Available online at www.sciencedirect.com
ScienceDirect
Environmental Research 107 (2008) 170–177
Environmental Research
www.elsevier.com/locate/envres

Age-related lung cell response to urban Buenos Aires air particle soluble fraction

Agustín Ostachuk[a], Pablo Evelson[b], Susana Martin[a], Laura Dawidowski[c], J. Sebastián Yakisich[d], Deborah R. Tasat[a,*]

[a]School of Science and Technology, National University of General Martín, Martín de Irigoyen 3100, 1650 San Martín, Buenos Aires, Argentina
[b]PRALIB-CONICET, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina
[c]National Atomic Energy Commission, Avda. Gral. Paz y Constituyentes, 1499, 1650, Buenos Aires, Argentina
[d]Department of Molecular and Cellular Medicine, Texas A&M University, HSC, 446 Reynolds Medical Building, College Station, TX 77843, USA

Received 22 May 2007; received in revised form 25 October 2007; accepted 14 January 2008
Available online 3 March 2008

Abstract

Exposure to particulate matter (PM) may alter lung homeostasis inducing changes in fluid balance and host defense. Bioavailability of soluble PM compounds like polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and transition metals has been shown to play a key role in lung injury. We have previously characterized the size, shape, and chemical components of urban air particles from Buenos Aires (UAP-BA) and their biological impact on lungs. Herein, we evaluate the possible toxic effect of UAP-BA-soluble fraction (UAP-BAsf) on pulmonary cells obtained from young (1–2 months old) and aged (9–12 months old) Wistar rats using phagocytosis, oxidant–antioxidant generation, and apoptosis as endpoints. UAP-BA were collected in downtown BA and residual oil fly ash (ROFA), employed as a positive control, was collected from Boston Edison Co., Mystic Power Plant, Mystic, CT, USA. Both particle-soluble fractions (sf) were employed at concentrations ranging from 0 to 100 μg/mL. UAP-BAsf and ROFAsf even at the lowest dose assayed (10 μg/mL) showed in both lung cell populations the ability to stimulate phagocytosis and increase superoxide anion (O_2^-) generation. Both types of air particles caused a marked intracellular oxidant stress in aged pulmonary cells that may contribute to subsequent cell activation and production of proinflammatory mediators, leading to cell dysfunction. These data suggest that the impact of UAP-BAsf on phagocytosis, oxidant radical generation, and apoptosis is clearly dependent on the maturational state of the animal and might have different mechanisms of action.

Keywords: Air pollution; Particulate matter; Soluble fraction; Aging; Lung

1. Introduction

Particulate matter (PM) composition is complex and varies with several factors such as the emission source, season of the year, location site, etc., all of which contribute to and may be responsible for their associated health effects. It is well known that ambient atmosphere contains organic (microorganisms, pollen, etc.) and inorganic (sulfur, nitrates, metals, etc.) particles (Lioy and Daisey, 1986; May et al., 1992) coming from natural sources and anthropogenic activities. The many different types of particles in the airborne mixture reflect a wide biological array of the observed epidemiological findings (Brunekreef et al., 2000).

Constant exposure to potentially harmful environmental substances and exposure to inhaled air PM have been correlated with diminished lung function or increased mortality (Dockery et al., 1993; Koenig et al., 1993; Longphre et al., 2000; Schwartz et al., 1994). As pointed out by Campbell and Campbell (2007), urban growth and increasing motor vehicles will make air pollution more common than in the past. Consequently, associated adverse health effects will augment in urban areas, especially for susceptible populations (children, the elderly, and individuals with preexisting respiratory pathologies).

*Corresponding author. Fax: +54 11 4580 7552/3.
E-mail address: deborah.tasat@unsam.edu.ar (D.R. Tasat).

0013-9351/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.envres.2008.01.007

Buenos Aires is Argentina's largest city, one of the largest urban centers in South America and the third most populated in Latin America. According to the 2001 Census (Instituto Nacional de Estadística y Censos (INDEC), http://www.indec.gov.ar), the city proper has a population of 2.77 million inhabitants and the Greater Buenos Aires conurbation has more than 8.1 million inhabitants. The high population density, the relatively elevated level of motor vehicle ownership, the large number of public transport vehicles, the high concentration of truck traffic, and the major industrial and thermoelectric complexes all contribute to air and noise pollution levels. In agreement with Borthagaray et al. (2001), we have recorded in downtown Buenos Aires a 72 μg/(m^3/day) particle average concentration of particulate matter less than 2.5 ($PM_{2.5}$) during wintertime (Martin et al., 2007).

A particularly atypical fact regarding Buenos Aires population is that 13.8% of the total population is elderly (adults over 65 years of age). Population studies estimate a sharp increase of the elderly reaching ~16.63% by 2025, making Buenos Aires a city with an aged population (people >60 years representing >7% of the total population) (Anzola Pérez et al., 1994; G. Urroz, personal communication). Since the proportion of the population that is older rises constantly, episodes of particulate air pollution in the elderly have become an important healthcare problem (Sandstrom et al., 2003).

We have previously analyzed the morphology of urban air particles from Buenos Aires (UAP-BA) by scanning electron microscopy and studied their chemical composition by energy-dispersive X-ray analysis and capillary gas chromatography. Regarding size, surface area, and distribution, UAP-BA proved to be small spherical ultrafine particles, free (0.2 μm) or grouped in clusters and associated to a matrix (40 μm). Chemical analysis showed high levels of total polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and almost no metal traces. We demonstrated that these <2.5 μm particles cause inflammation in the upper and lower respiratory tract of mice and suggested that the elevated content of PAHs and PCBs bound to UAP-BA might come from vehicular emissions (Martin et al., 2007). However, the potential physiological damage of Buenos Aires particulate matter soluble fraction to health has not been studied to date. The complex interactions of multiple factors are probably one of the main reasons leading to controversies regarding the mechanism of action of PM. This would be particularly true for the impact of particle-soluble fractions on health.

