Proteasome pathway operates for the degradation of ornithine decarboxylase in intact cells

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Ornithine decarboxylase (ODC) is degraded in an ATP-dependent manner *in vitro* by the 26 S proteasome in the presence of antizyme, an ODC destabilizing protein induced by polyamines. In the present study we examined whether the proteasome catalyses ODC degradation in living mammalian cells. Lactacystin, the most selective proteasome inhibitor, strongly inhibited the degradation of ODC that had been induced in hepatoma tissue-culture (HTC) cells by refeeding with fresh medium. Furthermore the inhibitor inhibited the rapid degradation of ODC that had been induced by hypotonic shock. Interestingly, hypertonic shock was found to increase the proportion of ODC present as a complex with antizyme (the ratio of ODC–antizyme complex to total ODC). Cycloheximide, which partly inhibits rapid ODC degradation caused by hypertonic shock, also partly inhibited the increase in the ratio of ODC–antizyme complex to total ODC. These results suggest that a common ODC degradation pathway, namely the antizyme-dependent and 26 S proteasome-catalysed ODC degradation pathway, is also operating in intact cells for osmoregulated ODC degradation.

INTRODUCTION

The degradation of many key regulatory proteins is rapid and highly regulated [1–3]. Recently, some of these proteins have been shown to be degraded by the 26 S proteasome in an ATP-ubiquitin-dependent pathway [4,5]. The physiological importance of this pathway has been suggested by genetic approaches. However, several inhibitors of the proteasome have been developed recently [6,7] and made available for examining the role of the proteasome in intact cells. Thus the proteasome was found to participate in the degradation of both long-lived and short-lived cytosolic proteins and in the generation of most peptides presented on major histocompatibility complex class I molecules [8].

Ornithine decarboxylase (ODC) is a key enzyme in the polyamine biosynthesis pathway. ODC is not only short-lived, but its turnover is regulated. ODC in cultured cells is in general stabilized during the ascending phase of its induction and destabilized during the descending phase. Elevation of cellular polyamine levels induces an ODC inhibitory protein, antizyme [9]. We have reported that the 26 S proteasome degrades ODC bound with antizyme [10], and it causes exhaustive endoproteolysis in a multicatalytic manner [11]. This antizyme-dependent rapid degradation of ODC seems to occur in living cells, because forced expression of antizyme in cells accelerates ODC degradation [12]. It is unknown, however, whether the proteasome operates for ODC degradation in intact mammalian cells. This point is interesting because ODC is a characteristic natural substrate that is degraded *in vitro* by the 26 S proteasome in a ubiquitin-independent fashion [10].

In contrast, the stability of ODC in cells is known to fluctuate with changes of ambient osmolarity. Hypotonic stress stabilizes ODC and reversal to isotonicity causes rapid degradation of the enzyme [13–16]. The protease responsible and the mechanism of destabilization remain unclear. In general, cell swelling is known to inhibit autophagic proteolysis [17,18]. This supports the idea that lysosomal enzymes also operate on ODC degradation in intact cells.

In the present study, by using lactacystin, the most specific proteasome inhibitor [7] originally isolated by Ōmura from Actinomyces [19,20], we have demonstrated that the proteasome is operating for both antizyme-stimulated and osmoregulated degradations of ODC in intact cells, suggesting the operation of a common ODC degradation pathway.

EXPERIMENTAL

Materials

Lactacystin was dissolved in DMSO. The solution was added to culture medium or ODC degradation mixture, at a dilution of 1:100 (v/v). An equivalent amount of DMSO was added to controls; 1% DMSO did not affect ODC induction or ODC degradation in hepatoma tissue-culture (HTC) cells, but inhibited ODC degradation by purified 26 S proteasome (14% inhibition). The 26 S proteasome was purified to near homogeneity from rat liver by sequential chromatographic operation [21].

Cell culture, ODC induction and osmotic shock experiments

HTC cells were cultured in Dulbecco's minimum essential medium supplemented with 2% (w/v) fetal calf serum, 4% (w/v) new-born calf serum, 2 mM L-glutamine and antibiotics. ODC activity was induced by refeeding with fresh medium or with fresh hypotonic medium. Hypotonic medium (about 150 mosm/l) was prepared by 2-fold dilution of the culture

Abbreviations used: HTC, hepatoma tissue-culture cells; ODC, ornithine decarboxylase.

