Activation of p53 and the pro-apoptotic p53 target gene PUMA during depolarization-induced apoptosis of chromaffin cells

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Received 10 May 2005; revised 8 July 2005; accepted 14 July 2005
Available online 19 August 2005

Abstract

The pathogenesis of non-glutamatergic, depolarization-induced cell death is still enigmatic. Recently, we have shown that veratridine induces apoptosis in chromaffin cells, and we have demonstrated protective effects of antioxidants in this system, suggesting a role for Na+ channels and oxidative stress in depolarization-induced cell death. We examined the possible contribution of p53, a transcription factor that has a major role in determining cell fate, and the mitochondrial apoptosis pathway in veratridine-induced cell death of cultured bovine chromaffin cells. Nuclear condensation and fragmentation were detected several hours after a 60-min exposure to 30 μM veratridine. Apoptosis was associated with a transitory increase in p53 protein levels. Veratridine induced transcription of the pro-apoptotic p53 target gene PUMA, but not of bax or pig3. Using transient transfection experiments, we found that wild-type p53, but not the mutant form p53-273H, was sufficient to induce cell death in the chromaffin cells, which was caspase-9 dependent. The down-regulation of either p53, by overexpressing p53-273H, or caspase-9 activity using a dominant-negative caspase-9 mutant protected chromaffin cells against veratridine-induced toxicity. Our data demonstrate the importance of p53 and the downstream activation of the mitochondrial apoptosis pathway in depolarization-induced apoptosis.

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Introduction

Apoptosis is an essential physiological process for the selective elimination of cells. The apoptotic pathway can be divided into three stages: a phase of induction (premitochondrial), a phase of decision (mitochondrial), and a phase of degradation (post-mitochondrial). Mitochondria that during years were postulated to be the energy supply of eukaryotic cells have emerged as the headquarter of apoptosis signaling pathway. The decision phase, which contains the point of no return, corresponds to the permeabilization of the mitochondrial membranes and the release of pro-apoptotic proteins, such as the cytochrome c, AIF, pro-caspases, and Smac/DIABLO, into the cytosol. Once in the cytoplasm Apaf-1, caspase-9 and cytochrome c assemble and form the apoptosome, a large caspase-activating complex that plays a central role in the initiation and execution phases of apoptosis. Indeed, active forms of caspase-9 or Apaf-1 are required to form a functional apoptosome complex, and cells expressing mutant caspase-9 are insensitive against apoptotic stimuli, including chemotherapeutics (Osaki et al., 1997). In cellular necrosis, mitochondria also may undergo permeability transition (Ravagnan et al., 2002; Baines et al., 2005; Nakagawa et al., 2005). A large, specific pore—the mitochondrial permeability transition pore (MPTP)—opens, and solutes of up to 1.5 kDa pass freely in and out of the mitochondrial matrix (Bernardi, 1999; Crompton, 1999; Jordan et al., 2003; Saelens et al., 2004).

Veratridine is an alkaloid obtained from sabadilla seeds and from the rhizome of hellebore that inhibits the complete inactivation of sodium channels, maintaining the channel open with a small but steady sodium current by generating a change in the three-dimensional conformation of the sodium channels (Sutro, 1986). Veratridine as a depolarizing agent can also be used in the...
study of neuronal death, and provides a model system to study glutamate-independent, depolarization-induced cell death pathways which can be relevant for white matter injury (LoPachin et al., 2001; Stys et al., 1992; Stys and LoPachin, 1998). We have already shown that in bovine chromaffin cell cultures, a well-established model to study secretory machinery (Bader et al., 2002), veratridine induces a delayed cellular death, which has the features of apoptosis such as chromatin condensation and DNA fragmentation, mitochondrial depolarization, cytochrome c release, and caspase activation (Jordan et al., 2000, 2002). Conversely, the molecular mechanisms underlying the overall signaling response of chromaffin cells to veratridine toxicity are not well characterized. Wild-type p53 protein has been shown to be capable of inducing apoptosis (Yonish-Rouach et al., 1991; Ramqvist et al., 1993; Jordan et al., 1997). Recently, the roles of several p53 targets genes in mediating the p53 apoptotic response have been queried through loss-of-function analysis using knockout mouse models. These studies have demonstrated that the p53 targets including bax and PUMA (p53 up-regulated modulator of apoptosis) play cell-type-specific roles in p53-mediated apoptosis (Schuler and Green, 2001). PUMA encodes a BH3-only protein and it could be a principal mediator of cell death in response to diverse apoptotic signals (Jeffers et al., 2003).