Vulnerability to harmful environmental agents undoubtedly rises with increasing adult age as most physiological functions deteriorate. As aging is associated to a progressive drop in the lung function, elderly face the same challenges as the younger people, albeit with a compromised respiratory system (Kelly et al., 2003). Numerous changes occur in the immune system with advancing age, probably contributing to the decreased immunoresponsiveness, associated with increased susceptibility to infection and cancer incidence in the elderly population (Castle, 2000). Previous reports have shown controversial age-related functional differences in macrophages recovered from different tissues and species (De La Fuente, 1985; Kojima et al., 1994; Lavie et al., 1992; Martin et al., 1995). Our group has demonstrated clear metabolic differences between aged and young animals. We have reported augmented levels of superoxide anion, nitric oxygen, antibody-dependent cellular cytotoxicity (ADCC), and secretion of tumor necrosis factor-alpha (TNFα) in lung macrophages from aged rats when compared to younger animals (Goldman et al., 2004; Tasat et al., 2003). Therefore, we hypothesized that alveolar macrophages (AM) (the major cell constituent in bronchoalveolar lavage) obtained from aged animals may be more susceptible to PM-soluble fraction exposure than cells obtained from younger animals. The aim of this study was to investigate the possible toxicity of UAP-BA soluble fraction (UAP-BAsf) and to elucidate if the biological response to UAP-BAsf in lung cells is age dependent.

2. Materials and methods

2.1. Chemicals

Nitrobluetetrazolium (NBT), phosphate-buffered solution (PBS), polyvinylpyrrolidone (PVP), luminol, 2,2-azobis-2-amidinopropane (ABAP), RPMI-1640 media, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), Hoechst 33258, and GenElute mammalian genome DNA Kit were purchased from Sigma Chemical Co. (St. Louis, MI, USA). Fluorescent latex beads (2.2 μm) were purchased from Molecular Probes.

2.2. Animals

Young (1–2 months old) and aged (9–12 months old) male Wistar rats were housed at the National Atomic Energy Commission, Argentina. Food and water were provided ad libitum. Animals were obtained from the animal breeding facility of the same institution. All experiments complied with local ethical guidelines.

2.3. Environmental particles

Urban air particles from downtown Buenos Aires (UAP-BA) were collected during a 3-month measuring campaign, from June to August (wintertime in the Southern Hemisphere). In order to select the monitoring place (58°23′49″W and 34°35′58″S) for collecting airborne particles, we identified an area in Buenos Aires characterized by its high population density with a high exposure to diesel exhaust due mainly to buses. UAP-BA were collected using a Mini Vol sampler (Airmetrics, Eugene, OR, USA) with 2.5 μm cut-point impactors (Baldauf et al., 2001) and a flow rate of 5 L/min onto Teflon filters (47 mm, Sartorious, 0.2 μm pore size). The average total sampling time was 48 h (approximately from noon to noon of consecutive days), yielding an average collected air volume of 14.4 m^3. Each filter was placed in a clean plastic cassette during transport and storage. The filters were weighed (after moisture equilibration) before and after sampling to determine the net particulate mass gain with a microbalance (Mettler M3, weighting accuracy of 1 μg), using an alpha source to remove the electrostatic charge. Selection of representative monitoring points, operation, treatment and handling of samples, and data validation were carried out according to QA/QC guidelines of the World Health Organization. Residual oil fly ash (ROFA) collected from

Boston Edison Co., Mystic Power Plant, Mystic, CT, USA, was kindly provided by Dr. J. Godleski (Harvard School of Public Health, MA, USA).

2.4. Preparation and characterization of particle-soluble fraction

Particle-soluble fraction (sf) was freshly prepared by resuspending collected particles in sterile phosphate-buffer saline, pH 7.2–7.4 (PBS) at a concentration of 1 mg/mL. Then, this particle suspension was sonicated for 20 min in an ultrasonic water bath and centrifuged for 10 min at $10{,}000 \times g$ (Goldsmith et al., 1998). The supernatant obtained after centrifugation was considered the soluble fraction employed in all experiments at the chosen concentration. The spectral composition from ROFA and UAP-BA particles was previously reported by our group. ROFA showed high content of Si, Al, S, and V. UAP-BA revealed a carbon core with traces of ground dust particles composed by geological elements such as Si, Al, and Ti, no V, Ni, or Pb were detected (Martin et al., 2007).

2.5. Cell isolation by bronchoalveolar lavage (BAL)

Animals were killed and their lungs were lavaged as described elsewhere (Tasat and de Rey, 1987). Briefly, the thoracic cavity was partly dissected, the trachea was cannulated with an 18-gauge needle, and infused 12 times with 1 mL of cold PBS. The BAL fluid was immediately centrifuged at $800 \times g$ for 10 min at 4 °C and the total cell number was determined using a hemocytometer. BAL was employed when, based on morphological criteria, the proportion of AM was >95% and more than 98% were viable according to the trypan blue exclusion test. Cell viability was determined by the trypan blue exclusion test under an inverted phase-contrast microscope for all the experimental conditions assayed. In all in vitro experiments, a density of 0.5×10^6 cells/mL was maintained per tube.

2.6. Phagocytosis assay

Phagocytic ability of cells obtained by broncholaveolar lavage from young and aged rats was evaluated as described previously by Muller et al. (1986). Non-treated and UAP-BAsf or ROFAsf treated cells were incubated in RPMI-1640 medium with fetal calf serum (FCS) opsonized FITC-labeled latex beads (Molecular Probes, Eugene, OR, USA) in a ratio of 1:10 for 2 h at 37 °C under constant agitation. Then, in order to remove non-adherent beads, the cells were washed twice in PBS and the pellet was fixed in 4% *p*-formaldehyde. A total of 200 cells in each sample were counted by fluorescence microscopy (Axioplan Microscope, Carl Zeiss, Germany). To distinguish strongly absorbed beads from ingested ones, parallel experiments were performed at 4 °C, a temperature known to block phagocytosis. Percentage phagocytosis represents the difference between the beads associated with cells at 37 and 4 °C. Results are expressed as percentage of phagocytosis (macrophages with phagocytic capacity).

2.7. Generation of superoxide anion

Superoxide anion (O_2^-), a main reactive oxygen species (ROS) generated during the respiratory burst, was evaluated using the NBT reduction test (Segal, 1974) in control and UAP-BAsf or ROFAsf treated AM. Superoxide anion is originated from the reduction of O_2^- by NADPH oxidase during the respiratory burst that is localized on the surface of the plasma membrane. The intracellular release of this active oxygen species is evidenced by the amount of a blue formazan precipitate in the cells after NBT reduction. BAL cells were treated with NBT in the presence or absence of TPA, a known inductor of O_2^- generation. All tubes were incubated with NBT for 60 min at 37 °C. In positive controls, TPA was added at a concentration of 0.5 μg/mL for the last 15 min. Cells were scored by light microscopy as described elsewhere (Molinari et al., 2000).