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medium with water, and isotonic or hypertonic reversal was achieved by adding NaCl.

Determinations of ODC activity, amount of ODC–antizyme complex, and ODC degradation

ODC activity was determined as described previously [22]. The amount of ODC–antizyme complex was determined as the increase in ODC activity caused by the addition of an excess of antizyme inhibitor, which specifically binds to antizyme with high affinity and thus can release active ODC from the complex [22]. Total ODC (free ODC plus ODC complexed with antizyme) was determined by measuring ODC activity in the presence of antizyme inhibitor. ODC degradation *in vitro* was determined as described previously [10]. The 26 S proteasome or crude cell extract was preincubated with or without lactacystin for 60 min at 37 °C before the degradation assay. Degradation of ODC *in vivo* was determined by the decrease of total ODC activity after stopping ODC synthesis with cycloheximide (50 μg/ml).

RESULTS AND DISCUSSION

Effect of lactacystin on ATP- and antizyme-dependent ODC degradation *in vitro*

Previously ODC has been shown to be degraded by HTC cell extract in the presence of ATP and antizyme, and the 26 S proteasome has been identified as the enzyme responsible for the degradation [10]. Lactacystin is a specific and irreversible proteasome inhibitor that covalently binds to the highly conserved N-terminal threonine of mammalian proteasome subunit X(MB1) [7]. We examined whether lactacystin inhibits ODC degradation *in vitro*. As shown in Figure 1(a), lactacystin clearly, though only partly, inhibited degradation of ODC by either crude extract of HTC cells or purified 26 S proteasome, in the presence of both ATP and antizyme.

Effect of lactacystin on ODC degradation in intact cells

Next we tested whether lactacystin inhibited ODC degradation in intact cells. ODC was induced by refeeding fresh medium with or without lactacystin, and then 4 h later ODC decay was measured by stopping protein synthesis with cycloheximide. Lactacystin inhibited ODC decay in a dose-dependent manner (Figure 1b). The effect of lactacystin was much stronger on ODC degradation in intact cells than on ODC degradation *in vitro* by the HTC cell extract or purified 26 S proteasome. This phenomenon was consistent with the inhibition of proteolysis in intact lymphoblasts with MG115 or LLnL [8] or with the inhibition of ODC degradation in HTC cells by MG115 (result not shown). Conceivably, the inhibitor might be accumulated in cells, or the 26 S proteasome might be altered during isolation from cells. Alternatively some modification of lactacystin might be necessary for its stronger inhibitory effect on the 26 S proteasome.

It is unlikely that the inhibitory effect of lactacystin on ODC degradation is due to its cytotoxic effects, because lactacystin (5.0 μM) did not change the appearance of the cells and increased the level of ODC induction in three out of four separate experiments (171 $\pm$ 8.1 % of control, mean $\pm$ S.D., $n = 3$), owing to its stabilization effect.

ODC degradation by an extract of lactacystin-treated cells

We examined the ODC-degrading activity of extracts of lactacystin-treated cells. When cells were incubated with a higher concentration of lactacystin, the extract of the cells showed a lower activity for degradation of ODC *in vitro* (Figure 1c). The inhibitory effect of lactacystin, however, was somewhat weaker in an extract of lactacystin-treated cells than in intact cells. This might be due to a heterogeneous effect of lactacystin on the heterogeneously distributed proteasome in the cells. The inactivated proteasome may be diluted with the uninhibited proteasome during preparation of the cell extract. The inhibitory effect of lactacystin was not decreased by extensive dialysis of the cell extract. These results suggested that the ODC-degrading activity of the 26 S proteasome is irreversibly inhibited by lactacystin in intact cells. Thus lactacystin is a useful reagent for examining the participation of the proteasome in ODC degradation under various cellular conditions.

