Delayed oxidant-induced cell death involves activation of phospholipase A2

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Abstract Short-term (1 h) exposure of cells to a low steady-state concentration of H_2O_2 causes no immediate cell death but apoptosis occurs several hours later. This delayed cell death may arise from activation of phospholipases, in particular phospholipase A2 (PLA2), which may destabilize lysosomal and mitochondrial membranes. Indeed, the secretory PLA2 (sPLA2) inhibitor 4-bromophenacyl bromide diminishes both delayed lysosomal rupture and apoptosis. Furthermore, sPLA2 activation by mellitin, or direct micro-injection of sPLA2, causes lysosomal rupture and apoptosis. Finally, B-cell leukemia/lymphoma 2 (Bcl-2) over-expression prevents oxidant-induced activation of PLA2, delayed lysosomal destabilization and apoptosis. This supports a causal association between PLA2 activation and delayed oxidant-induced cell death and suggests that Bcl-2 may suppress apoptosis by preventing PLA2 activation.

Key words: Apoptosis; B-Cell leukemia/lymphoma 2; Lysosome stability; Oxidative stress; Phospholipase A2

1. Introduction

Cultured cells transiently exposed to very low concentrations of oxidants do not immediately die but do undergo apoptosis several hours later [1,2]. The processes which occur during this interesting delay are unknown as are the very earliest events in apoptosis. Of course, delayed apoptotic cell death caused by other agonists, such as serum or growth factor withdrawal, is well described. In many cases, such as apoptosis of neuronal cells triggered by withdrawal of nerve growth factor, there is a stringent requirement for protein synthesis [3], which helps to explain the delay in apoptosis.

In the particular case of oxidant-induced apoptosis, it has been previously suggested that an important upstream event in this process may be the destabilization of lysosomes with release of their potent digestive enzymes into the cell cytoplasm [4–8]. When cultured cells are exposed to minimally effective doses of pro-apoptotic agents such as H_2O_2, the process of lysosomal leakage appears to occur in two phases: an early and minor lysosomal destabilization followed some time later by a more massive lysosomal rupture and apoptosis [1,2,9]. This occurs even if the oxidant is removed after only a brief period but the processes involved in this delayed lysosomal rupture and apoptosis are presently unknown.

We hypothesized that this delayed oxidant-mediated cell death might be related to a progressive destabilization of the membranes of intracellular organelles, such as might be effected by the degradation of phospholipids by phospholipases. Several neurodegenerative conditions, such as Alzheimer's disease, stroke and spinal cord trauma, have been reported to involve activation of phospholipases [10]. Moreover, activation of phospholipase A2 (PLA2) has been observed in apoptosis triggered by agonists such as tumor necrosis factor and oxidative stress [11,12]. This raises the possibility that delayed cell death following oxidant challenge might similarly involve activated PLA2 which, in turn, could progressively destabilize the membranes of organelles, such as lysosomes and mitochondria, either through simple depletion of phospholipid from organellar membranes, or through the accumulation of chaotropic products, such as free fatty acids or lysophosphatides. The resulting rupture of these organelles would then constitute a sort of auto-digestive 'death spiral' culminating in delayed apoptosis.

We have, therefore, tested the possibility that the leakage of lysosomal contents might be associated with activation of PLA2. Here, we report evidence that PLA2 activation is intimately involved in delayed oxidant-induced lysosomal rupture and ensuing apoptosis, providing a possible explanation for the slow appearance of cell death following even brief exposure to low levels of oxidants.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. RPMI 1640 medium, fetal bovine serum, glutamine, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). The CaspACE FITC-VAD-FMK In Situ Marker was purchased from Promega Corporation (Madison, WI, USA), while the liposomal delivery vehicle, *N*-[1-(2,3-dioleoyl)-propyl]-*N*,*N*,*N*-trimethyl-ammonium salt (DOTAP), was obtained from Boehringer Mannheim (Indianapolis, IN, USA). PLA2 (P6534) was from Sigma. The plasmid (murine) B-cell leukemia/lymphoma 2 (Bcl-2)α/pMKITNeo, was a kind gift from Dr. W.S. May, University of Texas Medical Branch, Galveston, TX, USA.

