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SHORT COMMUNICATION

Calpain Involvement in Calphostin C-induced Apoptosis

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ABSTRACT. A major problem in assessing the role of calpains in apoptosis induction concerns the fact that calpain inhibitors can also impair the activity of the proteasome, also reported to be involved in apoptosis. Herein we showed that apoptosis induced by calphostin C in U937 human promonocytic leukemia cells was associated, at its onset, with enhanced protein (poly)ubiquitination. This observation prompted us to study whether protein degradation through the ubiquitin/proteasome pathway was involved in apoptosis induction. We found that N-acetyl-Leu-Leu-norleucinal (50 μM), a proteasome as well as a calpain inhibitor, was able to reduce calphostin C-induced apoptosis by approximately 60%, whereas lactacystin (10 μM), a specific proteasome inhibitor, was ineffective. These results suggest that calphostin C-induced apoptosis is partly calpain-mediated, but does not require protein degradation through the ubiquitin/proteasome pathway. BIOCHEM PHARMACOL **56**;11:1489–1492, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. apoptosis; calphostin C; proteasome; calpains; U937 cells

A role for calpains as effectors of programmed cell death has been suggested [1–6]. The inhibition of calpain blocks T cell receptor-triggered apoptosis in murine T cell hybridoma [1], in activated peripheral T cells [1], and in activated lymphocytes from HIV+ donors [2]. Calpain inhibitors have also been reported to rescue thymocytes from apoptosis induced by treatment with dexamethasone [3,4] and to prevent programmed cell death induced by different stimuli in neural cells [5, 6]. At present, however, a major problem in unequivocally assessing the role of calpains in apoptosis involves the fact that calpain inhibitors may not be strictly selective and may also inhibit the activity of the proteasome [7–9], whose involvement in apoptosis has also been reported [9, 10]. In dexamethasone-treated thymocytes for instance, the antiapoptotic properties of LLnL,† widely employed as a calpain inhibitor, have also been displayed by lactacystin, a specific proteasome inhibitor [9].

We now provide evidence for calpain, but not proteasome, involvement in apoptosis induced by calphostin C, a potent and selective protein kinase C inhibitor [11], in U937 cells. We found that in this cell system the onset of apoptosis was associated with protein (poly)ubiquitination; investigating whether the proteasome pathway was involved in apoptosis induction, we found that cell death could be partly prevented by LLnL, but not by lactacystin.

MATERIALS AND METHODS

Cell Culture, Calphostin C Administration and Apoptosis Detection

Materials for cell culture were from Seralab Ltd. U937 cells were grown at 37° in RPMI 1640 medium that was supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin under a humidified atmosphere containing 5% CO_2. Cells, maintained overnight in media containing 0.5% serum, were plated and pre-incubated with calphostin C (Calbiochem Novachem Co.) for 20 min in the dark; thereafter, cells were exposed to a bench light for an additional 20 min [11] and the incubation allowed to proceed at 37° for different times. Lactacystin, a gift from Dr. B. A. Osborne (University of Massachusetts, U.S.A.), or LLnL (Calbiochem) was added to cells 1 hr before treatment with calphostin C or carrier (0.1% DMSO). Apoptosis was monitored by evaluating the pre-G1 cell population after flow cytometric analysis [12]. Briefly, cells detached from the plates were washed with PBS (pH 7.4) and resuspended in 0.5 mL of a solution containing 50 μg/mL of propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Propidium iodide-stained cells were analysed using a FACScan Flow Cytometer (Becton-Dickinson); fluorescence was measured between 565 and 605 nm. The data were acquired and analysed by the Lysis II program (Becton-Dickinson).

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† *Abbreviations*: anti-Ub mAb, anti-human ubiquitin monoclonal antibody; and LLnL, N-acetyl-Leu-norleucinal.

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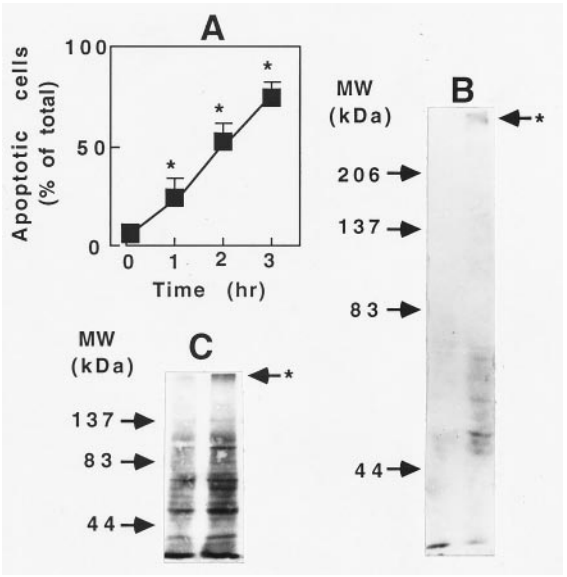

