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Proteasome function is required for activation of programmed cell death in heat shocked tobacco Bright-Yellow 2 cells

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Abstract **To find out whether and how proteasome is involved in plant programmed cell death (PCD) we measured proteasome function in tobacco cells undergoing PCD as a result of heat shock (HS-PCD). Reactive oxygen species (ROS) production, cytochrome *c* levels and caspase-3-like protease activation were also measured in the absence or presence of MG132, a proteasome inhibitor. We show that proteasome activation occurs in early phase of HS-PCD upstream of the caspase-like proteases activation; moreover inhibition of proteasome function by MG132 results in prevention of PCD perhaps due to the prevention of ROS production, cytochrome *c* release and caspase-3-like protease activation.**
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Keywords: Plant programmed cell death; Proteasome; Reactive oxygen species; Cytosolic ascorbate peroxidase; Cytochrome *c*; Caspase-like proteases

1. Introduction

Programmed cell death (PCD) is a genetically regulated process required for normal development and adaptation to a variety of stresses both in plants and in animals [1,2]. In contrast to the animal system, signalling pathways and molecular mechanism of PCD are largely unknown in plants. This applies to the proteasome system, a conserved multicatalytic proteolytic complex, which is known both to regulate a variety of cellular processes via degradation of regulatory proteins in mammals, yeast and plants [3] and to play a major role in regulating PCD in mammals [4–6]. In plants, although it was shown that upregulation of components of the ubiquitin-proteasome pathway occurs during diverse developmental PCD events [7–9], it has been also shown that disruption of proteasome function leads cells to PCD [10]. However, whether and how proteasome participates in PCD in plant cells remains to be fully elucidated, thus necessitating further investigation.

In vitro cultured tobacco (*Nicotiana tabacum*) Bright-Yellow 2 (TBY-2) cells heat shocked at 55 °C undergo PCD (HS-PCD) [11]. We have already shown that in HS-PCD generation of reactive oxygen species (ROS), a decrease in both the amount and the activity of the cytosolic ascorbate peroxidase (cAPX) and the release of cytochrome *c* (cyt *c*) occur [11,12] and that ATP production is modified [13].

In this paper, we investigated both whether and how proteasome function changes during HS-PCD and whether some features of HS-PCD are proteasome-dependent. We show that during HS-PCD there is an activation of the proteasome system that regulates ROS production, ascorbate peroxidase activity, cyt *c* release, and caspase-like protease activation, thus playing a major role for HS-PCD to occur.

2. Materials and Methods

2.1. Reagents

Suc-LLVY-MCA (succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin), Boc-LSTR-MCA (N-*t*-boc-Leu-Ser-threonin-arginin-7-amido-4-methylcournarin), Z-LLE-βNA (N-CBZ-Leu-Leu-Glu-β-naphthylamide), DCFH-DA (2′,7′-dichlorofluorescin diacetate) and MG132 (N-CBZ-Leu-Leu-Leu-Al) were from Sigma (St. Louis, MO, USA). MG132 was dissolved in dimethyl sulfoxide and added to the cells 30 min before the apoptotic treatment. Anti-cyt *c* antibody (7H8-2C12) was purchased from Pharmingen (San Diego, CA, USA). Anti-mitochondrial glutamate dehydrogenase (mGDH) antibody was kindly supplied by Dr. F. Rothe (Institut für Medizinische Neurobiologie, University of Magdeburg, Magdeburg, Germany).

2.2. Cell culture, growth conditions and PCD induction

The suspension of tobacco BY-2 cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) was routinely propagated and cultured at 27 °C according to [14]. A stationary culture was diluted 4:100 (v/v) and cultured for four days. PCD was induced by heat shock at 55 °C and investigated as in [11].

