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Quercetin modifies reactive oxygen levels but exerts only partial protection against oxidative stress within HL-60 cells

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Abstract

Quercetin may contribute to the protection afforded by fruit- and vegetable-rich diets against diseases for which excess production of reactive oxygen species (ROS) has been implicated as a causal or contributory factor. We examine the effect of short term (90 min) quercetin (1–100 μM) exposure on the progress of menadione induced oxidative stress within HL-60 cells. 2′,7′-dichlorofluorescein and rhodamine-123 fluorescence, resulting from oxidation of the ROS-sensitive dyes dichlorodihydrofluorescein and dihydrorhodamine-123 respectively, were utilised as indicators of general ROS levels. Ethidium fluorescence, resulting from oxidation of dihydroethidium, was used as a potentially more specific indicator of O_2^-. Exposure to quercetin alone induced a decrease in DCF and rhodamine fluorescence. Conversely, ethidium fluorescence was enhanced by treatment with ≥40 μM quercetin. Incubation with 1–100 μM quercetin reduced the extent of menadione-induced increase in DCF and rhodamine fluorescence but the menadione-induced increase in ethidium fluorescence was further elevated for cells treated with ≥25 μM quercetin. Exposure to ≥10 μM quercetin abrogated menadione-induced DNA single-strand breaks but, paradoxically, quercetin exacerbated membrane damage and failed to enhance the viability of menadione-challenged cells. In conclusion, quercetin exerts only site-specific protection against oxidative stress.

Keywords: Flavonoid; HL-60; Menadione; Oxidative stress; Quercetin; Cellular integrity

1. Introduction

Reactive oxygen species (ROS) are implicated in the aetiology of degenerative disease [1,2], the incidence of which is negatively correlated with the consumption of fruit and vegetable rich diets [3–6]. Such diets contain a complex mixture of non-nutritive compounds, macro and micronutrients including potential antioxidants, e.g., various polyphenols, carotenoids, vitamin C, etc. [3–5]. However, it is not clear whether single phytochemicals or antioxidant action per se are the primary agents and mechanism respectively responsible for patho-prevention.

The flavonoids constitute a group of phyto-polyphenols, categorised with their respective glycosides into flavones, flavonols, flavanones, flavanols, isoflavones and anthocyanins, to which an array of patho-preventative effects including anti-allergenic, anti-inflammatory, anti-proliferative and anti-viral as well as anti-carcinogenic and anti-athrogenic action have been attributed [6,7]. At the cellular level flavonoid effects on development and integrity include, but are not confined to, the mitigation of oxidative damage, anti-proliferative effects, promotion of differentiation, pro-apoptotic activity and inhibition of malignant transformation [6–8].

Amongst a range of specific effects on cellular function, the anti-oxidant activity of flavonoids has received particular attention [7,9–11]. By virtue of their extensively conjugated π orbitals, flavonoids are able to donate electrons, or hydrogen from hydroxyl groups to free radicals [7,12–14]. Certain flavonoids are also Fe^{2+}/Cu^{+} chelators, inhibiting the formation of the highly reactive hydroxyl radical, $HO^{\bullet}$ [7,15].

However, the patho-preventative benefit afforded by aspects of flavonoid cellular activity are ambiguous, with some activities being mutually opposing. For example, flavonoids may also exhibit pro-oxidant activity [16–18]. While such activity contributes to therapeutic functions attributed to the flavonoids [7,18–20], it may also be damaging to normal cell integrity and possibly act as a mech-

Abbreviations: DCF, 2′7′-dichlorofluorescein; DHR, dihydrorhodamine-123; HE, dihydroethidium; H_2DCFDA, dichlorodihydrofluorescein diacetate; LDH, lactate dehydrogenase; ROS, reactive oxygen species

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anism of the genotoxicity correlated with the addition of certain flavonoids to cultured cells [20,21].

A total flavonoid dietary intake (expressed as aglycones) of 650 mg/day comprising a combined flavonol, flavanone and flavone content of 110 mg/day is reported for the USA [7]. However, this may be an overestimate [7,22]. More generally, the 'European-style diet' provides flavonol/flavone intake (expressed as aglycones) ranges from 25 to 65 mg/day depending on local dietary habits [7,22,23]. For example, The Netherlands flavonol/flavone intake is 23–33 mg/day, of which 40–70% comprises the abundant flavonol, quercetin (3,5,7,3′,4′-pentahydroxyflavone) [22]. However, supplements may provide between approximately 10 to 20 times the quercetin intake of a typical vegetable/fruit-rich diet [24].

Here, we report the effect of quercetin on reactive oxygen production, DNA and membrane integrity and survival of non-stressed and oxidatively stressed HL-60 cells, which are derived from a patient with acute promyelocytic leukaemia [25]. Oxidative stress is induced by treatment with menadione (2-methyl-1,4-naphthoquinone; vitamin K_3).

2. Materials and methods

2.1. Cell culture

HL-60 cells (European Cell Culture Collection, 98070106) were cultured in RPMI medium (Gibco-BRL) supplemented with 5 ml l^{-1} 100× non-essential amino acids, 2 mM glutamine, 50 μg ml^{-1} streptomycin, 50 units ml^{-1} penicillin (Sigma Chemical Co., Poole, Dorset, UK) and 10% (v/v) foetal calf serum (Cyclone, Gibco BRL, Paisley, UK). Cells were seeded at a density of 5×10^5 cells ml^{-1} in 75-cm^2 flasks (Greiner, Gloucestershire, UK). All incubations were conducted in a humidified atmosphere of 5% CO_2 in air, at 37°C. Cells were maintained in culture for a maximum of four weeks. Absence of enhanced differentiation within the population was determined via monitoring CD11b expression and NBT reducing activity [25].

2.2. Menadione and quercetin

Menadione and quercetin (Sigma) were dissolved in DMSO. Cells (5×10^5 ml^{-1}) were incubated for 45 min with solvent vehicle or with quercetin (1–100 μM) followed by treatment, for a further 45 min, with 20 μM menadione or solvent vehicle. In certain experiments, cells treated with quercetin or solvent vehicle were centrifuged at 300×*g* for 5 min at 4°C, resuspended in 50 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl (PBS), recentrifuged and suspended in fresh culture medium prior to the addition of menadione.