FIG. 1. Calphostin C induces apoptosis and protein ubiquitination in U937 cells. (A) Apoptosis evaluation, by flow-cytometric analysis, after cell treatment with 250 nM calphostin C for the indicated times. Data are means ± SD of four different experiments. *Statistical significance: $P < 0.01$, as compared to untreated samples, as from Student's *t*-test. (B) Western blot analysis of ubiquitinated peptides on lysates from U937 cells treated for 1 hr with carrier (left lane) or 250 nM calphostin C (right lane). (C) Autoradiographic analysis of peptides immunoprecipitated with anti-Ub mAb from cells prelabelled with [^{35}S]methionine and treated for 1 hr with carrier (left lane) or 250 nM calphostin C (right lane). In panels B and C, the arrows on the right indicate the formation of polyubiquitinated high MW products.

Cell Labelling, Immunoprecipitation, and Western Blotting

Equal amounts of cells per sample (15×10^6 cells) were labelled for 24 hr with 300 μCi of [^{35}S]methionine (Amersham). After treatment with calphostin C or carrier, cells were lysed on ice for 30 min in 1.0 mL of lysis buffer (50 mM Tris HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% sodium lauryl sulfate; 10 mM benzamidine hydrocloride; 10 μM chymostatin; 1 mM EDTA; 1 μM pepstatin A), and cellular debris was removed by centrifugation at 15 000× g for 20 min. The resulting lysates were precleared with 10% (v/v) protein A-Sepharose (Pharmacia Biotech) for 30 min, an anti-Ub mAb (YLEM) was added overnight, and immunoprecipitates were captured by incubating with 10% (v/v) protein A-Sepharose for 60 min. Proteins were separated by SDS-PAGE and [^{35}S]methionine-labelled proteins revealed by autoradiography. Western blotting was carried out as previously described [12]. Proteins were measured according to Lowry *et al* [13].

RESULTS AND DISCUSSION

We have demonstrated that calphostin C induces apoptosis in U937 cells [12]. As shown in Fig. 1A, the phenomenon was already evident at 1 hr from the administration of a 250 nM concentration of the drug, and after 3 hr cell viability was reduced by over 70%. We found that apoptosis, at its onset, was associated with enhanced levels of cell protein (poly)ubiquitination. Western blot analysis of whole cell lysates with the anti-Ub mAb demonstrated that cell treatment for 1 hr with 250 nM calphostin C led to the accumulation of various immunopositive bands within the 40–80 kDa area; moreover, the formation of high MW products migrating slightly below the gel top was also observed (Fig. 1B).

Calphostin C-induced protein (poly)ubiquitination was confirmed by experiments in which lysates obtained from cells prelabelled with [^{35}S]methionine were immunoprecipitated by the anti-Ub mAb and analysed by autoradiography after SDS-PAGE development (Fig. 1C). We observed that calphostin C-induced apoptosis was partially prevented by pre-incubation of U937 cells with LLnL, a proteasome as well as a calpain inhibitor [3, 7, 9].

Figure 2 shows that LLnL was active within the low micromolar concentration range, with fairly maximal rescuing activity being observed at approximately 25 μM (*ca.* 60% decrease in drug-induced apoptosis). Although the data reported in Fig. 2 refer to a 3-hr cell treatment with calphostin C, the percent of cell rescue from apoptosis produced by 50 μM LLnL also held at around 50–60% when assessed 1 hr or 2 hr after calphostin C administration (not shown).

To elucidate whether protein ubiquitination was relevant to apoptosis induction by enhancing the degradation of proteins through the proteasome pathway, we studied whether lactacystin, a specific proteasome inhibitor [9], was also able to prevent calphostin C-induced cell death. We observed that lactacystin, at a concentration as high as 10 μM [9, 10], was ineffective in preventing calphostin C-induced apoptosis; moreover, it did not display toxic effects when added alone to U937 cells (Fig. 3).