2.3. Proteasome activity assay

TBY-2 cells were ground in liquid nitrogen and lysed in ice-cold homogenization buffer containing 20 mM Tris/HCl (pH 7.2), 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM ATP, 20% glycerol, 0.04% Nonidet P-40. After centrifugation, 40 μg of cell lysates was incubated at 37 °C with the fluorescent substrates Suc-LLVY-MCA (50 μM), Boc-LSTR-MCA (40 μM) and Z-LLE βNA (400 μM) in 150 μL of 50 mM HEPES–Tris, (pH 8.0), 5 mM EGTA, for 20, 30 and 60 min,

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Abbreviations: PCD, programmed cell death; HS-PCD, programmed cell death induced by heat shock; MG132-HS cells, MG132-treated heat shocked cells; ROS, reactive oxygen species; cyt *c*, cytochrome *c*; cAPX, cytosolic ascorbate peroxidase; mGDH, mitochondrial glutamate dehydrogenase

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respectively. The reactions were stopped by adding 1350 μL of 1% SDS. The proteasome function was monitored by measuring the hydrolysis of the above substrates by means of a LS50 Perkin Elmer spectrofluorimeter (380 nm excitation and 460 nm emission for MCA derivatives and 355 nm excitation and 410 nm emission for βNA compounds).

2.4. Detection of cyt c release by immunoblot analysis

Immunoblot analysis was performed on cytosolic and mitochondrial extracts from control and HS-PCD cells as in [12].

2.5. Oxygen uptake

Oxygen consumption was measured polarographically at 25 °C by means of a Gilson 5/6 oxygraph, using a Clark electrode as in [11]. The instrument sensitivity was set to a value which allowed us to follow rates of oxygen uptake as low as 0.5 natoms O/min mg protein.

2.6. Caspase 3-like activity assay

TBY-2 cells were collected from 1 mL of cell suspension by centrifugation ($10000 \times g$, 20 s, 4 °C), ground in liquid nitrogen and lysed at 4 °C in 100 μL of lysis buffer (BD Biosciences, Palo Alto, CA) 10 μg of cell lysate was assayed for caspase 3-like activity with 50 μM of Ac-DEVD-pNA as substrate as in [12].

2.7. ROS production measurement and cAPX assay

To measure intracellular ROS production TBY-2 cells were incubated with the probe DCFH-DA [15] (10 μM) at 27 °C in the dark for 1 h. The fluorescence of the sample (5 μL of cells in 2 mL of PBS) was measured by means of a LS50 Perkin Elmer spectrofluorimeter with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The fluorescence intensify at 528 nm, normalized to the protein content, was used to determine the relative ROS production. A qualitative analysis of ROS production was also performed by viewing the cells under a fluorescent microscope (Axioplan 2 immaging, Zeiss) with an excitation filter of 450–490 nm and a barrier filter of 520 nm.

cAPX activity was assayed on cell extract as described in [11].

2.8. Statistics

The data are reported as the means ± S.E. for the indicated experiments. Statistically significant differences between mean values of control and treated cells were determined using the Student's *t* test.