2.3. Reactive oxygen detection

To detect as broad a spectrum of ROS as possible, cells were loaded with three dyes (Molecular Probes, Leiden, The Netherlands) which fluoresce on oxidation by ROS. Hydroethidine (dihydroethidium, HE) is rapidly oxidised by O_2^- to yield ethidium which intercalates into DNA. Dichlorodihydrofluorescein diacetate (H_2DCFDA) passively diffuses into cells, is deacetylated ($DCFH_2$) and subsequently oxidised by a H_2O_2-peroxidase/Fe^{2+}/cytochrome *c* catalysed reaction, and possibly directly by other ROS to yield fluorescent 2′7′-dichlorofluorescein (DCF). Dihydrorhodamine-123 (DHR) also reacts with H_2O_2 in a peroxidase-like reaction to yield rhodamine-123 which localises to the mitochondria. DHR may also detect peroxynitrite [26–29].

Following treatment cells were incubated in 10 μM H_2DCFDA, 5 μM DHR or 5 μM HE, prepared in DMSO (final concentration 0.1% v/v). Incubation with H_2DCFDA and DHR was for 30 min and for HE, 15 min at 37°C. To prevent light accelerated oxidation, samples were maintained in the dark prior to and during analysis. Fluorescence within dye-loaded cells was analysed by fluorimetry (H_2DCFDA, DHR; Perkin Elmer, Wellesley, MA, USA) and flow cytometry (H_2DCFDA, DHR and HE; FACS Calibur; Becton Dickinson, Oxford, UK).

For flow cytometry the excitation wavelength for DCF, rhodamine-123 and ethidium fluorescence was 488 nm, and DCF and rhodamine-123 emission at 530 nm (FL-1 detector: DCF, 350 V; rhodamine, 330 V) and ethidium emission at 585 or 670 nm (FL-2 detector, 420V; FL-3 detector, 590 V). Signals were processed using a logarithmic amplifier and fluorescence distributions plotted on a 4-decade logarithmic scale (1024 channels). Fifteen thousand events were counted and, as a semi-quantitative indicator for ROS levels, the geo mean and mean linear fluorescence values were calculated using Cell Quest Software (Becton Dickinson, Oxford, UK). For fluorimetry, following dye loading, cells were centrifuged at 300×*g* for 5 min at room temperature, and the cell pellet treated with 1 ml of lysis buffer comprising 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA and 0.1% (v/v) Triton X-100. The lysate was centrifuged at 16 000×*g* for 5 min (4°C). The fluorescence of DCF or rhodamine within the lysate, the acellular incubation medium or of a lysate-acellular incubation medium mixture was analysed at excitation 488 nm and emission 530 nm.

For both cytometry and fluorimetry, non-dye-loaded cells with and without menadione and/or quercetin were used to determine and correct for changes to background fluorescence. To determine potential quenching and dye–menadione interactions, free DCF (excitation 480 nm, emission 530 nm) or ethidium (emission 585 nm) were incubated with 1–100 μM quercetin and/or menadione in

either solvent vehicle alone or in combination with cell culture medium.

2.4. Glutathione determination

Cells were centrifuged at 300×*g* for 5 min at 4°C, media removed and the pellet resuspended in PBS and recentrifuged. The cell pellet was suspended in 500 μl of 0.8% (w/v) sulphosalicylic acid and 4.2 mM EDTA, vortex mixed and centrifuged at 16 000×*g* for 5 min (4°C). The supernatant was used to determine total cellular glutathione as described [30].

2.5. DNA damage

DNA single-strand breaks were assessed by incubating 1.8×10^7 cells ml^{-1} with 0.1 μCi [^{3}H]thymidine ml^{-1} for 16 h at 37°C, 5% CO_2 within a humidified atmosphere. Cells were then washed by centrifugation (300×*g*, 5 min, room temperature) and resuspended (5×10^5 cells ml^{-1}) in fresh culture media for treatment with quercetin and/or menadione. Following cell lysis, the proportion of DNA that was single-stranded, as a measure of strand breaks, was estimated following alkali treatment and hydroxyapatite (DNA grade; BioRad, Hertfordshire, UK) chromatography as described in Burkitt et al. [31].

2.6. Membrane integrity

The release of lactate dehydrogenase (LDH) was used to determine membrane integrity. Cell suspensions were centrifuged at 500×*g* for 5 min (4°C). Cell pellets were extracted by sonication in 50 mM Na_2HPO_4/NaH_2PO_4 containing 0.1% (v/v) Triton X-100, centrifuged at 16 000×*g* (4°C) for 5 min and the supernatant used for the assay of cellular LDH. The remaining cell culture medium was centrifuged at 1000×*g* for 5 min (4°C) and the supernatant used as the source of leaked LDH. The percentage of LDH release into the culture medium was assayed and calculated, with correction for volume and serum LDH, as described previously [32].

2.7. Cell viability

Fluorescein diacetate (FDA) was used to determine immediate post-treatment changes in viability [33]. Cells were centrifuged at 300×*g* for 5 min (4°C), resuspended in

