In vitro folate supplementation alleviates oxidative stress, mitochondria-associated death signalling and apoptosis induced by 7-ketocholesterol

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Folate has recently been proposed as a new antioxidant. Folate supplementation may have a protective effect in counteracting oxidant-induced apoptotic damage. The present studies were undertaken to examine whether there is a direct link between folate levels, antioxidant capability and reduced apoptotic damage. Using an *in vitro* cellular model of 7-ketocholesterol (KC)-induced apoptosis, U937 cells were pre-cultured with a folate-deficient medium supplemented with various levels of folate ($2–1500\ \mu mol/l$) before treatment with 7-KC. Apoptotic markers, mitochondria-associated death signals and levels of reactive oxygen species were assayed. After treatment with 7-KC for 30 h, low and high levels of folate supplementation significantly ($P<0.05$) reduced nuclear DNA loss. Only high levels of folate supplementation ($>1000\ \mu mol/l$) were effective in counteracting 7-KC-promoted apoptotic membrane phosphatidylserine exposure and DNA laddering. The attenuation of 7-KC-induced apoptotic damage by high-dose folate supplementation coincided with a partial normalization of mitochondria membrane potential dissipation, a suppression of cytochrome *c* release and an inhibition of procaspase 3 activation. The prevention of mitochondrial dysfunctions and apoptotic processes was associated with antioxidant actions of high-dose folate by a marked scavenging of intracellular superoxide. Collectively, our present results demonstrate that *in vitro* folate supplementation exerts differentially protective effects against 7-KC-induced damage. High-dose supplementation alleviates oxidative stress, mitochondria-associated death signalling and apoptosis induced by 7-KC. However, the *in vivo* relevance is not clear and requires further study.

Folate supplementation: Antioxidant: Apoptosis: Mitochondria-associated death signalling: 7-Ketocholesterol

It has recently been proposed that folate scavenges peroxyl radicals, azide radicals and hydroxyl radicals in an *in vitro* radical reaction model system (Joshi *et al.* 2001). However, the antioxidant action of folate against cellular damage is not fully understood. Huang *et al.* (2001) reported that folate depletion accompanied by elevated homocysteine concentrations was correlated with increased lipid peroxidation in rat livers. Numerous investigations have shown that folate deficiency promoted H_2O_2 generation and lipid peroxidation in HepG2 cells (Chen *et al.* 2001; Chern *et al.* 2001). In human subjects, increased folate intake was found to improve endothelial function in patients with coronary artery disease (Doshi *et al.* 2001). Reduction of intracellular endothelial superoxide may have contributed to the effect. These studies raise the possibility that folate supplementation may protect cells against oxidative damage, yet a direct link has not been established.

Generation of reactive oxygen species can activate apoptosis, a programmed cell death (Palmer & Paulson, 1997; Chandra *et al.* 2000). Apoptotic damage in cells is characterized by chromatin condensation, nucleosomal DNA fragmentation and membrane phosphatidylserine translocation (Kroemer *et al.* 1995; Granville *et al.* 1998). Although the mechanisms by which reactive oxygen species induce

apoptosis are not fully defined, it has been suggested that mitochondria play a central role (Kroemer *et al.* 1997; Bernardi *et al.* 2001). In the oxidant-promoted apoptosis, the depolarization of mitochondrial membrane potential has been attributed to the mitochondrial release of pro-apoptotic proteins such as cytochrome *c*, which activates the apoptotic executor of caspase 3 to initiate apoptotic DNA damage or membrane disorder (Li *et al.* 1997; Zamzami & Kroemer, 2001; Lim *et al.* 2002). These oxidative apoptotic insults may play a pivotal role in atherosclerosis, because appreciable amounts of apoptotic cells are evident in human atheromatous lesions (Han *et al.* 1995; Isner *et al.* 1995). 7-Ketocholesterol (KC), one of the prominently oxidized cholesterols and the most abundantly formed oxysterol in human atherosclerotic plaque (Brown *et al.* 1997; Garcia-Cruset *et al.* 1999), has been shown to induce apoptosis in endothelial cells (Lizard *et al.* 1997, 1999), smooth muscle cells (Nishio *et al.* 1996; Nishio & Watanabe, 1996) and monocytic cells (Aupeix *et al.* 1995; O'Callaghan *et al.* 2001). The loss of mitochondrial transmembrane potential and the cytosolic release of cytochrome *c* occurred during 7-KC-induced apoptosis (Lizard *et al.* 2000). Antioxidants such as glutathione and vitamin E can prevent 7-KC-induced apoptosis

by the disruption of mitochondrial transmembrane potential and cytochrome *c* release (Lizard *et al.* 2000).