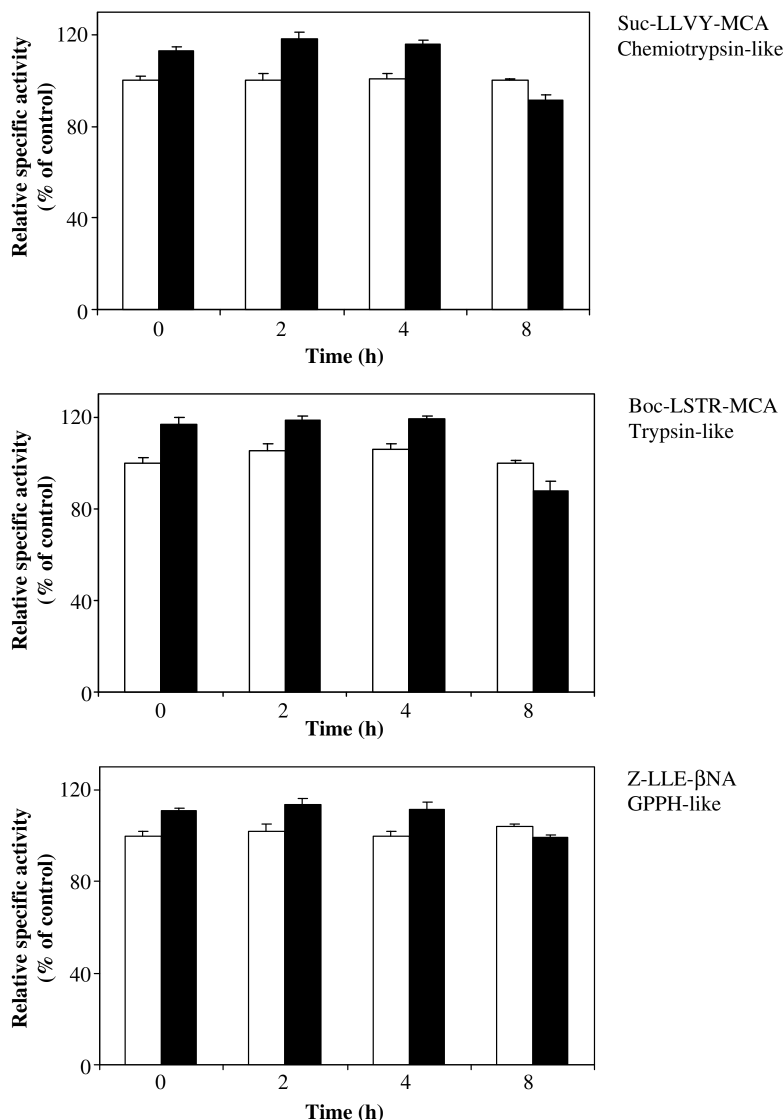

Fig. 1. Proteasome activities in TBY-2 cells during HS-PCD. Cell cultures were heat shocked and returned to 27 °C for a 0–8 h period. 40 μg of cell extract, obtained at the indicate time intervals from both control (□) and HS (■) cells, was incubated with the fluorogenic substrates specific for chymotrypsin-like (Suc-LLVY-MCA), trypsin-like (Boc-LSTR-MCA) and GPPH-like (Z-LLE-βNA) activities at 37 °C (see Section 2). Proteasome activities are expressed as the percentage of activities of control cells at 0 h. Results are means (±S.E.) of three independent experiments.

3. Results

To investigate the involvement of proteasome in plant PCD, use was made of TBY-2 cells, which undergo PCD when heat shocked at 55 °C [11]. To ascertain the presence of proteasome function in these cells as well as to find out whether the proteasome function changes en route to HS-PCD, proteasome function was measured in TBY-2 cell extracts obtained at different times from heat shock, by using Suc-LLVY-MCA, Boc-LSTR-MCA and Z-LLE βNA, which, as a result of the proteasome chymotrypsin-, trypsin-, and peptidylglutamylpeptide (PGPH)-like activities, respectively, produce fluorescent compounds (Fig. 1). Proteasome function was found in control cells; a slight (15–20%) but statistically significant ($P < 0.01$) increase in all three proteasome activities was found in the early phase of PCD (0–4 h). However, a reduction of about 15% of the control was found 8 h after HS.

To investigate the proteasome involvement in HS-PCD further, we used MG132, an inhibitor of proteasome both in animal and plant cells [16,17] and checked whether and how some of the processes occurring during HS-PCD were modified in the cells incubated with MG132. First we confirmed that MG132 inhibits proteasome function in our experimental system. MG132 (10 μM) was added to both control (MG132-C) and HS (MG132-HS) samples 30 min before heat treatment and proteasome activities were measured immediately after HS. All three proteasome activities (see above) were inhibited although at a different degree both in control and HS cells. The activities measured in MG132-HS cells were 25%, 45%, and 39% of those in control HS cells for chymotrypsin-, trypsin-, and PGPH-like activities, respectively (Fig. 2A).

