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Anthraquinone Cytotoxicity and Apoptosis in Primary Cultures of Rat Hepatocytes

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We compared three different anthraquinones, rhein (4,5-dihydroxy-anthraquinone-2-carboxylic acid), danthron (1,8-dihydroxy-anthraquinone) and chrysophanol (1,8-dihydroxy-3-methylanthraquinone), with respect to their toxicity and ability to induce apoptosis in primary cultures of rat hepatocytes. Rhein was the most effective in producing free radicals, and was the only one of the tested anthraquinones that could induce apoptosis. Addition of 50 μM rhein to hepatocyte cultures led to depletion of intracellular reduced glutathione (GSH) and ATP and accumulation of lipid peroxidation products. The substances N,N′-diphenyl-*p*-phenylenediamine (DPPD), dithiothreitol (DTT), nifedipine and desferal all protected the hepatocytes, i.e. prevented viability loss and ATP depletion, and decreased the GSH depletion.

Cultures exposed to rhein for 15 min and subsequently rinsed and incubated for 16 h under normal culture conditions (complete medium) exhibited apoptosis, as shown by DNA fragmentation, nuclear condensation and positive TUNEL reaction. Pretreatment with the antioxidant DPPD and the iron-chelator desferal gave complete protection against apoptosis.

No signs of oxidative cell damage were detected when the cultures were exposed to danthron or chrysophanol. All three anthraquinones did, however, cause an immediate increase in the intracellular Ca^{2+} concentration.

We conclude that rhein, which contains one carboxyl group, is a suitable substrate for one-electron-reducing enzymes and an effective redox cycler, which leads to the production of oxygen-derived free radicals that eventually induce apoptotic cell death.

Keywords: Anthraquinones, oxidative stress, redox cycling, hepatocytes, antioxidants, apoptosis

Abbreviations: DMSO, dimethyl sulfoxide; DPPD, N,N′-diphenyl-*p*-phenylenediamine; DTT, dithiothreitol; GSH, reduced glutathione; HBSS, Hanks' balanced saline solution supplemented with 10 mM HEPES; MDA, malondialdehyde; OTC, L-2-oxothiazolidine-4-carboxylic acid; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated dUDP biotin nick-end labelling

INTRODUCTION

The quinoid structure is widespread in nature, and several quinones e.g., ubiquinone and vitamin K_1, are found as constituents of cellular electron transport systems. Anthraquinones have

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been used as colorants in food, drugs, cosmetics, and textiles and as hair dyes. Rhein and danthron possess phytotherapeutic, laxative and cathartic properties,[1] and rhein also exhibits antibacterial, anti-inflammatory and antirheumatic effects.[2,3] It was recently hypothesised that rhein displays anti-neoplastic activity, because it affects glycolysis and mitochondrial functions.[4–6]

The cytotoxicity of quinones has often been explained as due to enzyme-catalysed one-electron reduction and subsequent autoxidation, resulting in the production of reactive oxygen species (ROS).[7–9] ROS are involved in various biological processes, including activation of gene expression, regulation of proliferative events, and cellular response to cytokines.[10] When the production of ROS overwhelms the antioxidative defence system, various macromolecules are subjected to oxidative damage that eventually leads to cell injury and death.[9–11] Quinones are also electrophiles, which enable them to form adducts with cellular nucleophiles. Both the capacity to produce ROS and the electrophilicity of quinones are dramatically influenced by the substituents present on the molecule.[12] Substituent position and characteristics determine the one-electron potential as well as the steric circumstances for efficient enzymatic reduction.[13]

Apoptosis is a gene-controlled self-destruction program that occurs in most cell types.[14] The onset of the apoptotic program can be mediated through the TNF receptor super family[15] or through several other stimuli, such as exposure to glucocorticoid hormones, Ca^{2+} ionophores, and toxins.[15,16] In recent years, several studies have shown that free oxygen radicals may be mediators of apoptotic cell death. This oxidative stress hypothesis is supported by data showing that spin traps and antioxidants can inhibit apoptosis induced by free radicals.[17–19] The regulation of apoptosis in the cell is presently a matter of intense investigation, and several events have been identified in the reaction sequence: elevation of Ca^{2+} and cAMP levels,[20] activation of proteases and endonucleases,[21,22] release of cytochrome c from mitochondria,[23] and phosphatidyl serine exposure.[24]

The aim of the present study was to elucidate the relationship between the chemical structure of anthraquinones and the ability to exhibit cytotoxicity and cause apoptosis in primary cultures of rat hepatocytes. Furthermore, the mechanism of apoptosis induced by free radicals was studied by using antioxidants and Ca^{2+}-channel blocker.