Folate deficiency was shown previously to induce apoptotic damage in several cell lines (James *et al.* 1994; Koury *et al.* 1997; Huang *et al.* 1999) and in animals (James *et al.* 1997; Craciunescu *et al.* 2004). The underlying mechanisms relating to the antioxidant capability of folate and its role in the signalling of mitochondria-associated apoptotic death are largely unknown. 7-KC-induced apoptosis provides us with a unique model to investigate whether there is a direct link between folate levels, antioxidant capability, mitochondria-associated death signalling and reduced apoptotic damage. U937 cells were chosen as an *in vitro* cellular model, because it is one of the most commonly used cell lines to study the molecular mechanisms of oxysterol-induced apoptosis (Lizard *et al.* 1998, 2000). U937 cells are also frequently used as macrophage reference models that are sensitive to oxysterols toxicity in the same range of concentrations observed in other vascular cells (Lizard *et al.* 1996, 1997, 1998). We added various levels of folate to folate-deficient (FD) medium and measured apoptotic variables, mitochondrial transmembrane potential, cytochrome *c* release, procaspase 3 activation and intracellular reactive oxygen species levels in U937 cells in the presence or absence of 7-KC.

Materials and methods

Materials

7-KC, folate (pteroylmonoglutamic acid), rodamine 123, propidium iodide (PI), 2′,7′-dichlorofluorescin diacetate and hydroethidine were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT, USA). Complete FD Roswell Park Memorial Institute 1640 medium and antibiotics were purchased from GIBCO Laboratories (Grand Island, NY, USA). The rabbit antihuman CPP32 and cytochrome *c* antibody were from BD Biosciences (San Jose, CA, USA) and R&D Systems (Minneapolis, MN, USA). Multimers of 100 bp DNA were from Pharmacia Biotech (Piscataway, NJ, USA).

Cell culture

U937 cells, a human promonocytic leukaemia cell line, were purchased from the cell bank of NRIH (Taipei, Taiwan; CCRC 60027). U937 cells were regularly maintained in complete Roswell Park Memorial Institute 1640 medium supplemented with heat-inactivated fetal bovine serum (100 ml/l) and antibiotics (2000 U penicillin/l and 20 mg streptomycin/l). Cells were maintained in suspension cultures in a humidified 5 % CO_2 atmosphere at 37°C. Cellular viability was determined by Trypan Blue exclusion.

Experimental treatments

For all experiments, a 7-KC stock solution was prepared according to the methods of Lizard *et al.* (2000). Briefly, 800 μg 7-KC was dissolved in 50 μl absolute ethanol and 950 μl FD medium was added. The solution was then sonicated. The ethanol level in culture medium of final concentration 40 μg 7-KC/ml had no effect on cell viability when it was measured with Trypan Blue. A stock solution of folate was prepared at 10 mmol/l by dissolving 44 mg folate in 10 ml $NaHCO_3$ (10 g/l) solution. To make up media with various levels of folate, folate stock was added to FD medium and the pH was adjusted to 7·4 before sterilization by filtration. All stock solutions were freshly prepared before experiments.

The doubling time for U937 cells is 26 ± 2 h. Viability and growth rate of cells were frequently checked using Trypan Blue exclusion. Cells in the log growth phase were sub-cultured into various sets of plates for conducting each experiment. U937 cells were pre-incubated in FD medium with various levels of folate for 6 h and then treated with 7-KC to induce apoptosis. Cultures without 7-KC treatment at the designated experimental time points were used as additional controls. After the treatments were terminated, cells were harvested for the assay.

Analysis of apoptotic cells with membrane phosphatidylserine exposure

The Annexin-V-Fluos kit (Roche Diagnostics, Mannheim, Germany) was used to measure the apoptotic cells with membrane phosphatidylserine translocation. Cells were suspended in incubation buffer that contained 1 mg PI/l and a 1:50 dilution of Annexin-V-Fluos labelling solution. The mixed solution was incubated for 15 min on ice, and green Annexin-V and red PI fluorescence intensities were analysed on a Coulter EPICS XL-MCL flow cytometer (Coulter Corporation, Miami, FL, USA). Cells with Annexin-V-positive and PI-negative fluorescence were defined as apoptotic cells. Cells with Annexin-V-negative and PI-positive fluorescence (PI permeable) were defined as necrotic cells.

Analysis of apoptotic cells with hypodiploid DNA contents

Cells were fixed in ice-cold ethanol. RNase A (500 mg/l) and Triton (5 g/l) were added to samples at 37°C for further incubation for 60 min. Cells were then incubated with PI (50 mg/l) for 20 min at 37°C. After centrifugation, cellular DNA (red fluorescence) in 10 000 cells was analysed on a flow cytometer with the System II software program (Coulter, Corporation). The percentage of cells in different cell cycle phases was estimated from PI histograms using WinMDI 2.8 software (Coulter Corporation). Hypodiploid cells, i.e. those with lower G0/G1 DNA contents, were defined as apoptotic cells, as described by Endresen *et al.* (1994).