Thus, we compared cell viability up to 8 h in control cells and in HS-PCD cells, both of them either in the absence or in the presence of MG132 (Fig. 2B). No effect on the viability

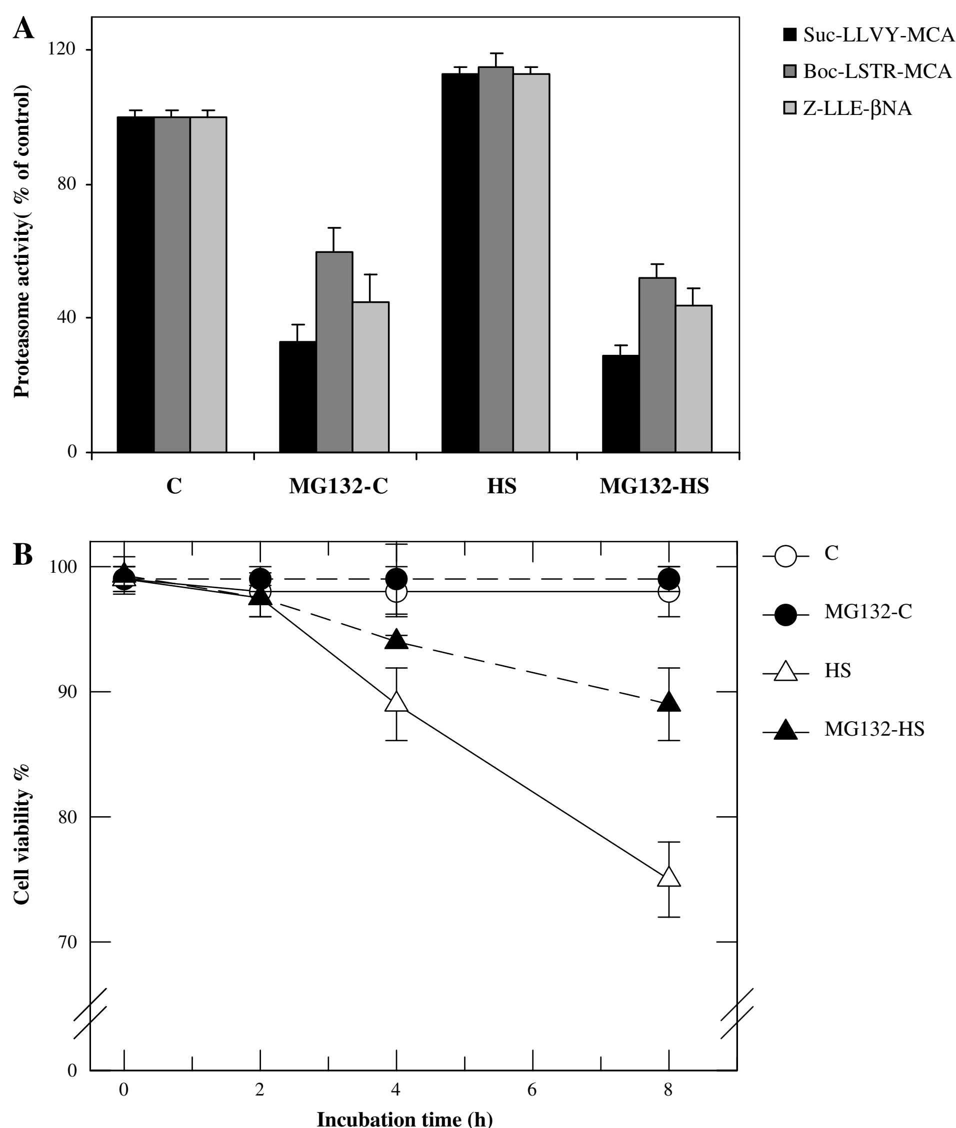

Fig. 2. Effect of MG132 on proteasome activities and cell survival in HS-TBY-2 cells. Both control and HS cells were kept at 27 °C in the absence (C, HS) or in the presence of MG132 (MG132-C, MG132-HS). (A) Immediately after HS, cells were collected and proteasome activities were assayed on cell extracts as described in the legend of Fig. 1. Data are expressed as percentage of activities in control cells. Results are means (±S.E.) of four independent experiments. (B) At the indicate intervals after HS, viability of the cells was measured as reported in Section 2. The percentage (±S.E.) of viable cells was counted in a population of at least 1000 cells in four separate experiments.

of control cells, which was about 98% up to 8 h, was found as a result of MG132 treatment. In HS cells viability decreased to 75%, but was about 90% in MG132-HS cells, this showing that the impairment of the proteasome function results in HS-PCD prevention.

To gain some insight into the mechanism by which this occurs we investigated in MG132-HS cells some PCD features, including ROS production, the antioxidant activity of cAPX, cyt *c* release and caspase 3-like activation [11,12].

Intracellular generation of ROS occurred immediately after HS (Fig. 3A), about 8-fold increase compared to the control (Fig. 3B), as shown by the bright fluorescence resulting from staining with DCFH-DA. By contrast, in MG132-HS cells the increase in fluorescence was only 50% of that observed with control-HS cells (Fig. 3A and B). Because the steady-state level of ROS depends on the balance between ROS-producing