Effect of lactacystin on osmoregulated ODC degradation

We used lactacystin to test whether the proteasome is necessary for osmoregulated ODC degradation. To induce ODC, HTC cells were cultured for 4 h in hypotonic medium with or without 5 μM lactacystin, and then NaCl was added to the culture

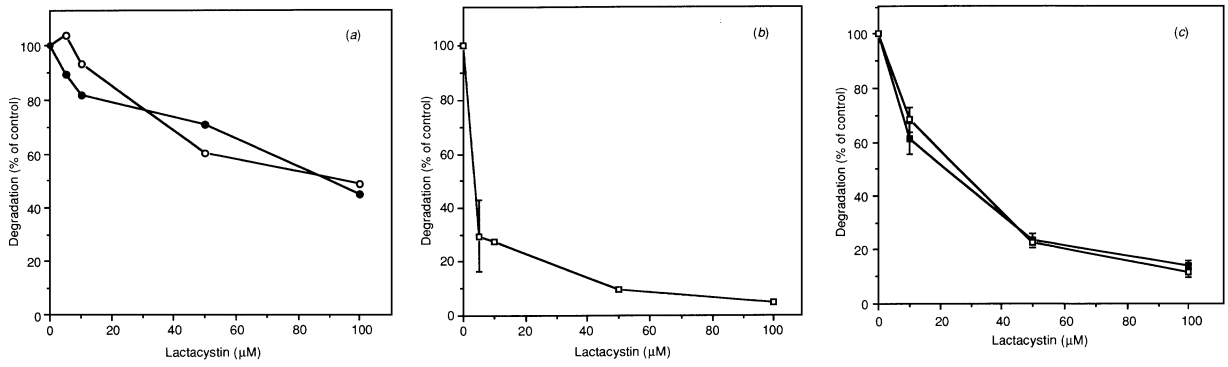

Figure 1 Effect of lactacystin on ODC degradation *in vitro* and *in vivo*

(**a**) ODC degradation by purified 26 S proteasome (○) or crude extract of HTC cells (●). Results are for a single experiment. Similar results were obtained in three other experiments. The control degradation of ODC in the absence of lactacystin was 8.9% per 90 min (○) and 79% per 30 min (●). (**b**) ODC degradation in HTC cells. Results are for a single experiment except for 5 μM lactacystin. The control degradation of ODC in the absence of lactacystin was 81% per 90 min. The value at 5 μM lactacystin is a mean $\pm$ S.D. for four separate experiments. (**c**) ODC degradation *in vitro* by an extract of HTC cells cultured in the presence of the indicated concentration of lactacystin. Purified [^{35}S]-labelled ODC from cultured mouse cells [10] was degraded by undialysed (□) or dialysed (■) crude extract of HTC cells. Values are means $\pm$ S.D. for three determinations. Degradations by control cell extract were 40% per 20 min (□) and 39% per 20 min (■).

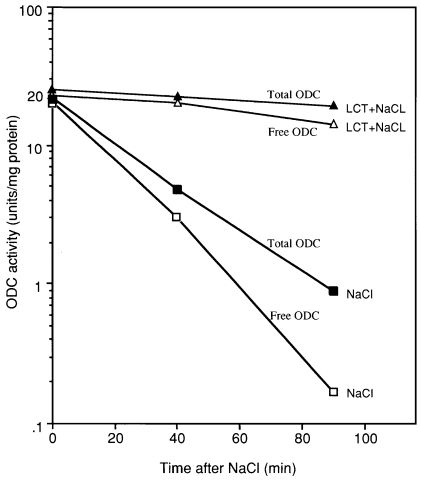

Figure 2 Effect of lactacystin on osmoregulated degradation of ODC in HTC cells

HTC cells were incubated in hypotonic medium with or without 5 μM lactacystin for 4 h, and subjected to hypertonic shock by the addition of NaCl to a final osmolarity of 450 m-osm/l. Cells were harvested at the indicated times after the additions. Free and total ODC were determined as described in the Experimental section. LCT, lactacystin.

Table 1 ODC destabilization and increase of the ratio of ODC–antizyme complex to total ODC caused by the increase in osmolarity

HTC cells were incubated in fresh hypotonic medium for 4 h, and then cycloheximide (50 μg/ml) and/or NaCl was added. Cells were harvested at 0 and 60 min after the addition. Free, total ODC and the amount of ODC–antizyme complex were determined as described in the Experimental section. Values are expressed as the means $\pm$ S.D. for three dishes. A dash indicates that ODC–antizyme complex was below the level of sensitivity of the assay.