2.8. Total reactive antioxidant potential (TRAP)

TRAP or total antioxidant capacity was measured by chemiluminescence as previously described (Lissi et al., 1992). Briefly, the reaction medium used contained 20 mM 2,2-azobis-2-amidinopropane (ABAP) and 40 μM luminol. ABAP is a source of free radicals that react with luminol yielding chemiluminescence that was measured in an LKB liquid scintillation counter. The addition of a sample aliquot decreases the chemiluminescence for a period proportional to the amount of antioxidants present in the sample to basal levels until luminol radicals are regenerated (induction time, δ). The system was calibrated against Trolox, a vitamin E hydrosoluble analog. A comparison of the induction time after the addition of known concentrations of Trolox and the sample allows for the obtention of the TRAP as the equivalent of Trolox concentration necessary to produce the same induction time. TRAP values are obtained employing the following equation: TRAP (μM Trolox) $= D \times (\delta_s/\delta_T)$, where D is a dilution factor, δ_s is the induction time of the sample, and δ_T is the induction time that corresponds to the addition of 1 μM Trolox. Results were expressed as μM Trolox.

2.9. Apoptosis

Morphological and biochemical evaluation of apoptosis were performed on BAL cells from young and aged Wistar rats exposed to UAP-BAsf or ROFAsf.

2.9.1. Analysis of macrophage nuclear morphology

Twenty-four hours post-incubation with UAP-BAsf or ROFAsf, cells were washed twice with PBS, fixed in ice-cold acetic-methanol (1:3) for 15 min and stained with 5 μg/mL of Hoechst 33258 in PBS (1 mg/mL) for 10 min. Morphological features such as pyknosis and nuclear fragmentation were examined under 460 nm under a fluorescent light microscope (Axioplan, Carl Zeiss).

2.9.2. DNA fragmentation

For each experimental condition, 0.5×10^6 cells incubated in duplicate tubes were pooled and trypsinized. Cells were centrifuged at $2000 \times g$ and the cell pellet was resuspended in PBS. DNA was extracted using the Sigma GenElute mammalian genomic DNA kit. Recovered fragments of DNA were separated by electrophoresis in a 2.0% agarose gel and visualized by staining with ethidium bromide.

2.10. Statistical analysis

The results corresponding to the end-points for control and treated samples were compared employing ANOVA test and post-Newmann–Kueles test. Statistical significance was set at $p<0.05$.

3. Results

In order to normalize for potential differences in number and cell types obtained by BAL, we first characterized the cell populations present in the BAL fluid from young and aged rats. The percentage of AM of the BAL cells obtained from young animals was $99.75 \pm 0.25\%$, while a lower percentage of AM with a concomitant increase in the percentage of polymorphonuclear cells (PMN) was observed in BAL from aged rats (AM: $78.4 \pm 3.74\%$, PMN $17.45 \pm 2.19\%$, $p<0.001$). In all the experiments performed during this study, macrophages from young and aged animals were incubated at the same cell density (0.5×10^6 cells/mL).

3.1. UAP-BA decreases cell viability of BAL cells

Cell viability determined by the trypan blue exclusion test was evaluated in BAL cells obtained from young and aged animals exposed ex vivo to the soluble fractions of 10 and 100 μg/mL UAP-BAsf or ROFAsf (positive control). As expected, a significant decrease in cell viability was observed in BAL cells exposed to the lower dose of ROFAsf assayed (10 μg/mL, $p<0.05$). The higher ROFAsf concentration (100 μg/mL) depicted a sustained reduction in viability in both young and aged cells to approximately 75.7±0.55% and 77.5±1.53% of the control values (Table 1). When young and aged BAL cells were incubated with UAP-BAsf, cell viability was also reduced (Y: 88.93±0.37%, A: 90.59±0.11%, and Y: 81.35±1.67%, A: 79.09±0.48% for 10 or 100 μg/mL, respectively). Both types of particles tested showed a dose-dependent effect. However, the comparison with ROFAsf showed that the drop in cell viability induced by UAP-BAsf was less pronounced.

3.2. Phagocytosis rate of UAP-BAsf treated BAL cells is age dependent

We next tested the phagocytic capacity of BAL cells exposed to UAP-BAsf or ROFAsf. Phagocytosis in control, non-treated cells proved to be significantly higher for the macrophage population recovered from aged animals (young: 58.8±6.1% vs. aged: 72.9±2.5%, $p<0.05$). As shown in Fig. 1, exposure to UAP-BAsf or ROFAsf (10 μg/mL) significantly increased the percentage of phagocytosis in both young and aged cells population. However, the response of aged cells always depicted higher values than young cells.

3.3. UAP-BAsf induces apoptosis in AM cells obtained from aged animals

To test whether the UAP-BAsf decreased cell viability by activating apoptotic pathways, we evaluated morphologic features of apoptotic cells by Hoechst 33258 staining. ROFAsf treatment induced apoptosis in aged alveolar cells, serving as a positive control and setting the criteria to identify and quantitate apoptotic cells (Fig. 2A). The percentage of young and aged alveolar cells exhibiting similar morphological changes indicative of apoptosis after UAP-BAsf (10–100 μg/mL) treatment is shown in Fig. 2B. For all treatments, significant differences were found between young and aged BAL cells. The percentage of apoptotic cells between ROFAsf or UAP-BAsf vs. control young cells showed significant differences with $p<0.001$ or $p<0.01$, respectively. When aged cells were exposed to either ROFAsf or UAP-BAsf, the percentage of apoptotic cells markedly increased. However, we were only able to observe the characteristic electrophoretical DNA pattern ("ladder") indicative of apoptosis in BAL cells obtained from the ROFAsf aged treated animals (Fig. 2C) but not in UAP-BAsf treated cells (data not shown).