Agarose electrophoresis of DNA fragmentation

As described by Huang *et al.* (2002), 3×10^6 cells at each indicated time point were harvested and suspended in ice-cold lysis buffer (100 mmol NaCl/l, 10 mmol Tris-HCl/l, 25 mmol EDTA/l, 5 g SDS/l and 0·3 g proteinase K/l) for 15 h in a water-bath at 50°C. The standard phenol–chloroform–isoamyl alcohol (25:24:1, by vol.) method was used to remove protein and extract DNA. RNA was digested with 1 mg ribonuclease A/l for 1 h at 37°C. DNA fragments

were electrophoresed on an agarose (20 g/l) mini-gel at 100 V for 40 min and visualized with ethidium bromide staining under UV illumination.

Quantification of depolarized cells with mitochondrial membrane potential dissipation

Rodamine 123 has been widely used as a flow cytometric probe for mitochondrial membrane potential (Juan *et al.* 1994). Rodamine 123 was added directly to the culture medium (5 mg/l) and incubated for 30 min at 37°C. After centrifugation, cells were analysed with a flow cytometer (excitation 488 nm, emission 625 nm). Dead cells and debris were excluded from the analysis by electronic gating of forward- and side-scatter measurement.

Determination of intracellular reactive oxygen species levels

Intracellular reactive oxygen species were assayed using the fluorescent dyes 2′,7′-dichlorofluorescin diacetate and hydroethidine (Robinson *et al.* 1994). Intracellular H_2O_2 or NO oxidizes 2′,7′-dichlorofluorescin to the highly fluorescent compound. Hydroethidine oxidation is particularly sensitive to superoxide anions. Thirty minutes before 7-KC treatment was terminated, 2′,7′-dichlorofluorescin diacetate (5 μmol/l) or hydroethidine (5 μmol/l) was added into cultured cells. At each indicated time point, cells were harvested and the fluorescence intensity of intracellular dichlorofluorescin (emission 530 nm) or hydroethidine (emission 585 nm) was monitored on a flow cytometer (Coulter Corporation).

Subcellular fractionation and Western blot analysis

Cytochrome *c* release into the cytosol and degradation of procaspase 3 were investigated by Western blot analysis. The analysis of cytochrome *c* release to cytosol was performed according to the method of Lizard *et al.* (2000). In brief, cells were suspended in lysis buffer (20 mmol HEPES–KOH/l (pH 7·5), 10 mmol KCl/l, 1·5 mmol MgCl/l, 1 mmol EDTA/l, 1 mmol sodium ethylene glycol tetraacetic acid/l, 1 mmol dithiothreitol/l, 250 mmol sucrose/l and a mixture of protease inhibitors) and were homogenized by successive passages through a 27-guage fine needle. By centrifuging the homogenate at 1000 *g* at 4°C for 10 min, the resulting supernatant fraction was subjected to 15 500 *g* centrifugation at 4°C for 30 min to generate cytosol. For analysis of procaspase 3 (CPP32), cells were lysed in lysis buffer (50 mmol Tris-HCl/l (pH 8·0), 150 nmol NaCl/l, 1 g SDS/l, 1 mmol sodium ethylene glycol tetraacetic acid/l, 1 mmol dithiothreitol/l, 250 mmol sucrose/l and a mixture of protease inhibitors) for 30 min on ice and then centrifuged at 4°C (14 000 *g* for 15 min). The protein concentration was measured by Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard. Proteins (50 μg) were incubated in loading buffer, separated by SDS–PAGE, and electroblotted to a polyvinylidine difluoride membrane. Proteins were probed with human monoclonal antibody directed against cytochrome *c* and caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by peroxidase-labelled anti-mouse antibodies and visualized using an enhanced chemoluminescence detection kit (Amersham International Amersham, Bucks., UK) by autoradiography.

Statistical analysis

All results were presented as mean values and standard deviations. One-way ANOVA and Duncan's test were used for comparisons among folate-supplemented groups. Statistical significance was set at $P < 0.05$.

