Journal of Cellular Biochemistry 103:877–885 (2008)

# Proteasomal Inhibition and Apoptosis Regulatory Changes in Human Isolated Lymphocytes: The Synergistic Role of Dopamine

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**Abstract** Abnormal deposition of protein aggregates and increased susceptibility to apoptotic cell death may result from defects in the activity of the ubiquitin-proteasome system (UPS); neurotoxicity related to UPS defects seems to require dopamine to be fully expressed. The aim of this study was to investigate the pro-apoptotic effects caused by proteasomal activity inhibition, as well as the synergistic effect of dopaminergic stimulation in human lymphocytes isolated from healthy volunteers. Cells were incubated 20 h at 37°C, with: (1) lactacystin, (2) increasing concentrations of dopamine or (3) mixture of dopamine and lactacystin. Activities of proteasome 20S and pro-apoptotic caspases-3 and -9 and levels of anti-apoptotic Bcl-2 were measured with fluorimetric or immunochemical assays, while a ''DNA diffusion'' assay was used to determine the apoptosis. Incubation of lymphocytes with lactacystin, which caused reduction of proteasomal activity, was associated with activation of caspases. A clear, dose-dependent reduction of proteasomal activity was also seen in the presence of increasing doses of dopamine, which was accompanied by a slight dose-dependent increase of caspases activities and Bcl-2 levels. Both effects on proteasome and caspase activities were enhanced when cells were simultaneously exposed to lactacystin and elevated concentrations of dopamine. Apoptosis was detected in all treated samples, but not in controls, without significant differences among the treatment groups; however, the association of dopamine and lactacystin induced a clear reduction in the number of cells being analyzed, pointing to marked cytotoxicity. Our data confirm the potentiation of cytotoxicity related to proteasome inhibition, in the presence of dopaminergic stimulation. J. Cell. Biochem. 103: 877–885, 2008. 

**Key words:** impaired proteasome activity; lactacystin; dopamine; caspase activity; neurodegeneration

The ubiquitin-proteasome system (UPS) is implicated in the regulation of numerous key cellular processes in mammalian cells, including cell cycle and apoptosis [Lang-Rollin et al., 2005; Staropoli and Abeliovich, 2005; Tsukamoto et al., 2006]. The proteasome is a multicatalytic proteinase complex with a highly ordered ring-shaped 20S core structure. The core structure is composed of 4 rings of 28 non-identical subunits, two of which comprising 7 alpha subunits, and the remainders including 7 beta subunits. Proteasomes are found in the cytosol and in the nucleus of eukariotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway: they are responsible for the majority of intracellular protein degradation [Lee and Goldberg, 1998; Di Napoli and McLaughlin, 2005; Voorhees and Orlowski, 2006].

Increasing evidence suggests that proteasome defects may play a causal role in promoting neuronal death [de Pril et al., 2004; Surgucheva et al., 2005], thus intervening in the pathogenesis of a neurodegenerative disorder such as Parkinson's disease (PD) [Ding et al., 2003; McNaught et al., 2003a]. In this

Abbreviations used: PD, Parkinson's disease; UPS, ubiquitin-proteasome system; PBLs, peripheral blood lymphocytes; ANOVA, analysis of variance; AMC, free 7-amino-4-methylcoumarin, AFC, fluorescent 7-amino-4-trifluoromethyl coumarin; LACTA, lactacystin.

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Received 8 March 2007; Accepted 31 May 2007
DOI 10.1002/jcb.21457

regard, postmortem analyses of adult rats chronically exposed to intrastriatal injection of proteasome inhibitors, such as lactacystin [Miwa et al., 2005] for 2 weeks, showed striatal dopamine depletion and dopaminergic cell death with apoptosis and inflammation in the substantia nigra pars compacta [McNaught et al., 2004]. On the contrary, other authors support that enhanced formation of intracellular inclusions due to prolonged administration of proteasome inhibitors protects against dopaminergic cell death in rat PD model [Setsuie et al., 2005].