MATERIALS AND METHODS

Chemicals

Rhein (4,5-dihydroxy-anthraquinone-2-carboxylic acid), danthron (1,8-dihydroxy-anthraquinone), and chrysophanol (1,8-dihydroxy-3-methylanthraquinone) were obtained from Sigma-Aldrich (Steinheim, Germany). NADPH cytochrome P-450 reductase from rabbit liver was from Sigma (C-4839; St. Louis, MO, USA). Fluo-3-acetoxymethyl ester (Fluo-3-AM) was from Molecular Probes (Eugene, OR, USA); NADPH, collagen (type I) and reduced glutathione (GSH) were from Boehringer Mannheim (Mannheim, Germany). Fetal bovine serum, Hank's buffered saline solution, glutamine and Williams E medium were from GIBCO Ltd (Paisley, Scotland, UK). Percoll was from Pharmacia (Uppsala, Sweden), and desferal (desferrioxamine mesylate) from Ciba (Basel, Switzerland).

Isolation and Culture of Hepatocytes

Male Wistar rats (weight 150–200 g) were purchased from B&K Universal AB (Sollentuna, Sweden). The rats were fed *ad libitum* upon arrival to our laboratory. Food was withheld 16 h before use.

Hepatocytes were isolated by collagenase perfusion and purified by centrifugation in a Percoll solution (1.09 mg/ml), as previously described.[25] The cells were resuspended in Williams E medium containing 5 μg/ml insulin, 0.335 μM dexamethasone, 2 mM glutamine, and

10% fetal bovine serum (referred to as "culture medium"). Cell viability was >95%, as determined by trypan blue dye exclusion test. Hepatocytes were plated at a density of 70,000 cells/cm^2 in Petri dishes (Costar, Cambridge, MA, USA) that were precoated with 1.7 μg/cm^2 type I collagen and cultured at 37°C in an atmosphere of 5% CO_2 and air. After 2 h of incubation, the culture medium was changed to remove unattached cells.

Anthraquinone Exposure and Plasma Membrane Integrity

Rhein, danthron and chrysophanol were dissolved in DMSO and then diluted in HBSS (Hanks' balanced saline solution with 10 mM HEPES; pH 7.4) immediately prior to use in experiments. All incubations were started 5 h after seeding. Pretreatment of hepatocytes with the iron-chelator desferal (1 mM) or the glutathione-synthesis-increasing substance OTC (5 mM)[26] were started 3 h before rhein was introduced. The lipid peroxidation inhibitor DPPD (2 μM), the dithiol DTT (500 μM), and the Ca^{2+}-channel blocker nifedipine (1 mM) were added together with rhein. Following exposure to an anthraquinone, cultures intended for DNA fragmentation and apoptosis analyses were rinsed in HBSS and returned to ordinary culture conditions for an additional 16 h.

The effect of different oxygen pressures was assessed by keeping hepatocytes in airtight chambers (Flow Laboratories, Isvine, UK) filled with 100% nitrogen gas, air, or 100% oxygen gas. All incubation mixtures were bubbled with the respective gas for 20 min before use. This procedure resulted in oxygen concentrations in the medium of 16, 172, and 834 μM when bubbled with nitrogen, air and oxygen, respectively.[12] Controls were incubated in HBSS without added anthraquinone in parallel to all experiments.

Plasma membrane integrity was determined by analysis of lactate dehydrogenase activity in the medium and in the cell layer.[27]

Biochemical Analysis

For analysis of intracellular GSH, samples were prepared by adding 0.5 M $HClO_4$ supplemented with 1 mM EDTA to Petri dishes containing 700,000 cells. The supernatant was analysed by HPLC as previously described.[28] GSH concentrations were calculated from standard curves, and protein was determined as described by Lowry *et al.*[29]

ATP was analysed in cell homogenates prepared in 2.5% (w/v) trichloroacetic acid by using an ATP monitoring reagent (144-041, BioThema AB, Dalarö, Sweden).

