Cardioprotective and cardiotoxic effects of quercetin and two of its in vivo metabolites on differentiated H9c2 cardiomyocytes

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Abstract

Whilst mitotic rat embryonic cardiomyoblast-derived H9c2 cells have been widely used as a model system to study the protective mechanisms associated with flavonoids, they are not fully differentiated cardiac cells. Hence the aim of this study was to investigate the cardioprotective and cardiotoxic actions of quercetin and two of its major *in vivo* metabolites, quercetin 3-glucuronide and 3'-*O*-methyl quercetin, using differentiated H9c2 cells. The differentiated cardiomyocyte-like phenotype was confirmed by monitoring expression of cardiac troponin 1 following 7 days of culture in reduced serum medium containing 10 nM *all-trans* retinoic acid. Quercetin-induced cardiotoxicity was assessed by monitoring MTT reduction, LDH release, caspase 3 activity and reactive oxygen species production following prolonged flavonoid exposure (72 h). Cardiotoxicity was observed with quercetin and 3'-*O*-methyl quercetin but not quercetin 3-glucuronide. Cardioprotection was assessed by pre-treating differentiated H9c2 cells with quercetin or its metabolites for 24 h prior to 2 h exposure to 600 μM H_2O_2, after which oxidative stress-induced cell damage was assessed by measuring MTT reduction and LDH release. Cardioprotection was observed with quercetin and 3'-*O*-methyl quercetin but not with quercetin 3-glucuronide. Quercetin attenuated H_2O_2-induced activation of ERK1/2, PKB, p38 MAPK and JNK but inhibitors of these kinases did not modulate quercetin-induced protection or H_2O_2-induced cell death. In summary, quercetin triggers cardioprotection against oxidative stress–induced cell death and cardiotoxicity following prolonged exposure. Further studies are required to investigate the complex interplay between the numerous signalling pathways that are modulated by quercetin and which may contribute to the cardioprotective and cardiotoxic effects of this important flavonoid.

Keywords: differentiation, flavonoids, H9c2 cells, hypoxia, oxidative stress, protein kinases, quercetin, 3'-*O*-methyl quercetin, quercetin 3-glucuronide

Introduction

Flavonoids are a large group of compounds (>4000) that occur naturally in fruits, vegetables, nuts, seeds, flowers and other plant matter and as such they are an integral part of the human diet [1]. They all share a common three-ring structure but are sub-divided into flavonols, flavons, flavanols and flavanons according to their substituents [2]. Epidemiological studies indicate that diets rich in flavonoids are associated with reduced incidences of several chronic diseases including cardiovascular disease, asthma, type II diabetes and certain types of cancer [3]. The cardioprotective properties of flavonoids are multi-faceted involving anti-oxidant, anti-hypercholesterolemia, anti-inflammatory, and inhibition of platelet aggregation effects [3]. The anti-oxidant property of flavonoids was thought, until relatively recently, to underlie the majority of their protective cellular effects. However, it is becoming increasingly apparent that flavonoids also influence cellular function by modulating the activity of many enzymes including the inhibition of protein kinases and lipid kinases [1,4].

The inhibitory effect of flavonoids on protein kinase activity is primarily due to their ability to function as competitive inhibitors of the ATP-binding domain located in the active site of these enzymes [1,4]. Protein kinases directly inhibited by flavonoids include protein kinase C (PKC), myosin-light chain kinase (MLCK), and MEK1 [1,4]. MEK1 is an up-stream kinase responsible for the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), a member of the mitogen-activated protein kinase (MAPK) family of protein kinases [5]. This family also includes p38 MAPK and JNK signalling pathways, both of which are targets for modulation by flavonoids [6-8]. It is also notable that flavonoids such as quercetin also inhibit (via competitive inhibition of the ATP-binding site) the lipid kinase PI-3Kγ [9], which plays a prominent role in the activation of protein kinase B (PKB; also known as Akt).

The MEK/ERK1/2 and the PI-3K/PKB pathways are important signalling pathways which mediate cell survival and cardioprotection against ischaemia/reperfusion injury

[10,11]. In contrast, p38 MAPK and JNK cascades are typically associated with inflammation and cell death; however there is also evidence that they mediate cardioprotection [11]. Hence the cardioprotective effects of flavonoids may relate to their ability to modulate MAPK and PI-3K/PKB signalling cascades. Indeed, recent studies have shown that grape seed proanthocyanidin extract protects cardiomyocytes from ischaemia/reperfusion injury via PKB activation [12]. Similarly, quercetin (the most abundant dietary flavonoid) protected H9c2 cardiomyoblasts from H_2O_2-induced cell death via enhancement of ERK1/2 and PI-3K/PKB signalling [13]. In contrast, isorhamnetin (a flavonol isolated from sea buckthorn) prevented H_2O_2-induced activation of apoptosis in H9c2 cells through ERK1/2 inactivation [14].

It is notable that rat embryonic cardiomyoblast-derived H9c2 cells [15] have been widely used as a model system to study the protective mechanisms associated with flavonoids [6,7,13,14,16,17]. However, these studies have all been performed using mitotic H9c2 cardiomyoblasts, which although possessing similar morphological, electrophysiological, and biochemical properties to primary cardiomyocytes [18] are not fully differentiated cardiac cells. H9c2 cardiomyoblasts can be differentiated towards a more cardiomyocyte-like phenotype by culturing cells in reduced serum medium containing *all-trans* retinoic acid [19,20]. These conditions promote the expression of cardiac specific L-type Ca^{2+} channels and troponin 1 and induce the appearance of cardiomyocyte-like ultra-structural features [19,20].

The primary aim of this study was to investigate the cardioprotective actions of quercetin and two of its major *in vivo* metabolites (quercetin 3-glucuronide and 3'-*O*-methyl quercetin*)* using differentiated H9c2 cells. The ability of quercetin and its metabolites to elicit cell survival were assessed by monitoring oxidative stress-induced cell death. The potential role of MAPK (e.g. ERK1/2, p38 MAPK and JNK) and PKB signalling pathways in mediating the protective effects of quercetin were also explored. The main findings of the study indicate that quercetin protects differentiated H9c2 cardiac cells from oxidative stress induced death possibly via the inactivation of MAPK and PKB signalling.

Materials and Methods

Materials

Quercetin, quercetin 3-glucuronide, and *all-trans* retinoic acid were purchased from Sigma-Aldrich l Co. Ltd. (Poole, Dorset, UK). 3'-*O*-methyl quercetin was obtained from Extrasynthese (France). LY 294002, PD 98059, SB 203580, SP 600 125 and wortmannin were obtained from Tocris Bioscience (Bristol, UK). Dulbecco's modified Eagle's Medium (DMEM), foetal bovine serum (FBS), trypsin (10×), L-glutamine (200 mM), penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) were purchased from Lonza Group Ltd. Antibodies were obtained from the following suppliers: monoclonal phospho-specific ERK1/2 (Thr^{202}/Tyr^{204}) from Sigma-Aldrich; polyclonal phospho-specific PKB (Ser^{473}), polyclonal total unphosphorylated PKB, monoclonal total unphosphorylated ERK1/2, polyclonal total unphosphorylated JNK, polyclonal total unphosphorylated p38 MAPK, monoclonal phospho-specific p38 MAPK, and polyclonal cleaved active caspase 3 were from New England Biolabs (UK) Ltd; monoclonal phospho-specific JNK was from Santa Cruz Biotechnology Inc and cardiac specific troponin 1 from Abcam (Cambridge, UK). All other chemicals were of analytical grade. Stock concentrations of quercetin (100 mM) were dissolved in DMSO, which was present in all treatments including the control at a final concentration of 0.1% (v/v).

Cell culture

Rat embryonic cardiomyoblast-derived H9c2 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). Undifferentiated cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 10% (v/v) FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were maintained in a humidified incubator (95% air/5% CO_2 at 37°C) until 70-80% confluent, detached using trypsin (0.05% w/v)/EDTA (0.02% w/v) and sub-cultured (1:5 split ratio. Differentiation of H9c2 cells was induced by culturing the cells for 7 days in DMEM supplemented with 1% FBS (v/v) and 10 nM *all-trans* retinoic acid [19,20]. The

medium was replaced every two days and differentiation into a more cardiomyocyte-like phenotype confirmed by monitoring the expression of cardiac troponin 1 by immunocytochemistry and Western blotting, as described previously [20].

Immunocytochemistry

The differentiation of H9c2 cells was visually analysed by monitoring the expression of cardiac specific troponin 1 via immunocytochemistry. H9c2 cells were seeded in 8-well chamber slides (BD Falcon™ CultureSlide) at a density of 1.5 $\times 10^4$ cells/well and cultured for 24 h, after which the medium was removed, replaced with differentiation medium and incubated for a further 7 days, changing the medium every two days. The medium was then removed and adherent differentiated cells washed with phosphate buffered saline (PBS) pre-warmed to 37°C . Cells were fixed with 3.7% (w/v) paraformaldehyde (Sigma-Aldrich, UK) in PBS, for 15 min at room temperature without agitation and then washed gently three times for 5 min with PBS. After fixation, permeabilisation was performed by incubating cells for 15 min at room temperature with 0.1% (v/v) Triton X-100 in PBS followed by washing three times for 5 min with PBS. Cells were then incubated for 1 h at room temperature with 3% (w/v) bovine serum albumin (BSA) in PBS to prevent non-specific antibody binding. Cells were then incubated overnight at 4°C in a humidified chamber with anti-cardiac specific troponin 1 antibody (1:1000) in 3% (w/v) BSA in PBS. Unbound primary antibody was then removed and the wells washed three times for 5 min with PBS. Cells were then incubated for 2 h at 37°C in a humidified chamber with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (Abcam), diluted 1:1000) in 3% (w/v) BSA in PBS. The chamber slides was subsequently washed three times for 5 min with PBS, air dried and mounted with Vectashield® medium (Vector Laboratories Ltd, Peterborough, UK) containing DAPI counterstain for nuclei visualisation. Finally slides were sealed using clear, colourless nail varnish and stained cells visualised using an Olympus DP71 epifluorescence microscope system equipped with an argon/krypton laser (FITC: E_{495}/E_{278}; DAPI: E_{360}/E_{460}).

Oxidative stress-induced cell death

Differentiated H9c2 cells in fully supplemented DMEM were treated for 2 h with 600 μM H_2O_2. To assess the effects of quercetin, cells were treated for 24 h in fully supplemented DMEM prior to 2 h H_2O_2 exposure. Where appropriate, cells were also treated for 30 min with the following protein kinase inhibitors; PD 98059 (50 μM; MEK1/2 inhibitor), LY 294002 (30 μM; PI-3K inhibitor); wortmannin (100 nM; PI-3K inhibitor), SB 203580 (30 μM; p38 MAPK inhibitor) or SP 600125 (10 μM; JNK1/2 inhibitor) before the addition of quercetin (24 h in the continued presence of the kinase inhibitors). Medium containing quercetin and inhibitors was removed and replaced with fresh fully supplemented DMEM prior to H_2O_2 treatment. In a separate set of experiments cells were treated for 24 h with the above protein kinase inhibitors prior to H_2O_2 treatment.