and -scavenging reactions, we measured the activity of cAPX, a central component of the ROS scavenging system in plants [18] (Fig. 3C). A 40% of reduction of cAPX activity was found immediately after HS followed by a further progressive decrease up to 8 h. In MG132-HS cells, significantly smaller decreases (18–20%) in cAPX activity were observed in the time range from 0 to 4 h after HS ($P < 0.01$). Notice that 8 h after HS, there was no significant difference between cAPX activity of MG132-HS cells and control-HS cells ($P > 0.01$).

Cyt *c* release was measured in both cytosolic and mitochondrial fractions from HS cells and in MG132-HS cells by immunoblotting analysis using a monoclonal antibody against cyt *c* (Fig. 4A). Two hours after HS, when, due to the technical reasons the first analysis could be carried out (see [12]), the cytosolic cyt *c* content of HS cells was approximately twice that in control with a concomitant decrease in the mitochondrial content. Thereafter, the amount of cyt *c* remains constant in the mitochondria, but is progressively decreased with time in the cytosol, thus showing a cytosolic degradation of the released cyt *c*. In MG132-HS cells, both the cytosolic and mitochondrial cyt *c* content remained the same as that of the control over the whole 8 h period showing that no cyt *c* release was found. Such a conclusion was confirmed by a densitometric analysis of several blots after mGDH normalization (Fig. 4B). In another set of experiments cyt *c* release was monitored by means of polarographic measurements of cyt *c*-dependent ascorbate oxidation in cell homogenates: since ascorbate itself (5 mM) cannot permeate the outer mitochondrial membrane [19], the ability of the cytosolic fraction of HS cells to oxidize ascorbate at a higher rate than that mea-