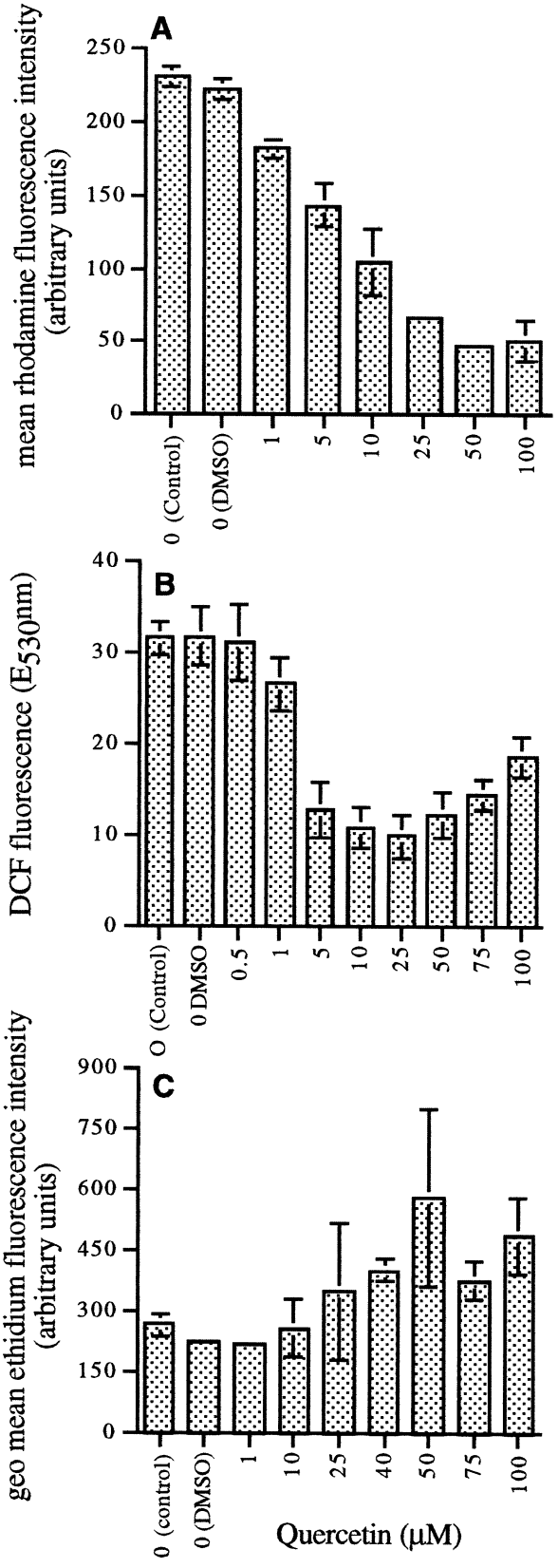

Fig. 1. Effect of quercetin on HL-60 cellular ROS levels. HL-60 cells were exposed to quercetin, water or DMSO prior to incubation with the ROS-sensitive dyes DHR (A) H_2DCFDA (B) or HE (C). Analysis of intracellular fluorescence was conducted by flow cytometry (A,C) and fluorescence within combined cell lysates and acellular culture media analysed by fluorimetry (B). Data are mean ± S.D. ($n\geq6$). Small errors are contained within the histograms.

0.5 ml of PBS comprising 0.2% (w/v) FDA prepared in acetone (final acetone concentration, 0.2% v/v) for 15 min at 37°C and analysed by flow cytometry (excitation 488 nm, emission 530 nm; FL-1, 310 V).

A longer term effect on viability was assessed by the delayed thymidine incorporation assay. Following treatment, cells were washed twice by centrifugation (350×*g*, 5 min) and resuspended in fresh culture medium without quercetin or menadione. After 24 h, cells were exposed to 1 μCi ^{3}H thymidine ml^{-1} for 30 min at 37°C, then centrifuged (350×*g*, 5 min), washed twice with PBS, fixed with 1 ml 5% (w/v) trichloroacetic acid (30 min at RT) and again washed twice (PBS and centrifugation; 350×*g*, 5 min). Samples were warm air-dried and 1 M NaOH (250 μl) added to digest acid-insoluble material for 14 h. The digest (100 μl) was added to 5 ml of scintillation fluid (Packard Ultima Gold; Packard 1900TR Liquid Scintillation Counter, Packard Bioscience, Groningen, The Netherlands). The mean radioactivity count from treated cells was expressed as a percentage of radioactivity from control water-treated cells, referred to as the survival index [34].

3. Results

3.1. Reactive oxygen

Treatment of HL-60 cells for 90 min with 1–100 μM quercetin alone lowered rhodamine fluorescence (Fig. 1A) and DCF fluorescence decreased in cells incubated with 5–100 μM quercetin (Fig. 1B). Cellular ethidium fluorescence, indicative of O_2^-, was largely unaltered with 1–25 μM quercetin treatment but enhanced in cells exposed to 40–100 μM quercetin (Fig. 1C).

Menadione treatment (Fig. 2) elevated rhodamine-123 (Fig. 2A), DCF (Fig. 2B) and ethidium (Fig. 2C) fluorescence, indicative of heightened ROS production. For cells exposed to menadione (Fig. 3), whether compared by flow cytometric analysis of intracellular fluorescence (Fig. 3A,B) or fluorimeter based measurements of fluorescence within cell extracts alone or combined with acellular dye-incubation medium (Fig. 3C), prior and continued exposure to quercetin (1–100 μM) resulted in diminished rhodamine (Fig. 3A) and DCF (Fig. 3B,C) fluorescence compared to menadione treatment alone. Removal of extracellular quercetin (45 min incubation) prior to menadione exposure also resulted in a lowering of menadione enhanced DCF (Fig. 3D) or rhodamine fluorescence (data not shown). Although the washing procedure appeared to encourage greater experimental variability, the extent of fluorescence decrease was generally less than that observed for cells maintained in quercetin-supplemented media during menadione exposure (Fig. 3B).

Prior to treatment with menadione, cells incubated for 45 min with 10–100 μM quercetin also possessed consis-