MTT reduction assay

Undifferentiated H9c2 cells were plated in 24-well flat bottomed plates (15,000 cells per well) and cultured for 24 h in fully supplemented DMEM. Cells were subsequently induced to differentiate for 7 days as described above. Cell viability was determined by measuring the metabolic reduction of MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a coloured formazan product. Briefly, cells were incubated for 1 h in 0.5 mg/ml MTT (in the continued presence of H_2O_2 or hypoxia) after which the medium was removed and replaced with 200 μl DMSO. The magnitude of the reduction reaction was determined by monitoring the absorbance of the solubilised formazan product at 570 nm.

Lactate dehydrogenase assay

H9c2 cells were plated in 96-well flat bottomed plates (5,000 cells per well) and cultured for 24 h in fully supplemented DMEM. Cells were subsequently induced to differentiate for 7 days prior to experimentation. The activity of lactate dehydrogenase (LDH) released into the culture medium was detected colourimetrically using the CytoTox 96® Non-

Radioactive Cytotoxicity assay (Promega, Southampton, UK). Assays were performed according to the manufacturer's instructions and changes in absorbance monitored at 490 nm.

Western blot analysis of caspase 3 activity and protein kinase phosphorylation

Analysis of caspase 3 activity and protein kinase phosphorylation was performed using differentiated H9c2 cells (7 days) cultured in 25 cm^2 tissue culture flasks. Following experimentation, cell supernatants were removed and the cells washed twice with warm PBS (37°C) to remove serum proteins, after which 300 μl of hot sodium dodecyl sulphate buffer (0.5% w/v SDS in Tris buffered saline: TBS; 20 mM Tris base, 150 mM NaCl) were added and the resultant cell lysate boiled for 5 min prior to storage at -20°C. Protein concentration was determined using Bio-Rad *DC* Protein assay (Bio-Rad laboratories, Hertfordshire, UK) with BSA as the standard. Protein samples (30 μg) were separated by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) in a 10% w/v polyacrylamide resolving gel, using a Bio-Rad Mini-Protean III system. Proteins were transferred to nitrocellulose membrane filters using a Bio-Rad Trans-Blot system (1 h at 100 V in 25 mM Tris, 192 mM glycine and 20% (v/v/) MeOH). Following transfer, the membranes were washed with TBS and blocked for 1 h at room temperature in blocking buffer (5% w/v skimmed milk powder and 0.1% v/v Tween-20 in TBS). Blots were then incubated overnight at 4°C in blocking buffer with the following primary antibodies (1:1000 dilutions unless otherwise indicated): phospho-specific ERK1/2, phospho-specific PKB (1:500), phospho-specific p38 MAPK, phospho-specific JNK or cleaved active caspase 3 (1: 500). The primary antibody was removed and the blot extensively washed three times for 5 min in TBS/Tween 20. Blots were then incubated for 1 h at room temperature with the appropriate secondary antibody (1:1000) coupled to horseradish peroxidase (DAKO Ltd, Cambridge, UK) in blocking buffer. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Ultra Chemiluminescence Detection System (Cheshire Sciences Ltd, Chester, UK) and quantified by densitometry using Advanced Image Data Analysis Software (Fuji; version

3.52). The uniform transfer of proteins to the nitrocellulose membrane was routinely monitored by transiently staining the membranes with Ponceau S stain (Sigma Chemical Co.) prior to application of the primary antibody. In addition, replicate samples from each experiment were analysed on separate blots using primary antibodies that recognise total unphosphorylated ERK1/2, PKB, p38 MAPK and JNK (all 1:1000 dilution) in order to confirm the uniformity of protein loading.

Determination of intracellular oxidative stress

Oxidative stress was determined using 2',7'-dichlorofluorescein diacetate (DCFDA), a reactive oxygen species (ROS) sensitive fluorescent probe (Abcam, Cambridge, UK). Analysis of ROS production was performed using H9c2 cells differentiated for 7 days in 25 cm^2 tissue culture flasks. Following 100 µM quercetin treatment (24, 48 and 72 h) cells were detached using trypsin (0.05% w/v)/EDTA (0.02% w/v), centrifuged and the resulting pellet re-suspended in 500 µl PBS containing 5 µM DCFDA and incubated for 30 min at 37°C. Following incubation, the cell suspension was transferred to FACS tubes (Fisher Scientific, Loughborough, UK) and DCFDA fluorescence (excitation 495 nm; emission 529 nm) measured using a Gallios™ flow cytometer (Beckman Coulter). The flow cytometer was set to count 10,000 cells in each treatment, and positive fluorescence was gated in relation to the untreated control. Results are reported as the percentage of cells showing positive fluorescence.

Data analysis

Statistical significance was determined by ANOVA with a post hoc Dunnett's test ($P<0.05$ was considered statistically significant). All data are presented as means $\pm$ S.E.M. The *n* in the text refers to the number of separate experiments.

Results

3.1 Differentiation of H9c2 cells and quercetin cardiotoxicity

Mitotic H9c2 cells derived from embryonic rat heart tissue [15] are increasingly used as an *in vitro* model for studies exploring cardioprotection since they display similar morphological, electrophysiological and biochemical properties to primary cardiac myocytes [18]. However, mitotic H9c2 cells can be differentiated into a more cardiomyocyte-like phenotype when cultured in DMEM supplemented with 1% FBS (v/v) and 10 nM *all-trans* retinoic acid [19,20]. In this study mitotic H9c2 cells were cultured in differentiation medium for 1, 3, 7 and 9 days. As shown in Figure 1 the expression of cardiac troponin 1 increased markedly after 7 days, confirming H9c2 cell differentiation into a more cardiomyocyte-like phenotype. Hence, H9c2 cells differentiated for 7 days were used throughout the study and initial experiments investigated whether quercetin induced cardiotoxicity.

Differentiated H9c2 cells were exposed to quercetin for 24, 48 and 72 h, after which cell viability was assessed by monitoring MTT reduction and LDH release. As shown in Figure 2a and 2b, exposure to quercetin (1-100 μM) for 24 h had no significant effect on cell viability. In contrast, increasing quercetin exposure time to 48 h resulted in the inhibition of MTT reduction at quercetin concentrations of 100 μM and 30 μM (Figure 2c) and increased LDH release at 100 μM (Figure 2d). Increasing the exposure time to 72 h resulted in cardiotoxicity at 10 μM quercetin, when assessed by the inhibition of MTT reduction (Figure 2e). Similarly after 72 h exposure, increased LDH release was observed at 30 μM quercetin (30 μM) when compared to 48 h exposure to the same concentration of flavonoid (Figure 2f). Overall, these observations indicate that quercetin-induces cardiotoxicity at a concentration (10 μM) achievable in the plasma through dietary supplements or quercetin-enriched foods [4]. Since MTT reduction and LDH release do not discriminate between apoptotic and necrotic forms of cell death, the effect of quercetin on caspase 3 activity (a marker of apoptotic cell death) was determined. As shown in Figure 3, exposure to 100 μM

quercetin (48 h and 72 h) induced a significant increase in caspase 3 activity, suggesting that quercetin-induced apoptosis contributes to cardiotoxicity.

The cardiotoxicity of quercetin may be a consequence of its intracellular metabolism to a semiquinone and/or quinone, both of which function as pro-oxidants [21,22]. Hence we determined whether the cardiotoxic effect of quercetin was due to its pro-oxidant properties. Levels of ROS were measured using the fluorescent probe DCFDA. As shown in Figure 4, exposure to 100 μM quercetin (48 h and 72 h) induced a significant increase in ROS, suggesting that quercetin-induced oxidative stress contributes to cardiotoxicity.

In order to understand the mechanism(s) of quercetin-induced cardiotoxicity more fully, the effect of quercetin on protein kinase cascades associated with cell survival and cell death e.g. ERK1/2, p38 MAPK, JNK and PKB was investigated. Modulation of protein kinase activity was assessed by Western blotting using phospho-specific antibodies that recognise phosphorylated motifs within activated ERK1/2 (pTEpY), p38 MAPK (pTGpY), JNK (pTPpY) and PKB (S^{473}). As shown in Figure 5a, 100 μM quercetin attenuated ERK1/2 phosphorylation following 24 h exposure. Similarly, 100 μM quercetin inhibited the level of phosphorylated PKB after 24 h exposure, whereas later time points (48 and 72 h) displayed significant increases in PKB phosphorylation (Figure 5b). Finally, the levels of phosphorylated p38 MAPK and JNK were both significantly increased following 72 h treatment with 100 μM quercetin (Figure 5c and 5d).

We also investigated the cardiotoxicity of two major *in vivo* quercetin metabolites; 3'-*O*-methyl quercetin and quercetin 3-glucuronide. As shown in Figure 6, exposure to quercetin 3-glucuronide for up 72 h had no significant on H9c2 cell viability, as judged by MTT reduction and LDH release. In contrast, 3'-*O*-methyl quercetin-induced cardiotoxicity was clearly evident at a concentration of 3 μM following 48 h and 72 h treatment, when assessed using MTT reduction (Figure 7). However, when monitoring LDH release, 3'-*O*-methyl quercetin-induced cardiotoxicity was only evident at a concentration of 100 μM following 24 and 48 h exposure.

3.2 Effect of quercetin on oxidative stress-induced cell death

As outlined in the Introduction, previous studies have used mitotic H9c2 cells to explore the cardioprotective mechanisms of flavonoids [6,7,13,14,16,17]. In this study we investigated the ability of quercetin and two of its major in vivo metabolites (quercetin 3-glucuronide and 3'-*O*-methyl quercetin*)* to protect differentiated H9c2 cells from H_2O_2-induced cell death. The effects of 24 h flavonoid pre-treatment on H_2O_2-induced cell damage were assessed by monitoring MTT reduction by cellular dehydrogenases and LDH activity released into the medium. As shown in Figure 8a, quercetin-induced a concentration-dependent attenuation of H_2O_2 (600 μM; 2 h) induced inhibition of MTT reduction, with significant protection still evident at a concentration of 3 μM. Similarly, quercetin at concentrations of 100 μM and 30 μM inhibited H_2O_2-induced release of LDH (Figure 8b). Next, the effects of two major *in vivo* quercetin metabolites (3'-*O*-methyl quercetin and quercetin 3-glucuronide) on H_2O_2-induced cell damage were investigated. As shown in Figure 9, pre-treatment of differentiated H9c2 cells for 24 h with quercetin 3-glucuronide had no significant effect on H_2O_2-induced inhibition of MTT reduction or LDH release. In contrast, 3'-*O*-methyl quercetin elicited statistically significant protection at 100 μM. The partition co-efficient (LogP) values for quercetin 3-glucuronide (-0.49), quercetin (1.68) and 3'-*O*-methyl quercetin (1.96) indicate that the glucuronide conjugate is less lipophilic, which may explain the lack of effect (both cardiotoxicity and cardioprotection) of quercetin 3-glucuronide observed in this study.