3.4. Superoxide anion (O_2^-) generation of UAP-BAsf treated BAL cells is age dependent

Since ROFA is a known inductor of ROS, we wondered if UAP-BAsf was also able to stimulate ROS production. TPA, a known inductor of superoxide anion generation, and ROFAsf were employed as positive controls. It is important to mention that TPA is not PM and thus serves

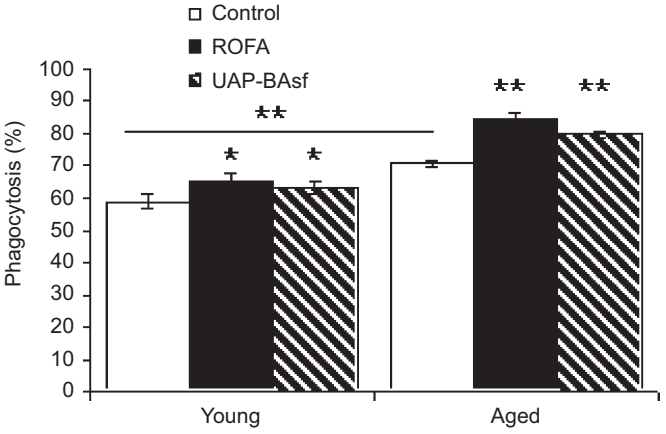

Fig. 1. Age-dependent phagocytosis activity in rat BAL cells. BAL cells (0.5×10^6 cells/mL) recovered from young and aged Wistar rats were treated with ROFAsf (10 μg/mL) or UAP-BAsf (10 μg/mL). Phagocytic activity was tested as described in Section 2. Statistically significant differences are indicated by an asterisk ($^*p<0.05$; $^{**}p<0.01$) for each treatment vs. the corresponding control. The asterisks on top of the line show that phagocytosis was significantly higher in control macrophages recovered from aged animals when compared to young animals. Each column is the mean±S.D. for a set of four experiments performed in duplicates.

Table 1
Effect of UAP-BAsf and ROFAsf on cell viability

	ROFA			UAP-BA		
	0 (μg/ml)	10 (μg/ml)	100 (μg/ml)	0 (μg/ml)	10 (μg/ml)	100 (μg/ml)
Young	97.45±0.68	86.46±0.97	75.71±0.55	98.61±0.42	88.93±0.37	81.35±1.67
Adult	97.46±0.36	89.25±0.91	77.55±1.53	96.87±0.16	90.59±0.11	79.09±0.48

Cellular viability from young ($n=5$) and aged ($n=5$) BAL cells evaluated by means of the trypan blue exclusion test. Data are expressed as percentage of viable cells. Values are mean±S.E.

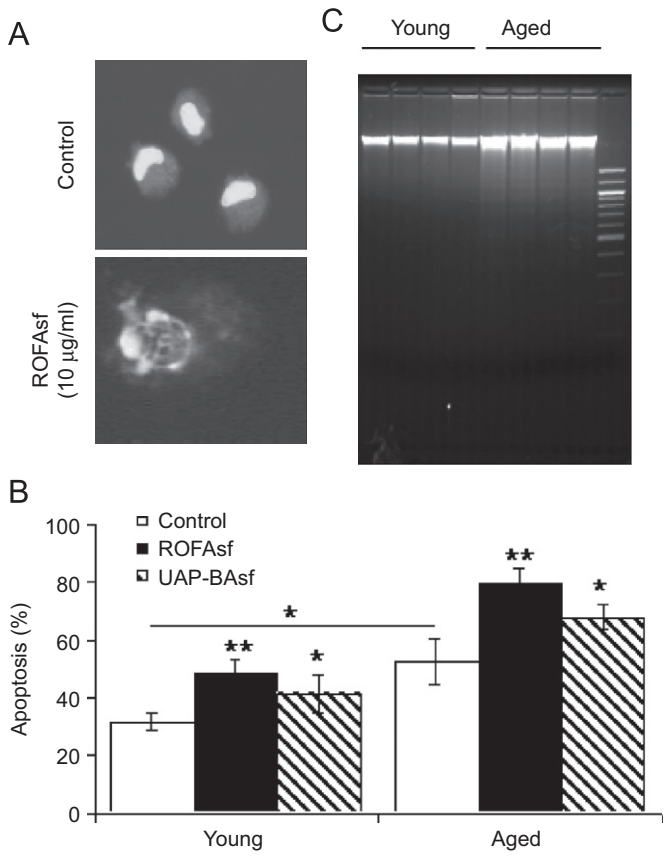

Fig. 2. UAP-BAsf induces apoptosis in alveolar macrophages from BAL: (A) Microphotographs of BAL cells with Hoechst 33258 staining. Upper panel shows aged control, non-treated macrophages showing homogeneous nuclear staining. Lower panel shows aged ROFAsf (10 μg/mL) treated macrophages revealing irregular staining of their nuclei as a result of chromatin condensation and nuclear fragmentation. (B) Percentage of apoptotic alveolar macrophages after treatment with UAP-BAsf (10 μg/mL) or ROFAsf (10 μg/mL). The asterisk on top of the line show statistically significant differences between control macrophages recovered from young and aged animals. Other asterisk(s) indicate the p-value ($^{*}p>0.01$; $^{**}p<0.001$) for each treatment vs. the corresponding control. Each column represents the mean ± S.D. for a set of 4–6 experiments performed in duplicates. (C) DNA fragmentation analysis of young and aged rat BAL cells treated with ROFAsf. DNA laddering was visualized by DNA electrophoresis in a 2% agarose gel and fluorescent staining by ethidium bromide. The last lane corresponds to a 200-bp DNA ladder molecular weight marker.

