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Arachidonic acid-induced apoptosis in rat hepatoma AS-30D cells is mediated by reactive oxygen species

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Arachidonic acid at micromolar concentrations produced a drastic increase of the generation of reactive oxygen species (ROS) in rat hepatoma AS-30D cells cultivated *in vitro* along with an increase in the incidence of apoptotic cell death. Both processes were prevented by trolox, a water-soluble tocopherol derivative, and tempol, a known antioxidative agent. A synthetic hybrid of lipoic acid and trolox or preincubation with *N*-acetylcysteine were less effective. Preincubation of the cells with etomoxir, a known highly specific irreversible inhibitor of carnitine-palmitoyltransferase I, partly decreased the ROS formation induced by arachidonic acid but it did not affect the increase in apoptosis. Cumulatively, these results indicate that apoptosis induced in hepatoma cells by arachidonic acid is mediated by ROS. They also suggest that this effect is due to arachidonic acid as such and not to its mitochondrial oxidative metabolites.

Keywords: antioxidants, apoptosis, arachidonic acid, hepatoma, oxygen free radicals, reactive oxygen species (ROS)

INTRODUCTION

Nonesterified long-chain fatty acids, in particular polyunsaturated ones, at micromolar concentrations are known to produce cell death of the apoptotic type. This has been shown for several types of cell lines cultivated *in vitro* (reviewed by Bernardi *et al.*, 2002). In a previous publication (Dymkowska *et al.*, 2006) we showed that arachidonic acid induced apoptosis in rat hepatoma AS-30D cells along with a drastic increase of the production of reactive oxygen species (ROS). An increased ROS generation induced by arachidonic acid and some other long-chain polyunsaturated fatty acids has been observed by other authors and ascribed to a partial inhibition of the respiratory chain, in particular at the sites of complexes I and III (for references see Schönfeld & Wojtczak, 2008). Apoptotic cell death induced by arachidonic acid was accompanied by the release of cytochrome *c* from mitochondria to the cytosol, activation of caspase-3 and association of the pro-apoptotic protein Bax with mitochondria, thus pointing to the mitochondrial mode of programmed cell death. We also showed that trolox, a water-soluble tocopherol derivative with potent antioxidant properties, prevented arachidonic acid-induced apoptosis along with diminution of ROS production. This latter observation points to a possible role of ROS as mediators of apoptosis induced by arachidonic acid in this cell line. To check this hypothesis we examined the effect of several other antioxidants and free radical scavengers on ROS production and the incidence of apoptotic death in AS-30D hepatoma cells. Apart from trolox, we chose two other free radical scavengers: tempol and a hybrid of trolox and lipoic acid characterised by a powerful scavenging activity (Koufaki *et al.*, 2004). In addition, we also used *N*-acetylcysteine that is a substrate for glutathione biosynthesis and thus indirectly supports the cell antioxidant defence system.

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Abbreviations: PBS, phosphate-buffered saline; ROS, reactive oxygen species.

MATERIALS AND METHODS

Cell culture and treatment. Hepatoma AS-30D cells were cultivated in RPMI-1640 medium containing 20 mM Hepes/NaOH (pH 7.4) and supplemented with 2 mM L-glutamine, 10% foetal calf serum and 40 μg/ml gentamycin at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were handled essentially as described previously (Dymkowska *et al.*, 2006). To investigate the effect of various antioxidants on cellular ROS production, the cells were preincubated with the investigated compounds in the complete cultivation medium for 30 or 60 min at a density of 5×10^6 cells/ml corresponding to about 1 mg protein/ml. Thereafter, they were separated by centrifugation for 3 min at $150\times g$ at room temperature and suspended in PBS supplemented with 2 mM L-glutamine and 40 μg/ml gentamycin. The antioxidants were added again to maintain their concentration at the same level, followed by arachidonic acid, and ROS production was measured as specified below.

To investigate the effect of antioxidants on arachidonic acid-induced apoptosis, the cells were treated as described above. Then, after 30 min incubation in PBS in the presence of arachidonic acid and the antioxidants, a four-fold volume of RPMI-1640 medium containing 10% foetal calf serum, 2 mM L-glutamine and gentamycin was added and the cells were cultivated under standard conditions for 24 h, when they were subjected to apoptosis measurement.

It has to be noted that the treatment of the cells with arachidonic acid was always performed in PBS instead of the full cultivation medium to avoid binding of this fatty acid by serum albumin present in foetal calf serum.

Flow cytometry. This was performed using a FACSCalibur instrument (Becton-Dickinson, Warszawa, Poland) equipped with an argon laser (488 nm excitation) and using CellQuest software. The photomultipliers used will be specified for the individual procedures (see below).