3.3 Effect of quercetin on oxidative stress-induced protein kinase activation

In order to understand the mechanism(s) of quercetin-induced protection against H_2O_2-induced cell damage, the effect of quercetin on H_2O_2-induced protein kinase activation (e.g. ERK1/2, p38 MAPK, JNK and PKB) was investigated using western blotting. Previous studies have shown that reactive oxygen species, including H_2O_2, activate an array of intracellular signalling pathways that are associated with cell death and survival [23]. Indeed, studies have reported H_2O_2-induced activation of ERK1/2, p38 MAPK, JNK and PKB pathways in cardiomyocytes [24-26]. In this study, treatment of differentiated H9c2 cells with 600 μM

H_2O_2 for 2 h triggered the activation of ERK1/2, PKB, p38 MAPK and JNK (Figure 10). Pre-treatment with 30 μM quercetin (24 h) significantly attenuated H_2O_2-induced activation of ERK1/2, PKB, p38 MAPK and JNK (Figure 10). These observations suggest that the protective actions of quercetin may involve the inactivation of signalling pathways involving ERK1/2, PKB, p38 MAPK and JNK. It should be noted that 24 h pre-treatment with 30 μM quercetin did not significantly attenuate the control levels of phosphorylated ERK1/2 and PKB (Figure 10). These data are surprising, since quercetin is a competitive inhibitor of the ATP-binding domain in MEK1 (an up-stream kinase associated with ERK1/2 activation) and PI-3Kγ (a lipid kinase involved in PKB activation). However, as shown in Figure 11, increasing the concentration of quercetin to 100 μM (24 h treatment) significantly inhibited basal phosphorylation of ERK1/2 and PKB. For comparison 30 min pre-treatment with PD98059 (50 μM) and LY 294002 (30 μM), inhibitors of MEK1 and PI-3K respectively, attenuated basal levels of phosphorylated ERK1/2 and PKB (Figure 11). These data would seem to suggest that 30 μM quercetin-induced attenuation of H_2O_2-induced ERK1/2 and PKB signalling is independent of MEK1 and PI-3K inhibition.

Previous studies have shown that quercetin protects mitotic H9c2 cells against H_2O_2-induced cell death via the activation of ERK1/ 2 and PI-3K/PKB signalling pathways [13]. In the current study, 24 h treatment of differentiated H9c2 cells with PD 98059 (50 μM; MEK1/2 inhibitor), LY 294002 (30 μM; PI-3K inhibitor); wortmannin (100 nM; PI-3K inhibitor), SB 203580 (30 μM; p38 MAPK inhibitor) or SP 600125 (10 μM; JNK1/2 inhibitor) had no significant effect on cell viability (Figure 12a) or quercetin mediated protection (Figure 12b). Similarly, these protein kinase inhibitors had no significant effect on H_2O_2-induced cell death (Figure 12c). Western blot analysis using phospho-specific ERK1/2, p38 MAPK, JNK, and PKB antibodies confirmed inhibition of kinase activation by the above protein kinase inhibitors (data not shown) .

Discussion

Quercetin-induced cardiotoxicity

Initial experiments explored the potential cytotoxic effects of quercetin exposure using differentiated H9c2 cells. In agreement with previous studies, albeit using mitotic H9c2 cells [13], 24 h exposure to quercetin (up to 100 μM) had no significant effect on the viability of differentiated H9c2 cells. However, quercetin-induced cardiotoxicity was evident following 48 h exposure with significant inhibition of MTT reduction at quercetin concentrations of 100 μM and 30 μM and increased LDH release at 100 μM. At 72 h cardiotoxicity was evident at 10 μM quercetin when assessed by the inhibition of MTT reduction. The difference in sensitivity between MTT and LDH assays is in agreement with previous studies which have reported the MTT assay as being more sensitive in detecting cytotoxic events [27]. To our knowledge this is the first report describing cardiotoxic effects of quercetin following prolonged (>48 h) exposure and importantly occurring at concentrations (10 μM) achievable in the plasma through ingestion of dietary supplements or quercetin-enriched foods [4]. These cytotoxic effects are consistent with previous studies showing that quercetin triggers or enhances drug-induced apoptosis in leukaemia cell lines [28], human prostate cancer cells [29] and mouse cortical neurons [30]. The cytotoxic effects of quercetin may relate to its intracellular metabolism to pro-oxidative metabolites [21,22]. In this study prolonged quercetin treatment of H9c2 cells (>48 h) resulted in the generation of ROS, which supports a role for pro-oxidative damage in the mechanism of quercetin-induced cardiotoxicity.

We also explored the potential cardiotoxicity effects of two major *in vivo* quercetin metabolites; 3'-*O*-methyl quercetin and quercetin 3-glucuronide. The observation that quercetin 3-glucuronide had no significant effect on the viability of differentiated H9c2 cells following 72 h exposure at concentrations up to 100 μM is in agreement with a previous study, which reported no damage to primary neuronal cells following exposure to quercetin 7-glucuronide for 6 h [30]. The lack of effect of quercetin 3-glucuronide may reflect the reduced lipophilicity of glucuronide conjugates. In marked contrast, the

metabolite 3'-*O*-methyl quercetin triggered cardiotoxicity following 48 and 72 h exposure with significant inhibition of MTT reduction evident at 3 μM. These data are in agreement with Angeloni et al. [13] who reported no decrease in the ability of mitotic H9c2 cells to reduce MTT following 24 h treatment with 3'-*O*-methyl quercetin (1-30 μM). However, as seen in this study cellular toxicity to 3'-*O*-methyl quercetin is evident in differentiated H9c2 cells following prolonged (> 48h) exposure. In contrast, cellular toxicity of 3'-*O*-methyl quercetin has been observed in primary neuronal cells following 6 h exposure [30]. When monitoring the effect of 3'-*O*-methyl quercetin and using LDH release as a marker of cell viability, cardiotoxicity was only evident at 24 and 48 h at a concentration of 100 μM. It is not clear why there is no release of LDH following 72 h treatment with 3'-*O*-methyl quercetin despite the significant inhibition of MTT reduction at the same time point. It may be that 3'-*O*-methyl quercetin disrupts mitochondrial function without triggering necrotic cell death which is detected when monitoring LDH release. Similar to quercetin, the cytotoxic effects of 3'-*O*-methyl quercetin may also relate to its metabolism to pro-oxidative metabolites. Alternative assays for cell viability/cytoxicity, such as the neutral red assay which is based on the incorporation of the dye into lysosomes [31] or caspase-3 detection to assess apoptosis, may provide further clues as to the possible mechanism(s) of 3'-*O*-methyl quercetin-induced cardiotoxicity.

In neuronal cells, quercetin-induced cell death involves the inhibition of cell survival signalling pathways (PKB and ERK1/2) and activation of caspase-3 [30]. In this study quercetin (100 μM) also triggered the activation of caspase-3 following 48 h and 72 h exposure, suggesting quercetin-induced cardiotoxicity involves apoptotic cell death. In order to understand more clearly the mechanism(s) of quercetin-induced cardiotoxicity the effect of prolonged quercetin exposure on protein kinase cascades associated with cell survival (ERK1/2 and PKB) and cell death (p38 MAPK and JNK) was investigated. In view of their respective roles in cell death and cell survival, it would be predicted that quercetin-induced cardiotoxicity may involve attenuation of ERK1/2 and PKB signalling and/or activation of p38 MAPK and JNK. Since the molecular actions of quercetin include inhibition of the protein kinase MEK1 and the lipid kinase PI-3Kγ, two critical up-stream kinases

involved in the activation of pro-survival ERK1/2 and PKB signalling cascades, respectively [32,33], pre-treatment of H9c2 cells with quercetin would be expected to attenuate levels of phosphorylated ERK1/2 and PKB. Indeed, quercetin attenuates MEK1 activity more effectively than the widely used MEK1 inhibitor PD98059 [34]. In this current study treatment with 100 μM quercetin for 24 h attenuated ERK1/2 phosphorylation. The effect of prolonged quercetin (100 μM) exposure on PKB phosphorylation revealed a biphasic response with inhibition observed following 24 h treatment, whereas later time points displayed increases in PKB phosphorylation. Previous studies have revealed conflicting data regarding the effects of quercetin on ERK1/2 and PKB signalling. For example, 24 h treatment with quercetin (30 μM) activates ERK1/2 and PKB in mitotic H9c2 cells [13]. Activation of ERK1/2 by quercetin has also been observed in macrophages (>50 μM for 40 min; [35]) and pancreatic β cells (20 μM for 1 h; [36]). Finally quercetin (10 μM for 60 min) induces increases in ERK1/2, JNK, p38 MAPK and PKB phosphorylation in HepG2 cells [8]. In contrast, quercetin inhibits ERK1/2 and PKB phosphorylation in neuronal cells (30 μM up to 6 h; [30] and the anti-proliferative effect of quercetin on cancer cells is via PKB inhibition [37]. In this study, using differentiated H9c2 cells, 24 h exposure to quercetin (30 μM) had no significant effect on the basal levels of phosphorylated (active) ERK1/2, p38 MAPK, JNK or PKB. This presumably reflects the cellular metabolism of quercetin which is undetectable in mitotic H9c2 cells after 24 h exposure to 30 μM having peaked at 1 h [13]. In contrast, 24 h exposure to 100 μM quercetin significantly reduced basal levels of phosphorylated ERK1/2 and PKB. These data are comparable to results obtained using selective up-stream inhibitors of these protein kinases, namely PD98059 (MEK1 inhibitor) and LY 294002 (PI-3K inhibitor).

In this study prolonged treatment with quercetin (100 μM for 72 h) resulted in significant increases in the levels of phosphorylated p38 MAPK and JNK in differentiated H9c2 cells. Given the pro-apoptotic role of these kinases it is conceivable that their activation contributes to quercetin-induced cardiotoxicity. Although it is interesting to note that quercetin-induced neuronal damage appears to be independent of JNK [28]. Although it is not clear how quercetin induces an increase in JNK and p38 MAPK phosphorylation in

differentiated H9c2 cells, a recent study has revealed that quercetin (100 μM; 6 h) stimulates TAK1 and MKK3/6, two upstream kinases associated with p38 MAPK activation, in macrophages [38]. Further experiments are clearly needed to explore the connection between quercetin-induced activation of p38 MAPK/JNK and cell death, possibly through the use of selective pharmacological inhibitors of these kinases, although prolonged (72 h) treatment with such inhibitors may also induce cell death. Thus, quercetin-induced cardiotoxicity potentially involves the modulation of multiple protein kinase cascades associated with cell survival (ERK1/2 and PKB) and cell death (p38 MAPK and JNK). Although beyond the scope of the present study, future experiments will explore in more detail the concentration and time dependent effects of quercetin on these kinases and their upstream activators.

Quercetin-induced cardioprotection

In this study, we investigated the ability of quercetin and two of its major *in vivo* metabolites (quercetin 3-glucuronide and 3'-*O*-methyl quercetin*)* to protect differentiated H9c2 cells from H_2O_2-induced cell death. The data presented indicate that 24 h exposure to quercetin induced a concentration-dependent attenuation of H_2O_2 induced inhibition of MTT reduction, with significant protection still evident at a concentration of 3 μM. At concentrations of 100 μM and 30 μM, quercetin also induced protection when monitoring H_2O_2 induced LDH release. These observations are comparable to previous reports indicating that MTT assays are more sensitive for detecting cytotoxic insults when compared to LDH release assays [27]. Whilst comparable to previous studies reporting quercetin-induced cardioprotection using mitotic H9c2 cells, ours is the first study to report protection using differentiated H9c2 cells. A notable feature of the data presented in this study concerns the temporal nature of quercetin's cardioprotective versus cardiotoxic effects. Treatment of H9c2 cells for 24 h with 10, 30 or 100 μM quercetin induces cardioprotection against H_2O_2 induced inhibition of MTT reduction (with no observable toxicity) whereas 72 h exposure to 10, 30 or 100 μM quercetin results in cardiotoxicity. The switch from a cardioprotective to cardiotoxic action may reflect a switch from anti-

oxidant to pro-oxidant effects due to the accumulation of oxidative metabolites and/or a temporal modulation of multiple kinase pathways associated with cell death and survival.