Addition	Change in osmolarity (m-osm/l)	Time after additions (min)	Total ODC activity (units/mg of protein)	Ratio of antizyme–ODC complex to total ODC (%)
None	150 to 150	0	24.2 $\pm$ 1.9	–
None	150 to 150	60	32.2 $\pm$ 3.5	–
Cycloheximide	150 to 150	60	21.2 $\pm$ 3.4	–
NaCl	150 to 300	60	2.74 $\pm$ 0.40	82.2 $\pm$ 3.3
NaCl + cycloheximide	150 to 300	60	7.39 $\pm$ 0.39	10.0 $\pm$ 5.2
NaCl	150 to 450	60	1.00 $\pm$ 0.12	78.4 $\pm$ 5.6
NaCl + cycloheximide	150 to 450	60	2.81 $\pm$ 0.07	21.6 $\pm$ 9.5

medium. NaCl caused rapid decreases in free and total ODC (free ODC plus ODC–antizyme complex), both of which were nearly completely inhibited by lactacystin (Figure 2). This result showed clearly that the proteasome is essentially involved in osmoregulated degradation of ODC. Because lactacystin (100 μM) is reported to have no effect on cysteine proteases calpain, papain and cathepsin B and serine proteases chymotrypsin and trypsin [7], the present result suggested that neither calpain nor lysosomal enzymes is concerned with the osmoregulated degradation of ODC in the cells. We also confirmed that neither E64 (100 μM) nor leupeptin (500 μg/ml) has a detectable effect on osmoregulated ODC degradation in HTC cells. Existence of multiple degradation pathways was suggested for c-Jun [23], but interestingly, at least antizyme-stimulated and osmoregulated-ODC degradation are both catalysed by the proteasome.

Thus hypertonic shock is a trigger for rapid degradation of ODC by the proteasome; what, then, is the direct driving force of rapid degradation? Degradation of ODC catalysed by the 26 S proteasome is dependent on antizyme, and the ODC degradation rate in HTC cells also correlates with the proportion of ODC present as a complex with antizyme (ratio of ODC–antizyme complex to total ODC) [24]. Therefore the increase of antizyme is a possible candidate for the driving force. The partial inhibitory effect of cycloheximide on the rapid ODC decay caused by hypertonic shock [16] also suggests an involvement of synthesis of the short-lived protein antizyme. The effect of NaCl on the ratio of ODC–antizyme complex to total ODC was determined. Both reversals from hypotonic to isotonic and hypotonic to hypertonic increased the ratio concomitantly with a rapid decrease in total ODC (Table 1). The simultaneous addition of cycloheximide and hypertonic reversal partly inhibited both the increase of the ratio and the decrease of ODC. These results suggest that hypertonic shock recruited antizyme directly for ODC degradation, and at the same time stimulated the synthesis of new antizyme molecules. Most polyamines are known to exist in a bound form with cellular components such as RNA, DNA, proteins or phospholipids [25]. Antizyme itself also exists partly in a bound form with insoluble fractions and can be extracted, at least partly, with polyamines [26]. An increase in ambient osmolarity might increase the level of the intracellular free pool of polyamines, which stimulates the synthesis and release of antizyme. Cycloheximide can inhibit synthesis of antizyme but not its release from the binding site, resulting in the partial inhibition of ODC degradation by cycloheximide.

Thus the present results strongly suggest that the antizyme-dependent and 26 S proteasome-catalysed ODC degradation pathway operates also in osmoregulated ODC degradation and the increase in the ratio of ODC–antizyme complex to total ODC caused by an increase in osmolarity is a direct trigger of rapid ODC degradation.

Recently, by using a genetic approach, Mamroud-Kidron et al. [27,28] have demonstrated that the 26 S proteasome rapidly degrades mouse and yeast ODC in intact yeast cells. Interestingly, this suggests that the yeast proteasome can rapidly degrade mammalian ODC in the absence of antizyme, because antizyme has not been detected in yeast cells. However, purified mammalian 26 S proteasome degrades ODC in the absence of antizyme at only approx. 10 % of the maximal rate stimulated by antizyme [10], and the present results also support the generality of antizyme-dependency in mammalian ODC degradation by the proteasome *in vivo*. The proteasomes may be different between yeast and mammals, as recently suggested for the proteasome of Trypanosoma [29], and recognize and degrade ODC by distinct mechanisms.

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