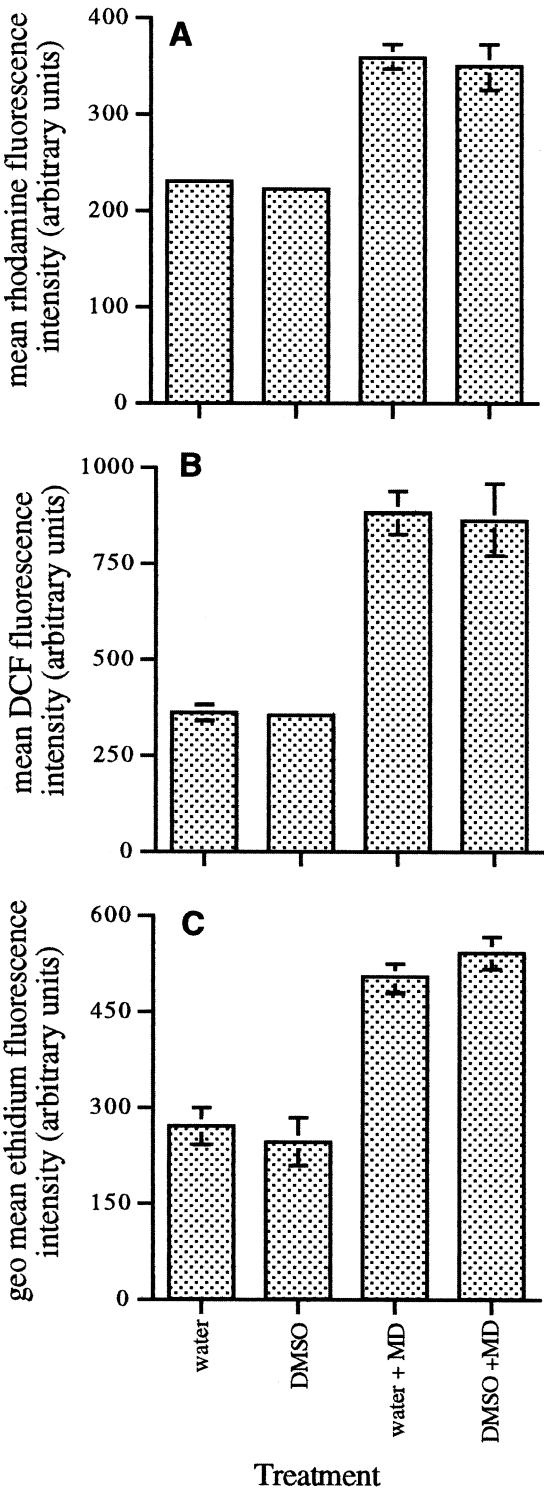

Fig. 2. Effect of menadione treatment on ROS levels within HL-60 cells. Cells were incubated for 45 min with water (control) or DMSO (solvent control) then exposed, for a further 45 min, to menadione (MD) or further additions of water or DMSO. ROS/oxidative stress levels were determined via flow cytometric analysis of fluorescence within cells loaded with either DHR 123 (A), $DCFH_2DA$ (B) or HE (C). Data are mean ± S.D. ($n = 3$).