Recent studies have emphasized the presence of interesting correlations between dopaminergic neurotransmission and proteasome activity [Keller et al., 2000a]. Administration of dopamine to neural cell line cultures induced a dose- and time-dependent decrease in proteasome activity, which occurred prior to cell death and enhanced formation of protein aggregates [Yoshimoto et al., 2005].

Peripheral blood lymphocytes have been used as a convenient and accessible model to study the involvement of oxidative stress and/or apoptosis in the pathogenesis of numerous pathological conditions, including neurodegenerative disorders [Migliore et al., 2001, 2002; Blandini et al., 2004; Straface et al., 2005]. Recently, we have shown that proteasome 20S activity is reduced in peripheral blood lymphocytes (PBLs) of PD patients and that such reduction is associated with increased caspase activation. Proteasome 20S reduction was observed in PD patients under treatment with dopaminergic drugs (L-Dopa and dopamine agonists), who also were the most compromised, in terms of disease severity [Blandini et al., 2006]. Thus, the exact roles played by disease progression and dopaminergic stimulation on the proteasomal defect described in PD patients remained to be clarified.

In this study, we investigated the effects of proteasome inhibition, induced by lactacystin [Nomoto and Nagai, 2005], and/or dopamine on pro-apoptotic (caspase-3 and -9) and anti-apoptotic (Bcl-2) proteins, as well as on proteasome 20S activity, in isolated PBLs from normal subjects. We also investigated the presence of apoptotic cells in all the groups analyzed. The aim was to determine how the two conditions—proteasome inhibition and dopaminergic stimulation—affect apoptotic regulation and whether any synergistic or antagonizing effect can be detected when PBLs are exposed, simultaneously, to both conditions.

## MATERIALS AND METHODS

Five healthy volunteers, including three female and two male subjects, aged between 20 and 50 years, were recruited for this study. Each of them signed an informed consent before being enrolled.

About 40 ml of peripheral blood were drawn by venipuncture into a vacuum tubes containing EDTA, as anti-coagulant (Terumo Europe, Belgium), then processed for separation of lymphocytes no later than 2 h after drawing. The mixture of blood and 15 ml of phosphate-buffered saline solution (PBS), pH 7.4, was layered over half of its volume of Lympholite®-H (Cedarlane Laboratories Limited) and the discontinuous gradient, thus formed, was centrifuged at 800*g* for 20 min.

After removal of the upper layer, the lymphocyte band at the interface was removed and washed twice with PBS (Sigma). PBLs were counted using an automated cell counter (MAX-M, Beckman Coulter Inc.) and re-suspended in modified RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum and 0.5% penicillin–streptomycin solution.

Different aliquots (each containing $4 \times 10^6$ lymphocytes/ml) were prepared from each sample in 50 ml sterile tubes.

Increasing amounts of dopamine were added to the samples, to obtain a final concentrations of 1, 5, 20, 100 μM. In order to have positive controls, proteasomal inhibition was selectively induced spiking a separated set of PBLs with 10 μM lactacystin, which was previously shown to induced marked inhibition of proteasome activity [Schwarz et al., 2000]. In addition, aliquots of lymphocytes were treated with a mixture of increasing dopamine concentration and lactacystin. PBLs incubated only with medium represented a reference control. All groups of cells were incubated for 20 h at 37°C in the dark. After incubation, all groups of cells were tested for viability with Trypan Blue. Cells used to evaluate the presence of apoptosis were centrifuged at 250*g* for 10 min and re-suspended at $1 \times 10^5$ cells/ml in ice cold 1× PBS ($Ca^{++}$ and $Mg^{++}$ free), after the removal of culture media. For the other assays, cells were pelleted by final centrifugation at 1,000*g* for 10 min, and frozen

at −80°C, after discarding the supernatant, until the assays were carried out.

### Apoptosis Detection

Presence of apoptotic cells was evaluated with a new comet assay kit (Trevigen). Lymphocytes were embedded in low melting agarose on a pretreated slide, lysed under alkaline conditions and the DNA of the remaining nuclear region was precipitated in the agarose. DNA was then visualized using Sybr Green I nucleic acid stain. When excited (425–500 nm), the DNA-bound fluorescent agent emitted green light. In healthy cells, the fluorescence was confined to the nucleus; while, in apoptotic cells, the lysis treatment unwinded the DNA, releasing fragments that diffused away from the center of the nucleus, giving the appearance of a halo with a hazy outline.