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Abbreviations: AA, arachidonic acid; AO, acridine orange; Bcl-2, B-cell leukemia/lymphoma 2; BPB, 4-bromophenacyl bromide; DOTAP, *N*-[1-(2,3-dioleoyl)propyl]-*N*,*N*,*N*-trimethyl-ammonium salt; PLA2, phospholipase A2

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2.2. Cell cultures

J774 cells (a murine histiocytic lymphoma cell line) and AG-1518 cells (a human foreskin fibroblast cell strain) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 1% non-essential amino acids. Cells were maintained in plastic flasks and sub-cultivated at confluence twice a week. Medium was changed daily. For experiments, cells were transferred into 35 mm six-well plates (5×10^5 cells/well) 24 h before use.

2.3. Over-expression of Bcl-2

J774 cells were sub-cultivated and grown in six-well plates (5×10^5 cells/well) as described above. The cells were transfected with Bcl-2α/pMKITNeo expression plasmids, which contain a neomycin resistance element, using liposomal delivery by DOTAP according to the manufacturer's directions. Ten clones of stably transfected cells were selected by their neomycin resistance. Bcl-2 expression levels were assayed by Western blot analysis (vide infra). Compared to the parental J774 cells, three clones of the transfected cells had at least a five-fold increase of immuno-reactive Bcl-2 protein [1] and one of these was chosen for further experiments. The Bcl-2 over-expressing J774 cell line is hereafter termed 'J774-Bcl-2'.

2.4. Pre-treatment of cells with desferrioxamine (Des)

In some experiments, J774 cells were pre-treated with 1 mM (final concentration) Des for 1 h at 37°C in complete medium. The cells were then rinsed and incubated in complete medium under standard culture conditions (without Des) for another 30 min and, finally, exposed to H_2O_2 as described below.

2.5. Exposure to H_2O_2

Parenteral J774 cells and J774-Bcl-2 cells, cultured in six-well plates (5×10^5 in 2 ml of complete medium) as described above, were exposed for 1 h at 37°C to a steady-state concentration (40 μM) of H_2O_2 as described previously [1,2,13]. To abruptly obtain this steady-state concentration of H_2O_2, 37 mU/ml glucose oxidase (1.5 μg/ml) was added together with an initial bolus of H_2O_2 to bring the starting H_2O_2 concentration to 40 μM. Following 1 h of oxidative stress, under otherwise standard culture conditions, catalase was added to clear the H_2O_2, and the medium was replaced (but not when medium was assayed for arachidonic acid (AA) release, see below).

2.6. Lysosomal stability assessment by acridine orange (AO)

Cells were assessed for lysosomal stability using the (i) AO uptake and (ii) AO relocation methods as previously described [1,2,14]. The number of remaining intact lysosomes was evaluated by assaying red fluorescence (AO uptake method) following AO-staining of the cells at particular times after exposure to oxidative stress. To detect early and minor lysosomal destabilization, the progressive release of AO from cells which had been pre-incubated with AO prior to oxidant challenge was monitored (the AO relocation method). AO fluorescence was measured by flow cytometry using a Becton-Dickinson FACScan (Becton-Dickinson, Mountain View, CA, USA) equipped with a 488 nm argon laser. Cells were detached by scraping with a rubber policeman and collected for flow cytometric assessment of red (AO uptake) or green (AO relocation) fluorescence.

2.7. Assessment of apoptosis

1. The DNA fragmentation associated with apoptosis was followed using propidium iodide staining of nuclear DNA (the 'Nicoletti' assay) as described elsewhere [15].
2. Caspase activation associated with apoptosis was evaluated by microscopic analysis of living cells stained according to the manufacturer's directions with a FITC-conjugated broad spectrum inhibitor of caspases, CaspACETM FITC-VAD-FMK In Situ Marker, that irreversibly binds to activated caspases. Briefly, cells were seeded at 5×10^5 cells/ml, exposed to H_2O_2 as described above, and then incubated under standard culture conditions for another 4 h. The marker was then added to the medium at a final concentration of 10 μM and the cells were incubated in the dark for 20 min, rinsed three times (5 min in total) in phosphate-buffered saline (PBS; 20 mM Na_3PO_4, 130 mM NaCl, pH 7.4) and observed, counted and photographed using a Nikon fluorescence microscope.