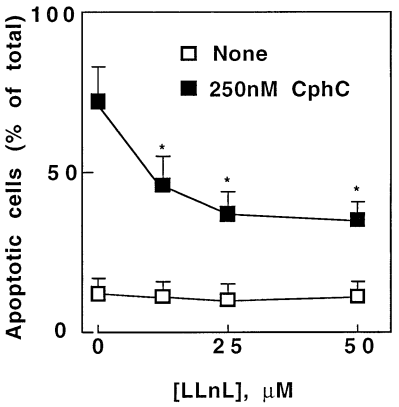

FIG. 2. Effect of LLnL on calphostin C-induced apoptosis. Cells were pre-incubated for 1 hr with the indicated concentration of LLnL and then exposed for 3 hr to 250 nM calphostin C (CphC) or carrier (None). Apoptosis was quantified by flow cytometric analysis. Data are means ± SD of four different experiments. *Statistical significance: $P < 0.01$, as compared to samples untreated with LLnL, as from Student's *t*-test.

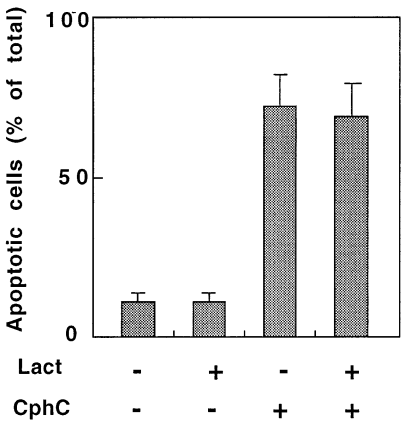

FIG. 3. Effect of lactacystin on calphostin C-induced apoptosis. Cells were pre-incubated for 1 hr either in the presence (+Lact) or absence (−Lact) of 10 μM lactacystin. Thereafter, 250 nM calphostin C (+CphC) or carrier (−CphC) were added for a further 3 hr and apoptosis was quantified by flow-cytometric analysis. Data are means ± SD of four different experiments. Lactacystin did not significantly affect apoptosis, either in cells treated with carrier or with calphostin C, as assessed by Student's *t*-test.

The evidence that apoptosis induced by calphostin C in U937 cells was largely prevented by LLnL, but not by lactacystin, clearly indicates that calpain activation is a major event for the completion of the death programme. Thus, although the onset of apoptosis was accompanied by the appearance of a pattern of (poly)ubiquitinated products, it appears that protein degradation through the ubiquitin/proteasome pathway is not relevant to apoptosis induction. Calphostin C is a potent and specific protein kinase C inhibitor; intriguingly, other protein kinase C inhibitors have been reported to induce forms of apoptosis that involve, at least partly, calpain activation [5, 6].

Elucidation of the mechanism(s) through which protein kinase C inhibition brings about calpain activation and identification of the downstream protease targets in apoptosis are two major issues to be addressed. In the latter respect, calpains have been reported to act on a broad spectrum of substrates, including cytoskeletal proteins (reviewed in [14]); calpain effects on the cytoskeleton could be relevant to the occurrence of events such as cell shrinkage, loss of plasma membrane integrity, and blebbing that are characteristic of apoptosis. Intriguingly, calpains have also been shown to induce the activation of some transglutaminase forms [15]. Because the latter enzyme is now regarded as a major effector of apoptosis in various cell systems [16, 17], the possibility is raised that transglutaminase may provide, in some instances, an apoptosis effector after calpain activation.

The question arises as to the mechanism through which calphostin C drives protein (poly)ubiquitination and to the significance of the phenomenon. We entertained the possibility that calphostin C could elicit the accumulation of (poly)ubiquitinated proteins by blocking steady-state proteasome activity; however, we observed that cell treatment for up to 3 hr with 50 μM LLnL or 10 μM lactacystin, in the absence of calphostin C, did not result in a substantial accumulation of (poly)ubiquitinated products (not shown). These results indicate that calphostin C acts by enhancing protein (poly)ubiquitination rather than by impairing proteasome activity. In this respect, however, it is difficult to hypothesize that the above-mentioned drug brings about its effects through protein kinase C inhibition because the latter kinase has been reported to positively modulate protein ubiquitination [18]. Intriguingly, recent results indicate that protein ubiquitination is enhanced in response to oxidative stress, thus favouring the rapid elimination of damaged proteins through the proteasome pathway [19]. Such a mechanism could also account for protein (poly)ubiquitination after calphostin C treatment because it is now well established that the above-mentioned drug generates free radicals after light activation [20] and inhibits protein kinase C by irreversible oxidative inactivation [21].

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