Previous studies have shown that 3'-*O*-methyl quercetin (up to 30 μM) protects dermal fibroblasts against H_2O_2-induced cell damage, whereas quercetin 7-*O*–β-D-glucuronide had no protective effects [21]. Similar results were obtained in the current work, although the protective effects of 3'-*O*-methyl quercetin were only observed at a concentration of 100 μM. The difference between the two quercetin metabolites may reflect their uptake into differentiated H9c2 cells. However, Angeloni et al. [13] reported that 3'-*O*-methyl quercetin did not protect mitotic H9c2 cells against oxidative damage.

Mechanisms of quercetin-induced cardioprotection

A previous report has shown that quercetin protects mitotic H9c2 cells against H_2O_2-induced cell death via the activation of ERK1/ 2 and PI-3K/PKB signalling pathways [13]. These data were based on the observation that pre-treatment of cells with PD98059 (MEK1 inhibitor) and LY 294002 (PI-3K inhibitor) attenuated quercetin-induced protection [13]. In this study treatment of cells with PD98059 (50 μM) and LY 294002 (30 μM) had no significant effect on 30 μM quercetin-induced protection. Similarly, inhibitors of p38 MAPK (SB203580; 30 μM) and JNK (SP 600125; 10 μM) also had no effect on quercetin-induced protection. The apparent discrepancy between this study and Angeloni et al. [13] may reflect the use of mitotic versus differentiated H9c2 cells. However, it is important to note that in this study 24 h quercetin (30 μM) pre-treatment did not activate PKB, ERK1/2, p38 MAPK or JNK and hence inhibitors of these kinases would not be expected to reverse the protective effect of quercetin.

The mechanism(s) of quercetin-induced protection against H_2O_2-induced cell damage may involve modulation of H_2O_2-induced protein kinase activation e.g. ERK1/2, PKB, p38 MAPK, and JNK, since these signalling pathways are associated with redox-mediated cell death and survival [39]. However, in the current study, inhibitors of these kinases had no significant effect on H_2O_2-induced cell death. Previous studies have shown H_2O_2-induced activation of ERK1/2, p38 MAPK, JNK and PKB pathways in cardiomyocytes [24-26]. We

also observed significant activation of these kinase cascades by H_2O_2 (600 μM; 2 h) in differentiated H9c2 cells. Previous studies using mitotic H9c2 cells have shown that H_2O_2 (150 μM; 1 h) attenuated PKB phosphorylation, whereas levels of phosphorylated ERK1/2 were not modulated [13]. The disparity between the results obtained in this study and those of Angenloni et al. [13] may again be a consequence of the use of mitotic versus differentiated cells or concentration/exposure time to H_2O_2. The data presented in our study also reveals that pre-treatment of differentiated H9c2 cells with 30 μM quercetin attenuated H_2O_2-induced activation of ERK1/2, p38 MAPK, JNK and PKB. Previous studies have shown that H_2O_2 triggers apoptosis in H9c2 cells via activation of the mitochondria-dependent or intrinsic apoptotic pathway [14]. Interestingly, the flavonol isorhamnetin attenuated H_2O_2-induced activation of mitochondrial-dependent apoptosis in H9c2 cells via ERK inactivation [14]. These observations are in agreement with the data obtained in the current study, suggesting that quercetin protects against H_2O_2-induced oxidative cell death via ERK1/2 inactivation. The attenuation of H_2O_2-induced p38 MAPK, JNK and PKB activation by quercetin is comparable to previous studies, which have reported quercetin inhibiting lipoteichoic acid (constituent of the cell wall of gram-positive bacteria) induced ERK1/2, JNK, p38 MAPK and PKB activation in mitotic H9c2 cells [6]. Similarly, quercetin inhibited LPS-induced JNK and p38 MAPK activation in H9c2 cells [7]. Overall these observations indicate that quercetin is capable of attenuating protein kinase activation (ERK1/2, JNK, p38 MAPK and PKB) triggered via a range of different mediators (H_2O_2, lipoteichoic acid and LPS) in H9c2 cells. The inhibition of ERK1/2 and PKB may relate to the actions of quercetin as an inhibitor of MEK1 and PI-3Kγ, although the observation that basal levels of ERK1/2 and PKB phosphorylation are not inhibited by 24 h treatment with 30 μM flavonoid suggests otherwise. Hence, although the attenuation of H_2O_2-induced cell death by quercetin may be via ERK1/2 and PKB inhibition, these data suggest it is independent of MEK1 and PI-3Kγ inhibition. The attenuation of p38 MAPK and JNK signalling presumably reflects the inhibition of up-stream components of these two protein kinases. The potential mechanisms of how quercetin may modulate JNK and p38 MAPK signalling are discussed in detail by Spencer [40]. However, it is important to note that quercetin is known to inhibit a

multitude of other protein kinases that include CK2, AMPK, RSK2, S6K1, IKK, Hck and 16 cell-cycle related kinases [4,41,42]. Although this study has shown that quercetin attenuates H_2O_2-induced PKB, ERK1/2, p38 MAPK and JNK activation and promotes cell survival, inhibitors of these kinases had no significant effect on H_2O_2-induced cell death. The discrepancy between these observations suggests that the protective effect of quercetin may be a consequence of the simultaneous inhibition of multiple kinase pathways as opposed to the lack of protection observed with inhibitors of individual kinase pathways.

It is important to note that quercetin in foods is glycosylated and, as such, plasma levels of the aglycone are very low or undetectable [4]. However, following ingestion, quercetin glycosides are rapidly hydrolysed in the gut to quercetin aglycone, which is subsequently metabolized into glucuronidated, sulphated or methylated derivatives via phase II conjugation reactions. It has also been suggested that many of the major metabolites of quercetin exhibit weaker in vitro effects than the parent compound [43]. The data presented is this study confirms this view, since quercetin 3-glucuronide did not elicit cardioprotection, whereas 3'-*O*-methyl quercetin was only effective at 100 μM. Furthermore, recent studies have suggested that circulating quercetin glucuronides in the plasma function as quercetin carriers, which when taken up by cells are de-conjugated into the more active aglycone [44]. Hence in this study it is notable that quercetin 3-glucuronide was unable to produce either cardiotoxic or cardioprotective effects, suggesting that this metabolite may not be de-conjugated in H9c2 cells.

In conclusion, this study has shown for the first time that 24 h treatment with quercetin induces cardioprotection against oxidative stress triggered cell death in differentiated H9c2 cells, a cell-based model system that displays a more robust cardiomyocyte-like phenotype. In contrast, the *in vivo* metabolite quercetin 3-glucuronide displayed no protective effects against H_2O_2-induced cell damage. Although quercetin attenuated H_2O_2-induced activation of ERK1/2, PKB, p38 MAPK and JNK, inhibitors of these kinases had no significant effect on quercetin-induced protection or H_2O_2-induce cell death. Prolonged exposure to quercetin (72 h) triggered cardiotoxicity which appears to involve apoptotic cell death and modulation of cell survival (PKB) and cell death (p38 MAPK and JNK) signalling

pathways. Cardiotoxicity was also observed with 3'-*O*-methyl quercetin but not quercetin 3-glucuronide. Clearly further studies are required to explore the complex interplay between the numerous signalling pathways that are modulated by quercetin and thus may contribute to the observed cardioprotective and cardiotoxic effects of this important flavonoid.

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References

1. Middleton Jr E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. Pharmacol Rev 2000; 52: 673-751.

2. Beecher GR. Overview of dietary flavonoids: nomenclature, occurrence and intake. J Nutr 2003; 133: 3248S-54S.

3. Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal 2013; 18: 1818-92.

4. Russo M, Spagnuolo C, Tedesco I, Bilotto S, Russo GL. The flavonoid quercetin in disease prevention and therapy: facts and fancies. Biochem Pharmacol 2012; 83: 6-15.

5. Roskosko Jr R. ERK1/2 MAP kinases: Structure, function and regulation. Pharmacological Res 2012 66: 105-43.

6. Gutiérrez-Venegas G, Bando-Campos CG. The flavonoids luteolin and quercetagetin inhibit lipoteichoic acid actions on H9c2 cardiomyocytes. Int Immunopharmacol 2010; 10: 1003-09.

7. Angeloni C, Hrelia S. Quercetin reduces inflammatory responses in LPS-stimulated cardiomyoblasts. Oxid Med Cell Longev 2012; 2012: 583901.

8. Weng CJ, Chen MJ, Yeh CT, Yen GC. Hepatoprotection of quercetin against oxidative stress by induction of metallothionein expression through activating MAPK and PI3K pathways and enhancing Nrf2 DNA-binding activity. New Biotechnol 2011; 28: 767-77.

9. Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP et al. Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. Mol Cell 2000; 6: 909-19.

10. Armstrong SC. Protein kinase activation and myocardial ischaemia/reperfusion injury. Cardiovasc Res 2004; 61: 427-36.

11. Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: united at reperfusion. Pharmacol Ther 2007; 114: 208-21.

12. Shao Z-H, Wojcik KR, Dossumbekova A, Hsu C, Mehendale SR, Li C-Q et al. Grape seed proanthocyanidins protect cardiomyocytes from ischemia and reperfusion injury via Akt-NOS signaling. J Cell Biochem 2009; 107: 697-705.

13. Angeloni C, Spencer JPE, Leoncini E, Biagi PL, Hrelia S. Role of quercetin and its *in vivo* metabolites in protecting H9c2 cells against oxidative stress. Biochimie 2007; 89: 73-82.

14. Sun B, Sun GB, Xiao J, Chen RC, Wang X, Wu Y et al. , Isorhamnetin inhibits H_2O_2-induced activation of the intrinsic apoptotic pathway in H9c2 cardiomyocytes through scavenging reactive oxygen species and ERK inactivation. J Cell Biochem 2012; 113: 473-85.

15. Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. Exp Cell Res 1976; 98: 367-81

16. Kim DS, Kwon DY, Kim MS, Lee YC, Park SJ, Yoo WH et al. The involvement of endoplasmic reticulum stress in flavonoid-induced protection of cardiac cell death caused by ischaemia/reperfusion. J Pharm Pharmacol 2010; 62: 197-204.

17. Mojzisová G, Sarisský M, Mirossay L, Martinka P, Mojzis J. Effects of flavonoids on daunorubicin-induced toxicity in H9c2 cardiomyoblasts. Phytother Res 2009; 23: 136-39.

18. Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circ Res 1991; 69: 1476-86.

19. Ménard C, Pupier S, Mornet D, Kitzmann M, Nargeot J, Lory P. Modulation of L-type channel expression during retinoic acid-induced differentiation of H9c2 cardiac cells. J Biol Chem 1999; 274: 29063-070.

20. Comelli M, Domenis R, Bisetto E, Contin M, Marchini M, Ortolani F et al. Cardiac differentiation promotes mitochondria development and ameliorates oxidative capacity in H9c2 cardiomyoblasts. Mitochondrion 2011; 11: 315-326.