Lipid peroxidation was measured in dishes containing 1.75×10^6 cells. First, the incubation medium was collected and analysed separately. Next, the cell layer was collected in 200 μl of water, sonicated, and centrifuged at 500× *g* for 10 min. The supernatant was mixed with reagents from the LPO-586 colorimetric assay (Bioxytech, R&D Systems, Abingdon, UK), incubated at 45°C for 40 min and then monitored spectrophotometrically at 586 nm. The concentration of malondialdehyde (MDA) and 4-hydroxy alkenals (4-HA) was calculated from standard curves.

Cytosolic free Ca^{2+} was measured in hepatocytes seeded on Lab-Tec objective slides (Miles Scientific, Naperville, IL, USA) precoated with collagen and loaded with Fluo-3-AM (2 μM) as described elsewhere.[26] Anthraquinones were added, and the immediate increase in fluorescence intensity was analysed using an LMS 410 confocal laser scan microscope (Zeiss, Genua, Germany) equipped with an argon laser; all specimens were scanned under constant conditions.

Anthraquinone Redox Reactions

One-electron reduction of anthraquinones by NADPH cytochrome P-450 reductase and oxygen consumption was determined in 0.1 M phosphate buffer supplemented with 1 mM EDTA to which 10 μM anthraquinone, 200 μM NADPH, and 0.7 μg enzyme had been added. The reaction rate

was monitored as NADPH oxidation at 340 nm ($\varepsilon = 6.22\ mM^{-1} \cdot cm^{-1}$). Oxygen consumption was measured using a thermostatted cuvette containing a Clark-type electrode (Techtum); the O_2 concentration was 254 μM in buffer at 37°C.

Detection of Apoptosis

DNA fragmentation was determined in samples containing $1.4 \cdot 10^6$ cells. Cells and culture medium were collected with a cell scraper and centrifuged at 300× *g* for 10 min. The pellet was lysed in 400 μl of 10 mM Tris-HCl supplemented with 10 mM EDTA and 2% SDS (pH 8.3), and the fraction containing large and small DNA fragments was separated by precipitation, as described by Olive.[30] DNA was hydrolysed in 0.8 M HCl at 80°C for 30 min, and an aliquot was mixed with 2 volumes of 87 mM diphenylamine containing acetaldehyde to detect nucleotides, as described by Burton.[31] The tubes were incubated in the dark overnight and analysed spectrophotometrically at 600 nm. The DNA content was calculated using a standard curve and calf thymus DNA as standard.

Pycnotic nuclei were assessed in cultures fixed in 4% neutral-buffered formalin and stained with 5% Giemsa. The number of condensed nuclei was determined in a light microscope. Apoptotic cells were also identified with the ApopTag *in situ* apoptosis detection kit according to the manufacturer's instructions (Oncor Inc., Gaithersburg, MD, USA). Briefly, the cells were fixed for 20 min in 4% neutral-buffered formalin containing 0.5% Triton X-100 and then incubated with the terminal deoxynucleotidyl transferase enzyme for 1 h. Thereafter, anti-digoxigenin-peroxidase was added, and the cultures were incubated for an additional 30 min. The cells were stained for 10 min with 0.05% diaminobenzidine solution containing 0.02% H_2O_2 and then rinsed in water and examined under a light microscope at 250× magnification.

Statistical Methods

Cell culture experiments were generally repeated 4–5 times using hepatocytes isolated from different rats. All values are given as arithmetic means ± SD. Statistical significance was calculated by *t* test.

RESULTS

Anthraquinone Redox Properties

Of the studied anthraquinones, rhein was found to be the best substrate for NADPH cytochrome P-450 reductase one-electron reduction (Table I). It was also the best redox cycler, consuming oxygen in a time-dependent manner.