The slides were analyzed by means of fluorescence microscopy, using a AxioSkop 2 microscope connected to a computerized image analysis system (AxioCam MR5) equipped with a dedicated software (Axio Vision Rel 4.2) (Zeiss, Germany). The apoptotic cells could be easily differentiated from the normal cells; we counted, on randomly three selected fields for each slide, the lymphocytes in apoptosis and normal cells and calculated the ratio between the apoptotic and the total cells visualized. For each slide, we considered a mean of the fields analyzed.

For the other assays, pellets were re-suspended in ice-cold PBS and lysed by repeated thawing/freezing cycles. Homogenates were centrifuged at 15,000*g*, for 10 min at room temperature, and resulting supernatants were used for the biochemical determinations.

### Proteasome Activity

The proteolytic activity of 20S proteasome was evaluated with the BIOMOL AK-740 QuantiZyme Assay System (BIOMOL), which detects the release of free 7-amino-4-methylcoumarin (AMC) fluorophore, upon Suc-LLVY-AMC fluorogenic peptide substrate cleavage.

### Caspase-3 and -9 Activity

We also performed fluorometric assays for the determination of caspase-3 (Molecular Probes, Inc.) and caspase-9 (Oncogene) activities. Concerning caspase-3, which has a substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD), the assay was based on the proteolytic cleavage of AMC-derived substrate Z-DEVD-AMC, which yields a fluorescent product; for caspase-9 activity, we used a method based on the specificity of the enzyme for cleavage after aspartate residues in a particular peptide sequence (LEHD). The LEHD substrate is labeled with fluorescent 7-amino-4-trifluoromethyl coumarin (AFC) and reaction is monitored by a blue to green shift in fluorescence upon cleavage of the AFC fluorophore. Positive controls were used for all determinations, as expressly indicated by the assay procedures; standard samples treated with a proteasome inhibitor (lactacystin) or pro-apoptotic agents (Camptothecin or Actinomycin D) were always added to batches when proteasome 20S or caspases-3 and -9 activities were measured. All readings were performed using a fluorometric microplate reader (Spectramax Gemini XS), (Molecular Devices).

### BCl -2 Levels

Bcl-2 levels were assayed using a commercially available ELISA kits (Bender MedSystems DiagnosticsGmbH, Austria) on a microplate reader "ELx800" (BIO-TEK INSTRUMENTS, Inc.). Samples and Bcl-2 standards reacted with monoclonal antibody coating microplate wells, then were complexed with biotin-conjugated monoclonal anti-Bcl-2 antibody and, subsequently, with Streptavidin-HRP. Finally a specific substrate was added to complexes until color development. The reaction was quenched by addition of acidic solution and absorbance was measured at 450 nm.

## STATISTICS

Comparisons among groups were carried out using the analysis of variance (ANOVA) coupled to the Fisher's post hoc test and Tukey's test. The presence of correlations among variables was evaluated by calculating the Pearson's ($r$) correlation coefficient. The minimum level of significance was set at $P < 0.05$.

## RESULTS

Our results show a clear dose-dependent reduction of the proteasomal activity in lymphocytes incubated with increasing concentrations of dopamine, which reached statistical significance at higher concentrations, in particular 100 μM; 10 μM lactacystin,

as expected, reduced proteasome 20S activity; this effect was further enhanced when the inhibitor was combined with dopamine, especially at the highest concentration (100 μM) (Fig. 1). Conversely, a slight, non significant, dose-dependent increase of caspase-3 and -9 activities was observed in lymphocytes treated with increasing concentrations of dopamine compared with control cells. Caspase activation was also observed when the cells were incubated with lactacystin; incubation with the association of lactacystin and dopamine (Figs. 2 and 3) induced further significant increase in the activity of both caspase-3 and -9, with respect to control cells.