21. Spencer JPE, Kuhnle GGC, Williams RJ, Rice-Evans C. Intracellular metabolism and bioactivity of quercetin and its *in vivo* metabolites. Biochem J 2003; 372: 173-81.

22. Metodiewa D, Jaiswak AK, Cenas N, Dickancaité E, Sequra-Aguilar J. Quercetin may act as a cytotoxic pro-oxidant after its metabolic activation to semiquinone and quinoidal product. Free Radic Biol Med 1999; 26: 107-16.

23. Kamata H, Hirata H. Redox regulation of cellular signalling. Cell Signal 1999; 11: 1-14.

24. Clerk A, Fuller SJ, Michael A, Sugden PH. Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and

p38-mitogen-activated protein kinases) in perfused hearts by oxidative and other stresses. J Biol Chem 1998; 273: 7228-34.

25. Pham FH, Sugden PH, Clerk A. (2000) Regulation of protein kinase B and 4E-BP1 by oxidative stress in cardiac myocytes. Circ Res 2000; 86: 1252-58.

26. Takeishi Y, Abe J, Lee J-D, Kawakatsu H, Walsh RA, Berk BC. Differential regulation of p90 ribosomal S6 kinase and Big-mitogen-activated protein kinase 1 by ischemia/reperfusion and oxidative stress in perfused guinea-pig hearts. Circ Res 1999; 85: 1164-72.

27. Fotakis G, Timbrell JA. In vitro cytotoxic assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cells following exposure to cadmium chloride. Toxicology Letts. 2006; 160: 171-77.

28. Kim YH, Lee YJ. TRAIL apoptosis is enhanced by quercetin through Akt dephosphorylation. J Cell Biochem 2007; 100:998-1009.

29. Russo M, Spagnuolo C, Volpe S, Mupo A, Tedesco I, Russo GL. Quercetin induced apoptosis in association with death receptors and fludarabine in cells isolated from chronic lymphocytic patients. Br J Cancer 2010; 103: 642-8.

30. Spencer JPE, Rice-Evans C, Williams RJ. Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its *in vivo* metabolites underlie their action on neuronal viability. J Biol Chem 2003; 278: 34783-93.

31. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature Protocols 2008; 3: 1125-31.

32. Hers I, Vincent EE, Tavaré JM. Akt signalling in health and disease. Cell Signal 2011; 23: 1515-27.

33. Lu Z, Xu S. ERK1/2 MAP kinases in cell survival and apoptosis. IUBMB Life 2006; 58: 621-31.

34. Lee KW, Kang NJ, Heo YS, Rogozin EA, Pugliese A, Hwang MK et al. Raf and MEK protein kinases are direct molecular targets for the chemopreventive effect of quercetin, a major flavonol in red wine. Cancer Res 2008; 68: 946-55.

35. Chow J-M, Shen S-C, Huan Sk, Lin H-Y, Chen Y-C. Quercetin, but not rutin and quercitrin, prevention of H_2O_2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. Biochem Pharmacol 2005; 69: 1839-51.

36. Youl E, Bardy G, Magous R, Cros G, Sejalon F, Virsolvy A et al. Quercetin potentiates insulin secretion and protects INS-1 pancreatic β-cells against oxidative stress damage via the ERK1/2 pathway. Br J Pharmacol 2010; 161: 799-814.

37. Gulati N, Laudet B, Zohrabian VM, Murali R, Jhanwar-Uniyal M. The antiproliferative effect of quercetin in cancer cells is mediated via inhibition of the PI3K-Akt/PKB pathway. Anticancer Res 2006; 26: 1177-82.

38. Chang YC, Lee TS, Chiang AN. Quercetin enhances ABCA1 expression and cholesterol efflux through a p38-dependent pathway in macrophages. J Lipid Res 2012; 53: 1840-50.

39. Kamata H, Hirata H. Redox regulation of cellular signalling. Cell Signal 1999; 11: 1-14.

40. Spencer JPE. The interactions of flavonoids within neuronal signalling pathways. Genes Nutr. 2007; 2: 257-73.

41. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000; 351: 95-105.

42. Boly R, Gras T, Lamkami T, Guissou P, Sertyn D, Kiss R et al. Quercetin inhibits a large panel of kinases implicated in cancer biology. Int J Oncol 2011; 38: 833-42.

43. Williamson G, Barron D, Shimoi K, Terao J. In vitro biological properties of flavonoid conjugates found in vivo. Free Radic Res 2005; 39: 457-69.

44. Kawai Y, Nishikawa T, Shiba Y, Saito S, Murota K, Shibata N, et al. Macrophage as a target of quercetin glucuronides in human atherosclerotic arteries: implication in the anti-atherosclerotic mechanism of dietary flavonoids. J Biol Chem 2008; 283: 9424-34.

Figure Legends

Figure 1. Expression of cardiac troponin 1 during differentiation of H9c2 cells. Levels of cardiac troponin 1 were monitored following 1, 3, 7 and 9 days cell culture in 1% FBS (v/v) and 10 nM *all-trans* retinoic acid. (a) Immunocytochemistry by fluorescence microscopy was performed using cardiac troponin 1 specific antibody (green) and DAPI counterstain for nuclei visualisation (blue). Images presented are from one experiment and representative of three. (b) Levels of cardiac troponin 1 assessed by western blot. Data are expressed as the percentage of control undifferentiated cells (=100%) and represent the mean ± SEM of three independent experiments. *$P<0.05$ versus control response.

Figure 2. Effect of quercetin on the viability of differentiated H9c2 cells monitored by MTT reduction and LDH release. Differentiated H9c2 cells (7 day) were exposed to the indicated concentrations of quercetin for 24, 48 and 72 h. Following quercetin exposure cell viability was assessed by measuring (a, c, and e) metabolic reduction of MTT by mitochondrial dehydrogenases and (b, d and f) the release of LDH. Data are expressed as the percentage of control cells (=100%) and represent the mean ± SEM of five independent experiments each performed in quadruplicate (MTT) or sextuplicate (LDH). *$P<0.05$ versus control response.

Figure 3. Effect of quercetin on caspase 3 activation. Differentiated H9c2 cells (7 day) were exposed to 100 μM quercetin for (a) 24 h, (b) 48 h and (c) 72 h. Following quercetin exposure caspase 3 activation was assessed via Western blotting using anti-active caspase 3 antibody. Data are expressed as the percentage of control cells (72 h =100%) and represent the mean ± SEM of three independent experiments. *$P<0.05$ versus untreated control cells.

Figure 4. Effect of quercetin on reactive oxygen species production. Differentiated H9c2 cells (7 day) were exposed to 100 μM quercetin for (a) 24 h, (b) 48 h and (c) 72 h. Following quercetin exposure, ROS production was assessed via flow cytometry using the fluorescent probe DCFDA. Data are expressed as the percentage of fluorescent positive cells (10,000 sampled) in each condition and represent the mean ± SEM of three independent experiments. *$P<0.05$, **$P<0.01$ versus untreated control cells.

Figure 5. Effect of prolonged quercetin exposure on protein kinase activation. Differentiated H9c2 cells (7 day) were exposed to 100 μM quercetin for (a) 24 h, (b) 48 h and (c) 72 h. Following quercetin exposure cell lysates were analysed by Western blotting for activation of (a) ERK1/2, (b) PKB, (c) p38 MAPK and (d) JNK using phospho-specific antibodies. Samples were subsequently analysed on separate blots using antibodies that recognise total ERK1/2, PKB, p38 MAPK and JNK. Time-matched control experiments (24, 48 and 72 h in the absence of quercetin) revealed no significant change in activated ERK1/2, PKB, p38 MAPK or JNK (data not shown). Data are expressed as the percentage of control cells (72 h =100%) and represent the mean ± SEM of three or four independent experiments. *$P<0.05$ versus untreated control cells.

Figure 6. Effect of quercetin 3-glucuronide on the viability of differentiated H9c2 cells monitored by MTT reduction and LDH release. Differentiated H9c2 cells (7 day) were exposed to the indicated concentrations of quercetin 3-glucuronide (Q3G) for 24, 48 and 72 h. Following quercetin 3-glucuronide exposure cell viability was assessed by measuring (a, c, and e) metabolic reduction of MTT by mitochondrial dehydrogenases and (b, d and f) the release of LDH. Data are expressed as the percentage of control cells (=100%) and represent the mean ± SEM of six independent experiments each performed in quadruplicate (MTT) or sextuplicate (LDH).

Figure 7. Effect of 3'-*O*-methyl quercetin on the viability of differentiated H9c2 cells monitored by MTT reduction and LDH release. Differentiated H9c2 cells (7 day) were exposed to the indicated concentrations of 3'-*O*-methyl quercetin (Q3M for 24, 48 and 72 h. Following 3'-*O*-methyl quercetin exposure cell viability was assessed by measuring (a, c, and e) metabolic reduction of MTT by mitochondrial dehydrogenases and (b, d and f) the release of LDH. Data are expressed as the percentage of control cells (=100%) and represent the mean ± SEM of six independent experiments each performed in quadruplicate (MTT) or sextuplicate (LDH). **P*<0.05, ***P*<0.01, ****P*<0.005 versus control response.

Figure 8. Effect of quercetin on H_2O_2-induced cell death. Differentiated H9c2 cells (7 day) were exposed to the indicated concentrations of quercetin for 24 h prior to 2 h exposure to 600 μM H_2O_2 in the continued presence of quercetin. Cell viability was assessed by measuring (a) metabolic reduction of MTT and (b) LDH released into the culture medium. Data are expressed as the percentage of control cells (=100%) and represent the mean ± SEM of six independent experiments each performed in (a) quadruplicate and (b) sextuplicate. **P*<0.05 versus H_2O_2 alone.

Figure 9. Effect of quercetin 3-glucuronide and 3'-*O*-methyl quercetin on H_2O_2-induced cell death. Differentiated H9c2 cells (7 day) were exposed to the indicated concentrations of quercetin 3-glucuronide (Q3G) or 3'-*O*-methyl quercetin (Q3M) for 24 h prior to 2 h exposure to 600 μM H_2O_2 in the continued presence of quercetin 3-glucuronide or 3'-*O*-methyl quercetin. Cell viability was assessed by measuring (a and c) metabolic reduction of MTT and (b and d) LDH released into the culture medium. Data are expressed as the percentage of control cells (=100%) and represent the mean ± SEM of five independent experiments each performed in quadruplicate (MTT) and sextuplicate (LDH). **P*<0.05 versus H_2O_2 alone.

Figure 10. Effect of quercetin on H_2O_2-induced protein kinase activation. Differentiated H9c2 cells (7 day) were exposed to 30 μM quercetin for 24 h prior to 2 h exposure to 600 μM H_2O_2 in the continued presence of quercetin. Cell lysates were analysed by Western blotting for activation of (a) ERK1/2, (b) PKB, (c) p38 MAPK and (d) JNK using phospho-specific antibodies. Samples were subsequently analysed on separate blots using antibodies that recognise total ERK1/2, PKB, p38 MAPK and JNK. Data are expressed as the percentage of control cells (=100%) and represent the mean ± SEM of three independent experiments. *P<0.05, **a** versus control cells and **b** versus H_2O_2 treated cells.