In the present study, we examined the role of p53 in veratridine-induced cell death and its implication in apoptosis of bovine chromaffin cell death.

Materials and methods

Chromaffin cell culture

Bovine chromaffin cells were isolated as previously described (Galindo et al., 2003; Neco et al., 2004). After washing the gland with a Ca$^{2+}$-free Locke’s solution (Locke’s medium) containing (in mmol L$^{-1}$): NaCl 154, KCl 5.6, MgCl$_2$ 1, HEPES 10, glucose 10, pH 7 to remove remaining erythrocytes, adrenal glands were incubated with Ca$^{2+}$-free Locke’s medium containing 0.2% collagenase (Boehringer-Mannheim, Indianapolis, IN) and 0.5% bovine serum albumin (Calbiochem, La Jolla, CA) for 45 ($3 \times 15$) min. Following medulla dissection and further incubation in collagenase solution for 30 additional minutes, chromaffin cells were separated from erythrocytes using a Percoll gradient. Cells were plated either onto poly-L-lysine (Sigma, St. Louis, MO; 0.5 mg/mL in borate buffer, pH 8.0)-coated 15-mm round glass coverslips (2–3 $\times 10^5$ cells/coverslips) for cell viability experiments or in 25 cm$^2$ flasks (5–8 $\times 10^6$ cell/culture flasks) for reporter assays, in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, penicillin (100 IU/mL) and streptomycin (50 $\mu$g/mL) at 37°C under an atmosphere of 5% CO$_2$.

Veratridine exposures

Chromaffin cell cultures were rinsed twice with Krebs HEPES buffer (K–H) with the following ionic composition (in mM): NaCl 140, KCl 5.9, MgCl$_2$ 1.2, HEPES 15, glucose 10, CaCl$_2$ 2.5, pH 7.4, incubated for 1 h either in K–H or in K–H containing 30 $\mu$M veratridine at room temperature. Exposure was terminated by washing the cells three times with K–H solution. For vitamin E and cycloheximide treatments, drugs were added 12 h before veratridine exposure and maintained until the end of the experiment.

Cell viability experiments

To assess the cell viability, coverslips containing chromaffin cells were treated with 1 $\mu$g/mL Hoechst 33342 for 1 min. The chromatin of GFP-positive cells stained with Hoechst 33342 were examined with a standard epi-illumination fluorescence microscope (Axiophot, Zeiss, Germany). Cells with condensed or fragmented chromatin represented dead cells. A blinded observer counted the number of dead and alive cells in 10 microscopic fields (under 40$\times$ magnifications) for each coverslip and the mean was regarded as the representative value for the coverslip. The percentage of dead cells was determined in 3 or 4 coverslips for each experimental condition. The average percent apoptotic cells from at least three separate experiments for each condition is expressed in the text and figures as the mean ± SEM. Statistical significance was determined by Student’s $t$ test.

Immunoblotting

Chromaffin cell cultures were washed with cold PBS twice and then collected by mechanical scraping with 1 mL of PBS per tissue culture dish. The protein suspension was centrifuged at 12,000–14,000 rpm for 5 min. The supernatant was discarded, and the protein pellet was brought up in 150 $\mu$L of sample buffer. The protein from each condition was quantified spectrophotometrically (Micro BCA Protein Reagent Kit, Pierce, Rockford, IL), and an equal amount of protein (30 $\mu$g) was loaded onto each lane of the 10% SDS-PAGE, which was then run at 90 mV. After electrophoresis, proteins were transferred to Immobilon PVDF membranes. Non-specific protein binding was blocked with Blotto [4% w/v non-fat dried milk, 4% bovine serum albumin (Sigma) and 0.1% Tween 20 (Sigma)] in PBS for 1 h. The membranes were incubated with anti-p53 [1:50 dilution of anti-mouse monoclonal (Pab240) sc-99 Santa Cruz] or anti-PUMA (1:1000 dilution of polyclonal, Oncogene Research Products, San Diego CA) overnight at 4°C. After washing with Blotto, the membranes were incubated with a secondary antibody (1:5000 dilution of peroxidase-labeled anti-mouse, Promega, Madison, WI) in Blotto. The signal was detected using...
an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 Kit). Immunoblots were developed by exposure to X-ray film (Eastman-Kodak, Rochester, NY).