Anthraquinone Cytotoxicity

The cellular uptake of the anthraquinones was determined by the partition coefficients in mixture of octanol and water. The partition coefficients (log *P*) were 1.930, 2.031, and 2.422 for danthron, rhein, and chrysophanol, respectively, analysed by applying the computer program *C* log *P* (BioByte Corporation). Figure 1A illustrates plasma membrane integrity of hepatocytes

TABLE I NADPH cytochrome P-450 reductase catalysed one-electron-reduction of anthraquinones and oxygen-consumption

Anthraquinone	NADPH oxidation (nmol/min · μg enzyme)[a]	Rate of oxygen consumption (μM/min)[b]
Rhein	1.83 ± 0.08	1.44 ± 0.05
Danthron	0.61 ± 0.04	1.09 ± 0.39
Chrysophanol	0.28 ± 0.07	0.35 ± 0.05

Reaction mixture contained 0.7 μg NADPH cytochrome P-450 reductase, 200 μM NADPH, and 10 μM anthraquinone in 0.1 M phosphate buffer supplemented with 1 mM EDTA (pH 7.4).
[a] The reaction was followed spectrophotometrically at 340 nm. Values are means ± SD, $n = 3$;
[b] The reaction was monitored with a Clark-type electrode at 37°C. Values are means ± SD, $n = 3$.

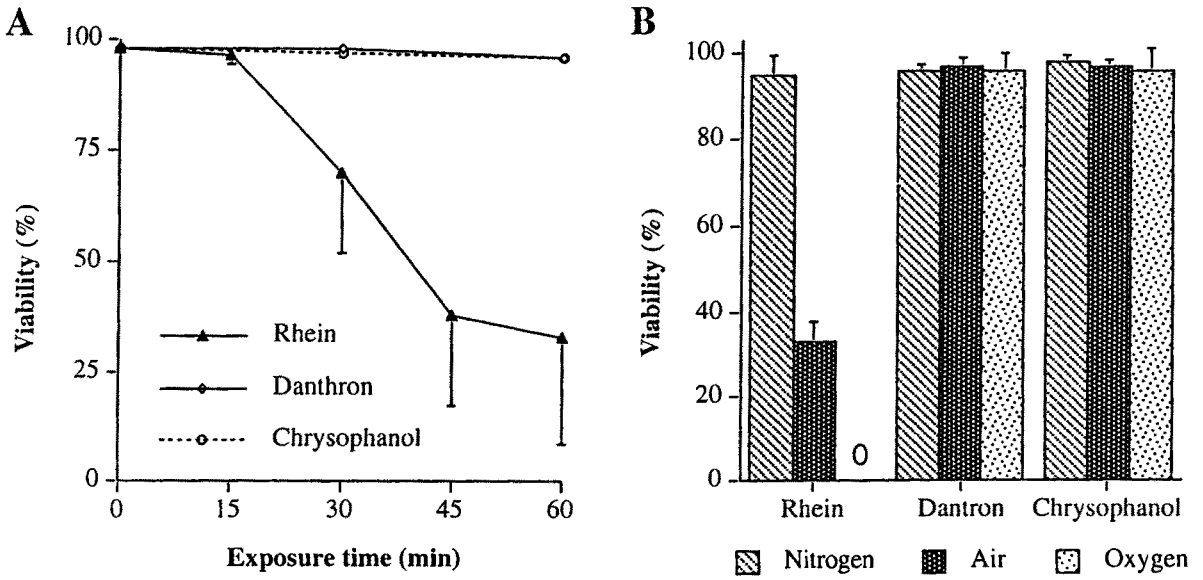

FIGURE 1 Plasma membrane integrity in primary rat hepatocytes exposed to anthraquinones. The membrane integrity was determined by lactate dehydrogenase activity measurements. (A) Time course of the toxicity of rhein, danthron, and chrysophanol (all 50 μM). (B) Plasma membrane integrity at different oxygen pressures after exposure to rhein, danthron or chrysophanol for 60 min. In B, the medium was saturated with nitrogen, air, and oxygen, resulting in concentrations of 16, 172 and 835 μM O_2, respectively. Values are means ± SD, $n = 3–4$.

exposed to 50 μM rhein, danthron and chrysophanol, respectively. The plasma membranes were intact during the first 15 min of treatment with rhein but thereafter rapidly lost their ability to resist leakage. Cultures exposed to danthron and chrysophanol did not show any signs of plasma membrane damage, even if the exposure was extended to 18 h (results not shown). When exposure to rhein was done in an atmosphere of 100% oxygen, all of the cells died within 60 min, whereas only limited cell death was detected after 60 min of incubation in 100% nitrogen (Figure 1B). Moreover, no effects were seen when hepatocytes were exposed to danthron or chrysophanol in an atmosphere of 100% oxygen for 60 min.

