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SHORT COMMUNICATION

The Effect of Polbax Extract on Lipofuscin Accumulation in Cultured Neonatal Rat Cardiac Myocytes

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Polbax®, a water-soluble extract of fresh pollen grains and pistils, was tested for its ability to influence the accumulation of lipofuscin (age pigment) in cultured neonatal rat cardiac myocytes. Exposure for 3 weeks to Polbax at concentrations of 0.1, 1.0 or 10 mg/L decreased lipofuscin accumulation morphometrically assayed using laser scanning microscopy images (green excitation light) of formaldehyde-fixed cells, by 24%, 41% or 43%, respectively. Based on the knowledge that oxidative stress and iron-catalysed peroxidation play an important role in lipofuscinogenesis, we suggest that Polbax may slow lipofuscin formation due to antioxidant activities, perhaps involving intralysosomal dismutation of superoxide produced by autophagocytosed mitochondria and/or iron-chelation. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: aging; lipofuscin; oxidative stress; superoxide dismutase.

INTRODUCTION

Oxidative stress is known to play an important role in aging (Harman, 1956; Sohal and Weindruch, 1996) and, specifically, in the formation of lipofuscin, a hallmark of senescent postmitotic cells (Brunk *et al.*, 1992). Lipofuscin accumulation and other age-related changes can be retarded by the administration of various antioxidants and iron-chelators, while enhanced oxidative stress and/or antioxidant deficiency may accelerate aging (Brunk *et al.*, 1992). Polbax (also known as Baxtin or Prozyme), a water-soluble extract of fresh pollen grains and pistils harvested from plants of the family *Gramineae spp.*, has been shown to contain superoxide dismutases (SOD), a group of antioxidant metalloenzymes which accomplish the dismutation and reduction of $O_2^{\cdot-}$ into H_2O_2 (Halliwell and Gutteridge, 1999). Polbax possesses copper-zinc SOD (SOD1, cytosolic) and, to a lesser degree, manganese SOD (SOD2, mitochondrial) activities (Oden *et al.*, 1992). The importance of SOD as an anti-aging agent was clearly demonstrated by experiments on transgenic *Drosophila*. Transfected fruit flies overexpressing SOD1 and/or catalase lived significantly longer than their wild counterparts (Orr and Sohal, 1994; Parkes *et al.*, 1998). The purpose of our study was to test whether Polbax would retard lipofuscin accumulation in cultured neonatal rat cardiac myocytes, a type of postmitotic cells which are known to exhibit characteristic senescent changes *in vitro* (Terman and Brunk, 1998).

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MATERIALS AND METHODS

Culture conditions. Primary neonatal rat cardiac myocyte culture was prepared according to a previously described technique (Galaris *et al.*, 1980), unless otherwise stated. Briefly, small pieces of heart ventricles obtained from 2–3 day old Sprague-Dawley rats were incubated in 0.05% collagenase type II (Worthington, Lakewood, NJ, USA) dissolved in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, at 37 °C. Disaggregated cardiac myocytes were purified from other cell types by centrifugation of the cell suspension in a Percoll gradient (1.05 g/mL) (Ollinger and Brunmark, 1994). The myocytes were plated in collagen-coated 35 mm plastic petri dishes (Costar, Cambridge, MA, USA) at a density of 80 000 cells/cm^2 and cultivated in Eagle's minimum essential medium (EMEM) containing 10% dialysed calf serum, 2 mm glutamine, 100IU/mL penicillin-G and 100 μg/mL streptomycin in a humidified atmosphere of 8% O_2 and 5% CO_2, at 37 °C. The culture medium, supplemented with 10 μg/mL cytosine-β-d-arabinofuranoside (to prevent fibroblast proliferation), was changed every second day. Cardiac myocytes were continuously treated with Polbax® extract (Pharmacia & Upjohn Allergon AB, Ängelholm, Sweden) at concentrations of 0.1, 1.0 or 10 mg/L for a period of 3 weeks. Untreated cells of the same culture age were used as controls.

Microscopy, morphometry and statistical analysis. During cultivation, the cells were inspected in a Nikon TMS phase contrast inverted microscope (Tokyo, Japan). For lipofuscin measurement, 3-week-old cultures were fixed overnight in 4% neutral buffered formaldehyde, rinsed in water, air dried, mounted in glycerol and examined by a LSM-410 inverted confocal laser scanning

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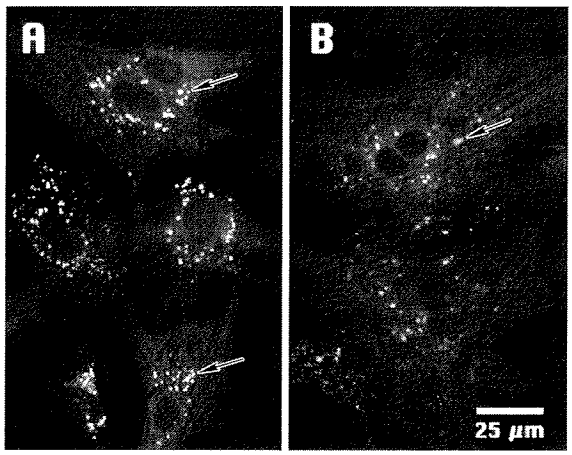

Figure 1. Laser scanning microscopy images of neonatal rat cardiac myocytes: A, cultured for 3 weeks at normal conditions; B, exposed to 1.0 mg/L Polbax extract (under otherwise normal conditions) for the same period of time. Note the decreased content of lipofuscin granules (arrows) in Polbax-treated cells.

microscope (Carl Zeiss, Jena, Germany). Images were obtained with a 63x/1.25 Plan-Neofluar lens using 543 nm (green) excitation light, a 590 nm barrier filter and a 20 pinhole. The intracellular content of lipofuscin was determined as the area occupied by pigment granules on confocal images of the cells using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). The experiments were done in triplicate for all groups.