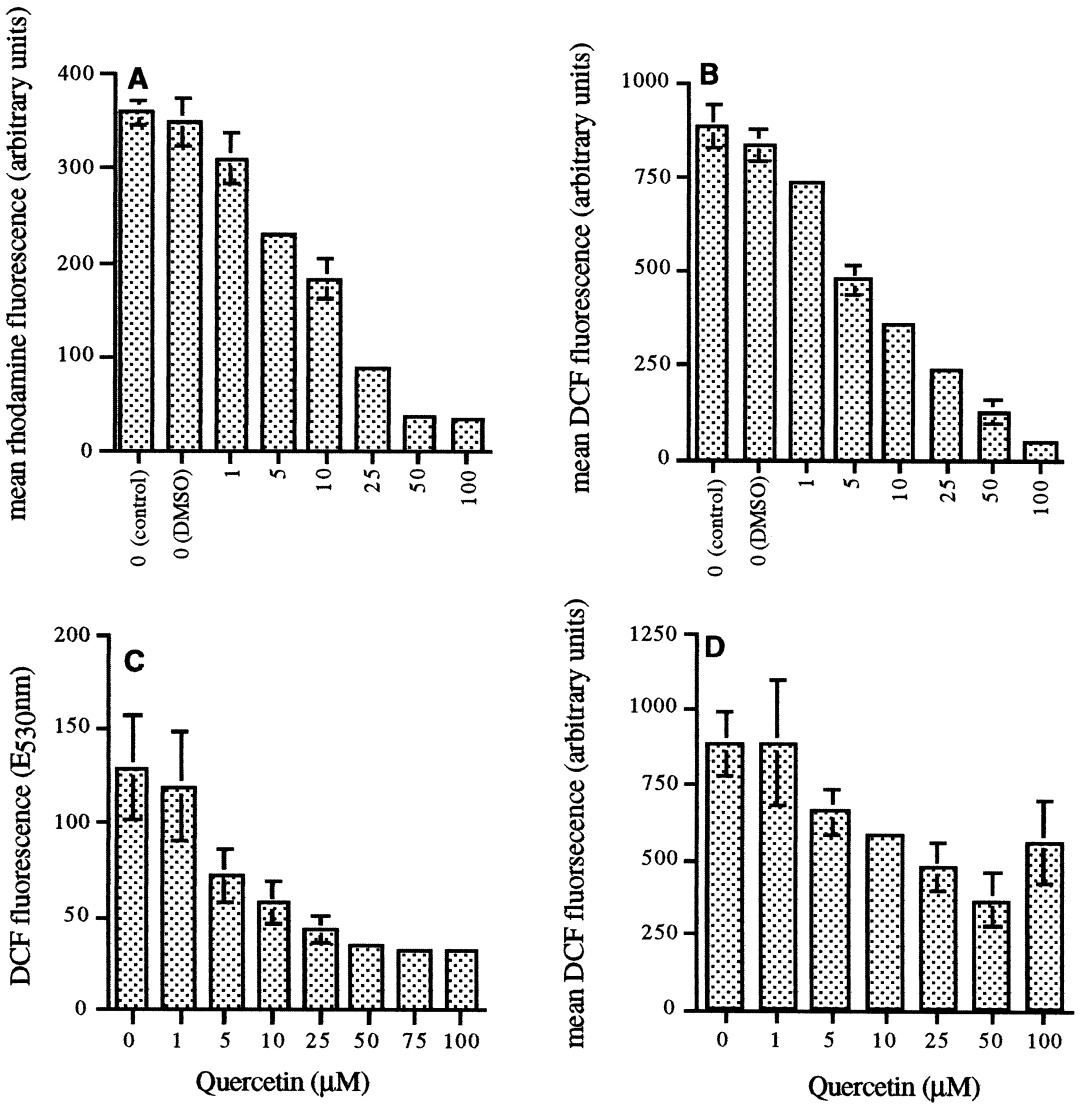

Fig. 3. Influence of quercetin on ROS/oxidative stress within menadione exposed cells. ROS production in cells subject to quercetin, water or DMSO treatment prior to and during exposure to menadione was determined by loading cells with either DHR 123 (A) or $DCFH_2DA$ (B,C). Intracellular fluorescence was analysed by flow cytometry (A,B). Fluorescence of cell lysates combined with acellular culture media, allowing for determination of any oxidised dye leakage, was analysed spectrofluorimetrically (C). Data (A–C) are mean ± S.D. ($n \geq 6$). Further replicates were conducted at altered FL-1 detector voltages and revealed an identical trend. Fluorescence was also flow-cytometrically determined within cultures from which DMSO or quercetin was removed prior to menadione exposure (D). Data (D) are mean ± S.D. from three independent cultures from a representative experiment. Small errors (A–D) are contained within the histograms.

tently decreased levels of DCF fluorescence relative to water- and DMSO-treated cultures (Fig. 4). This decrease may have contributed to, but could not account for the subsequent magnitude of the decrease in menadione-induced fluorescence in the presence of the respective concentration of quercetin (Fig. 4). However, the relative change (fold increase) in fluorescence over that measured immediately prior to menadione addition was in general, similar for non-quercetin-treated cells and cells incubated with 1–50 μM quercetin (Fig. 4). Comparable results were obtained for rhodamine fluorescence (data not shown).

For menadione-treated cells, the influence of quercetin on O_2^- indicative ethidium fluorescence again contrasted with the effect of quercetin treatment on DCF and rhodamine fluorescence. Cells incubated with ≥ 25 μM quercetin prior to and during menadione exposure exhibited an enhanced ethidium fluorescence relative to that induced by menadione addition to non-quercetin-treated cultures (Fig. 5).

Preliminary investigations confirmed an absence of direct quercetin and or menadione influence on rhodamine-123 or ethidium fluorescence emissions in vitro in the range 1–100 μM. However an approximate 14% decrease in DCF fluorescence was observed with 100 μM quercetin alone, and an approximate 16% increase in fluorescence emissions for a combination of 100 μM quercetin with menadione. This may suggest the potential for a slight under- and overestimate of ROS levels in H_2DCFDA

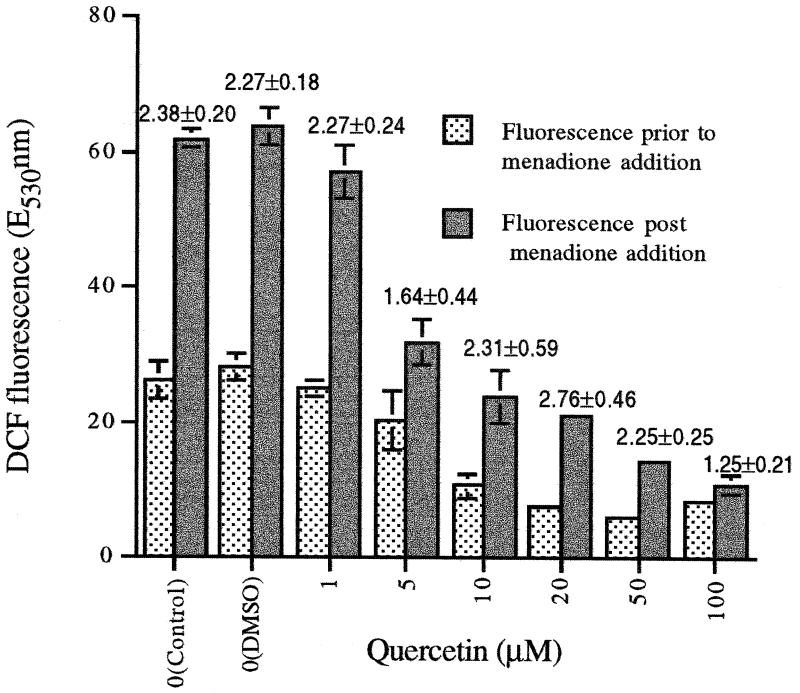

Fig. 4. Effect of quercetin treatment on ROS levels prior to menadione exposure. DCF fluorescence was spectrofluorimetrically determined in cells treated for 45 min with water, DMSO or quercetin (light bars) and compared to fluorescence following the subsequent 45 min incubation with menadione (dark bars). Numbers above the histogram refer to the fold increase in fluorescence induced by menadione relative to the fluorescence level induced by the respective treatment prior to menadione exposure. Data are mean ± S.D. ($n = 3$) from three independent cultures from a representative experiment. Small errors are contained within the histogram.

loaded cells exposed to quercetin alone or in combination with menadione, respectively. Addition of 8.8M H_2O_2 to oxidise all $DCFH_2$ revealed no change in this dye's uptake between treatments (data not shown).

3.2. Glutathione

Incubation for 90 min with 1 μM quercetin alone increased cellular glutathione levels (Fig. 6). Correspondingly, there was less depletion of glutathione for cells treated with 1 μM quercetin prior to and during menadione exposure. There was no alteration of glutathione content with 10 and 100 μM quercetin treatment alone and 10 and 100 μM quercetin treatment also had no effect on the extent of menadione-induced glutathione depletion (Fig. 6).

3.3. DNA damage

Incubation with quercetin alone did not induce increased DNA single-strand breakage (Fig. 7). Exposure to menadione induced extensive single-strand DNA breaks, which were completely prevented by treatment with 10 or 100 μM quercetin (Fig. 7).

3.4. Membrane integrity

LDH leakage, indicative of membrane damage, was elevated following menadione treatment. Prior and continued treatment with 1 μM quercetin was ineffective at preventing LDH leakage and treatment with 10 or 100 μM quercetin exacerbated the extent of leakage from menadione-treated cells (Fig. 8).