Figure 11. Effect of quercetin on ERK1/2 and PKB phosphorylation. Differentiated H9c2 cells (7 day) were exposed to quercetin (100 μM and 30 μM) for 24 h or PD 98059 (50 μM) and LY294002 (30 μM) for 30 min. Cell lysates were then analysed by Western blotting for phosphorylated (a) ERK1/2 and (b) PKB, using phospho-specific antibodies. Samples were also analysed on separate blots using antibodies that recognise total ERK1/2 and PKB. Data are expressed as the percentage of control cells (=100%) and represent the mean ± SEM of three independent experiments. *P<0.05 versus control response.

Figure 12. Effect of protein kinase inhibitors on quercetin-induced cardioprotection. (a) Differentiated H9c2 cells were exposed to PD 98059 (50 μM; MEK1/2 inhibitor), wortmannin (100 nM; PI-3K inhibitor), LY 294002 (30 μM; PI-3K inhibitor); SB 203580 (30 μM; p38 MAPK inhibitor) or SP 600125 (10 μM; JNK1/2 inhibitor) for 24 h. (b) Differentiated H9c2 cells were pre-treated for 30 min with the protein kinase inhibitors prior to 24 h quercetin (30 μM) followed by 2 h exposure to 600 μM H_2O_2. (c) Differentiated H9c2 cells were pre-treated with protein kinase inhibitors for 24 h prior to 2 h exposure to 600 μM H_2O_2.

Figure 1

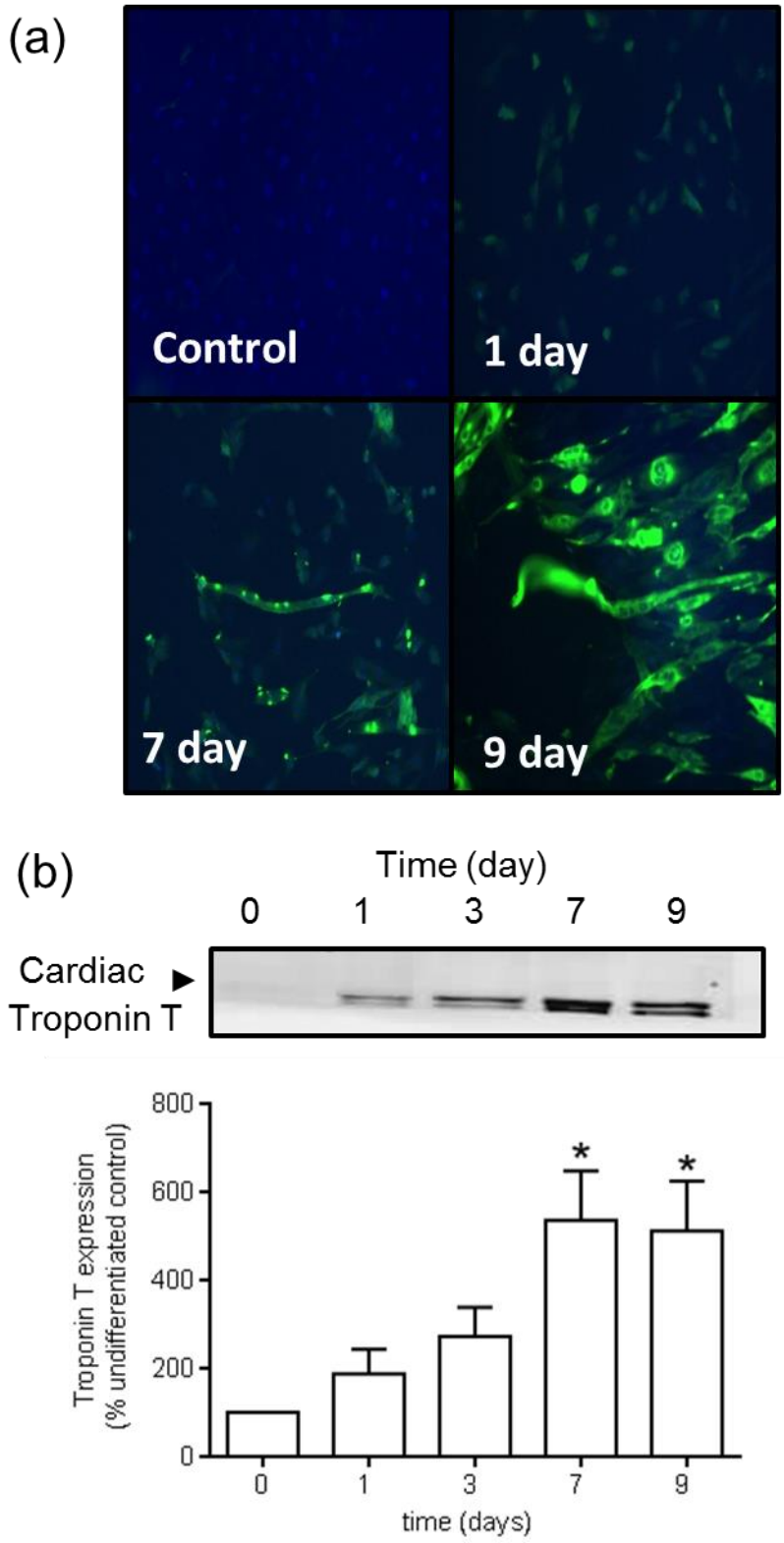

(a)
Control
1 day
7 day
9 day
(b)
Time (day)
0
1
3
7
9
Cardiac
Troponin T
Troponin T expression
(% undifferentiated control)
800
600
400
200
0
*
*
0
1
3
7
9
time (days)

Figure 2

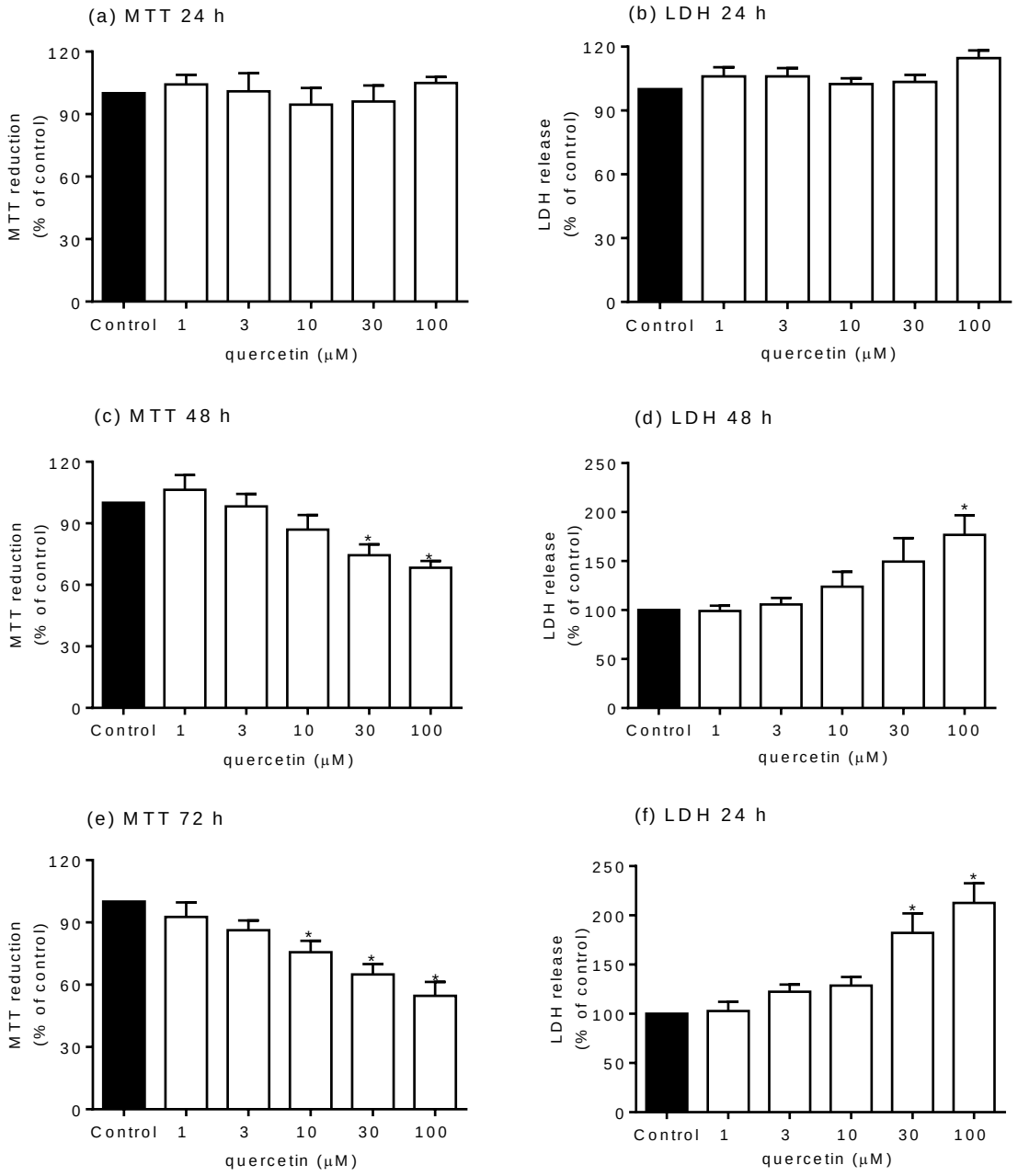

(a) MTT 24 h
MTT reduction (% of control)
120
90
60
30
0
Control 1 3 10 30 100
quercetin (μM)
(b) LDH 24 h
LDH release (% of control)
120
90
60
30
0
Control 1 3 10 30 100
quercetin (μM)
(c) MTT 48 h
MTT reduction (% of control)
120
90
60
30
0
*
*
Control 1 3 10 30 100
quercetin (μM)
(d) LDH 48 h
LDH release (% of control)
250
200
150
100
50
0
*
Control 1 3 10 30 100
quercetin (μM)
(e) MTT 72 h
MTT reduction (% of control)
120
90
60
30
0
*
*
*
Control 1 3 10 30 100
quercetin (μM)
(f) LDH 24 h
LDH release (% of control)
250
200
150
100
50
0
*
*
Control 1 3 10 30 100
quercetin (μM)

Figure 3

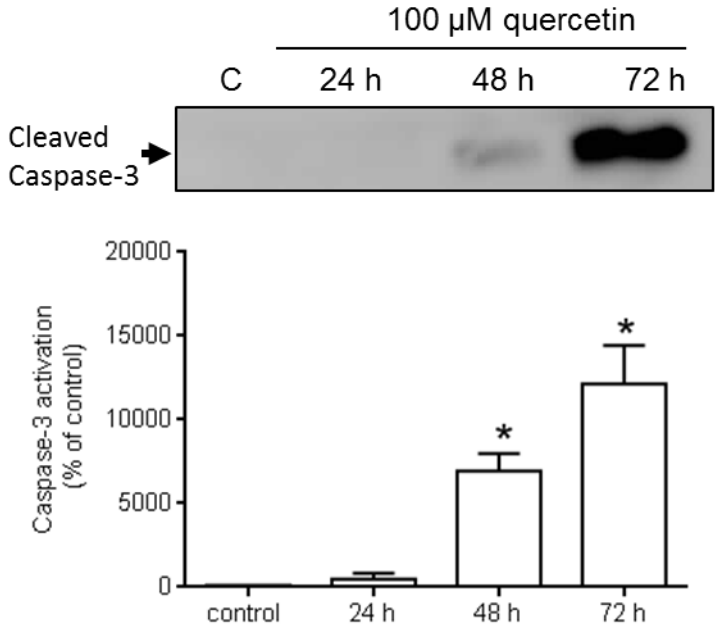

100 μM quercetin
C
24 h
48 h
72 h
Cleaved
Caspase-3
Caspase-3 activation
(% of control)
20000
15000
10000
5000
0
control
24 h
48 h
72 h
*
*

Figure 4.