Preparation of cytosolic and nuclear extracts

Cells (8 × 10^6) were washed with PBS and collected by centrifugation. Cell pellets were homogenized with 100 μL of buffer A (10 mM HEPES [pH 7.9], 1 mM EDTA, 1 mM EGTA, 100 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 10 μg/mL leupeptin, 2 μg/mL N-p-tosyl-L-lysine chloromethyl ketone, 5 mM NaF, 1 mM NaVO₄, 10 mM Na₂MoO₄). After 10 min at 4°C, Nonidet P-40 was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s, and nuclei were collected by centrifugation at 8000 × g for 15 min (Diaz-Guerra et al., 1996). The supernatants were stored at 80°C (cytosolic extracts); the pellets were resuspended in 50 μL of buffer A supplemented with 20% glycerol–0.4 M KCl and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000 × g for 15 min, and the supernatant was stored at 80°C. Protein content was assayed using the Bio-Rad protein reagent. All cell fractionation steps were carried out at 4°C.

Immunostaining

Cells were fixed in 4% paraformaldehyde before permeabilization in 0.2% Triton X-100. Primary antibody used was anti-p53 anti-mouse monoclonal (Pab240, sc-99 Santa Cruz). Secondary antibody was FITC-conjugated for mouse IgG (Jackson immunoresearch). All images were collected by a standard epi-illumination fluorescence microscope (Axioskop, Zeiss, Germany) and processes using Adobe Photoshop software.

Transfections

For transient transfections, we used 2-day-old in vitro bovine chromaffin cells that by 24 h before transfections were grown in antibiotic-free media. Transfections were achieved using Lipofectamine™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, 3 μL of lipofectamine reagent were pre-incubated for 20 min with 4 μg of DNA/10⁶ cells of plasmids encoding full-length, wild-type p53, p53-273H, p21^{WAF-1}, retinoblastoma (provided by A. Carnero, CNIO Spain), or caspase-9 dominant-negative mutant caspase-9 (C287A; casp 9DN, a gift from Ding HF, Medical College of Ohio, Toledo, Ohio; Cui et al., 2002), GFP (pGFP-C1; CLONTECH Laboratories, Inc.), the reporter for bax, pig3, and mdm2 were a gift from X. Lu (Ludwig Institute for Cancer Research, Imperial College School of Medicine, London, Bergamaschi et al., 2004) and PUMA from Dr. T. Chittenden (Immunogen Inc., Cambridge, MA). After 5 h of incubation, the transfection mixture was removed and replaced with fresh complete medium.

Luciferase assay

Luciferase activity was measured in protein extracts from cultured bovine chromaffin cells using a Luciferase detection kit (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Briefly, after a 6-h treatment with veratridine (1 h, 30 μM), cells were collected and centrifuged at 600 rpm for 10 min at room temperature, and the pellet was homogenized in 300 μL of Passive Lysis Buffer. Cell homogenates were spin down, and 20 μL of protein extracts were added to 100 μL of luciferase assay buffer containing the luciferase assay substrate and immediately measured in a luminometer. Luminescence was expressed in an arbitrary scale as relative light units (ALU). β-galactosidase (β-gal) activity was measured according to the manufacturer’s protocol (Promega, Madison, WI, USA) as an internal control by co-transfection of CMV-β-gal (Lecanda F, University of Navarra). Briefly, 30 μL of the same protein extracts obtained in the Luciferase assay and 20 μL of reporter lysis buffer were added to 50 μL of assay 2× buffer in a 96-well plate, and incubated at 37°C for 5 h. The reaction was stopped by adding 1 M sodium carbonate and the absorbance was measured at 415 nm in a microplate reader.