Measurements of the production of reactive oxygen species. ROS production within the cells was measured using the cell-penetrating probe 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA). Taken up by the cells, it was hydrolysed to non-fluorescent 2′,7′-dichlorodihydrofluorescein that was subsequently oxidised by ROS (mostly the superoxide anion radical $O_2^{-\bullet}$) to fluorescent 2′,7′-dichlorofluorescein. The cells, suspended in PBS supplemented with L-glutamine, were incubated with this probe (20 μM) for 30 min at 37°C and analysed by flow cytometry (Inoue *et al.*, 2004) using a green light-sensitive photomultiplier (FL1). The data were presented as the geometric mean of fluorescence.

Mitochondrial membrane potential. The mitochondrial transmembrane potential was measured using flow cytometry of cells stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanineiodide (JC-1) (Cossarizza *et al.*, 1993) as described previously (Dymkowska *et al.*, 2006).

Estimation of apoptosis. This was assayed by flow cytometry of cells stained with propidium iodide (Nicoletti *et al.*, 1991) as described by Bielak-Żmijewska *et al.* (2004), using orange-red light emission (FL2). In this procedure the DNA content histograms enabled discrimination between cells with normal (diploid) DNA and those forming a broad hypodiploid DNA peak, the sub-G_1 population.

Chemicals. Arachidonic acid, *N*-acetylcysteine, tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) and etomoxir {2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxylic acid} were from Sigma-Aldrich (Poznań, Poland). Arachidonic acid was used as 10 mM stock solution in dimethylsulphoxide. Solutions of arachidonic acid were kept tightly stoppered under nitrogen at –20°C. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Oxis International Inc. (Portland, OR, USA). $DCFH_2$-DA and JC-1 were from Invitrogen (formerly Molecular Probes, Eugene, OR, USA). 5-(1,2-Dithiolan-3-yl)-*N*-[(6-hydroxy-2,2,7,8-tetramethyl-3,4-dihydro-2*H*-chromen-5-yl)methyl]pentan-amide, compound "19" of Koufaki *et al.* (2004) here abbreviated as MK1, was kindly offered by Dr. Maria Koufaki of the Institute of Organic and Pharmaceutical Chemistry, National Hellenic Research Foundation (Athens, Greece). Other chemicals were of the highest purity grade commercially available. RPMI-1640 medium and PBS were supplied by the Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland).

RESULTS

To investigate the protective effect of antioxidants against increased ROS production induced by arachidonic acid, AS-30D cells were first preincubated for 30 or 60 min with individual antioxidants followed by incubation for 30 min with the same compounds plus micromolar concentrations of arachidonic acid (see Materials and Methods). The amount of arachidonic acid, 40 nmol/mg protein, was chosen as to produce a maximum or sub-maximum uncoupling and induce apoptosis in about 30% cells (Dymkowska *et al.*, 2006). Because arachidonic acid, as well as other long-chain fatty acids, are quantitatively taken up by intact cells or isolated mitochondria, the only relevant way of expressing their effective "concentration" is per mg protein of the biological material used rather than per volume

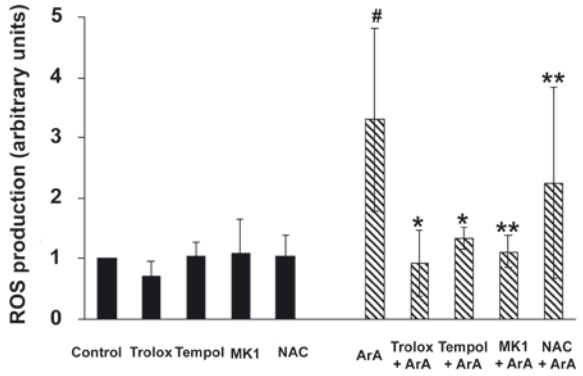

Figure 1. Effect of antioxidants on arachidonic acid-stimulated ROS generation.
Cells were preincubated with 2 mM trolox, 0.5 mM tempol or 1 μM MK1 for 30 min or with 1 mM *N*-acetylcysteine (NAC) for 60 min followed by incubation with arachidonic acid (ArA) for 30 min as described under Materials and Methods. ROS production is expressed in arbitrary units, the amount produced by cells without any additions (control) assumed as 1. Black columns, without arachidonic acid; dashed columns, with arachidonic acid, 40 nmol/mg protein. Mean values ±S.D. for 4–9 experiments are shown. Statistical significance evaluated using paired Student's *t*-test: $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ with respect to the control without arachidonic acid; $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ with respect to arachidonic acid alone.

of the incubation mixture (Dymkowska *et al.*, 2006; Schönfeld & Wojtczak, 2007). Under our experimental conditions (1 mg cell protein per ml incubation medium) the amount of arachidonic acid used corresponded to a formal concentration of 40 μM.