2.8. Measurement of ^{3}H-AA release

To assess intracellular activation of phospholipases, cells were pre-labeled with 1.5 μCi/ml ^{3}H-AA and incubated in serum-free medium at 37°C for 18 h, as previously described [16]. Unincorporated ^{3}H-AA was removed by washing (×3) with PBS. Cells were then resuspended in fresh serum-free medium containing 1% fatty acid-free albumin in 24-well plates in triplicate and incubated for another 5 h to let the cells attach well to the plates, exposed to H_2O_2 as described above, and then kept under normal culture conditions for different periods of time. Medium was not changed (in order to preserve any released AA) and catalase (90 U/ml) was added to stop further exposure to H_2O_2 following the 1 h period of oxidative stress.

For measurements of AA release, we used a procedure designed to avoid artifactual detection of ^{3}H-AA which might be associated with the very small membrane blebs typically produced by cells undergoing apoptosis. Cells and insoluble material such as larger membrane fragments were pelleted by centrifugation at $13\,000\times g$ for 10 min. The supernatant was then chromatographed on Cibracron blue 3GA-agarose and retained radioactivity (representing free ^{3}H-AA bound to the albumin) was eluted using 0.1 M guanidine–HCl. Total radioactivity in the starting cells and in the eluted liquids was measured by scintillation counting. The total incorporation of ^{3}H-AA into cell membrane phospholipids was also measured by scintillation counting (value *A*) and the percentage of radioactivity released into the medium and bound by albumin (value *B*) was expressed as the ratio of $B/A\times100$.

2.9. Micro-injection of PLA2

Micro-injection of the fibroblast cell strain, AG-1518, with purified sPLA2 was performed on the stage of a Zeiss Axiovert (Gena, Germany) inverted microscope using an Eppendorf pressure injector (model 5246) and a micro-manipulator ('Injectman®'). Micro-injection needles (Femtotips II; Eppendorf, Hamburg, Germany) with an inner diameter of less than 0.5 μm were loaded using Eppendorf micro-loaders. All injectates were freshly prepared and contained 5 U/ml sPLA2 as well as 1 mg/ml dextran-conjugated Texas Red in PBS (pH 7.4) as a tracer. The solution was injected into the cytoplasm of individual cells at a pressure of 100 h Pa, with an injection time of

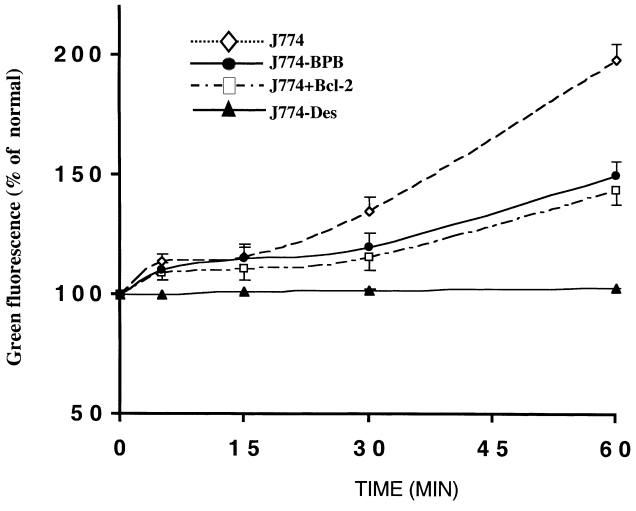

Fig. 1. Inhibition of PLA2 and Bcl-2 over-expression block the second phase of oxidant-induced lysosomal rupture. J774 and J774-Bcl-2 cells were pre-stained with AO as described in Section 2, then exposed to a low steady-state concentration of H_2O_2 and periodically analyzed by flow cytofluorometry for green AO-induced fluorescence (representing the appearance of previously lysosomal AO in the cell cytoplasm-relocation AO method) at indicated periods of time. The results are presented as percentage of values before the initiation of oxidative stress. Each point represents the mean ± 1S.D. of triplicate determinations. All points for the Des curve are statistically different ($P<0.01$) from each of those for the J774, J774-BPB, and J774-Bcl-2, while only the time points beyond 15 min differ between J774 and J774-Bcl-2, and between J774 and J774-BPB ($P<0.01$ in both cases).