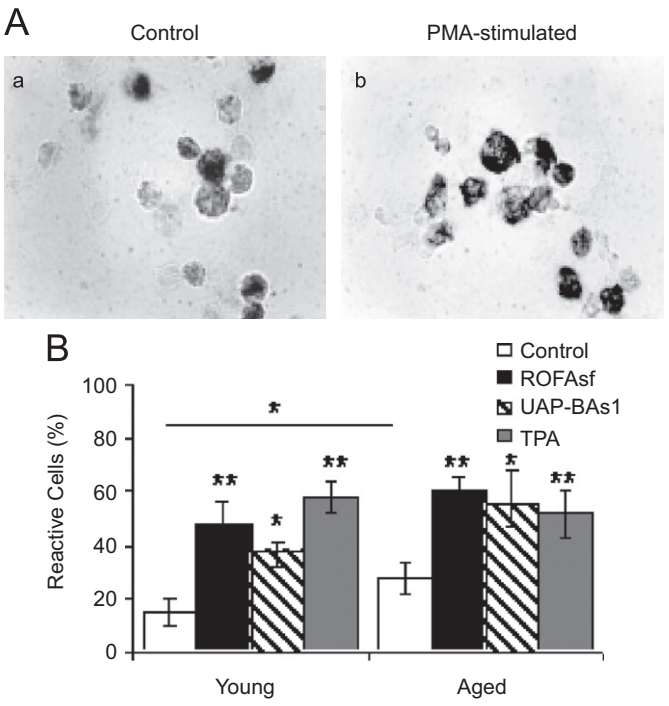

Fig. 3. Superoxide anion (O_2^-) generation is age dependent: (A) Light microphotographs of BAL cells obtained from aged animals. Control non-treated cells depict a basal NBT reaction with a heterogeneous degree of reaction. PMA-stimulated cells show a larger proportion of reactive cells with a more intense response to the stimulus. Bar = 10 μm. (B) Effect of UAP-BAsf (10 μg/mL) or ROFAsf (10 μg/mL) on O_2^- generation evaluated by the NBT reduction test. Exposed BAL cells (0.5×10^6 cells/mL) were treated with NBT alone or NBT and TPA-stimulated (0.5 μg/mL). Data are expressed as the mean ± S.D. for a set of four experiments performed in duplicates. The asterisk on top of the line show significantly higher O_2^- generation in macrophages recovered from control-aged animals in comparison to macrophages obtained from young animals. Differences in means are expressed as $^{*}p<0.05$ or $^{**}p<0.01$ compared to control non-treated cells.

as an unrelated positive control. Control non-stimulated cells were mostly non-reactive, although a few of them did exhibit scattered, insoluble dark formazan granules indicating intracellular NBT reduction. TPA-stimulated cells showed not only a larger proportion of cells responding to this stimulus but a higher degree of color intensity, clearly visible by light microscopy (Fig. 3A). The generation of basal levels of ROS was age dependent, since control non-treated BAL recovered from aged animals showed a higher percentage of dark reactive cells (color intensity is associated to cell ability to produce O_2^- when compared to BAL cells from young animals). As shown in Fig. 3B, the percentage of reactive cells observed in control young BAL was as low as 15.4 ± 5.06%, while in the BAL obtained from aged animals 27.9 ± 5.7% of the cell population showed a positive reaction to NBT. Despite the differences in the basal levels, UAP-BAsf (10 μg/mL) was able to produce a significant increase ($p<0.05$) in the percentage of reactive cells, similar to the value reached when ROFA (10 μg/mL) was added to the cell suspension. The percentage increase of reactive cells was observed both in young and aged BAL cells exposed to either 10 μg/mL UAP-BAsf (young: 37.2 ± 2.7% vs. aged: 54.9 ± 13.0%) or 10 μg/mL ROFAsf (young: 48.0 ± 8.9% vs. aged: 61.3 ± 4.7%). It is noteworthy that when treated with ROFAsf, the percentage of reactive cells reached values that were similar to those obtained when incubated in the presence of TPA, indicating that AM obtained from both populations still have a considerable ability to generate superoxide anion. Despite the low concentration of particulate matter used, a statistically significant increase in the amount of superoxide anion generated was induced in both populations studied.

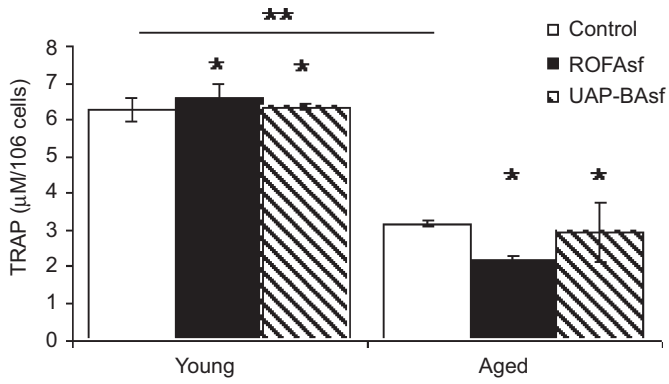

Fig. 4. Age-dependent antioxidant capacity in young and aged BAL cells: Total antioxidant reactive potential (TRAP) level in BAL cells treated with UAP-BAsf (10 µg/mL) or ROFAsf (10 µg/mL). Macrophages obtained from young animals revealed higher and unchangeable values for TRAP. This behavior held true for control or particle-treated cells. The asterisks on top of the line show a significant decrease in the antioxidant capacity in macrophages recovered from aged animals compared to cells obtained from young animals. Statistically significant differences are indicated by an asterisk ($^{*}p>0.05$; $^{**}p<0.01$) for each treatment vs. the corresponding control. Each column represents the mean ± S.D. for a set of three experiments performed in duplicates.

3.5. Reduced TRAP in aged cells

As ROS damage cells, defense mechanisms have evolved to protect them against oxidant injury. ROS not only are very reactive, but also have a short life. Hence, they are difficult to measure directly (Pryor and Godber, 1991). For this reason, most methods are indirect determinations of the levels of low-molecular-weight antioxidants or antioxidant enzymes. BAL cells from young rats exhibited higher values of TRAP as compared with those from aged animals (Y: 6.3 ± 0.3 vs. A: 3.2 ± 0.1). This behavior held true for control cells as well as for UAP-BAsf or ROFAsf treated cells (Fig. 4). The non-enzymatic antioxidant capacity of cell homogenates of young animals failed to show any antioxidant mobilization. However, when lung cell homogenates from aged rats were exposed to 10 µg/mL UAP-BAsf or ROFAsf, a slight decrease in TRAP values was observed.