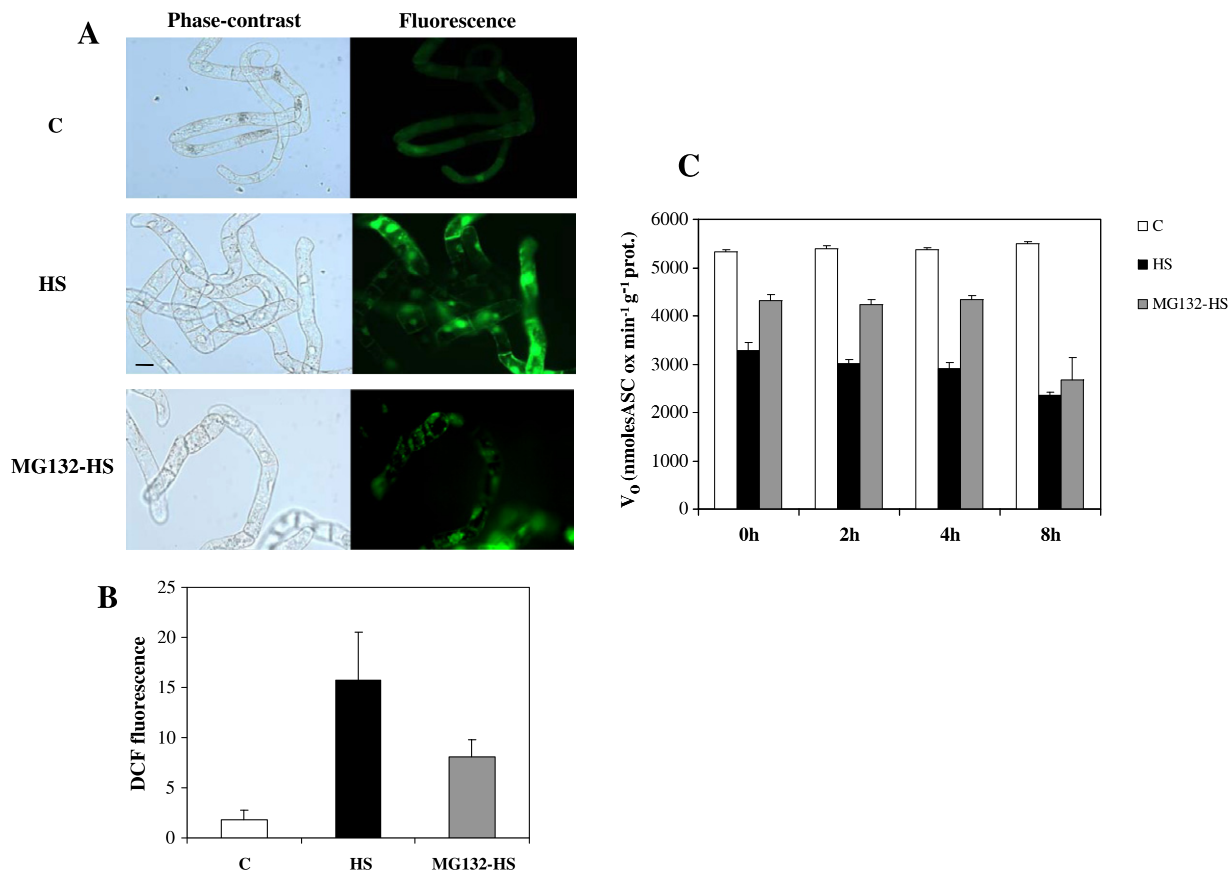

Fig. 3. Effect of proteasome inhibition on ROS production and cAPX activity in HS-TBY-2 cells. Cells were heat shocked in the absence (HS) or in the presence of 10 μM MG132 (MG132-HS). Control cells (C) were also used. (A) The cells immediately after HS were stained with DCFH-DA and ROS production was visualized by fluorescent microscopy as described in Section 2. Cells were photographed under both fluorescent field and phase contrast. Pictures represent typical examples. Bar = 20 μm. (B) Statistical analysis of intracellular ROS content measured as DCF fluorescence as described in Section 2. (C) Cell cultures were heat shocked and returned to 27 °C for a 8-h period. cAPX specific activity was measured on cell extracts and expressed as nmoles ASC oxidized per minute per milligram of protein. Values represent the means (±S.E.) of three independent measurements.