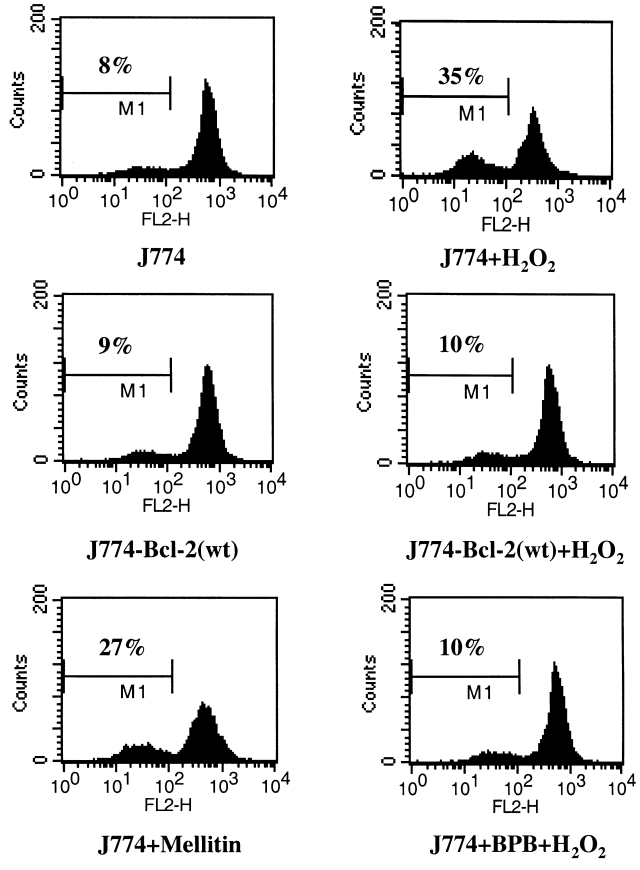

Fig. 2. Inhibition of sPLA2 and Bcl-2 over-expression block longer term oxidant-induced lysosomal rupture, whereas it is promoted by mellitin-induced PLA2 activation. J774 cells and J774-Bcl-2 cells were exposed to oxidative stress for 1 h, or to 0.5 μM mellitin for 3 h, as described in Section 2. After exposure to oxidative stress, or to mellitin, the cells were incubated under standard culture conditions for another 8 h, and then stained by AO and analyzed for red fluorescence (AO uptake method) by flow cytofluorometry as described in Section 2. Representative results from at least triplicate experiments are shown.

1.5 s. Injections were performed on 100–200 cells in each dish for each experimental condition. Control cells received Texas Red only. Data presented are representative of results obtained with at least three separate dishes. Note that the purity of this preparation of sPLA2 was assayed by SDS–PAGE electrophoresis and >95% was present at the 14 kDa band (as determined by densitometric scanning) (not shown).

2.10. Preparation of hepatic lysosomes

Livers were removed from 160–200 g female Wistar rats, homogenized in 9 volumes of 0.3 M sucrose and centrifuged at 450×*g* for 10 min. The supernatants were again centrifuged at 3500×*g* for 10 min, the pellet discarded, and the lysosome-containing supernatant centrifuged at 10 000×*g* for 10 min. The pellets were washed and re-centrifuged at 10 000×*g* for 10 min, and resuspended in the sucrose buffer to a protein concentration of ~15 mg/ml. The resultant lysosome enriched fraction (LEF) was found to be stable in the homogenization buffer at 4°C for up to 4 h.

2.11. Enzymatic detection of lysosomal integrity

The effects of sPLA2 on the integrity of lysosomes in the LEF preparation were assessed by measurement of the release of β-galactosidase. LEF (200 μl) was incubated with sPLA2 (0.2 U/ml) for various periods of time and then centrifuged at 14 000×*g* for 10 min. The supernatants were removed and 1 ml distilled water with Triton X-100 (final concentration 1%) was added to the pellets to cause lysis of the remaining intact lysosomes. The sediment (intralysosomal) and supernatant (released) activities of β-galactosidase were measured as described previously [17]. The results were expressed as percentage of total β-galactosidase released.

The integrity of lysosomes within individual cells micro-injected with sPLA2, was estimated by staining to cytoplasmic acid phosphatase activity (which is usually restricted to the lysosomal compartment). Fibroblast AG-1518 cells in 35 mm plastic Petri dishes were micro-injected with sPLA2 as described above and then kept under standard culture conditions for 1 h. The cultures were briefly rinsed in 0.25 M sucrose in 0.01 M Tris–HCl buffer (pH 7.4) at 37°C and fixed in 4% formaldehyde in 0.15 M Na cacodylate buffer, pH 7.4. Cultures were incubated at 37°C for 45 and 60 min with continuous agitation in a modified Gomori type medium containing 0.22 M sucrose and 10% DMSO [18]. Following rinsing in 0.1 M cacodylate buffer with 0.1 M sucrose (pH 7.2; 300 mOsm), the reaction product was visualized with 1% ammonium sulphide. The cultures were air-dried and mounted under coverslips in immersion oil.