Intracellular reduced glutathione was totally depleted after 10 min of treatment with rhein but reappeared after 15 min (Figure 2A). Danthron and chrysophanol decreased GSH slowly to about 60% of initial value after 60 min of exposure. Table II shows the GSH concentration in cells that were treated with the protective substances desferal, DPPD, DTT, nifedipine, and OTC and exposed to rhein for 60 min. Control cells pretreated with OTC for 3 h showed an initial 3-fold increase in glutathione concentration.[26] None of the protectors prevented the initial GSH depletion, but after 60 min the GSH level was higher in protector treated hepatocytes than in the cells exposed solely to rhein.

Rhein caused only a minor decrease in ATP levels during the first 15 min, but thereafter the concentration declined rapidly (Figure 2B). Table II shows the ATP level in protector-treated hepatocytes after 60 min of exposure to rhein. All of the protective substances, except OTC guarded against ATP, loss to a certain degree.

Figure 3 shows the accumulation of the lipid peroxidation products MDA and 4-HA in cells and medium during treatment of hepatocytes with rhein. MDA and 4-HA are water soluble and will therefore appear in the surrounding medium after rupture of the cell membrane. No increase in lipid peroxidation products was

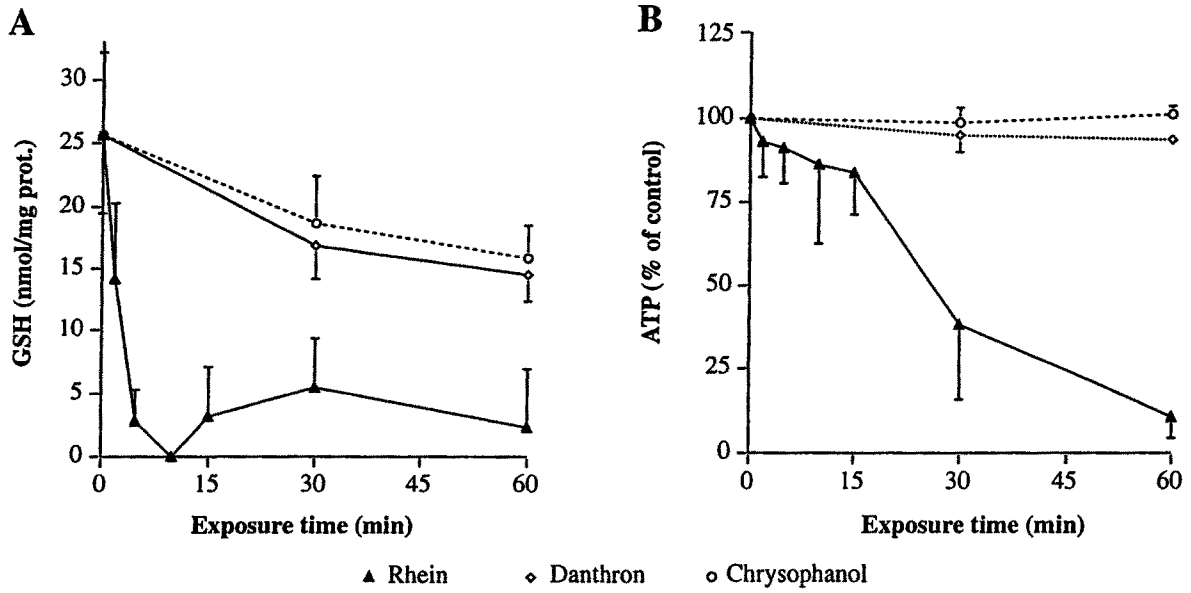

FIGURE 2 Intracellular levels of reduced glutathione (A) and ATP (B) in rat hepatocytes during exposure to anthraquinones for 60 min. Values are means ± SD, $n = 4–5$.