Bcl-2 levels appeared increased in cells incubated with all concentrations of dopamine, compared with control cells. In particular, the levels of Bcl-2 increased gradually when cells were incubated with low concentrations of dopamine (1 and 5 μM), then the values decreased with high concentrations of dopamine. The presence of lactacystin seemed not to affect this phenomenon: in fact, the values of Bcl-2 obtained incubating cells with increasing doses of dopamine were similar to those resulted from combining dopamine with lactacystin (Fig. 4).

As for the detection of apoptosis, we found a low, rather constant rate of apoptotic cell death in all the treated samples (Figs. 5 and 6), while no apoptosis was detected in the control samples. No significant differences were found among the groups analyzed. A trend toward a dose-dependent increase, tending to reach a plateau at the highest concentration (100 μM) was observed in cells exposed to dopamine alone (Fig. 6). However, in the group treated with lactacystin and, above all, in those treated with the association of lactacystin and dopamine (at all concentrations), we observed a relevant rate of nonspecific cell death—evidenced by the Trypan Blue reading (the method stains dead cells, without distinguishing apoptosis from necrosis)—with respect to the controls and lymphocytes treated only with dopamine. This markedly reduced the total number of lymphocytes being analyzed in these treatment groups (Fig. 7).

## DISCUSSION

The UPS plays a pivotal role in controlling intracellular short-lived proteins [Meriin and Sherman, 2005]. Recent studies have demonstrated that inhibition of the UPS causes cell death in various cell systems, including neuronal preparations [Fornai et al., 2003; Inden et al., 2005], by inducing accumulation of abnormal proteins that should be, otherwise, eliminated from the intracellular environment [Michel et al., 2002; Miwa et al., 2005].

Recent studies have suggested that impaired proteasomal function may be a common feature in PD [McNaught and Jenner, 2001; McNaught et al., 2002b, 2004; McNaught and Olanow, 2003b; Hattori et al., 2005; Nomoto and Nagai, 2005; Rideout et al., 2005], which is characterized by dopaminergic cell death in the substantia nigra pars compacta [Michel et al.,

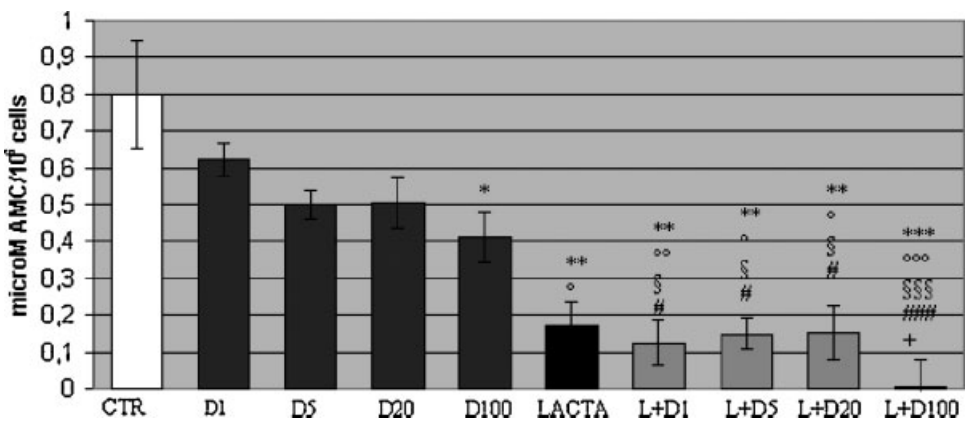


**Fig. 1.** 20S proteasome activity (means ± SEM) in PBLs isolated from healthy volunteers. Cells treated with: medium alone (controls, CTR); increasing concentrations of dopamine (D) 1, 5, 20 or 100 μM; 10 μM lactacystin (LACTA); lactacystin in association with different doses of dopamine (L + D 1, 5, 20, or 100 μM). $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ versus controls. $^{\circ}P < 0.05$; $^{\circ\circ}P < 0.005$; $^{\circ\circ\circ}P < 0.001$ versus D1. $^{\S}P < 0.05$; $^{\S\S\S}P < 0.001$ versus D5. $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ versus D20. $^{+}P < 0.05$ versus D100.