2.12. Statistical analysis

All experiments were repeated at least three times. Data are presented as arithmetic means ± 1S.D. Statistical significance of differences between groups were determined using Student's two-tailed *t*-test.

3. Results and discussion

Cells briefly exposed to oxidative stress, in the form of low steady-state concentrations of H_2O_2, do not die immediately

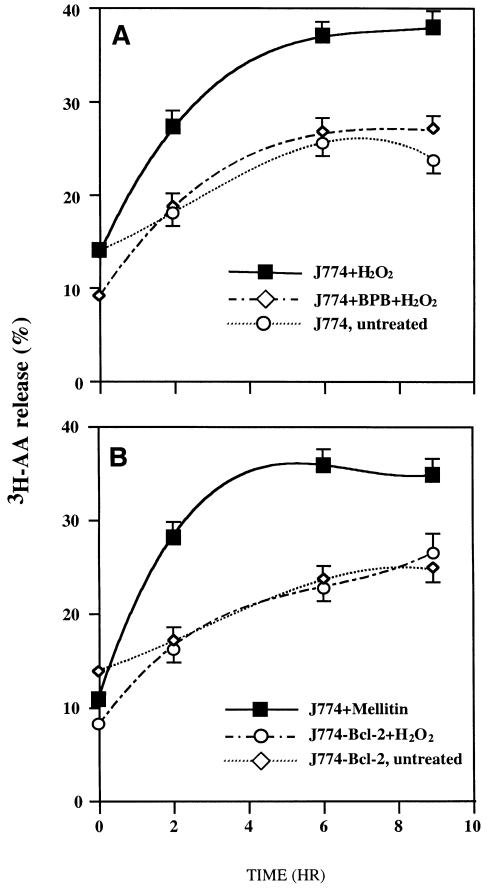

Fig. 3. Both oxidant stress and mellitin activate intracellular phospholipase(s), and Bcl-2 over-expression suppresses this activation. J774 and J774-Bcl-2 cells were pre-labeled with ^{3}H-AA and then exposed to oxidative stress for 1 h or to 0.5 μM mellitin for 3 h. The cells were then incubated under standard culture conditions for the indicated periods of time. Results shown represent mean ± 1S.D. of triplicate determinations.

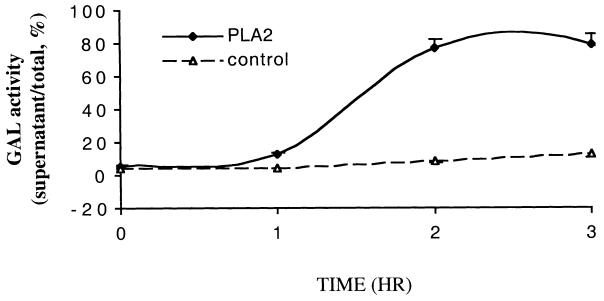

Fig. 4. Lysosomes are destabilized by sPLA2. LEFs (0.6 mg/ml) were incubated with sPLA2 (0.2 U/ml) for the indicated periods of time and sedimentable and unsedimentable activity of β-galactosidase (GAL) was measured. Results shown represent mean ± 1S.D. of triplicate determinations.

yet undergo apoptosis several hours later [1,2]. This delayed cell death is paralleled or, perhaps, preceded, by slow, progressive lysosomal rupture. Whereas only limited lysosomal destabilization is observed during the actual exposure to oxidative stress, a much more prominent rupture occurs several hours after return of the cells to standard culture conditions [1,2]. The early phase is probably a direct result of the oxidant stress and may be explained by preferential oxidant damage to the lysosomal compartment which is known to be particularly rich in low molecular weight, redox-active iron. In contrast, the second phase, which is inhibited by over-expression of Bcl-2 [1,9] may involve secondary actions of the released lysosomal enzymes which may promote further lysosomal rupture. We hypothesized that activation of cellular phospholipases, especially PLA2, might be involved in this second phase. PLA2 attack on the membranes of intracellular organelles such as lysosomes and mitochondria might explain both the progressive lysosomal destabilization and ultimate cell death which occur long after the oxidant challenge is removed.