Images of 30 cells from each specimen (culture dish) were analysed (i.e. 90 cells from each group). Values corresponding to the amounts of lipofuscin were averaged for every specimen and group. Different groups were compared using the two-tailed *t*-test. Data are presented as mean ± SD. Values of $p<0.05$ were considered significant.

RESULTS

Consistent with our previous observations (Terman and Brunk, 1998; Terman and Welander, 1999), the cardiac myocytes cultivated under normal conditions (controls) progressively accumulated autofluorescent, mainly perinuclearly located lipofuscin granules of assorted sizes (Fig. 1A). The administration of Polbax for 3 weeks partly prevented lipofuscin accumulation. Compared with the controls, the amount of pigment was reduced by 24%, 41%, or 43% due to Polbax treatment at concentrations of 0.1, 1.0 or 10 mg/L, respectively (Fig. 1B, 2). The treatment did not, however, change the overall pattern of lipofuscin accumulation (Fig. 1). Cardiac myocytes treated with Polbax did not show any morphological signs of damage.

DISCUSSION

Lipofuscin represents an undegradable intralysosomal substance formed due to iron- catalysed oxidative modification of autophagocytosed macromolecules (Brunk *et al.*, 1992). Potentially, the increase of reactive oxygen species (ROS) in any cellular compartment promote lipofuscin formation, since ROS either diffuse into lysosomes (uncharged ROS, such as H_2O_2 or $HO_2^{\cdot}$), or are produced within by autophagocytosed mitochondria or endoplasmic reticulum where they form (charged ROS, such as $O_2^{\cdot -}$). Moreover, ROS can contribute to lipofuscinogenesis by attacking and initiating peroxidation of biomolecules before they become autophagocytosed.

It is reasonable to believe that the observed reduction of lipofuscin accumulation by Polbax is due to its known SOD (mainly SOD1) activity. Potentially, SOD can be internalized by cardiac myocytes through endocytosis, the valid way for most proteins. SOD may then enter lysosomes as a result of their fusion with endosomes. Apparently, this will lead to a decrease of intralysosomal $O_2^{\cdot -}$ levels by enhancing its conversion to H_2O_2. H_2O_2 easily diffuses into the cytosol where it would be removed by catalase and glutathione peroxidase. The importance of dismutating $O_2^{\cdot -}$ is evident from its known capacity to convert Fe^{3+} into Fe^{2+} which, in turn, may induce Fenton- type chemistry. Even at physiological pH, $O_2^{\cdot -}$ is partially converted to $HO_2^{\cdot}$, an uncharged and more active radical which easily crosses biological membranes (Halliwell and Gutteridge, 1999).

There are a few other studies indicating an antioxidant property of Polbax. Oral treatment of humans with Polbax was found to prevent muscle soreness and lower the increase in malonyldialdehyde and lipid peroxides in both muscles and blood after exercise (Krotkiewski *et al.*, 1994). Polbax (either added to blood samples, or administered orally) decreased the production of malonyldialdehyde in human erythrocytes exposed to oxidative stress (Krotkiewski *et al.*, 1995).

Regarding the antioxidant effects of Polbax, the question arises of whether SOD activity is preserved, especially when the drug is administered orally and, thus, exposed to digestive enzymes. However, SOD1 (the main SOD fraction of Polbax) is known to be unusually stable

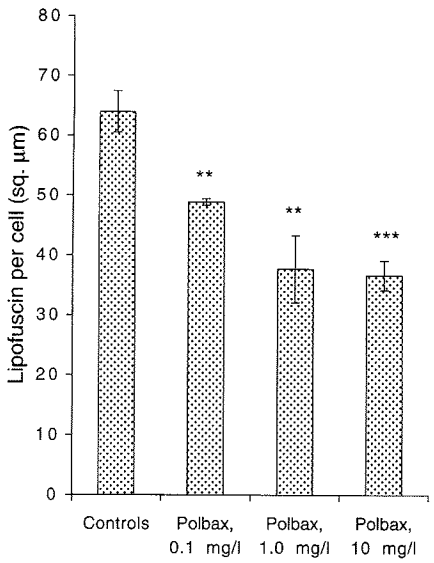

Figure 2. Accumulation of lipofuscin in neonatal rat cardiac myocytes cultured under normal conditions (controls) and exposed for 3 weeks to different concentrations of Polbax. Statistical significance (versus controls): * $p = 0.0018$; ** $p = 0.0023$; *** $p = 0.0004$.

compared with most enzymes, even to attack by proteases (Fridovich, 1995). It is not excluded that Polbax may contain other, as yet unidentified antioxidants (e.g. of non-protein nature and more stable) or iron chelators, which may contribute to the biological effects of Polbax in experimental and clinical studies.

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