TABLE II Effect of antioxidants on rhein-induced toxicity in primary cultures of rat hepatocytes

Treatment	GSH (nmol/mg prot.)	ATP (% of control)	Lipid peroxidation products (μM MDA and 4-HA)	DNA fragmentation (% of total DNA)
Control	25.8 ± 6.3	100	1.02 ± 0.05	17.6 ± 6.1
Rhein only	2.3 ± 4.7	10.8 ± 6.5	7.49 ± 0.54	58.8 ± 4.6
Rhein + DPPD	12.4 ± 0.4**	81.1 ± 17.0**	1.30 ± 0.18**	16.2 ± 5.0**
Rhein + OTC	9.1 ± 5.8	31.8 ± 9.0*	6.81 ± 1.49	56.5 ± 9.9
Rhein + DTT	15.8 ± 3.3**	93.8 ± 18.1**	2.25 ± 0.22**	18.7 ± 8.8**
Rhein + desferal	4.8 ± 0.7	79.4 ± 20.4**	1.08 ± 0.19**	12.7 ± 7.2**
Rhein + nifedipine	18.3 ± 4.2**	78.0 ± 9.3**	2.00 ± 0.52**	12.9 ± 5.6**

Hepatocytes were exposed to 50 μM rhein for a period of 60 min before analysis of GSH and ATP, and for 30 min before analysis of lipid peroxidation products. Cells exposed for 30 min were subsequently kept under normal culture conditions for 16 h, after which DNA fragmentation was analysed. The protective substances nifedipine (1 mM), DPPD (2 μM) and DTT (0.5 mM) were added together with rhein whereas desferal (1 mM) and OTC (5 mM) were added under normal culture conditions 3 h before exposure to the anthraquinone. Values are means ± SD, $n = 3–6$, values significantly different from that found in cultures exposed to rhein only are marked *$p \leq 0.05$, **$p \leq 0.001$.

detected in culture treated with DPPD and desferal, nifedipine and DTT before or during rhein exposure for 30 min (Table II).

Alteration of Ca^{2+} Homeostasis

Addition of 50 μM rhein caused an immediate increase in cytosolic free Ca^{2+} from 149 ± 31 to 1356 ± 74 nM, and this level was sustained for at least 5 min. Danthron and chrysophanol increased the Ca^{2+} concentration to 1125 ± 112 and 477 ± 73 nM, respectively. In a Ca^{2+}–Mg^{2+} free medium, the calcium level was increased 2.5-fold immediately after the addition of rhein (results not shown).

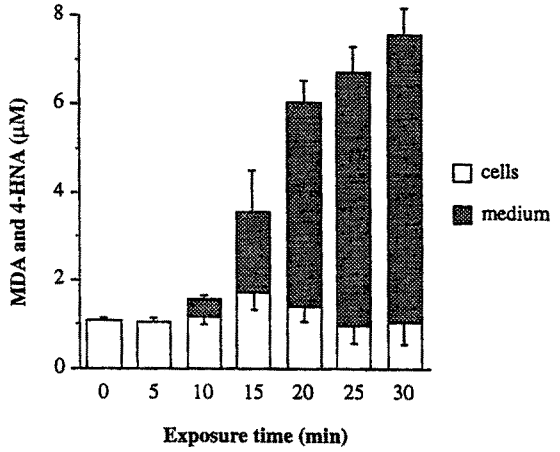

FIGURE 3 Lipid peroxidation products measured separately as water-soluble MDA and 4-HA in the culture medium and the cell layer during exposure to rhein. Values are means ± SE, $n = 4$.

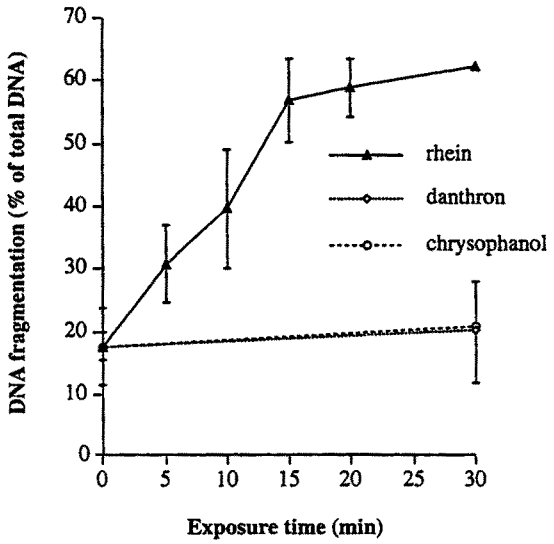

FIGURE 4 DNA fragmentation in rat hepatocytes exposed to anthraquinones for various length of time and subsequently cultured under normal conditions for 16 h. Values are means ± SD, $n = 4$ using cells from different rats.