In partial support of this hypothesis, inhibition of PLA2 (probably sPLA2) with 4-bromophenacyl bromide (BPB) appears to block preferentially the secondary phase of lysosomal destabilization (i.e. that which occurs more than 30 min following initiation of oxidant challenge). Interestingly, similar suppression of lysosomal AO release was seen in the cell line over-expressing Bcl-2 (Fig. 1). The protective effect of BPB and Bcl-2 over-expression persisted over a period of at least 8 h (Fig. 2). However, note that neither BPB pre-treatment nor Bcl-2 over-expression had significant effect on the primary phase of lysosomal destabilization (whereas pre-treatment of cells with Des, known to localize almost exclusively within the lysosomal compartment [2,19,20], had the expected protective effect on both phases of lysosomal rupture) (Fig. 1).

These observations suggested a possible causal relationship between intracellular PLA2 activity and lysosomal destabilization. Therefore, under identical experimental conditions, we measured the release of ^{3}H-AA from cells previously labeled with this isotope. ^{3}H-AA is presumed to be incorporated into membrane phospholipids, preferentially in the *sn*-2 position which is selectively hydrolyzed by PLA2. There were no differ-

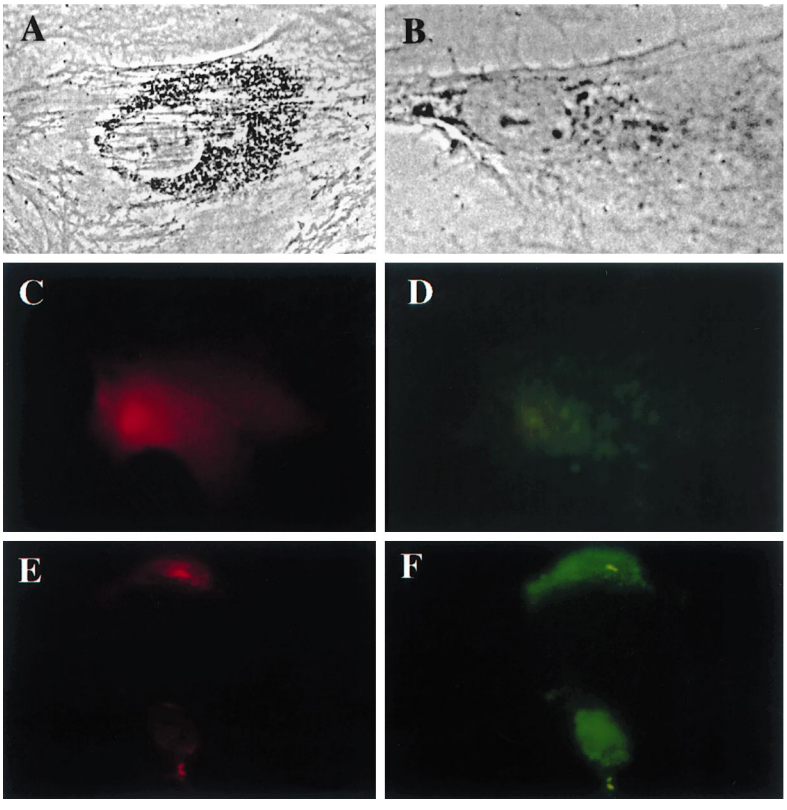

Fig. 5. Micro-injection of sPLA2 induces lysosomal leak and ensuing apoptosis. AG1518 fibroblasts were micro-injected with sPLA and then incubated under standard culture conditions for another 2 h before cyto-chemical staining for acid phosphatase. A: Control cells. B: sPLA2-exposed cells. Apoptotic cells were assayed after 3 h by the CaspACE™ FITC-VAD-FMK In Situ Marker staining. C: Control cells injected only with Texas Red Tracer (green-light excitation). D: Control cells (blue-light excitation to show the FITC-conjugated caspase inhibitor). E: sPLA2 micro-injected cells (green-light excitation). F: sPLA2 micro-injected cells (blue-light excitation). Representative examples are shown from experiments done at least in triplicate. For all treatments, at least 100 cells were examined in each experiment.