Results

Dose effects of folate supplementation on 7-ketocholesterol-induced apoptosis

Previous studies have demonstrated that treatment with 40 μg 7-KC/ml can induce apoptosis in the U937 cellular model (Lizard *et al.* 1998, 2000). We measured the dose effects of folate supplementation against apoptotic damage as a function of time after treatment with 40 μg 7-KC/ml. The results are shown in Table 1. In the absence of 7-KC treatment, cells cultured in FD medium did not display apoptosis within the experimental time periods. After 24 h of treatment with 7-KC, the apoptotic cells with hypodiploid DNA content were increased to 18 %. Folate supplementation at 100–1500 μmol/l significantly reduced the hypodiploid cellular fractions, with the greatest magnitude of reduction with high-folate supplementation. After 30 h of treatment with 7-KC, pre-incubation of 7-KC-treated cells with media that contained a normal folate level (2 μmol/l) and high folate levels (1000 and 1500 μmol/l) significantly reduced ($P < 0.05$) the hypodiploid cellular fractions (%). Folate at 1500 μmol/l markedly diminished 7-KC-promoted hypodiploid cellular fractions by half. The fraction of apoptotic cells with membrane phosphatidylserine translocation (Annexin-V-positive and PI-negative) was 25 % after 24 h of 7-KC treatment, and this increased to 49 % after 30 h of treatment with 7-KC. Folate supplementation (2–1500 μmol/l) did not affect 7-KC-induced apoptotic membrane disorder at 24 h, whereas supplementation with 1500 μmol folate/l significantly reduced ($P < 0.05$) Annexin-V-positive cells (by half) after 30 h of 7-KC treatment.

The protective effects of folate supplements against apoptotic DNA damage were further confirmed by the results of electrophoretic analysis (Fig. 1). After 30 h of 7-KC treatment, apoptotic DNA laddering was seen (lane 3). Folate supplements at 1000–2000 μmol/l (lanes 6–8), but not at 2–100 μmol/l (lanes 4 and 5), substantially decreased the 7-KC-induced DNA fragmentation. In the absence of 7-KC treatment, no DNA fragmentation was detected in cells cultured with complete or FD medium (lanes 1 and 2).

Effects of folate supplementation on 7-ketocholesterol-induced mitochondria membrane potential dissipation

To understand whether the impairment of 7-KC-induced apoptosis by folate might be associated with changes in

Table 1. Effects of folate supplementation on the percentages of apoptotic cells in the presence or absence of 7-ketocholesterol treatment*
(Mean values and standard deviations of three independent experiments in triplicate)

| | FD | | FD + 7-KC | | FD + 7-KC + folate (µmol/l) | | | | | | | | | |
| | | | | | 2 | | 10 | | 100 | | 1000 | | 1500 | |
Culture time (h)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cells with hypodiploid DNA contents (%)														
24	0·8[d]	0·1	18[a]	3	15[a]	2	15[a]	3	14[b]	1	11[c]	1	12[c]	1
30	0·9[e]	0·1	19[a]	1	16[b]	1	15[b]	2	16[b]	2	13[c]	1	10[d]	1
Annexin-V-positive (%)														
24	0·7[b]	0·1	25[a]	6	23[a]	2	23[a]	3	24[a]	2	25[a]	2	26[a]	1
30	0·6[c]	0·1	49[a]	3	50[a]	3	52[a]	5	46[a]	4	49[a]	3	20[b]	4

FD, folate deficient; KC, ketocholesterol.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).
* U937 cells were preincubated with FD medium or FD medium supplemented with various folate levels for 6 h before treatment with 7-KC (40 µg/ml) for the designated time periods. The proportions of apoptotic cells (Annexin-V-positive or hypodiploid cells) were analysed by flow cytometry as described on p. 888.

mitochondria membrane potential, the percentage of cells with depolarized mitochondria was evaluated using the rodamine 123 probe, by a flow cytometer analysis. Rodamine 123 distribution histograms of cells with FD, FD + 7-KC and FD + 7-KC + folate treatment are shown in Fig. 2(A)–(C). In FD cultures, the percentage of depolarized cells was 10 after 24 h of 7-KC treatment and had increased 3-fold after 30 h of treatment (Fig. 2(D)). Pre-incubation of cells with 2 or 100 µmol folate/l for 6 h did not affect the 7-KC-induced mitochondrial depolarization. Pre-incubation of cells with 1000 and 1500 µmol folate/l for 6 h significantly reduced the percentage of depolarized cells after 24 and 30 h of treatment with 7-KC. The results shown in Fig. 2(E) further revealed that the protective effects of high-dose folate supplementation were modulated by the length of