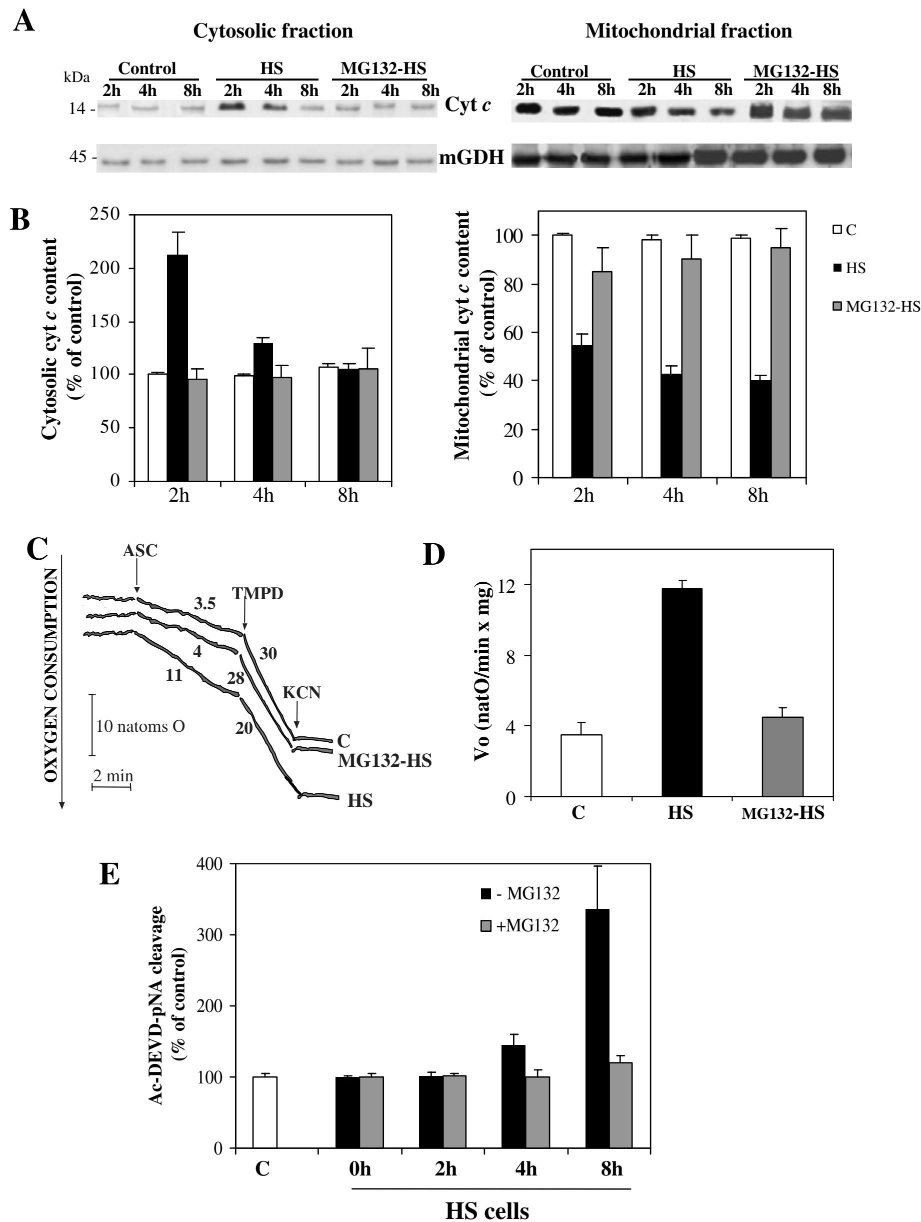

Fig. 4. Effect of proteasome inhibition on cyt *c* release from mitochondria to the cytosol and on caspase-3-like activity in HS-TBY-2 cells. Cell cultures were heat shocked and returned to 27 °C for a 0–8 h period. Where indicated, 10 μM MG132 was added to the cells. (A) Immunoblot analysis of cytosolic and mitochondrial fractions obtained 2, 4, and 8 h after by using anti-cyt *c* antibody. Antibody against mGDH was used to normalize the amount of protein loaded onto the gel. (B) Statistical analysis of cytosolic and mitochondrial cyt *c* content. Cyt *c* content was expressed as percentage of control (C). Values represent the means (±S.E.) of six independent measurements. (C) Activity of cyt *c* released from mitochondria. Homogenates (about 0.2 mg protein) were incubated at 25 °C in the presence of rotenone (3 μM), antimycin (0.8 μM) and myxothiazole (6 μM). Oxygen consumption was started by adding 5 mM ascorbate and the initial rate of the reaction was expressed in natoms O/min/mg cell protein. (D) Statistical analysis of ASC oxygen consumption rate measured as natoms O/min/mg cell protein. Results are the means (±S.E.) of three independent measurements. (E) Activity of caspase 3-like protease. Cells were collected at the indicated time intervals after HS, and caspase-3-like activity was assayed in 10 μg of cell lysate by following the hydrolysis of the specific caspase 3 substrate Ac-DEVD-pNA. Specific activity is expressed as the percentage of the activity of control cells (C). The variability of caspase 3-like activity in the control cells during the 0–8 h period was always less than 5%. Data represent the means (±S.E.) of three independent measurements.

sured in the control reflects the release of active cyt *c* from mitochondria (see [12]). This was found in the control cells, whereas no increase of oxygen consumption due to ascorbate was found in MG132-HS cells, further showing that proteasome impairment prevented cyt *c* release in cells undergoing PCD (Fig. 4C and D).

In parallel, we investigated the caspase-3-like activity in control-HS cells and MG132-HS cells as a function of time (Fig. 4E). No change in caspase 3-like protease activity was found up to 2 h after HS. Thereafter it progressively increased with time, and 8 h after HS was found to be 3.5-fold higher than that measured in control. In MG132-HS cells, the caspase-3-like activity remained the same as that of the control over the whole 8 h period ($P > 0.01$), thus showing that caspase-3-like activation requires the involvement of proteasome in the early phase of PCD.