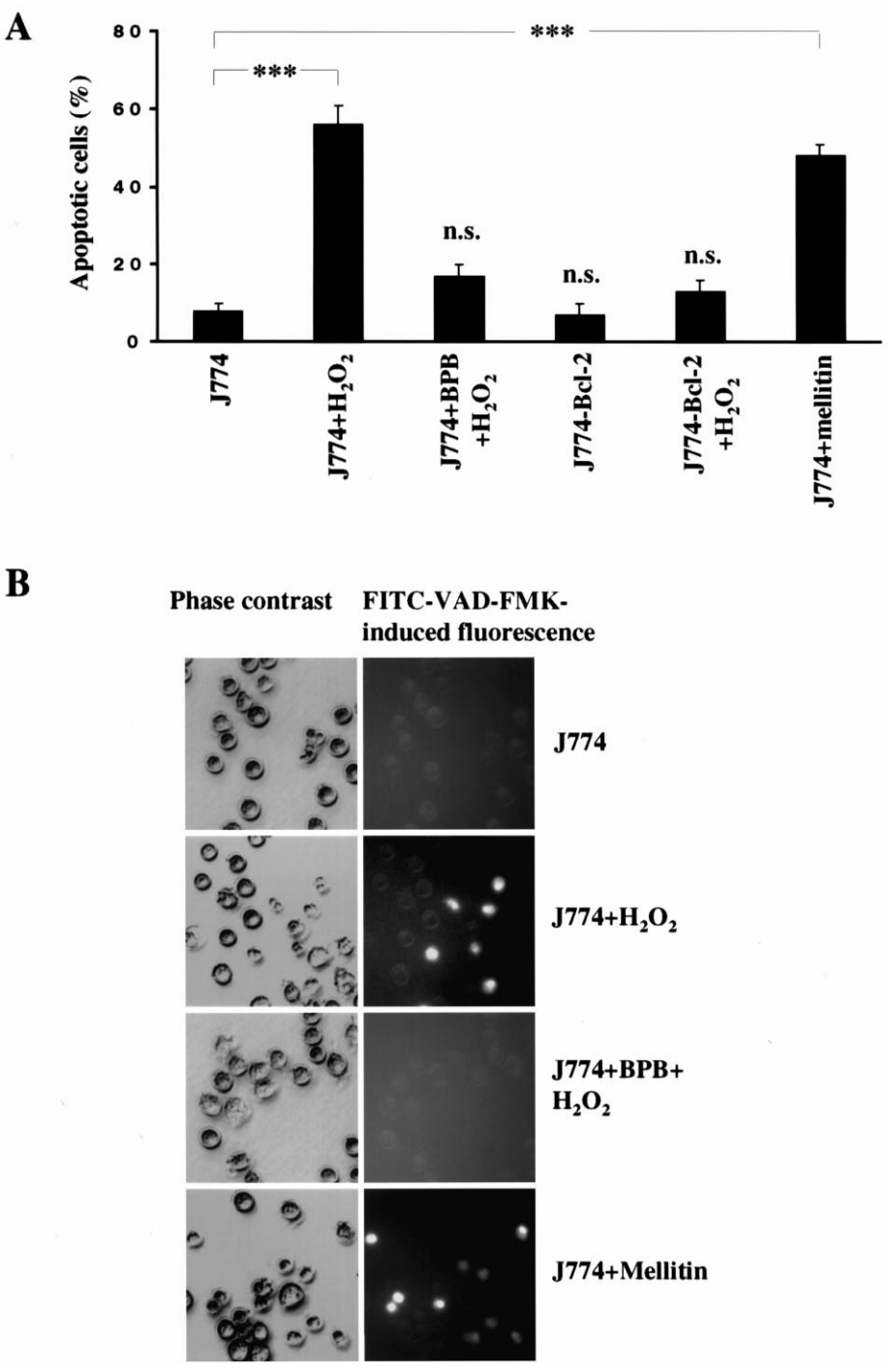

Fig. 6. Inhibition of sPLA2 or Bcl-2 over-expression make cells resistant to oxidant-induced apoptosis. J774 and J774-Bcl-2 cells were exposed to oxidative stress for 1 h, or to 0.5 μM mellitin for 3 h, and then kept under standard culture conditions for another 8 h (A) or 2 h (B), respectively. The cells were then assayed for apoptosis by the Nicoletti technique (A) or with CaspACE™ FITC-VAD-FMK In Situ Marker to determine caspase activation (B). n.s., no significant differences from non-oxidant exposed cells; $***P < 0.001$. Representative examples from at least three independent experiments are shown.

ences in the release of ^{3}H-AA following the 1 h exposure to oxidative stress but, over the following 8 h period, PLA2 activity increased significantly as judged by AA release (Fig. 3). Because our experimental conditions were designed to detect only free ^{3}H-AA release (as opposed to the production of small membrane fragments known to form during apoptosis), we are fairly confident that these results do reflect intracellular PLA2 activation.