Fig. 1. Effects of folate supplementation on apoptotic DNA laddering in the presence or absence of 7-ketocholesterol (KC) treatment. U937 cells were pre-incubated in folate-deficient medium supplemented with various levels of folate (2, 100, 1000, 1500 and 2000 µmol/l) for 6 h before treatment with 7-KC. After 30 h of 7-KC treatment (40 µg/ml), total DNA extracted from U937 cells was analysed by electrophoresis on an agarose (20 g/l) mini-gel at 100 V for 40 min. For details of procedures, see p. 888. Lane M: DNA markers of molecular size at 100 bp multimers. Lane 1: cells not treated with 7-KC in complete medium. Lane 2: cells not treated with 7-KC in folate-deficient medium. Lanes 3–8: cells treated with 7-KC and pre-incubated with folate levels at 0 (lane 3), 2 (lane 4), 100 (lane 5), 1000 (lane 6), 1500 (lane 7) and 2000 (lane 8) µmol/l.
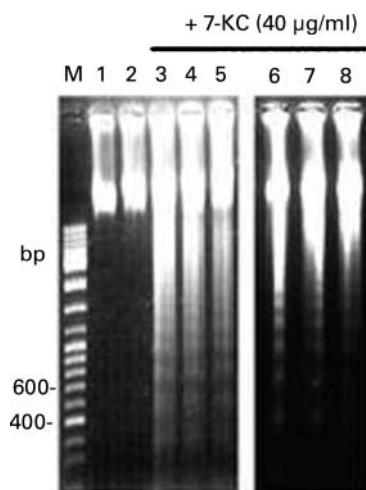
pre-incubation time. After 24 h of treatment with 7-KC, high-dose folate did not affect 7-KC-induced mitochondria depolarization if cells were not pre-cultured with folate (0 h pre-incubation). The reduction of mitochondria depolarization by high-dose folate supplementation was more remarkable when cells were pre-cultured with folate for 24 h, compared with the cells without folate pre-incubation or with 6 h folate pre-incubation (Fig. 2(D)).

Effects of folate supplementation on mitochondria-associated signalling
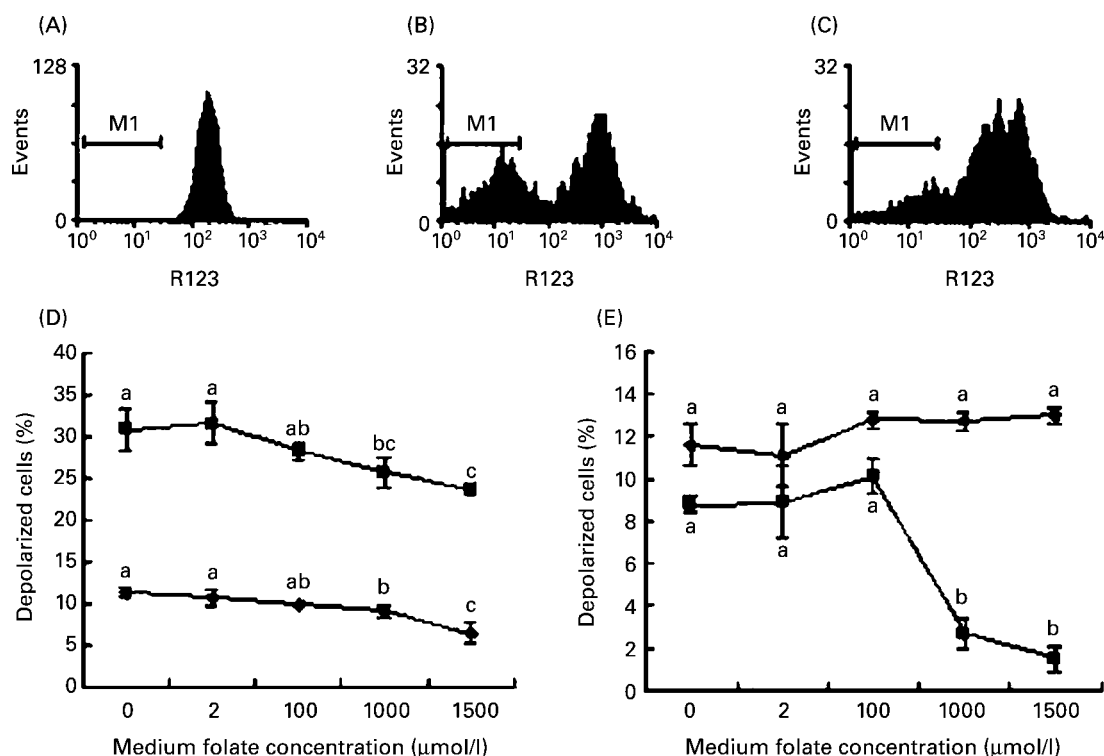
The apoptotic signalling proteins of cytochrome *c* release from mitochondria into the cytosol and the cleavage of the inactive precursor (CPP32) were assessed by Western blot analysis. The results are presented in Figs. 3 and 4. After treatment with 7-KC for 24 h, a release of cytochrome *c* to cytosol was apparent (Fig. 3(B), lane 2), whereas no CPP32 cleavage was observed (Fig. 3(A), lane 2). Folate supplementation at 1500 µmol/l inhibited the 7-KC-promoted cytochrome *c* release (Fig. 3(B), lane 5). This cytochrome *c* release in 7-KC-treated cells was followed by the activation of CPP32 after 30 h of treatment with 7-KC (Fig. 3(A), lane 6). Folate supplementation at 1000 and 1500 µmol/l partially suppressed CPP32 cleavage (Fig. 3(A), lanes 7 and 8). The protective effects of high-dose folate against cytochrome *c* release and caspase 3 activation persisted to 36 h of 7-KC treatment, as shown in Fig. 4 ((A) and (B), lanes 4 and 5).