Apoptosis

DNA fragmentation was determined in hepatocyte cultures that had been exposed to rhein and then returned to normal culture conditions for another 16 h. Figure 4 shows that the DNA fragmentation levelled off after 15 min of exposure to rhein. DNA fragmentation was not induced by treatment with danthron or chrysophanol.

Apoptosis is associated with a specific type of DNA cleavage that leaves free 3′-OH strands of DNA that can be detected by the TUNEL technique. Positive TUNEL reaction in combination with condensed nuclei are typical features of apoptotic cell death. In our experiments, the highest ratio of TUNEL-positive, pycnotic nuclei was found in hepatocytes exposed to rhein for 15 or 20 min (Table III). Incubation with danthron and chrysophanol did not cause nuclear condensation or positive TUNEL reactions. Exposure to 50 μM rhein for 20 min in the presence of DPPD or after pretreatment with desferal completely inhibited apoptosis. However, in the presence of OTC, DTT and nifedipine, considerable condensation of the nuclei was detected as well as positive TUNEL staining (Table III).

TABLE III Analysis of apoptosis

Exposure	Pycnotic nuclei (% of total)	TUNEL positive nuclei (% of total)
Control	0	0
Rhein (15 min)	73.2	73.4
Rhein (20 min)	83.7	87.5
Rhein (20 min) + DPPD	0	0
Rhein (20 min) + desferal	0	0
Rhein (20 min) + nifedipine	34.2	26.0
Rhein (20 min) + DTT	49.9	46.3
Rhein (20 min) + OTC	61.9	62.2

Hepatocytes were exposed to rhein with or without the addition of protectors. The cultures were then washed and returned to ordinary culture conditions for another 16 h before apoptosis analysis was performed. TUNEL values and percentage from analysis of pycnotic nuclei (Giemsa staining) are means of 2–4 assays.

DISCUSSION

Several of our results show that generation of free oxygen radical is the main cause of rhein toxicity: (i) rhein was the best substrate for NADPH cytochrome P-450 reductase reduction; (ii) rhein was the most effective redox cycler; (iii) rhein was

much less toxic to cells in an atmosphere of nitrogen; (iv) DPPD and desferal protected hepatocytes from rhein-induced cell death. Furthermore, we have recently noted,[26] that rhein exhibited a concentration-dependent toxicity and caused mitochondrial dysfunction in primary rat hepatocytes. In the present investigation, we also observed apoptotic cell death in hepatocytes initially exposed to rhein and then returned to normal culture conditions for 16 h. Unsubstituted and methyl-substituted anthraquinones (i.e., danthron and chrysophanol) did not induce any noteworthy generation of free radicals and, consequently, did not cause apoptotic cell death. Nevertheless, all three of the anthraquinones studied were probably taken up by the cells, as suggested by the partition coefficients. The more efficient redox cycling of rhein might be due to a π-electron-mediated mesomeric effect of the carboxyl group, which would increase the redox potential and stabilise the substance in a semianthraquinone form and cause higher oxidative stress.