4. Discussion

The healthy lung is capable of dealing effectively with a large number of particles deposited on its surface. However, there will come a point when the defense mechanisms are overwhelmed either by particle numbers or by the inherent toxicity of the particle. Exposure to components of air pollution may cause adverse effects on lung cellular and organ functions through several mechanisms, depending among other factors on the nature of the PM as well as the physiological condition (e.g. age) of lung cells (Seagrave and Nikula, 2000). Herein, we studied the response of AM, the major effectors of the first line of non-specific defense in the lower respiratory tract, against UAP-BAsf and compared to ROFAsf. Residual oil fly ash is a metallic by-product of the combustion of fossil fuel oil contributing with 49,000 tons to the US particle burden in 1992 (US EPA, 1993). Because ROFA is rich in metals with little organic component, it is widely used to study biological impact in experimental animal models where it proves to induce a variety of pro-inflammatory responses in lung cells and consequently lung injury (Antonini et al., 2004; Gardner et al., 2000; Gavett et al., 1999; Ghio et al., 2002). Therefore, an important factor related to PM is its composition which, in turn, might be dependent on its insoluble and/or soluble components (Dreher et al., 1997; Ghio et al., 1999). The direct correlation between PM extracts (soluble fraction) and cytotoxicity is controversial (Adamson et al., 1999; Dye et al., 1999; Huang et al., 2004; Lambert et al., 2000; Lewis et al., 2003; Prahalad et al., 2001). Recently, we collected, characterized, and demonstrated that UAP-BA provoke in vivo lung inflammation (Martin et al., 2007). UAP-BA are small spherical particles with PAHs and PCBs adsorbed onto the particle surface (Rehwagen et al., 2005) in much higher concentrations than the levels established as not dangerous to human health by the World Health Organization (WHO, 2004). PAHs are considered carcinogenic and mutagenic substances that can also exert cytotoxic effects (Muller et al., 2001) and alter cytokine production in immunological cells (macrophages, neutrophils, lymphocytes, etc.). However, whether UAP-BAsf induces cell lung toxicity remains unknown. The average concentration of particulate matter less than 2.5 ($PM_{2.5}$) recorded in downtown Buenos Aires by our group (72 µg/(m^3/day)) (Martin et al., 2007) was similar to the values recorded by other groups in Buenos Aires (Borthagaray et al., 2001) and other South American megacities like Santiago de Chile (84.88 µg/(m^3/day)) (Cakmak et al., 2007). Interestingly, Cakmak et al. (2007) found that a variation in 24-h mean PM_{10} of 10 µg/m^3 was associated with a 1% mortality change with stronger mortality effects in older age groups. Moreover, a 10 µg/m^3 increase for $PM_{2.5}$ was associated with a 1.4% raise in total mortality in Mexico City (Borja-Aburto et al., 1998). Due to airway geometry, particle characteristics, and ventilatory parameters (PM deposition and clearance), extrapolation of ambient PM concentration to the concentration of PM in lung cells is complex. ROFA and UAP-BAsf concentrations used in this study are standard for "in vitro" as well as "in vivo" studies in the field (Risom et al., 2005). Still, it has to be pointed out that these PM concentrations and their soluble fractions employed might be higher than the ambient "real" PM concentration. Future studies are required to establish a linkage between exposure and dose with special consideration to particle characteristics, definitions of dose metrics, and biological normalizing factors (Brown et al., 2005).

Relevant physiological changes in AM during aging, specifically related to ROS production and phagocytic activity (Tasat et al., 2003; Goldman et al., 2004) might have an effect on their ability to deal with inhaled particles.

Once macrophages have phagocytosed PM, they produce oxidative stress by generating ROS, and induce apoptosis and a decrease in cellular viability (Becker et al., 1996, 2002; Goldsmith et al., 1998; Holian et al., 1998; Soukup et al., 2000; Soukup and Becker, 2001). AM are very sensitive to changes in environmental conditions, releasing pro-inflammatory factors when they are challenged by PM (Sioutas and Kobzik, 1997; Imrich et al., 2007).

In this study, we showed that UAP-BAsf deleteriously affects several parameters of lung cells and that these effects are age dependent. When treated with UAP-BAsf, BAL cells obtained from aged rats showed reduced viability (Table 1), increased phagocytic activity (Fig. 1), and occurrence of apoptosis (Fig. 3). Apoptosis is a sequential process that can be divided into three phases: the initiation phase, the execution phase, and the elimination phase (Hengartner, 2000). Thus, more than one technique is required to perform an appropriate data interpretation of the apoptotic process. While some of them are able to detect mainly early events (Hoechst, Anexin V, etc.), others detect late events of the apoptotic cell death process (DNA electrophoresis, and TUNEL). In this study, the presence of apoptotic AM was determined by two complementary techniques that have become standard criteria for confirmation of apoptosis (Yuan, 1996). Both techniques detected apoptosis in AM treated with ROFAsf. On the other hand, although typical morphological features resembling apoptosis were clearly observed in UAP-BAsf-treated AM, the percentage of late apoptotic cells found might not be enough to be detected by DNA electrophoresis. Note that the "ladder" was only observed in ROFAsf treated cells obtained from aged animals.

We propose that a dysregulation in the redox balance might be responsible for the reduced cell viability, triggering apoptosis only in the cells obtained from the aged animal population of cells. Our observation that the UAP-BAsf induces a significant increase in the phagocytic activity of AM obtained from aged animals together with an increase of the oxidative status (Fig. 3) and a reduced antioxidant potential (Fig. 4) support this hypothesis. The finding that UAP-BAsf reduces cell viability by apoptosis is relevant, since this organized form of cell death is under the control of positive and negative regulators, and thus offers specific targets for potential therapeutic modalities. The age-dependent deleterious effect of UAP-BAsf suggests that the older the general population becomes, the higher is the probability to suffer damage by ambient PM. Antiapoptotic drugs might be useful to minimize lung cell damage due to PM in the elderly population. Finally, we believe that only a comprehensive study of the composition of the local PM as well as the elucidation of the mechanisms responsible for its harmful effects may help find strategies to (1) reduce the level of the most noxious pollutants in megacities and (2) develop treatments for existing environmental associated diseases in order to decrease the percentage of mortality and morbidity in vulnerable subpopulations.

Acknowledgment

This work was supported by the National University of General San Martin Grant A053.

References

Adamson, I.Y., Predicts, H., Vincent, R., 1999. Pulmonary toxicity of an atmospheric particulate sample is due to the soluble fraction. Toxicol. Appl. Pharmacol. 157, 43–50.