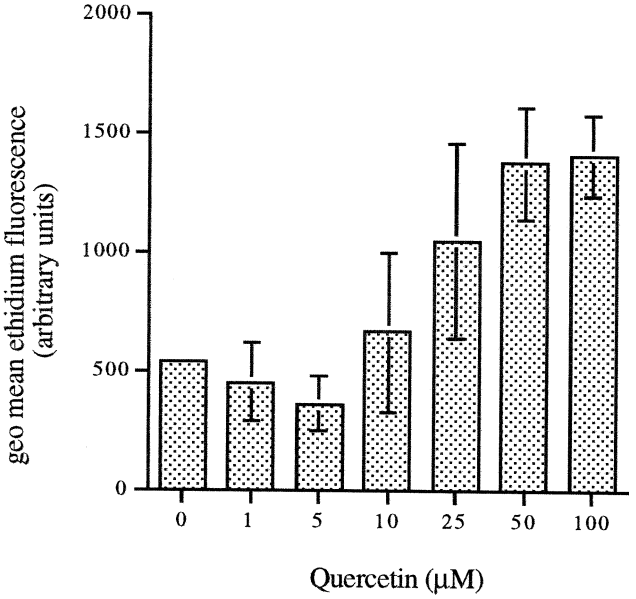

Fig. 5. O_2^- within menadione-exposed cells. Cells were incubated with DMSO or quercetin prior to and during menadione exposure. Intracellular O_2^- was detected by flow-cytometric analysis of ethidium fluorescence within HE loaded cells. Data are mean ± S.D. ($n = 6$).

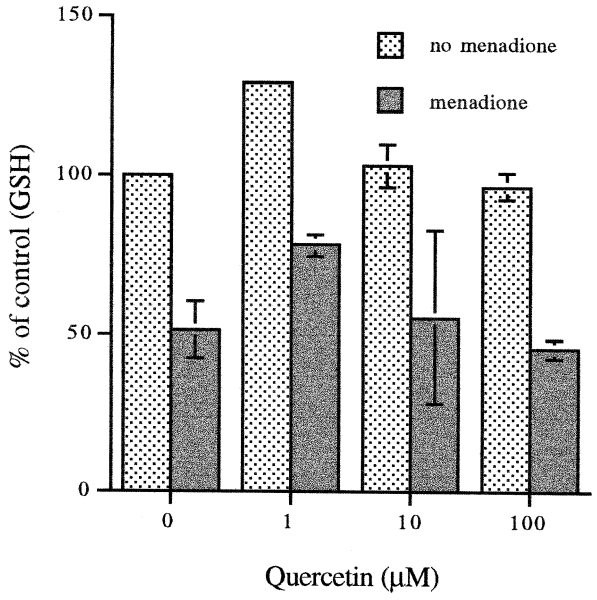

Fig. 6. Total glutathione. Cells were either treated with DMSO or quercetin for 45 min and then exposed to either DMSO or menadione for a further 45 min and changes in glutathione concentration determined. Data are mean ± S.D. ($n = 6$).

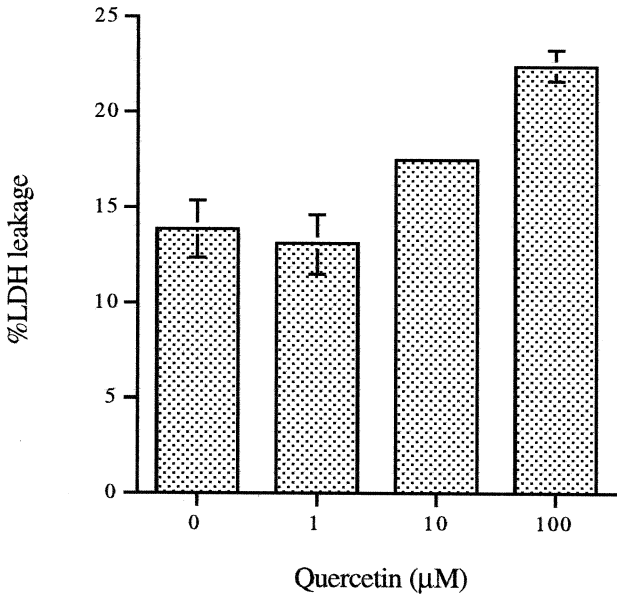

Fig. 8. Membrane damage. As an indicator of membrane integrity, LDH leakage was determined in cells treated with DMSO or quercetin prior to and during exposure to menadione. Data are mean ± S.D. from three independent cultures (assayed in triplicate) from a representative experiment. The experiment was repeated twice, revealing an identical trend. Small errors are contained within the histogram.

3.5. Cell viability

The intensity distribution of fluorescein fluorescence varied considerably following quercetin exposure alone but there was no significant decrease in the intensity of the fluorescence distribution within the cell population relative to non-quercetin-treated cells. This suggests no immediate effect of quercetin on membrane integrity and thereby gross cell viability (Fig. 9A). The extent of fluorescein fluorescence declined by approximately 33% after treatment with menadione. Loss of fluorescein fluorescence was not prevented by pre- and continued treatment with 1 μM quercetin and, in agreement with the exacerbation of LDH leakage from cells treated with quercetin and menadione (Fig. 8), ≥ 5 μM quercetin exposure resulted in a further loss of fluorescence (Fig. 9B).

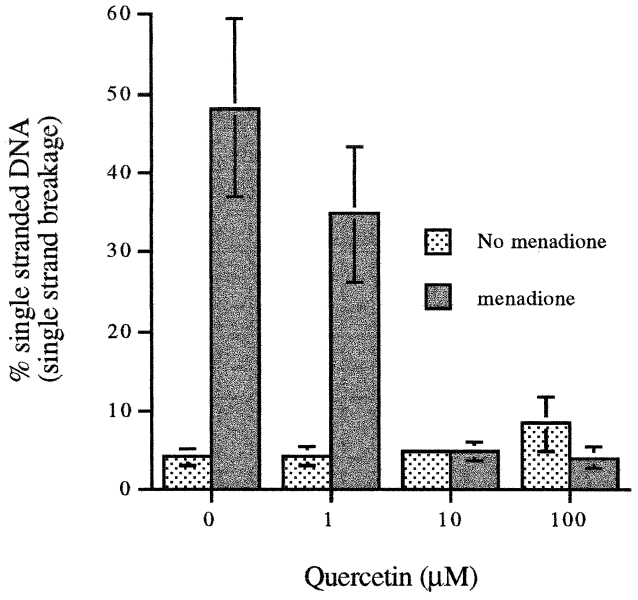

Fig. 7. DNA Damage. Cells were either treated with DMSO or quercetin for 45 min and then exposed to either DMSO or menadione for a further 45 min. Strand break formation was determined as the percentage of DNA single-stranded following treatment in alkali. Data are mean ± S.D. ($n = 6$). Small errors are contained within the histogram.