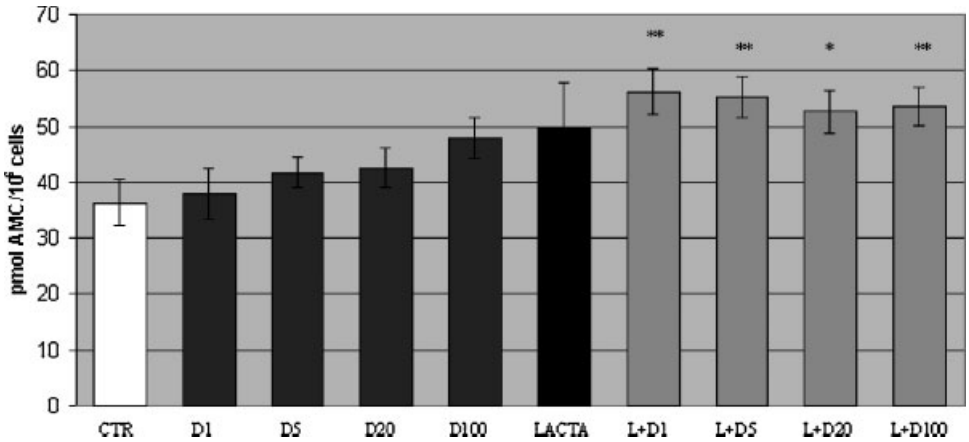


**Fig. 2.** Caspase-3 activity (mean ± SEM) in PBLs isolated from healthy volunteers. Cells treated with: medium alone (controls, CTR); increasing concentrations of dopamine (D) 1, 5, 20 or 100 μM; 10 μM lactacystin (LACTA); lactacystin in association with different doses of dopamine (L + D 1, 5, 20, or 100 μM). $^{*}P < 0.05$; $^{**}P < 0.01$ versus controls.

2002; Eriksen et al., 2005] and by the presence, in surviving neurons, of Lewy body-like inclusions, positive for alpha-synuclein and ubiquitin [Inden et al., 2005; Setsuie et al., 2005]. Several authors have hypothesized that defects in the UPS and proteolytic stress may be involved in the neurodegenerative process of sporadic and familial PD, as well as in the formation of the cytoplasmic inclusions [Schapira, 2001; McNaught et al., 2002a]. In this regard, it has been reported that rat ventral mesencephalic cultures and PC12 cells treated with proteasome inhibitors develop dopaminergic neuron degeneration and form alpha-synuclein/ubiquitin-containing intracytoplasmic inclusion bodies, as occurs in PD [McNaught et al., 2002b]. Fornai et al. [2003], after inhibition of proteasome with a microinfusion of lactacystin in the striatum of rats, have found that neurotoxicity was selective for striatal dopamine components and caused retrograde apoptosis within dopaminergic cell bodies of the substantia nigra. More recently, McNaught et al. [2004] have proposed that also the systemic administration of proteasome inhibitors is able to reproduce the cardinal features of PD in rodents, including the selective degeneration of nigral, dopamine-containing neurons, and presence of Lewy body-like inclusions.

Further supporting the view that dopaminergic transmission and UPS defect may be functionally linked, consistent experimental evidence has showed that dopamine induces a dose- and time-dependent decrease in proteasome activity [Keller et al., 2000a,b] in different