4. Discussion

We show that proteasome function is required to trigger the death process in HS-PCD. Such a conclusion derives essentially from: (i) the slight but consistent increase in the three peptidase activities of proteasome occurring in the early phase of HS-PCD and (ii) prevention of cell death found in cells with impaired proteasome function. Since in MG132-HS cells ROS production, cyt *c* release and caspase-3-like protease activation which occur in early phase of PCD [11,12] are somehow prevented, we conclude that proteasome function take place in the early phase of HS-PCD, definitely upstream caspase activation.

The origin of the changes in proteasome function in HS-PCD remains a matter of speculation. We might suggest that ROS can trigger proteasome activation, with a feed-back like effect in which, due to the proteasome dependent proteolysis of the antioxidant system including cAPX, ROS production increases en route of PCD. Since the activity of the cAPX, increases in MG132-HS cell as compared to HS cells, we suggest that a cAPX proteasome-dependent proteolysis occurs.

On the other hand, the decrease of proteasome function occurring in the late phase of PCD (8 h after HS) which occurs also in animal systems [5] might be due to other cellular proteases perhaps the caspase-like proteases [20]; consistently we found that caspase-3 like protease is activated 4 h after HS and its activity increases with the time.

The picture emerging from this and from our previously papers is the following: in the early phase of HS-PCD, when ROS production increases as a consequence of oxidative mitochondrial metabolism impairment [11,13], the cell components, including antioxidant enzymes and proteasome, are evoked to maintain sufficient ROS production for application of PCD. Cyt *c* release occurs from intact mitochondria probably to participate to the activation of the caspase-like cascade [12]. In the late phase of PCD, proteasome function decreases slowly, but caspase-like proteases activity increases resulting in degradation of cyt *c* and finally in cell death.

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