32 proteins which is similar to results in other cell types: prior to the present study proteasome inhibitors have been known to induce Hsp72, in several cell types including kidney cells [14], lung fibroblasts [19], yeast cells [20], U937 cells [21], HepG2 cells [22], and mouse embryonic fibroblasts [23]. Of note, induction of any Hsp (Hsp72, Hsp60 or Hsp32) by any proteasome inhibitor in cardiomyocytes has hitherto not been reported.

Hsp72 induction may result from enhanced levels of a specific, usually short-lived regulator protein (i.e. HSF2, heat shock transcription factor 2) which accumulates during proteasome inhibition. We provide evidence for the contribution of p38 MAP kinase. This is supported by the fact that in cardiomyocytes MG132 activated p38 MAP kinase in a time-frame and to an extent is reminiscent of earlier work in other cell types [14,21]. We have demonstrated that in cardiomyocytes MG132 induced Hsp72 and Hsp32 at least in part in a p38 MAP kinase-dependent fashion because the induction was attenuated by SB202190, a well known inhibitor of p38 MAP kinase activity (Fig. 5). The involvement of this MAP kinase pathway in the induction of Hsp72 and Hsp32 has been reported by others before in other cell types as well [24–26] including hyperthermic expression. What mechanisms might trigger the activation of p38 MAP kinase ? There is evidence for an unstable regulator protein which accumulates upon proteasome inhibition by MG132 and then activates p38 MAP kinase [27]. Activated p38 MAP kinase might contribute to activation of transcription factors that increase Hsp72 transcription. Although the present data do not unequivocally prove a direct p38 MAP kinase-dependent mechanism, they indicate that a functional p38 kinase is necessary for Hsp72 induction. Herbimycin A is a benzoquinoid ansamycin originally described as a tyrosine kinase inhibitor acting on src-kinases. In the present study herbimycin A did not induce Hsp72 protein after 4 h incubation time. However, it enhanced the MG132-induced Hsp72 expression. Earlier results of other reports showed that herbimycin A induced Hsp72 protein in cardiomyocytes that were incubated with herbimycin A for 4 h, followed by 20–24 h recovery [8,9]. Three mechanisms of action for herbimycin A are currently discussed: (1) Herbimycin A is a known inhibitor of Hsp90 function [28]. Herbimycin A tightly binds to Hsp90 and thus competes with HSF1 for binding to Hsp90. Once released from Hsp90, free HSF1 is activated even in the absence of stress and may induce Hsp72 induction. Thus, herbimycin A may indirectly activate HSF1 by disrupting Hsp90–HSF1 complexes in the cytosol [28]. (2) Herbimycin A might disrupt the protective function of Hsp90 on other proteins in the cell [29] resulting in enhanced levels of misfolded proteins. These cannot be degraded (inhibition of proteasomes by concomitantly applied MG132) and trigger Hsp induction. (3) Alternatively, herbimycin A might enhance Hsp72 induction by increasing the MG132-induced p38 MAP kinase activation. This is based on data in leukemic cells, where herbimycin A time- and concentration-dependently activated p38 MAP kinase [30]. Thus, herbimycin A and MG132 might act additively to induce Hsp72 and Hsp32.

This is the first report where MG132 protected cardiomyocytes against heat shock and oxidative stress. Severe heat shock at 46 °C leads to denaturation of cellular proteins and cell damage [31]. As we show here in cardiomyocytes, MG132 induced Hsp72 and Hsp32. Thus, MG132 may exert cardioprotection by Hsp induction. The overexpression of Hsp72 correlated with protection of the myocardium against injury (reviewed in [2]). Hsp72 may refold and thus repair damaged proteins (reviewed in [2]). Data from the literature clearly show, that heat-stressed cardiomyocytes exerted enhanced degradation of connexin 43 at 43.5 °C, which could be prevented by proteasome inhibitors [32]. Another report indicates enhanced proteolysis during hyperthermia in myotubes [33]. MG132 not only induced Hsp72 but also protected several cell types against hyperthermia: canine kidney cells [14], aged human lung fibroblasts [19], yeast cells [20], and U937 cells [21].

MG132 may protect cardiomyocytes via transient activation of p38 MAP kinase. In the present study inhibition of p38 MAP kinase by SB202190 treatment partially blocked the protective effect of MG132 (Fig. 6). Similarly, in isolated perfused rat hearts p38 MAP kinase inhibition by SB203580 abolished the hyperthermia-induced resistance against myocardial infarction [34]. Various reports support a protective function of p38 MAP kinase in cardiomyocytes (reviewed in [15]). Transient p38-MAP kinase activation might exert cardioprotection by enhanced phosphorylation of Hsp27 and αB-crystallin [35,36].

We assume that MG132 protected cardiomyocytes via the induction of Hsp72. However, other Hsps (i.e. Hsp32) as well as other mechanisms (i.e. p38 MAP kinase activation) may contribute to the cardioprotective effects of MG132. Clearly, Hsp60 did not mediate the cardioprotective effect observed here.

In subsequent work, it will be necessary to study whether MG132 induces Hsps not only in neonatal but also in adult cardiomyocytes. However, several groups have studied cardioprotective mechanisms and Hsp induction in neonatal cardiomyocytes [9,27]. Conceivably, these find-

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ings per se might be relevant at least in new-born infants. Furthermore, it will be worthwhile to investigate whether MG132 can also protect (neonatal or adult) cardiomyocytes from hypoxic/ischemic injury.

In summary, we show here for the first time that a proteasome inhibitor can protect cardiomyocytes from injury. This is accompanied and may be caused by induction of Hsp72 as well as Hsp32 and/or activation of p38 MAP kinase. Proteasome inhibition might be a valid novel therapeutic approach in order to minimize cellular injury during myocardial stress.

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