In a recent report Dypbukt *et al.* showed that exposure of RINm5F cells to low concentration of 2,3-dimethoxy-1,4-naphthoquinone caused proliferation. If the concentration was increased, apoptosis was induced and necrosis was detected after exposure to even higher concentration of the quinone.[32] Here we show a similar relationship concerning the time period of which the cultures were exposed for rhein before withdrawal and return to normal culture conditions. If the cultures were exposed to rhein for 15 min, apoptosis was induced. On the other hand, exposure to rhein for more than 15 min resulted in extensive loss of plasma membrane integrity, depletion of ATP, and accumulation of lipid peroxidation products. We believe that the generated oxidative stress overwhelmed the antioxidative defence system and led to an overall intracellular oxidation, which in turn elicited disturbance of membrane stability and functions.[8,9] Furthermore, typical signs of necrotic cell death, such as swollen mitochondria and degenerated endoplasmic reticulum, have previously been seen in hepatocytes exposed to 50 μM rhein for 30 min.[26] In accordance with that, we detected apoptosis before plasma membrane integrity was lost in hepatocytes exposed to rhein for shorter periods of time. This demonstrates that apoptosis in hepatocytes is a regulated and highly energy-demanding process that may involve protein synthesis.[33] DNA fragmentation occurs during both apoptosis and necrosis. In the apoptotic process the fragmentation is internucleosomal, resulting in a characteristic ladder pattern after DNA gel electrophoresis.[34] When DNA fragmentation was analysed 16 h after exposure to rhein, we detected an initial increase that levelled off at 55% fragmented DNA after 15 min or treatment. This agrees with the observation that nuclear DNA fragmentation during apoptosis is ATP dependent,[35] since no increase in DNA fragmentation was detected when ATP was depleted.

There is increasing evidence that free radicals take part in the apoptotic process, both as inducers and as mediators.[14,17] It has been proposed that the oncogene bcl-2, which can prevent apoptosis, codes for a protein that induces antioxidation.[36] It is also possible that growth-factor-dependent apoptosis is regulated by oxygen-derived free radicals. Apoptosis induced by TNF-α has been shown to be accompanied by an elevation in ROS in several different cell types.[37] Furthermore, the mitochondrial membrane potential ($\Delta\Psi_m$) seems to play a key role in apoptosis,[38] since apoptotic cells show a reduction in $\Delta\Psi_m$ before they exhibit nuclear DNA fragmentation and aberrant exposure of phosphatidylserine.[39] Since the mitochondria are responsible for cellular energy production, loss of $\Delta\Psi_m$ could be an indication of the point-of-no-return in the death program. In an earlier study,[26] we found that the $\Delta\Psi_m$ was lowered in hepatocytes exposed to 50 μM rhein for 30 min and that such a decrease did not occur when DPPD, nifedipine and DTT were added together with rhein. The mechanisms by which bcl-2

inhibits apoptosis have also been suggested to be due to stabilisation of the mitochondrial membrane potential and maintenance of Ca^{2+} homeostasis.[40]

The protective substances used in our experiments give some indications of the reaction sequence that eventually leads to apoptosis. Increasing the cellular level of GSH by pretreating with OTC did not prevent glutathione depletion nor did it protect the hepatocytes against plasma membrane rupture or apoptosis. However, the reducing agent DTT did prevent ATP depletion and plasma membrane rupture during 60 min of exposure to rhein, but it did not prevent apoptosis, although the number of apoptotic cells was decreased as compared to what was seen in cultures exposed to rhein alone. Several investigators have reported that increased influx of Ca^{2+} precedes apoptosis.[41] In our experiments, the Ca^{2+}-channel blocker nifedipine prevented plasma membrane rupture and ATP loss. Moreover, in our earlier study,[26] the intracellular free Ca^{2+} level was increased by only one-third when rhein was added together with nifedipine, as compared to rhein alone. In the present investigation, blocking of Ca^{2+} channels with nifedipine decreased the amount of apoptosis by about 70%. Complete protection against apoptosis was achieved by pretreatment with desferal, an effective iron-chelator that prevents production of hydroxyl radicals by Fenton chemistry, or by addition of DPPD, a lipid-soluble free radical scavenger that mainly removes carbon-centred radicals. The main task performed by both of these protectors is probably to prevent lipid peroxidation. Desferal inhibits production of the hydroxyl radical which is a very effective initiator of lipid peroxidation, and DPPD inhibits the progression of a peroxidation reaction in the cell membrane.

Following oxidative damage, the cell has to determine whether to repair the injury or to initiate the apoptosis program.[42] We suggest that rhein-induced apoptosis is caused by production of free radicals, which in turn starts the apoptotic program by disturbing of mitochondrial functions and depleting ATP. The critical events in this system seem to be the production of hydroxyl radicals and initiation of lipid peroxidation, which ultimately lead to execution of the apoptotic program.

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