It was found that under these conditions trolox, a water-soluble derivative of tocopherol with potent antioxidant properties, tempol, a known antioxidative agent, and MK1, a hybrid of lipoic acid and trolox, significantly decreased ROS production

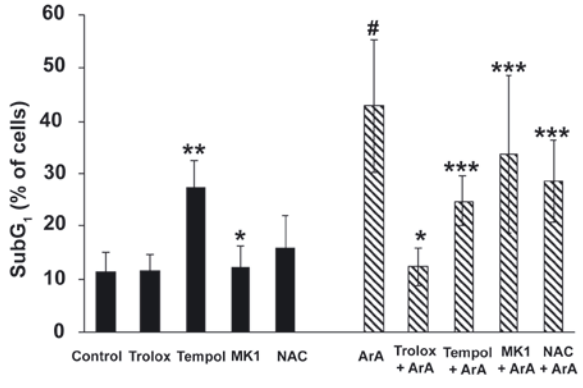

Figure 2. Effect of antioxidants on arachidonic acid-induced apoptosis.
Cells were treated as described under Materials and Methods. The concentrations of antioxidants and arachidonic acid were as in Fig. 1. Columns show mean values ±S.D. for 4–6 experiments. Abbreviations and statistical significance as in Fig. 1.

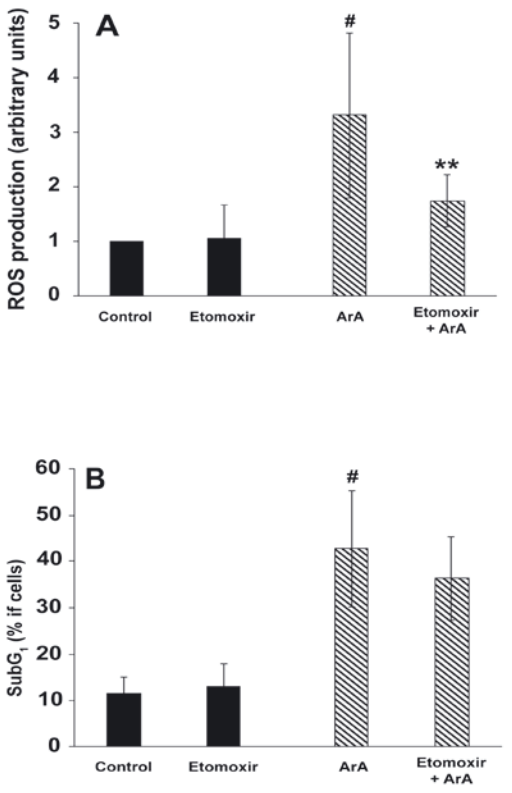

Figure 3. Effect of etomoxir on ROS production and apoptosis induced by arachidonic acid.
Cells were preincubated with 10 μM etomoxir in full incubation medium for 60 min followed by incubation in PBS with arachidonic acid, 40 nmol/mg protein, as described under Materials and Methods. Experimental conditions for ROS production (**A**) and apoptosis measurements (**B**) were as in Figs. 1 and 2, respectively. Columns show mean values ±S.D. for 6–9 experiments. Abbreviations and statistical significance as in Fig. 1.

induced by micromolar concentrations of arachidonic acid. *N*-acetylcysteine was less effective (Fig. 1).

It is known that uncouplers of oxidative phosphorylation diminish mitochondrial ROS generation (Skulachev, 1996). In fact, a decrease of ROS production in AS-30D cells by the potent uncoupler CCCP, both in the presence and absence of arachidonic acid, was described in our previous publication (Dymkowska *et al.*, 2006). Therefore, all the antioxidants used in the present investigation were checked for their possible uncoupling properties. Using JC-1 as a probe, it was found that none of them at concentrations applied affected mitochondrial membrane potential within the cells (not shown). It can be therefore concluded that they exerted their protective effects as true antioxidants and not by affecting mitochondrial energetics.