Following recovery in fresh culture media, treatment with 10 and 50 μM quercetin alone induced a decrease in [^{3}H]thymidine incorporation (Fig. 9C). Relative to control treatments (Fig. 9C) menadione treatment all but abolished [^{3}H]thymidine incorporation (Fig. 9D) and quercetin treatments (5, 10 and 50 μM) failed to increase the level of [^{3}H]thymidine incorporation in cells stressed with menadione (Fig. 9D).

4. Discussion

The flavonoids are proposed to act as in vivo anti-oxidants and thereby, in part, contribute to the patho-preventative activity correlated with the consumption of a fruit- and vegetable-rich diet [6,7]. Plant cell extracts, comprising complex polyphenol mixtures, and also individual flavonoids protect numerous cell types from oxidative stress [7]. Quercetin is a scavenger of O_2^-, NO$^\bullet$, HO$^\bullet$, peroxy radicals and an iron chelator [7,12–15]. Examples of potentially physiologically relevant quercetin anti-oxidant

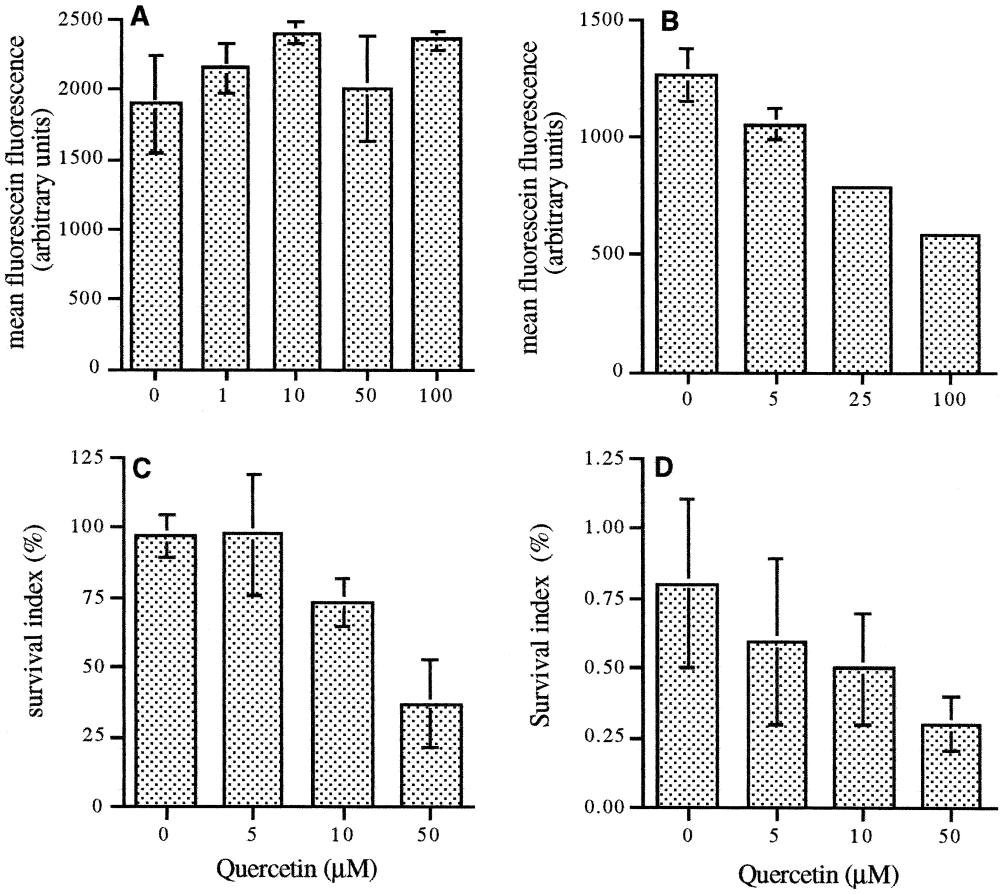

Fig. 9. HL-60 viability. The immediate effect of quercetin treatment on HL-60 viability in non-menadione-treated (A) and menadione-stressed (B) cells was assessed by flow cytometric analysis of fluorescein fluorescence within FDA-stained cells. In addition, a delayed thymidine incorporation assay determined longer term effects of quercetin on cell survival. Following treatment with quercetin or solvent vehicle alone (C) or in combination with menadione (D), cells were resuspended in fresh culture medium for a recovery period of 24 h prior to analysis. Data are mean ± S.D. ($n = 6$). Small errors are contained within the histograms. Note differences in y-axis scale.

action include the prevention of in vitro LDL peroxidation (reviewed in [7]), the protection (IC_{50} 0.1 μM) of lymphoid cell lines from the cytotoxic effects of oxidised LDL [35], at ≥10 μM protection of human lymphocytes from H_2O_2-induced DNA damage [36] and the abrogation (EC_{50} 20–40 μM) of oxidant injury within glutathione depleted cells [9].

To investigate quercetin anti-oxidant activity, HL-60 cells were loaded with the ROS-sensitive dyes H_2DCFDA and DHR. These dyes are effective indicators of total cellular ROS levels/oxidative stress status but cannot be considered specific for individual reactive oxygen species [26–29]. The use of DHR as a detector of ROS, in addition to H_2DCFDA, allows any effects of changes in esterase activity that might affect the release of $DCFH_2$ within H_2DCFDA-loaded cells to be negated. The spectrofluorimetric analysis of fluorescence within cell lysates combined with acellular culture media also allowed for any artefacts arising from oxidised dye leakage to be negated. HE is more selective for O_2^- [27], although it too can oxidised by peroxidase-H_2O_2 and is slowly oxidised by high concentrations of H_2O_2 [37]. These dyes have been widely used to monitor changes to ROS levels within cell cultures including following treatment with plant extracts and individual phyto-chemicals (e.g. [8,28,32,37–40]).