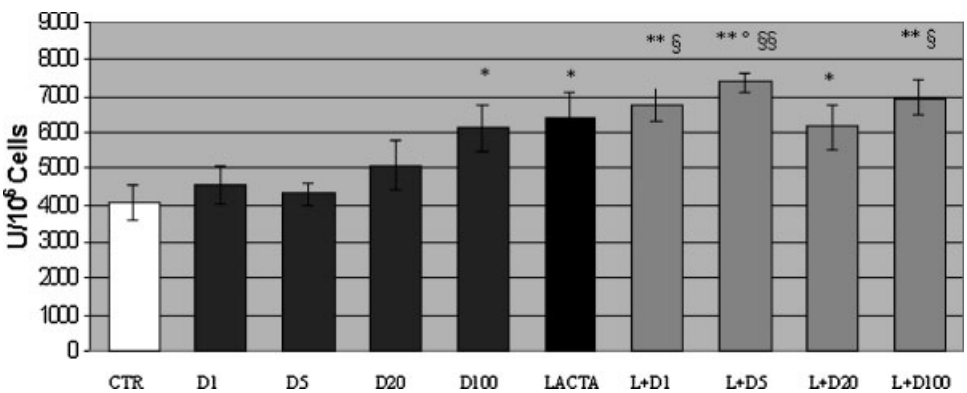


**Fig. 3.** Caspase-9 activity (mean ± SEM) in PBLs isolated from healthy volunteers. Cells treated with: medium alone (controls, CTR); increasing concentrations of dopamine (D) 1, 5, 20 or 100 μM; 10 μM lactacystin (LACTA); lactacystin in association with different doses of dopamine (L + D 1, 5, 20, or 100 μM). $^{*}P < 0.05$; $^{**}P < 0.005$ versus controls. $^{\circ}P < 0.05$ versus D1. $^{\S}P < 0.05$; $^{\S\S}P < 0.01$ versus D5.

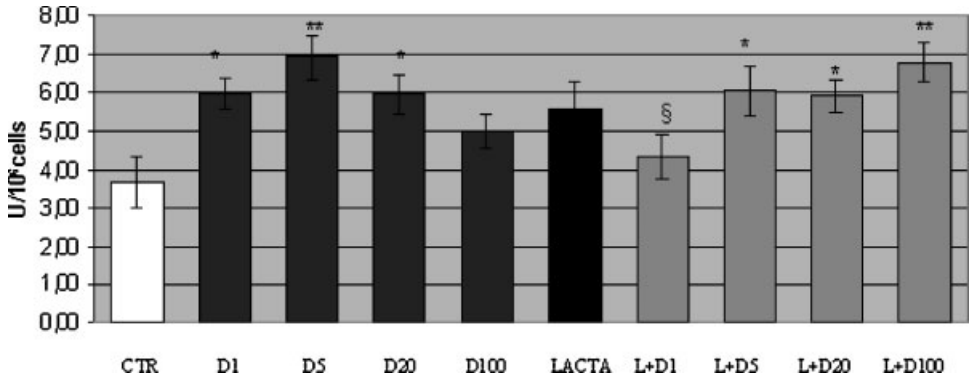


**Fig. 4.** Levels of Bcl-2 (mean ± SEM) in PBLs isolated from healthy volunteers. Cells treated with: medium alone (controls, CTR); increasing concentrations of dopamine (D) 1, 5, 20 or 100 μM; 10 μM lactacystin (LACTA); lactacystin in association with different doses of dopamine (L + D 1, 5, 20, or 100 μM).*$P < 0.05$; **$P < 0.005$ versus controls. §$P < 0.05$ versus D5.

cellular types and cause apoptosis, probably as a result of the increased production of reactive oxygen species (ROS) accompanying the autooxidation of this neurotransmitter [Stokes et al., 1999; Wang et al., 2005], which may interfere with regulation of proteasome activity [Ishii et al., 2005]. However, other researchers have showed a protective effect of proteasome inhibition on dopaminergic cells death in the 6-hydroxydopamine rat PD model, suggesting that accelerated formation of inclusions, via defective UPS, may represent an attempt of the cell to protect itself against potentially harmful proteic constituents, by confining them within circumscribed subcellular structures (inclusion) [Setsuie et al., 2005].