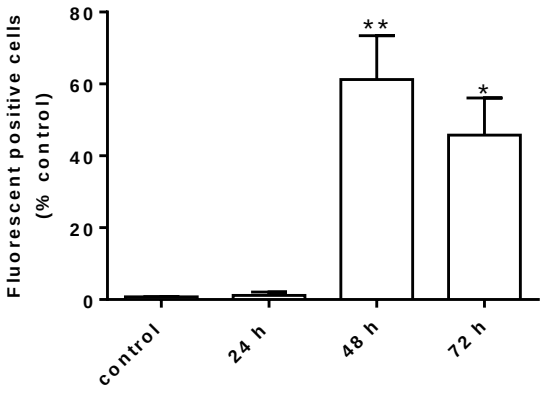

Fluorescent positive cells
(% control)
80
60
40
20
0
**
*
control
24 h
48 h
72 h

Figure 5

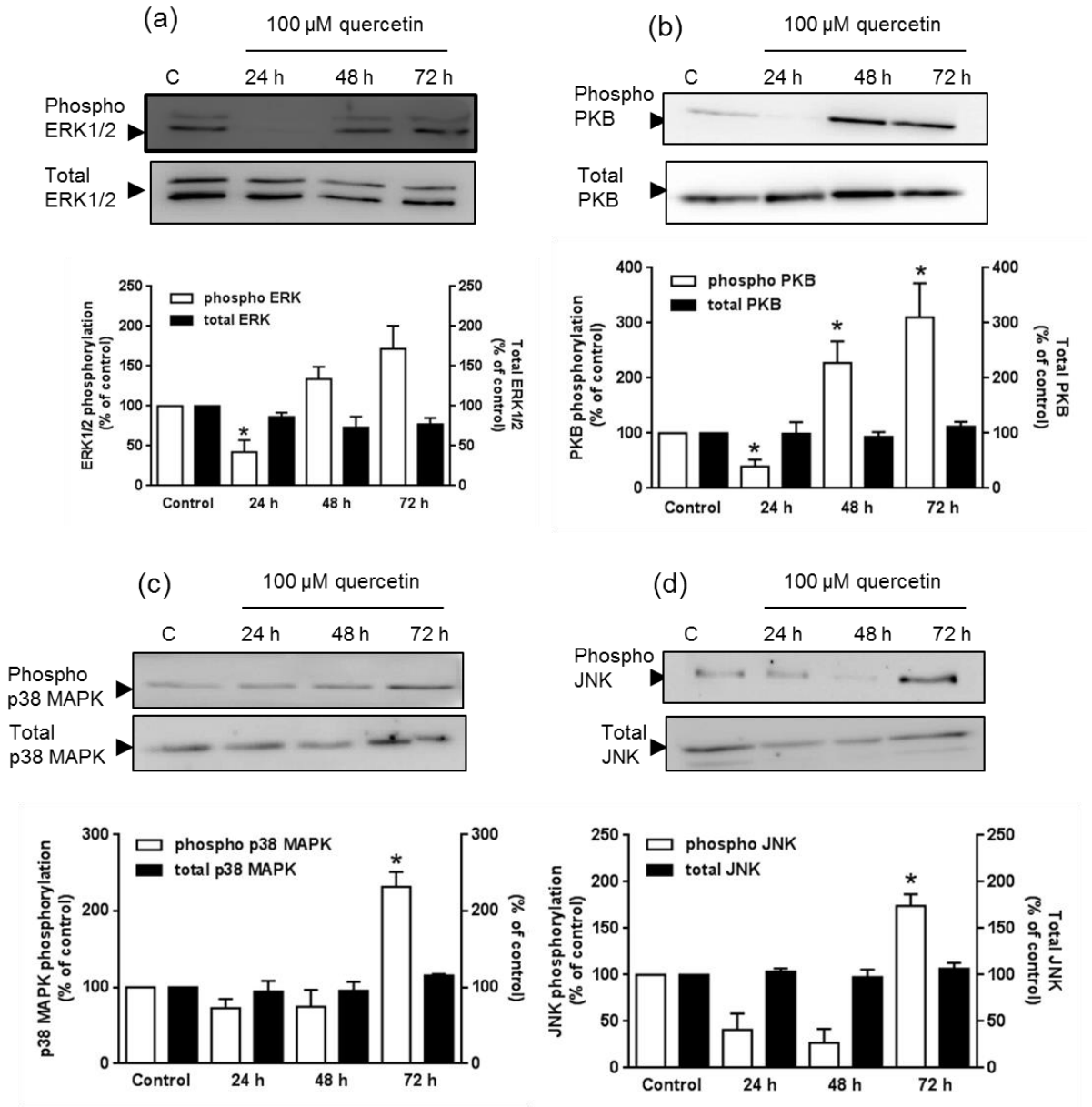

(a)
100 µM quercetin
C
24 h
48 h
72 h
Phospho ERK1/2
Total ERK1/2
phospho ERK
total ERK
ERK1/2 phosphorylation (% of control)
Total ERK1/2 (% of control)
Control
(b)
Phospho PKB
Total PKB
phospho PKB
total PKB
PKB phosphorylation (% of control)
Total PKB (% of control)
(c)
Phospho p38 MAPK
Total p38 MAPK
phospho p38 MAPK
total p38 MAPK
p38 MAPK phosphorylation (% of control)
(d)
Phospho JNK
Total JNK
phospho JNK
total JNK
JNK phosphorylation (% of control)
Total JNK (% of control)

Figure 6

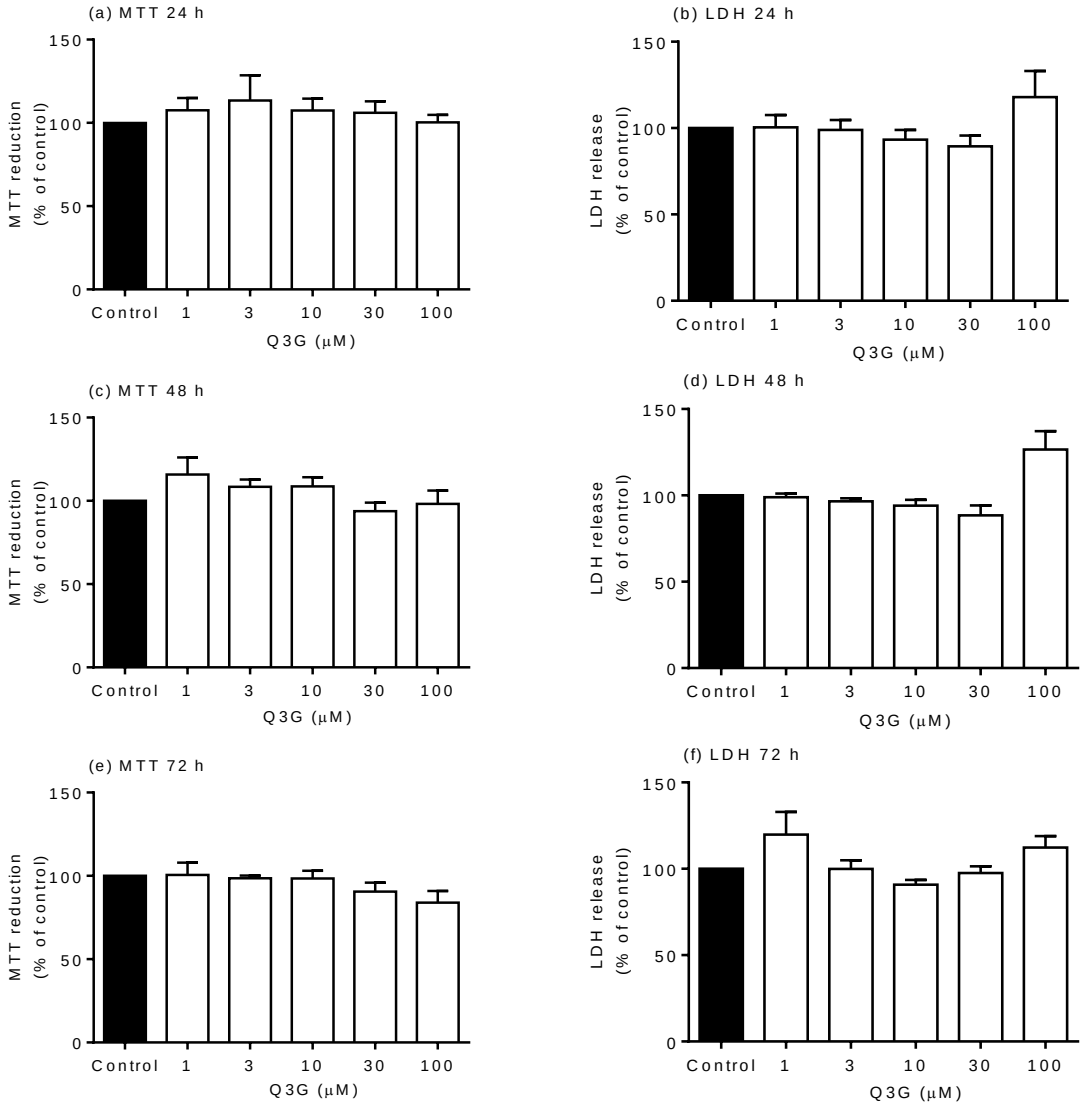

(a) MTT 24 h
MTT reduction (% of control)
150
100
50
0
Control 1 3 10 30 100
Q3G (μM)
(b) LDH 24 h
LDH release (% of control)
150
100
50
0
Control 1 3 10 30 100
Q3G (μM)
(c) MTT 48 h
MTT reduction (% of control)
150
100
50
0
Control 1 3 10 30 100
Q3G (μM)
(d) LDH 48 h
LDH release (% of control)
150
100
50
0
Control 1 3 10 30 100
Q3G (μM)
(e) MTT 72 h
MTT reduction (% of control)
150
100
50
0
Control 1 3 10 30 100
Q3G (μM)
(f) LDH 72 h
LDH release (% of control)
150
100
50
0
Control 1 3 10 30 100
Q3G (μM)