Effects of folate supplementation on 7-ketocholesterol-induced reactive oxygen species production

Table 2 presents results on superoxide anion scavenging by folate supplementation. Compared with 7-KC-untreated cells cultured in FD medium, folate supplementation at 2, 100, 1000 and 1500 µmol/l significantly reduced intracellular superoxide levels to 81, 75, 56 and 37 %. After 7-KC treatment for 12, 24 and 30 h, the intracellular superoxide levels of FD cells increased to 2-fold of those in 7-KC-untreated cells. Folate supplementation at 1500 µmol/l had a significantly greater ($P < 0.05$) capability to scavenge 7-KC-elicited superoxide generation (a 42 % reduction)

Fig. 2. Changes in mitochondrial membrane potential in cells treated with 7-KC and folate pre-incubation. U937 cells were pre-cultured with various levels of folate for 0, 6 and 24 h, treated with 7-ketocholesterol (KC; 40 μg/ml) for the indicated times, and stained with rodamine 123 (R123). The uptake of R123 was analysed by flow cytometry. For details of procedures, see p. 889. (A), (B) and (C), representative histograms of three independent experiments ((A), folate-deficient (FD); (B), FD + 7-KC; (C), FD + 7-KC + 1500 μM folate). Treatment with 7-KC lasted for 30 h. M1 is associated with the proportions of depolarized cells, which are characterized by low values of mitochondrial membrane potential. (D), effects of folate supplementation on the proportions of depolarized cells after 24 h (–◆–) and 30 h (–■–) treatment with 7-KC. Cells were pre-incubated with various levels of folate for 6 h before 7-KC treatment. (E), effects of the pre-incubation time on the depolarized cell percentage after 24 h treatment with 7-KC; –◆– no folate pre-incubation; –■– 24 h folate pre-incubation. [a,b]Mean values with unlike superscript letters on the same line were significantly different: $P < 0.05$.

compared with folate supplementation at 2 μmol/l (an 18 % reduction) after 30 h of treatment with 7-KC. 7-KC treatment and folate supplementation did not affect intracellular H_2O_2 generation as measured by dichlorofluorescin fluorescence (results not shown).

Discussion

Our present results confirm previous findings that 7-KC promotes apoptosis in U937 cells (Lizard *et al.* 1998, 2000; O'Callaghan *et al.* 2001). Treatment with 7-KC caused a concomitant loss in mitochondria membrane potential, release of cytochrome *c*, and subsequently, the activation of apoptotic executor caspase 3, which was in agreement with the proposed mechanisms in the transmission of death signalling (Lizard *et al.* 2000). In the absence of 7-KC treatment, however, no apoptotic damage was observed in cells cultured in FD medium during the experimental periods. This is in contrast to previous evidence, which linked folate deficiency to apoptotic promotion in Chinese hamster ovary cells (James *et al.* 1994), HepG2 cells (Huang *et al.* 1999) and erythroblasts (Koury *et al.* 1997). Different cell types and the duration of folate deficiency may be attributable to these observed discrepancies. A longer period of folate deficiency (days or weeks) was frequently used to induce apoptosis, whereas our experimental time period for U937 cells was about 12–36 h.

The present results demonstrate that *in vitro* folate supplementation protects against 7-KC-triggered apoptotic damage. Supplemented folate at a culture medium dose (2 μmol/l) was sufficient to diminish hypodiploid cellular fractions on 7-KC treatment, whereas only high-dose supplementation was able to ameliorate the apoptotic membrane disorder and DNA fragmentation through suppressing mitochondria-associated death signalling. These results suggest that folate supplementation counteracted the 7-KC-induced apoptotic damage at various intracellular targets and molecular levels. In early literature, the proposed links for folate and apoptosis included decreased thymidylate synthesis (Koury *et al.* 1997), deoxynucleotide pool imbalance (James *et al.* 1994, 1997), DNA mismatch repair (Gu *et al.* 2002), elevated homocysteine levels (Duan *et al.* 2002) and cell cycle-dependent processes (Huang *et al.* 1999). We found that folate may act as a modulator of mitochondria-associated death signalling to alleviate apoptotic damage, which, to our knowledge, has not been demonstrated previously.