In order to further test whether PLA2 activity might, in fact, be responsible for the delayed lyosomal rupture following oxidant challenge, we employed semi-purified rat liver lysosomes. Indeed, as judged by both flow cytometry (results not shown) and the release of lysosomal β-galactosidase activity, purified sPLA2 causes almost total lysosomal disruption over a period of 2 h (Fig. 4).

To determine whether PLA2 was, by itself, sufficient to

initiate both lysosomal destabilization and ensuing apoptosis, sPLA2 was micro-injected into AG1518 cells. As shown in Fig. 5, 2 h following the injection of sPLA2 there was a pronounced loss of lysosomal integrity as judged by the release of acid phosphatase activity from its normal intralysosomal location (Fig. 5A,B). Furthermore, 3 h after the microinjection of sPLA2, there was substantial activation of caspases as estimated by enhanced binding of the FITC-conjugated caspase inhibitor (Fig. 5F).

As additional evidence for a possible causal relationship between PLA2 activation, lysosomal rupture and apoptosis, pre-treatment of J774 cells with the PLA2 inhibitor BPB greatly decreased the numbers of apoptotic cells appearing 8 h following oxidant challenge (Fig. 6A). This protection coincides with the suppression of both lysosomal leakage and PLA2 activation reported above. Furthermore, cells treated with an activator of endogenous PLA2, mellitin, also became apoptotic after 8 h of further incubation (Fig. 6A). Mellitin treatment was also associated with both lysosomal destabilization and, as expected, activation of intracellular PLA2. Finally, as shown in Fig. 6B, caspase activation showed a similar pattern, being induced by both mellitin or H_2O_2 exposure and, in the latter case, being prevented by BPB pre-treatment.

The identity of the isoform(s) of PLA2 which might be activated by mild oxidant stress remains unknown. There are at least eight known isoforms of PLA2, broadly divided into two classes, secretory (or sPLA2) and cytoplasmic (or cPLA2), with the latter class being sub-divided into calcium-dependent and -independent types. Given that inhibitors with absolute specificity for these various isoforms are not now available, we are unable to identify those most likely responsible. However, the suppression of endogenous PLA2 activation, lysosomal rupture and apoptosis by BPB would support the tentative conclusion that activation of sPLA2 may be responsible inasmuch as this inhibitor is claimed to be relatively specific for this class [21].

Assuming that PLA2 activation is, indeed, causally involved in the delayed apoptosis following oxidant challenge (and, perhaps, apoptosis caused by other agonists), the mechanisms directly involved in this process are not yet clear. The possibilities include simple depletion of phospholipid from the bilayers of intracellular organelles such as lysosomes and mitochondria, leading to insufficient remaining phospholipid to constitute a stable bilayer. Alternatively, the products of PLA2 attack on phospholipid, lysophosphatides and free fatty acids which are known to have chaotropic and detergent actions, may be primarily responsible for lytic destruction of organellar membranes.

Overall, our results suggest that the activation of PLA2 arises from partial lysosomal rupture, but it is not clear whether PLA2 is directly activated by lysosomal enzymes or if this activation is indirect. It was suggested that PLA2 may be activated by caspases [22], which, in turn, may be activated by lysosomal proteases [6]. The apparent suppression of PLA2 activation/activity by over-expression of Bcl-2 implies that this well-known anti-apoptotic protein may exert its effects through previously unsuspected mechanisms. Indeed, Bcl-2 over-expression does somehow stabilize lysosomes against the secondary, later phase of mild oxidative stress-induced lysosomal rupture while, interestingly, having no effect on the short-term initial destabilization [1,9]. Such a downstream mechanism of action is consistent with the general idea that early lysosomal leakage itself leads to the later and much more pronounced lysosomal rupture, perhaps by direct or indirect activation of PLA2. By somehow interfering with the activation, or activity, of PLA2, Bcl-2 (as well as inhibitors such as BPB) suppresses the second phase of lysosomal rupture and the ensuing apoptotic process. We are hopeful that more detailed knowledge of these intracellular events may lead to more effective pharmacologic intervention, permitting the purposeful acceleration, or blockage, of apoptosis which might be useful in diseases ranging from cancer and neuro-degenerative diseases to inflammation.

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