In our study, we exposed human isolated lymphocytes to increasing concentrations of dopamine, in the presence or absence of lactacystin 10 μM, and measured modifications of proteasome 20S, caspase-3 and -9 activities and Bcl-2 levels, as well as the presence of apoptotic cells. This cell model was used to provide additional information that may complement recent data our group, showing reduced proteasomal activity associated with caspase activation in PBLs of PD patients under treatment with dopaminergic drugs (L-Dopa and dopamine agonists) [Blandini et al., 2006]. Since these patients were also characterized by a more severe expression of the disease, it remained unclear whether these changes were to be ascribed to disease progression or to the effects of dopaminergic stimulation of PBLs. The results of this study evidenced a clear synergistic effect of dopamine and lactacystin, particularly on proteasomal and caspase activities. In fact, at the highest concentration, dopamine was able to decrease proteasomal activity, while inducing an increase in caspase activity; this effect was considerably enhanced

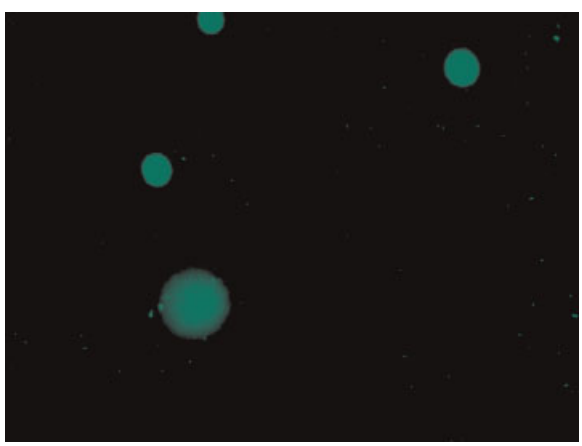

**Fig. 5.** Photomicrograph that shows one apoptotic cell and normal cells from lymphocytes treated with lactacystin and dopamine. Magnifications: 200×.

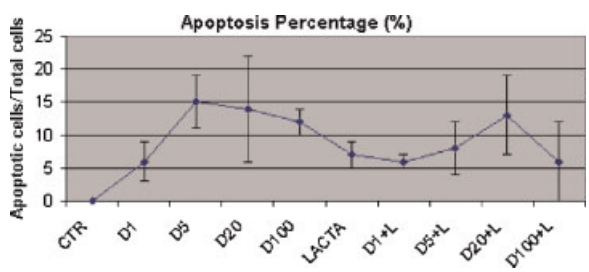


**Fig. 6.** Each data point is the mean and standard error of the percentage of apoptosis from all groups of lymphocytes analyzed. Cells were treated with: medium alone (controls, CTR); increasing concentrations of dopamine (D) 1, 5, 20 or 100 μM; 10 μM lactacystin (LACTA); lactacystin in association with different doses of dopamine (L + D 1, 5, 20, or 100 μM).

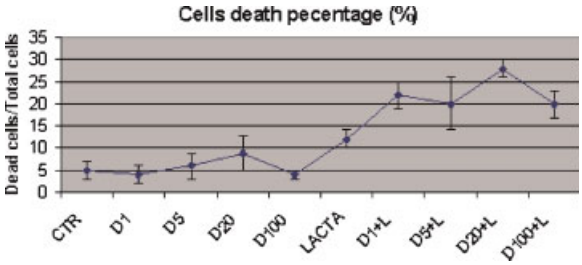


**Fig. 7.** Each data point is the mean and standard error of the percentage of cellular death calculated after the incubation from all groups of lymphocytes analyzed. Cells were treated with: medium alone (controls, CTR); increasing concentrations of dopamine (D) 1, 5, 20 or 100 μM; 10 μM lactacystin (LACTA); lactacystin in association with different doses of dopamine (L + D 1, 5, 20, or 100 μM).