The molecular mechanisms by which high-dose folate could suppress mitochondria-associated death signalling are largely unknown. One of the potential protective mechanisms could be radical scavenging. Reactive oxygen species, such as superoxide and H_2O_2, have been reported to be important regulators of mitochondria membrane potential and cytochrome *c* release in several kinds of
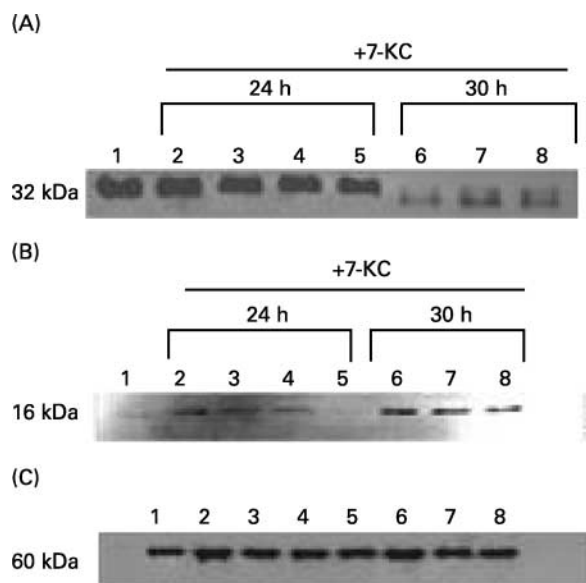
Fig. 3. Modulation of cytochrome *c* release and procaspase 3 activation by folate supplementation. The cleavage of caspase 3 precursor as procaspase (CPP32, (A)), the release of cytochrome *c* into the cytosol (B) and β-actin (C) were investigated by Western blot analysis. U937 cells were pre-incubated in folate-deficient medium supplemented with various levels of folate for 6 h before treatment with 7-KC. For details of procedures, see. p. 889. Lane 1: cells not treated with 7-ketocholesterol (KC). Lanes 2–5: cells treated with 7-KC for 24 h, and supplemented with folate at concentrations of 0 (lane 2), 2 (lane 3), 1000 (lane 4), and 1500 (lane 5) μmol/l. Lanes 6–7: cells treated with 7-KC for 30 h, and supplemented with folate at concentrations of 0 (lane 6), 1000 (lane 7), and 1500 (lane 8) μmol/l.

oxidative stress-promoted apoptosis (Cai & Jones, 1998; Stridh *et al.* 1998; Tada-Oikawa *et al.* 1999). By scavenging superoxide, antioxidants such as vitamin E and *N*-acetylcysteine could reverse mitochondria membrane depolarization and cytochrome *c* release to the cytosol in 7-KC-treated U937 cells (Lizard *et al.* 2000). Indeed, we found that high-dose folate supplementation significantly reduced intracellular superoxide levels during the early phase of 7-KC treatment, which coincided with the concomitant reverse of mitochondria dysfunction and apoptotic damage. These results suggest that folate antioxidant
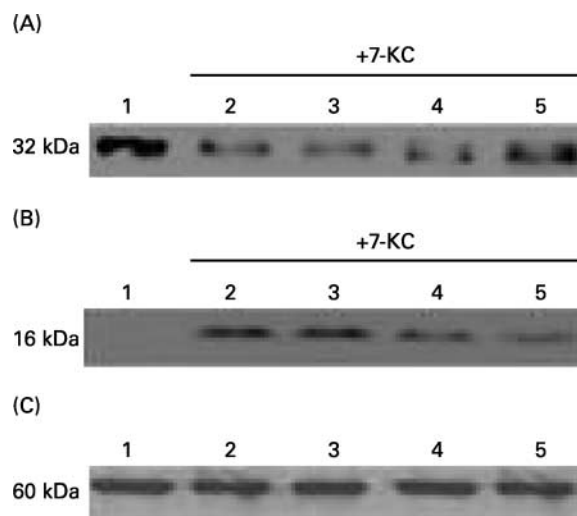
Fig. 4. Effects of folate supplementation on procaspase 3 activation (A), cytochrome *c* release (B) and β-actin (C) after 36 h of treatment with 7-ketocholesterol (KC). Folate pre-incubation is the same as described in Fig. 3. For further details of procedures, see p. 889. Lane 1: cells not treated with 7-KC. Lanes 2–5: cells treated with 7-KC and supplemented with folate at concentrations of 0 (lane 2), 2 (lane 3), 1500 (lane 4) and 2000 (lane 5) μmol/l.

capability, particularly for superoxide scavenging, as has been proposed by previous investigators (Doshi *et al.* 2001; Huang *et al.* 2002), can partially account for the impairment in mitochondria-associated death signals.