Results

Veratridine induces p53 protein levels

Previous studies (Callaway et al., 2001; Takahashi et al., 2000) have shown that veratridine is capable of inducing cell death in different cell types. We have previously shown that veratridine triggers DNA fragmentation and chromatin condensation in bovine chromaffin cells (Jordan et al., 2000, 2002). We have also demonstrated that veratridine induced the generation of reactive oxygen species in bovine chromaffin cells (Jordan et al., 2000). In the first set of experiments, we were interested in determining the contribution of p53 in this cell death model. Therefore, we asked whether the total p53 protein level changed in response to veratridine by immunocytofluorescence analysis. As shown in Fig. 1A, p53 protein was undetectable in these cells under control conditions, but 6 h after veratridine treatment (30 μM, 1 h), p53-like immunoreactivity was observed in about 15% of chromaffin cells (Fig. 1B). Western blotting technique reveals that exposing cultures to veratridine (30 μM, 1 h) had a marked transitory effect on p53 protein levels and changes in total p53 protein levels were already apparent 6 h after veratridine treatment (Fig. 1C).

Since p53 is frequently found in the cytoplasm of peripheral neurons under resting conditions and migrates to the nucleus upon activation by stress stimuli, we
performed subfractionation experiments to clarify the role of veratridine on p53 localization. As shown in Fig. 1 D, after 6 h of veratridine treatment, p53 protein was increased in the nuclear fraction. To determine whether the mechanisms of actions of veratridine on p53 depend on reactive oxygen species generation, we analyzed if a pre-treatment for 12 h with the antioxidant vitamin E (50 μM) modified the levels of p53 protein by 6 h after veratridine exposure. However, the antioxidant pre-treatment was ineffective in blocking the veratridine-induced p53 protein increase. Our results also suggested that p53 increases are mainly a consequence of post-translational mechanisms, since cycloheximide (12 h, 10 μg/mL) did not alter p53 protein levels after 6 h of veratridine treatment (Fig. 1 E).

**p53 Overexpression induces apoptosis in chromaffin cells**

Because p53 has been shown to promote cell death in several models, we tested whether p53 expression was sufficient to induce cellular death in bovine chromaffin cells. Cellular cultures were co-transfected with a plasmid encoding GFP and either wild p53 (wp53) or the p53 mutant form 273 (mp53) plasmids. Morphological chromatin analysis of GFP-positive cells by using Hoechst 33342 staining demonstrated that wp53-transfected cells showed an increase in apoptosis by 48 h after transfection (Fig. 2A); and were evident morphological characteristics of apoptosis including cell shrinkage and chromatin condensation/fragmentation (Figs. 2B–C). In contrast, mp53-transfected cultures remained cell viability largely intact (Fig. 2A).

p53 activation results in the up-regulation of different proteins including the cyclin kinase inhibitor p21\(^{\text{WAF1}}\), which regulate the activity of other transcription factors including the retinoblastoma gene product, Rb. However, overexpression of either p21\(^{\text{WAF1}}\) or Rb failed to induce significant cell death (Fig. 2A).
Effects of veratridine on p53-transcriptional activity

The next set of experiments was addressed to evaluate whether veratridine modulates some of these genes transcriptionally regulated by p53, in particular the pro-apoptotic genes bax, pig3, and PUMA. Two-day-old chromaffin cell cultures were transfected with reporter construct containing p53 binding sites upstream of the luciferase gene and 24 h later were exposed to veratridine (30 μM, 1 h). Luciferase activity in cell extracts was determined 6 h later. As shown in Fig. 3, we detected a significant induction of luciferase activity in extracts from chromaffin cultures transfected with an mdm2 reporter plasmid (2.13-fold increase), which served as a positive control. Interestingly, veratridine failed to increase the activity reporter of bax (1.13-fold increase) and pig3 (0.80-fold change).

In extracts from chromaffin cultures transfected with PUMA reporter and 12 h after veratridine treatment (30 μM, 1 h), we detected a significant induction of luciferase activity (1.98-fold increase). Consistently, veratridine induced PUMA protein levels in chromaffin cells cultures (Fig. 3B).