The partial mitigation of $DCFH_2$/DHR oxidation induced by quercetin treatment alone, or in conjunction with menadione exposure, indicates that this flavonol does exert, either directly or indirectly, an anti-oxidant effect. However, the increase in ethidium fluorescence at high (≥40 μM) quercetin concentrations in cells treated with flavonol alone and the elevation of ethidium fluorescence in cells exposed to a combination of ≥25 μM quercetin and menadione, indicates that quercetin treatment does not universally lower all ROS production.

The effects of quercetin treatment on cellular damage and survival are also contradictory. Quercetin provided considerable protection against DNA susceptibility to menadione-induced DNA single-strand breaks. This is in agreement with a decreased susceptibility of lymphocyte and colonocyte DNA to H_2O_2-induced single-strand breakage following short-term (30 min) incubation with 50 μM quercetin [36,41]. Incubation for 24 h with 50 μM quercetin also protected Caco-2 cells from menadione

(10 μM) and *tert*-butylhydroperoxide-induced DNA strand breaks [42]. However, for HL-60 cells, protection against menadione-induced damage is limited, as quercetin failed to prevent or even exacerbated membrane damage.

The mechanism(s) determining this paradoxical effect of quercetin on HL-60 cell integrity remain(s) to be determined. Metal chelation may be a factor in the differing effect of quercetin on DHR/rhodamine relative to ethidium fluorescence. The peroxidase-like activity involved in the H_2O_2 mediated oxidation of both DHR and $DCFH_2$ [26,28] may be sensitive to inhibition due to iron chelation. Although quercetin is potentially a scavenger of O_2^-, the absence of any appreciable decrease in ethidium fluorescence within menadione exposed cells implies that metal chelation, rather than general radical scavenging, may be the principal feature of the quercetin anti-oxidant activity [15]. Thus, quercetin may orchestrate an altered spectrum of ROS production, possibly with less propensity for $HO^{\bullet}$ generation. This may afford some selective protection against oxidative DNA damage which is generally dependent on iron/copper availability [15]. Other subcellular sites may be directly susceptible to other ROS (e.g., the hydroperoxyl radical and the plasma membrane) leading to the failure of quercetin to mitigate the toxicity of menadione despite providing DNA protection. Duthie and Dobson [41] have suggested that, in addition to acting as an anti-oxidant, quercetin may directly stabilise the DNA molecule.

The reported effects of flavonoids on cell function and integrity vary from cytoprotection through to geno- and cytotoxicity [7,21,24]. Sergediene et al. [18] report a 50% reduction in HL-60 cell viability following a 24h exposure to 100 μM quercetin. At concentrations ranging from 30 to 100 μM quercetin cytotoxicity within HL-60 cells most probably results from the induction of apoptosis [19,43]. Quercetin cytotoxicity, whether manifest through apoptosis or necrosis, may be associated with pro-oxidant activity [18,19] but, as reported here, a general pro-oxidant effect cannot account for the immediate influence of quercetin on menadione toxicity.

To what extent are effects with quercetin aglycone representative of those that may result from dietary intake? Although the free aglycone is present in red wines, quercetin is encountered predominantly as glycosides [6,7,44–46]. The site and mechanism of quercetin absorption, and that of flavonoids in general, is currently unresolved [45,46]. However, absorption in the small intestine as both the hydrolysed aglycone and glycosides is reported [47–49]. Colonic microflora may metabolise glycosides, possibly allowing absorption of free aglycones although further degradation may occur [44]. The absorption, from the small intestine, and presence in plasma of apparently intact quercetin glycosides is reported [47] but glycosides are generally thought to be deglycosylated either in the intestinal lumen or within the enterocyte following transport [45,46,49]. Further metabolism may include methylation, hydroxylation, conjugation with sulphate and/or glucuronic acid [45,46]. Such conjugates/metabolites possess varying degrees of anti-oxidant activity [22,45,50]. With regard to cell cultures, there have been few studies of flavonoid/quercetin metabolism. For Hep G2 hepatocarcinoma cells [51], absorbed quercetin is completely metabolised or degraded within 8 h. Formation of the methylated metabolite isorhamnetin and polymeric quercetin was detected. However, the decline in native quercetin primarily resulted from oxidative degradation leading to the formation of protocatechuic acid, also a potential anti-oxidant [51,52].

Thus, factors influencing the cellular response include the cell-flavonoid concentration, the cell and flavonoid type, the degree of flavonoid metabolic conversion and/or degradation into a range of potentially bioactive products, the nature of any pre-existing or subsequent stress and, for cells ex vivo, the culture conditions (e.g. [36,41,50,51,53,54]). The contrasting effects of quercetin on DNA and membrane integrity suggest that subcellular flavonol distribution and concentration may also be a determinant of the cellular response. Such a potential diversity of effects may both confound attempts to determine any patho-preventative or therapeutic benefit for quercetin/flavonoids, but also suggests that quercetin and other flavonoids are unlikely to exert a universally predictable effect within differing cell and tissue types in vivo.

In conclusion, for non-menadione stressed HL-60 cells, within a range of 1–100 μM, quercetin concentrations <10 μM are not overtly cytotoxic and do lower basal intracellular ROS levels. It remains to be determined whether this exerts any influence on cellular redox-sensitive signalling and function [55] but, for cells subsequently subject to menadione-induced oxidative stress, concentrations of <10 μM do not clearly act as a 'stress cytoprotectant'. At ≥ 10 μM, quercetin protects against menadione-induced DNA single-strand breaks but, conversely, exacerbates membrane damage. These apparent contradictory effects of quercetin on HL-60 integrity may reflect both diversity in and conflict between the various activities attributed to the flavonoids [7] and, under the experimental conditions described, quercetin cannot be considered as a general cytoprotectant.

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