The anti-apoptotic function by folate may not be limited to its antioxidant property. High-dose folate supplementation may enrich the mitochondria folate pool to strengthen respiratory function, the impairment of which leads to superoxide production and abnormal mitochondrial permeability transition during apoptosis (Cai & Jones, 1998; Crompton, 1999). This postulation is partially supported by the finding that a lipophilic folate antagonist, at high concentrations, can be accumulated in mitochondria to act as a respiration inhibitor (Sprecher *et al.* 1995). Alternatively, the suppression of mitochondrial cytochrome *c* release by folate may be attributable to a downstream effect of folate metabolites through mitochondrial C_1 metabolism. 10-Formyl-tetrahydrofolate, a major mitochondria folate coenzyme (Horne

Table 2. Effects of folate supplementation on superoxide production in the presence or absence of 7-ketocholesterol treatment*

(Values are means and standard deviations for three independent experiments in triplicate)

| | | FD | | | FD + folate supplementation (μmol/l) | | | | | | | | | | | |
| | | | | | 2 | | | 100 | | | 1000 | | | 1500 | | |
Culture time (h)	Treatment	Mean	SD	%†	Mean	SD	%†	Mean	SD	%†	Mean	SD	%†	Mean	SD	%†
HE fluorescent intensity																
12	− 7-KC	16[a]	2	100	13[b]	1	81	12[b]	1	75	9[c]	1	56	6[d]	1	37
12	+ 7-KC	33[a]	6	100	35[a]	7	106	26[b]	3	78	22[b]	4	67	19[b]	2	57
24	+ 7-KC	34[a]	3	100	27[b]	2	79	25[b]	3	73	26[b]	2	76	19[c]	3	55
30	+ 7-KC	39[a]	1	100	32[b]	5	82	31[b]	3	79	26[c]	1	66	23[c]	2	58

FD, folate deficient; HE, hydroethidine; KC, ketocholesterol.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).
* U937 cells were preincubated with FD-medium supplemented by 2, 100, 1000 and 1500 μmol/l folate/l for 6 h before treatment with 7-KC (40 μg/l). At the indicated time points, cells were labelled by HE fluorescent dye for 30 min, harvested, and analysed by flow cytometer. For details of procedures, see p. 889.
† Percentage of FD value.

et al. 1989), has been shown to support the respiration of rat mitochondria by reducing cytochrome *c* at the site of respiratory complex IV enzymes (Brookes & Baggott, 2002). The altered redox state of cytochrome *c* could modulate its release to the cytosol, and its activation to trigger programmed cell death (Calver *et al.* 1997; Hancock *et al.* 2001; Lim *et al.* 2002). More studies are warranted to elucidate the regulatory role of folate on mitochondria-associated death signalling.

In the current investigation, the effective dose of folate to counteract mitochondria dysfunction was found at mM levels, which are much greater than those encountered in the human physiological state. It is open to debate whether such *in vitro* experiments are meaningful in the context of the whole organism. However, it is also generally accepted that metabolic observations in cultured cell lines may not be directly applicable to the multicellular environment. Supraphysiological levels of treatment proved to be necessary to achieve the potential effects on cells *in vitro* over relatively short experimental time intervals (hours in the present study). We cannot exclude the possibility that, over a longer time course (weeks or months), high dietary folate supplementation could be sufficient to protect against mitochondria dysfunction *in vivo*. This possibility is supported by the observation that rats fed a high-folate diet had significantly lower percentages of depolarized cells and mitochondrial protein oxidative damage in their livers compared with rats fed a FD diet (Chang, 2004). In human subjects, folic acid supplements at a high dose of 5 mg/d (more than 10-fold the daily recommended value) for 6 weeks or 4 months significantly improved endothelial dysfunction in patients with coronary artery disease (Title *et al.* 2000; Doshi *et al.* 2001). Clinical intervention trials on folate and cancer have further revealed that supplementation with a pharmacological dose of folate (5–20 mg) may have a chemoprotective role (Kim, 1999). Although the mechanisms of long-duration high dietary folate supplementation to protect against human disease are far from understood, our present observations from an *in vitro* cellular model may provide potential insights into the possible role of high-dose folate in reducing oxidative stress and mitochondria dysfunction.

Given the assumptions and limitations of *in vitro* studies, the results in the present report suggest that folate supplementation exerts differentially protective effects against 7-KC-induced damage. High-dose supplementation alleviates oxidative stress, mitochondria-associated death signalling and apoptosis induced by 7-KC. However, the *in vivo* relevance is not clear and requires further study.

Acknowledgements

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