Caspase-9 knockdown blocks p53-induced cell death

We have previously shown that veratridine-induced cytochrome c release from mitochondria leads to caspase-3 activation in bovine chromaffin cells (Jordan et al., 2000, 2002). Caspase-9 participates in the formation of the apoptosome that results in caspase-3 activation (Srinivasula et al., 1998; Bratton et al., 2001). We therefore asked whether the mitochondrial/caspase-9 pathway was targeted by p53 in bovine chromaffin cells. To investigate the role of caspase-9, we used the well-characterized caspase-9 dominant-negative mutant caspase-9 (C287A; casp9 DN) (Cui et al., 2002). Chromaffin cells were co-transfected with GFP and either wp53 or casp9 DN or wp53 plus casp9 DN. As shown in Fig. 4, casp9 DN expression protected chromaffin cells from the p53-induced cell death. Therefore, a functional caspase-9 is essential for p53-induced apoptosis in bovine chromaffin cells.

Mutant p53 overexpression blocks veratridine-induced cell death

The present data point to p53 might play an important role in veratridine-induced chromaffin cell death. In order to establish whether p53 is a key step in this death pathway, cells were transfected with mp53 plasmid and 24 h later exposed to veratridine (30 μM, 1 h). As shown in Fig. 5, mp53 overexpression affords protection in chromaffin cultures against veratridine toxicity when cell viability was assayed 24 h after treatment.

Due to the fact that caspase-9 knockdown blocks p53-induced cell death in chromaffin cells, we analyzed the effect of casp9 DN overexpression on veratridine-induced cell death. Consistent with the idea that caspase-9 could participate in this pathway, the cultures transfected with Casp9 DN were more resistant to the veratridine exposure than those in which the native p53 was inhibited (Fig. 5). The simultaneous inhibition of both proteins, by overexpressing mp53 and casp9 DN, did not afford a higher protection than mp53 or casp9 DN alone (Fig. 5).

Discussion

We have previously shown that veratridine induces cell death in bovine chromaffin cells throughout a mechanism that involves an increase in mitochondrial permeability leading to cytochrome c release and caspase-3 activation (Jordan et al., 2000, 2002). In the present study, we gained insight in the apoptosis mechanisms involved in this model and present evidence for p53 activation and the activation of caspase-9-mediated, veratridine-induced cell death.

Bovine chromaffin cultures exposed to veratridine showed the typical hallmarks of apoptosis including changes in birefringence, chromatin condensation, and DNA fragmentation. The tumor suppressor gene p53 was increased in chromaffin cell cultures exposed to veratridine (30 μM, 1 h). This increase reached a maximum 6 h after the veratridine exposure, and this was associated with an increased nuclear localization of p53. Our results suggest that regulation of p53 by veratridine is mainly a conse-

Fig. 3. Effects of veratridine treatments on p53-transcriptional activity. (A) Two-day-old chromaffin cell cultures were transfected with reporter construct containing p53 binding sites upstream of the luciferase gene and 24 h later were exposed to veratridine (30 μM, 1 h). Luciferase activity in cell extracts was determined 6 h later. Luminescence was expressed in an arbitrary scale as relative lights units (ALU). β-galactosidase (β-gal) activity was measured as an internal control. Values are the mean ± SD of four experiments. **P < 0.01 versus control conditions; ANOVA and Tukey’s test. (B) Immunoblot analysis of PUMA protein levels in whole-cell extracts from control and veratridine-treated chromaffin cells at the indicated times points after exposures (30 μM, 1 h). Similar results were found in three separate experiments.
quence of a post-translational mechanism since cyclo-
heximide pre-treatment did no alter p53 protein levels (see
also Voelkerding et al., 1995). Interestingly, vitamin E was
unable to block the p53 induction in chromaffin cell
cultures, suggesting that the increase in ROS production is
downstream of p53 activation. Indeed, we have previously
demonstrated that the mitochondrial release of cytochrome
c triggered a caspase-dependent ROS production (Dussmann
et al., 2003). A positive correlation between p53 expres-
sion and chromatin condensation or fragmentation could be
observed, indicating that p53 expression is sufficient to
induce apoptosis in chromaffin cells (Fig. 2). It is
noteworthy that the overexpression of genes that have
been involved in p53-mediated cell cycle arrest, such as
p21\(^{Waf1}\) or Rb, failed to induce apoptosis in chromaffin cell
cultures (Attardi et al., 1996). We suggest that p53 is
involved in non-glutamatergic, depolarization-induced cell
death and demonstrate the ability of p53 to sense different
cellular stressors in neurons such as gamma irradiation,
glutamate receptor overactivation, ischemia, and prolonged
depolarization (Leker et al., 2004; Xiang et al., 1996).