Antonini, J.M., Taylor, M.D., Leonard, S.S., Lawryk, N.J., Shi, X., Clarke, R.W., Roberts, J.R., 2004. Metal composition and solubility determine lung toxicity induced by residual oil fly ash collected from different sites within a power plant. Mol. Cell. Biochem. 255, 257–265.

Anzola Pérez, E., Galinsky, D., Morales, M.F., Salas, A., Sánchez, M., 1994. La atención de los ancianos, un desafío para los años '90''. Organización Panamericana de la Salud, Publicación científica no. 546.

Baldauf, R.W., Lane, D.D., Marote, G.A., 2001. Ambient air quality monitoring network design for assessing human health impacts from exposures to airborne contaminants. Environ. Monit. Assess. 66, 63–76.

Becker, S., Soukup, J.M., Gilmour, M.I., Devlin, R.B., 1996. Stimulation of human and rat alveolar macrophages by urban air particulates: effects on oxidant radical generation and cytokine production. Toxicol. Appl. Pharmacol. 141, 637–648.

Becker, S., Soukup, J.M., Gallagher, J.E., 2002. Differential particulate air pollution induced oxidant stress in human granulocytes, monocytes and alveolar macrophages. Toxicol. In Vitro 1, 209–218.

Borja-Aburto, V.H., Castillejos, M., Gold, D.R., Bierzwinski, S., Loomis, D., 1998. Mortality and ambient fine particles in Southwest Mexico City, 1993–1995. Environ. Health Perspect. 106, 849–855.

Borthagaray, J.M., Prini, R.F., de Nistal Igarzabal, M.A., San Román, E., Tudino, M., 2001. Diagnóstico Ambiental del Area Metropolitana de Buenos Aires, pp. 70–105.

Brown, J.S., Wilson, W.E., Grant, L.D., 2005. Dosimetric comparisons of particle deposition and retention in rats and humans. Inhal. Toxicol. 17, 355–385.

Brunekreef, B., Hoek, G., Fischer, P., Spieksma, F.T., 2000. Relation between airborne pollen concentrations and daily cardiovascular and respiratory-disease mortality. Lancet 355, 1517–1518.

Cakmak, S., Dales, R.E., Vidal, C.B., 2007. Air pollution and mortality in Chile: susceptibility among the elderly. Environ. Health Perspect. 115, 524–527.

Campbell, T., Campbell, A., 2007. Emerging disease burdens and the poor in cities of the developing world. J. Urban Health 84 (1), 54–64.

Castle, S.C., 2000. Impact of age-related immune dysfunction on risk of infections. Z. Gerontol. Geriatr. 33, 341–349.

De La Fuente, M., 1985. Changes in the macrophage function with aging. Comp. Biochem. Physiol. A 81, 935–938.

Dockery, D.W., Pope, C.A.R., Xu, X., Spengler, J.D., Ware, J.H., Fay, M.E., Ferris, B.G.J., Speizer, F.E., 1993. An association between air pollution and mortality in six US cities. N. Engl. J. Med. 329, 1753–1759.

Dreher, K.L., Jaskot, R.H., Lehmann, J.R., Richards, J.H., McGee, J.K., 1997. Soluble transition metals mediate residual oil fly ash induced acute lung injury. J. Toxicol. Environ. Health 50, 285–305.

Dye, J.A., Adler, K.B., Richards, J.H., Dreher, K.L., 1999. Role of soluble metals in oil fly ash-induced airway epithelial injury and cytokine gene expression. Am. J. Physiol. 277, L410–L498.

Gardner, S.Y., Lehmann, J.R., Costa, D.L., 2000. Oil fly ash-induced elevation of plasma fibrinogen levels in rats. Toxicol. Sci. 56, 175–180.

Gavett, S.H., Madison, S.L., Stevens, M.A., Costa, D.L., 1999. Residual oil fly ash amplifies allergic cytokines, airway responsiveness, and inflammation in mice. Am. J. Respir. Crit. Care Med. 160, 1897–1904.

Ghio, A.J., Stonehuerner, J., Dailey, L.A., Carter, J.D., 1999. Metals associated with both the water-soluble and insoluble fractions of an