In long-term experiments, as those for investigating the apoptotic effect of arachidonic acid, a complete cultivation medium was required to ensure optimal growth conditions (Dymkowska *et al.*,

2006). Therefore, after preincubation of the cells with arachidonic acid in PBS with or without the antioxidants, a four-fold volume of the cultivation medium was added. Starting from this point, practically all free arachidonic acid that still remained in the medium and in the cells became complexed by serum albumin present in the medium (Dymkowska *et al.*, 2006). Quantitative evaluation of apoptosis by flow cytometry that was carried out 24 h later revealed an increasing proportion of cells recorded in the sub-G_1 phase with increasing concentrations of arachidonic acid (Dymkowska *et al.*, 2006). This was fully confirmed in the present investigation. All four antioxidative compounds mentioned above substantially protected the cells against apoptosis induced by arachidonic acid but, with the exception of tempol, had no effect on the proportion of apoptotic cells that appeared spontaneously after 24 h incubation. Tempol by itself increased the incidence of apoptosis to about 30%. However, it completely abolished the proapoptotic action of arachidonic acid (Fig. 2).

To check whether the increased ROS production and the observed proapoptotic effect of arachidonic acid could be ascribed to its metabolic products generated within mitochondria, etomoxir, the known highly specific irreversible inhibitor of carnitine-palmitoyltransferase I (Agius *et al.*, 1991), was applied. Although etomoxir only partly decreased ROS formation, it did not affect the proportion of apoptotic cells (Fig. 3). This result suggests that the proapoptotic effect of arachidonic acid did not depend on its mitochondrial oxidative metabolites.

DISCUSSION

ROS are well-known apoptosis-inducing factors in a variety of cells (Simon *et al.*, 2000; Bauer, 2002; Fleury *et al.*, 2002). On the other hand, arachidonic acid and other long-chain polyunsaturated fatty acids have been reported to induce generation of ROS in isolated mitochondria (Cocco *et al.*, 1999; Schönfeld & Wojtczak, 2007) and intact cells (for review see Schönfeld & Wojtczak, 2008) and to trigger apoptotic cell death (Bernardi *et al.*, 2002). As a consequence, we proposed that apoptosis induced in rat hepatoma AS-30D cells by arachidonic acid is mediated by ROS (Dymkowska *et al.*, 2006). This was supported by observations that the free radical scavenger trolox protected the cells against apoptosis along with diminution of arachidonic acid-stimulated ROS generation. The present report confirms these observations and shows that three other compounds, tempol, MK1 and *N*-acetylcysteine, share a similar protective effect.

Trolox is a synthetic water-soluble derivative of vitamin E shown to be particularly effective in preventing membrane lipid peroxidation (Wu *et al.*, 1990). It has been proved effective in protecting cells against oxidative stress-induced apoptosis (McClain *et al.*, 1995; Salgo & Pryor, 1996). In our previous study (Dymkowska *et al.*, 2006), 2 mM trolox completely prevented both ROS generation and apoptosis induced by arachidonic acid in AS-30D hepatoma cells. This was confirmed in the present investigation where trolox appeared the most effective among the antioxidants tested (Figs. 1 and 2).

The results obtained with tempol were complicated by the fact that this compound, which is a stable free radical by itself, doubled the proportion of apoptotic cells. Nevertheless, it also protected against the proapoptotic action of arachidonic acid (Fig. 2).

MK1, a hybrid of trolox and lipoic acid linked by a spacer, appeared extremely effective at micromolar concentrations as free radical scavenger (Koufaki *et al.*, 2001; 2004). In the present investigation it was as effective as trolox in protecting against arachidonic acid-induced ROS production (Fig. 1). However, it was less active in protecting against arachidonic acid-induced apoptosis (Fig. 2). Moreover, its effect was subject to large variations from experiment to experiment. It can be speculated that this discrepancy between the high effectiveness against ROS production and the poor protective action against apoptosis could be due to the fact that MK1 was applied at a very low concentration of 1 μM that was sufficient in short-term (30 min) preventing of ROS generation but was probably metabolized or otherwise decomposed during 24 h incubation required for apoptosis assays.

N-Acetylcysteine is a well-known physiological precursor of glutathione. It is easily taken up by cells and provides cysteine moiety for glutathione synthesis. Since glutathione participates in the main physiological system protecting cells against oxidative stress (Dickinson & Forman, 2002), *N*-acetylcysteine is often regarded as a factor strengthening the physiological antioxidative barrier. This can be, however, true under condition when the glutathione system is a limiting factor. This was, presumably, not the case in hepatoma cells used in this investigation, since *N*-acetylcysteine appeared the least active in protecting against both ROS generation and apoptosis induction.

In conclusion, the present investigation provides further arguments for the notion that arachidonic acid exerts its proapoptotic effect on hepatoma AS-30D cells by induction of ROS generation.

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