Our data also indicate that veratridine-induced p53
activation also triggers the transcription of mdm2 and
PUMA, but not bax or pig3. The mdm2 up-regulation
might explain the transient nature of the p53 protein
increase, since mdm2 functions as a p53 ubiquitin ligase
and triggers its degradation (Oren, 1999). On the other hand,
the lack of effect on bax shown herein is in the line of
previous observations where it has been reported that p53
overexpression failed to induce bax protein levels (Johnson
et al., 1998). However, we cannot exclude the participation
of this protein in veratridine-induced cell death as bax can
play its pro-apoptotic activity through a transcription-

act as a principal mediator of cell death in p53-mediated
apoptosis (Jeffers et al., 2003), and its absence strongly
inhibits p53-induced apoptosis (Macip et al., 2003). Our
data therefore suggest that PUMA might be the link between
p53 and the mitochondrial apoptosis pathway. In line with
this hypothesis, knockdown of caspase-9 activity by over-
expressing casp9 DN afforded protection against veratridine
toxicity and p53 overexpression. Our data therefore suggest
that caspase-9 functions as a limiting effector of p53-
mediated apoptosis in this pathway.

Modulation of mitochondria membrane permeability
seems to control the activation of cell death programs
through mechanisms that include the release of pro-
apoptotic proteins, leading to caspase activation, mitochon-
drial ROS production and dysfunction, and eventually cell
death. The cytochrome c triggered assembly of the
apoptosome complex represents the initiating step for the
activation of the caspase cascade. Catalytically active
caspase-9 is required for recruitment of caspase-3 to the
apoptosome (Bratton et al., 2001). Consistently, several
reports using gene knockout technologies or overexpression
of dominant-negative mutants of caspase-9 have demon-
strated a requirement for caspase-9 for most apoptotic cell
death paradigms (Twiddy et al., 2004; Hakem et al., 1998;
Kuida et al., 1998; Soengas et al., 1999; Cui et al., 2002;
Soengas et al., 1999).

In summary, our data show that the tumor suppressor p53
plays an essential role in veratridine-induced cell death, and
that this pathway requires the participation of a functional
caspase-9. Veratridine induces a loss of cellular ion homeo-

Fig. 4. Requirement for caspase-9 in p53-induced apoptotic death. Chromaffin cell culture viability was determined 48 h after transfection with GFP and either wp53 or casp9 DN or wp53 and casp9 DN plasmid. GFP was used as a marker to indicate transfected cells that were subsequently scored for viability by chromatin morphology by using Hoechst 33342 staining. By 24 h later, green cells were scored as either apoptotic or healthy in a blind manner. Each column represents the average obtained from four independent experiments ± SEM. **P < 0.01 versus control conditions; ANOVA and Tukey’s test.

Fig. 5. Role of p53 and caspase-9 in veratridine-induced cell death. Cell cultures were co-transfected with GFP and mp53, Casp9 DN, or mp53 plus Casp9 DN 48 h before veratridine exposures. Cell viability was performed by studding the state of chromatin using Hoechst 33342 staining in GFP-positive cells. Each column represents the average obtained from four independent experiments; results ± SEM. ***P < 0.001 versus control conditions; ANOVA and Tukey’s test.
Acknowledgments

We want to thank V. Guijarro for expert technical assistance and Prof. V. Ceña and Prof. L.M. Gutierrez (Universidad Miguel Hernández) for providing bovine chromaffin cells. This work has been supported, in part, by grants SAF2002-04721 from CICYT, 04005-00 from Junta de Comunidades de Castilla La Mancha (JCCM), Consejería de Sanidad of JCCM and SEF-Almirall Prodesfarma to J.J. M. G.-L., M.F.G., and F.J. F.-G. are JCCM fellows. We are greatly indebted to Prof. P. Tranque for critical reading of the manuscript.

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