by combination with lactacystin. On the other hand, when used alone, the effects of lactacystin were similar to those exerted by the higher concentrations of dopamine, with the obvious exception of proteasome activity, which was reduced in a more profound manner by lactacystin. Therefore, our results tend to confirm a direct, inhibitory effect of dopamine on proteasomal activity [Keller et al., 2000a,b], also in isolated human PBLs, which may be related to the pro-oxidant properties of the neurotransmitter [Stokes et al., 1999; Wang et al., 2005]. Indeed, Ishii et al. [2005] have recently demonstrated that, in SH-SY5Y cells, exposure to high rates of ROS formation induces protein carbonilation and that S6 ATPase, one of the regulatory subunits of the 26S proteasome, represents a major intracellular target for this process; thus, oxidative stress would impair S6 ATPase efficiency, decreasing the proteasome ability to recognize and degrade its substrates. A dopamine receptor-mediated mechanism may also be taken into consideration; indeed, dopamine receptors are well represented in lymphocytes, where they mediate numerous functions, including release of cytokines [Lachowicz and Sibley, 1997] chemotactic responses [Yasumoto et al., 2004], inhibition of the proliferation and cytotoxicity of $CD4^+$ and $CD8^+$ T-cells [Wolf and Kapatos, 1989], reduction of $CD4^+$ $CD25^+$ T-lymphocyte function [Malgaroli et al., 1987], inhibition of the intracellular synthesis of catecholamines [Rajda et al., 2005]; changes in dopamine receptor expression have also been reported in lymphocytes of parkinsonian patients [Barbanti et al., 1999]. However, there is no consistent evidence that activation of lymphocyte dopamine receptors may alter proteasome or caspase activity, potentially leading to cell death. On the contrary, Cosentino et al. [2004] hypothesized that a protective effect, against oxidative stress and apoptosis, may be exerted by low doses of dopamine, through the activation of a D1-like receptor-dependent pathway.

A different response was observed with Bcl-2 levels; in this case, dopamine induced significant expression of this anti-apoptotic protein at the lower concentrations, while Bcl-2 levels tended to return toward control values when PBLs were exposed to higher concentrations of the neurotransmitter, thus yielding a bell-shaped response. This trend was lost when lactacystin was added to the incubation medium and a moderate, dose-dependent increase in Bcl-2 levels was then observed. Given the protective role of Bcl-2, these findings seem to support previous data [Weinreb et al., 2003; Cosentino et al., 2004], showing an anti-apoptotic effect for low concentrations of dopamine, in isolated human PBLs. The initial increase of Bcl-2 levels, elicited by low concentrations of dopamine that caused slight, non significant increases in caspase-3 and -9 activity, may, in fact, represent an attempt of compensatory response of the cell to potentially pro-apoptotic stimuli of low intensity. Such response would decline with further progression in the intensity of the pro-apoptotic stimulus.

We observed low rates of apoptotic cell death in all treated samples, as expressed by the comet assay, with a slight, dose-dependent increase in the samples treated with dopamine alone; we had obtained similar results, in preliminary experiments, using a alternative method based on the use of the fluorescent probe Yopro-1 iodide [Plantin-Carrenard et al., 2003], which binds to apoptotic nuclei (data not shown). However, we observed a clear reduction in the total number of lymphocytes, in these latter samples. Thus, although no significant differences in the apoptosis rate were observed, in our experimental setting, between the two conditions (dopamine or dopamine plus lactacystin), the simultaneous exposure to both substances induced high levels of cytotoxicity, mirrored by the clear reduction in the absolute number of lymphocytes being analyzed. This may be related to necrotic cell death or apoptotic cell death occurred during the incubation period, which both may have gone undetected

under our experimental conditions (Comet assay carried out following 20 h incubation).

In conclusion, our data confirmed, in isolated human PBLs, a clear and interesting correlation between impaired proteasomal function and changes in caspase activation, as previously reported in different cell systems [Pasquini et al., 2000; Lang-Rollin et al., 2004; Yang et al., 2005]. More importantly, we showed that these effects are potentiated by dopamine; this observation lends further support to the view that high levels of this neurotransmitter may represent a favoring condition for the selective cytotoxicity in the presence of a generalized defect of proteasome activity, as it may occur in PD [Blandini et al., 2006]. Finally, these data further prove that human isolated PBLs represent a valid experimental tool to study, in vitro, various mechanisms potentially underlying severe neurodegenerative disorders.

## ACKNOWLEDGMENTS

This study was supported by a grant of the Italian Ministry of Health (Ricerca Corrente 2004) to the IRCCS Neurological Institute "C. Mondino".

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