ambient air pollution particle catalyze an oxidative stress. Inhal. Toxicol. 11, 37–49.
Ghio, A.J., Silbajoris, R., Carson, J.L., Samet, J.M., 2002. Biologic effects of oil fly ash. Environ. Health Perspect. 110, 89–94.
Goldman, A., Rubin, C., Gomez, S., Palermo, M.S., Tasat, D.R., 2004. Functional age-dependent changes in bronchoalveolar lavage rat cells. Cell Mol. Biol. (Noisy-le-grand) 50 (online pub:OL649-55).
Goldsmith, C.A., Imrich, A., Danaee, H., Ning, Y.Y., Kobzik, L., 1998. Analysis of air pollution particulate-mediated oxidant stress in alveolar macrophages. J. Toxicol. Environ. Health A 54, 529–545.
Hengartner, M.O., 2000. The biochemistry of apoptosis. Nature 407, 770–776.
Holian, A., Hamilton, R.F.J., Morandi, M.T., Brown, S.D., Li, L., 1998. Urban particle-induced apoptosis and phenotype shifts in human alveolar macrophages. Environ. Health Perspect. 106, 127–132.
Huang, Y.C., Li, Z., Harder, S.D., Soukup, J.M., 2004. Apoptotic and inflammatory effects induced by different particles in human alveolar macrophages. Inhal. Toxicol. 16, 863–878.
Imrich, A., Ning, Y., Laurence, J., Coull, B., Gitin, E., Knutson, M., Kobzik, L., 2007. Alveolar macrophage cytokine response to air pollution particles: oxidant mechanisms. Toxicol. Appl. Pharmacol. 218, 256–264.
Kelly, F.J., Dunster, C., Mudway, I., 2003. Air pollution and the elderly: oxidant/antioxidant issues worth consideration. Eur. Respir. J. Suppl. 40, 70s–75s.
Koenig, J.Q., Larson, T.V., Hanley, Q.S., Rebolledo, V., Dumler, K., Checkoway, H., Wang, S.Z., Lin, D., Pierson, W.E., 1993. Pulmonary function changes in children associated with fine particulate matter. Environ. Res. 63, 26–38.
Kojima, T., Hattori, K., Fujiwara, T., Hirata, Y., Kobayashi, Y., 1994. Maturational changes in superoxide anion production of rabbit alveolar macrophages. Life Sci. 55, 911–915.
Lambert, A.L., Dong, W., Selgrade, M.K., Gilmour, M.I., 2000. Enhanced allergic sensitization by residual oil fly ash particles is mediated by soluble metal constituents. Toxicol. Appl. Pharmacol. 165, 84–93.
Lavie, L., Weinreb, O., Gershon, D., 1992. Age-related alterations in superoxide anion generation in mouse peritoneal macrophages studied by repeated stimulations and heat shock treatment. J. Cell. Physiol. 152, 382–388.
Lewis, A.B., Taylor, M.D., Roberts, J.R., Leonard, S.S., Shi, X., Antonini, J.M., 2003. Role of metal-induced reactive oxygen species generation in lung responses caused by residual oil fly ash. J. Biosci. 28, 13–18.
Lioy, P.J., Daisey, J.M., 1986. Airborne toxic elements and organic substances. Environ. Sci. Technol. 20, 8–14.
Lissi, E., Pascual, C., Del Castillo, M., 1992. Luminol luminescence induced by 2,2-Azo-bis (2-aminopropane) thermolysis. Free Radical Res. Commun. 17, 299–311.
Longphre, M., Li, D., Li, J., Matovinovic, E., Gallup, M., Samet, J.M., Basbaum, C.B., 2000. Lung mucin production is stimulated by the air pollutant residual oil fly ash. Toxicol. Appl. Pharmacol. 162, 86–92.
Martin, T.R., Ruzinski, J.T., Wilson, C.B., Skerrett, S.J., 1995. Effects of endotoxin in the lungs of neonatal rats: age-dependent impairment of the inflammatory response. J. Infect. Dis. 171, 134–144.
Martin, S., Dawidowski, L., Mandalunis, P., Cereceda-Balic, F., Tasat, D.R., 2007. Characterization and biological effect of Buenos Aires urban air particles on mice Lungs. Environ. Res. 105, 340–349.
May, W.E., Benner, B.A., Wise, S.A., Schuetzle, D., Lewtas, J., 1992. Standard reference materials for chemical and biological studies of complex environmental samples. Mutat. Res. 276, 11–22.
Molinari, B.L., Tasat, D.R., Fernandez, M.L., Duran, H.A., Curiale, J., Stoliar, A., Cabrini, R.L., 2000. Automated image analysis for monitoring oxidative burst in macrophages. Anal. Quant. Cytol. Histol. 22, 423–427.
Muller, B., Franck, W., Joseph, K., von Wichert, P., 1986. Bronchoalveolar lavage on a quantitative basis? Eur. J. Respir. Dis. 146 (Suppl.), 177–182.
Muller, A., Alzuet, P., Herbarth, O., Ronco, A., 2001. Assessment of toxicity and mutagenicity in air particulate matter from an urban industrial area in the coast of the Rio de la Plata. Environ. Toxicol. 16, 151–157.
Prahalad, A.K., Inmon, J., Dailey, L.A., Madden, M.C., Ghio, A.J., Gallagher, J.E., 2001. Air pollution particles mediated oxidative DNA base damage in a cell free system and in human airway epithelial cells in relation to particulate metal content and bioreactivity. Chem. Res. Toxicol. 14, 879–887.
Pryor, W.B., Godber, S.S., 1991. Noninvasive measures of oxidative stress status in humans. Free Radical Biol. Med. 10, 177–184.
Rehwagen, M., Muller, A., Massolo, L., Herbarth, O., Ronco, A., 2005. Polycyclic aromatic hydrocarbons associated with particles in ambient air from urban and industrial areas. Sci. Total Environ. 348, 199–210.
Risom, L., Moller, P., Loft, S., 2005. Oxidative stress-induced DNA damage by particuate air pollution. Mutat. Res. 592, 119–137.
Sandstrom, T., Frew, A.J., Svartengren, M., Viegi, G., 2003. The need for a focus on air pollution research in the elderly. Eur. Respir. J. Suppl. 40, 92s–95s.
Schwartz, J., Dockery, D.W., Neas, L.M., Wypij, D., Ware, J.H., Spengler, J.D., Koutrakis, P., Speizer, F.E., Ferris, B.G.J., 1994. Acute effects of summer air pollution on respiratory symptom reporting in children. Am. J. Respir. Crit. Care Med. 150, 1234–1242.
Seagrave, J.C., Nikula, K.J., 2000. Multiple modes of responses to air pollution particulate materials in A549 alveolar type II cells. Inhal. Toxicol. 12 (Suppl.), 247–260.
Segal, A.W., 1974. Nitroblue-tetrazolium tests. Lancet 2, 1248–1252.
Sioutas, C., Kobzik, L., 1997. Alveolar macrophage interaction with air pollution particulates. Environ. Health Perspect. 105, 1191–1195.
Soukup, J.M., Becker, S., 2001. Human alveolar macrophage responses to air pollution particulates are associated with insoluble components of coarse material, including particulate endotoxin. Toxicol. Appl. Pharmacol. 171, 20–26.
Soukup, J.M., Ghio, A.J., Becker, S., 2000. Soluble components of Utah Valley particulate pollution alter alveolar macrophage function in vivo and in vitro. Inhal. Toxicol. 12, 401–414.
Tasat, D.R., de Rey, B.M., 1987. Cytotoxic effect of uranium dioxide on rat alveolar macrophages. Environ. Res. 44, 71–81.
Tasat, D.R., Mancuso, R., O'Connor, S., Molinari, B., 2003. Age-dependent change in reactive oxygen species and nitric oxide generation by rat alveolar macrophages. Aging Cell 2, 159–164.
US Environmental Protection Agency, 1993. National Air Pollutant Emission Trends, 1990–1992. EPA-454/R-93-032. US Environmental Protection Agency, Research Triangle Park, NC.
WHO (World Health Organization), 2004. Health-care management. Available 〈http://www.who.int/mediacentre/factsheets/fs281/en/〉.
Yuan, J., 1996. Evolutionary conservation of a genetic pathway of programmed cell death. J. Cell Biochem. 60, 4–11.