Figure 7

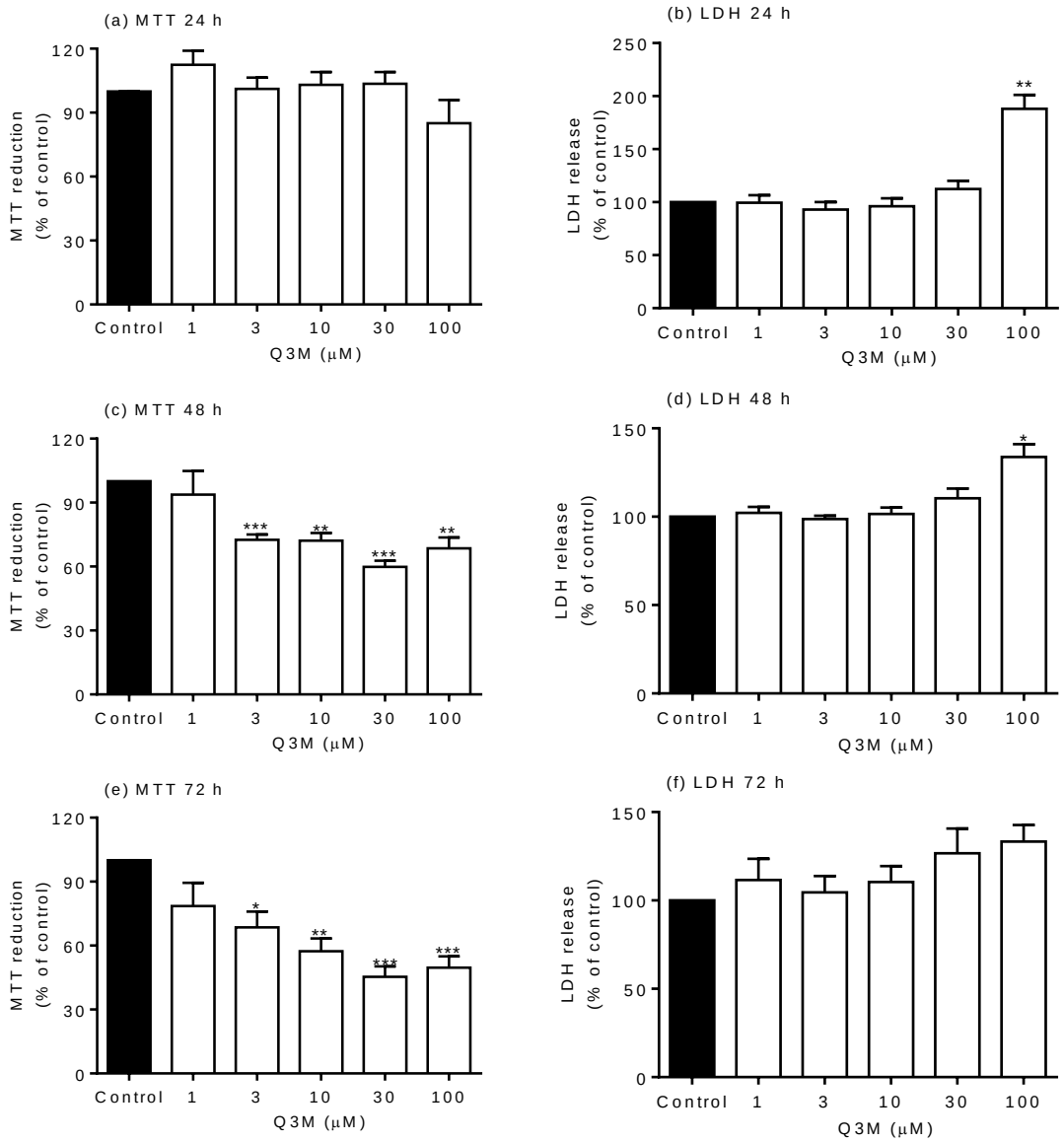

(a) MTT 24 h
MTT reduction (% of control)
120
90
60
30
0
Control 1 3 10 30 100
Q3M (μM)
(b) LDH 24 h
LDH release (% of control)
250
200
150
100
50
0
**
Control 1 3 10 30 100
Q3M (μM)
(c) MTT 48 h
MTT reduction (% of control)
***
**
***
**
Control 1 3 10 30 100
Q3M (μM)
(d) LDH 48 h
LDH release (% of control)
150
100
50
0
*
Control 1 3 10 30 100
Q3M (μM)
(e) MTT 72 h
MTT reduction (% of control)
*
**
***
***
Control 1 3 10 30 100
Q3M (μM)
(f) LDH 72 h
LDH release (% of control)
150
100
50
0
Control 1 3 10 30 100
Q3M (μM)

Figure 8

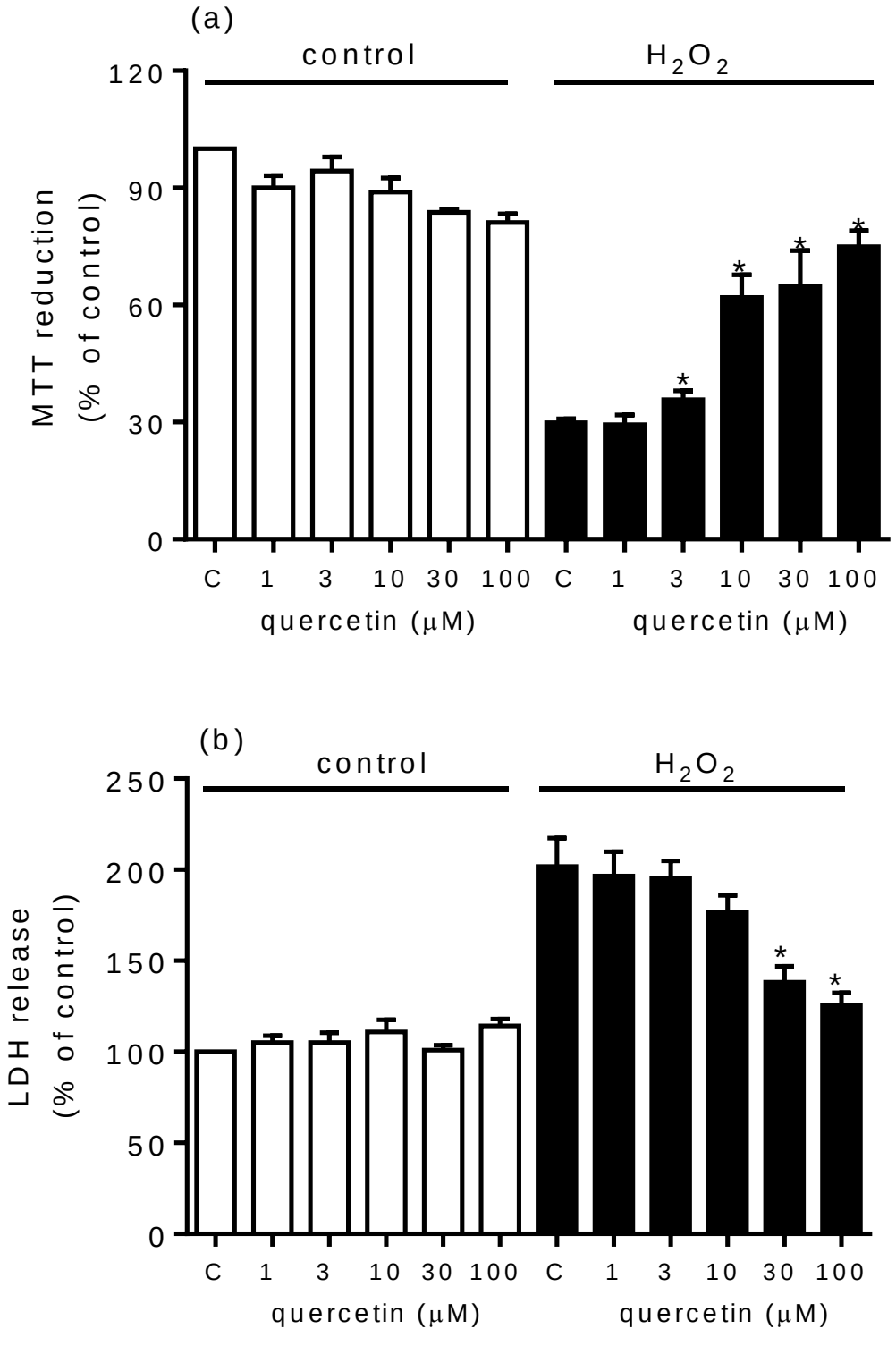

(a)
control
H_2O_2
MTT reduction
(% of control)
120
90
60
30
0
C 1 3 10 30 100 C 1 3 10 30 100
quercetin (μM)
quercetin (μM)
(b)
control
H_2O_2
LDH release
(% of control)
250
200
150
100
50
0
C 1 3 10 30 100 C 1 3 10 30 100
quercetin (μM)
quercetin (μM)

Figure 9

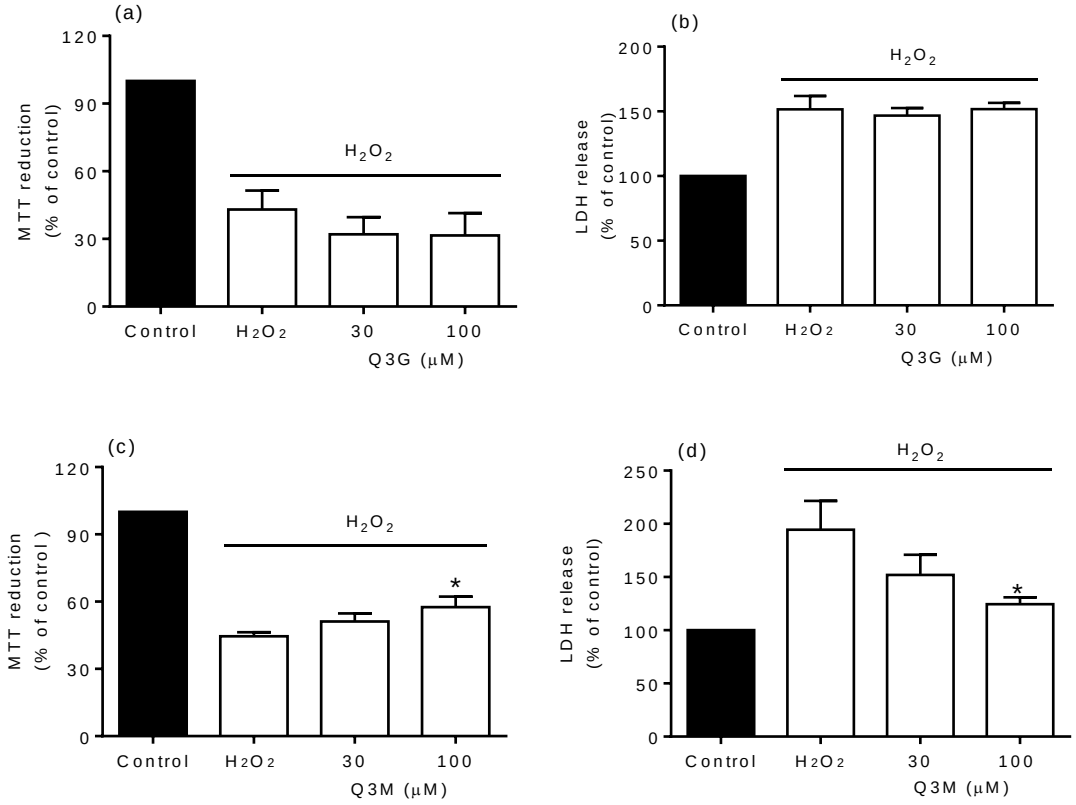

(a)
MTT reduction (% of control)
120
90
60
30
0
H_2O_2
Control
H_2O_2
30
100
Q3G (μM)
(b)
LDH release (% of control)
200
150
100
50
0
H_2O_2
Control
H_2O_2
30
100
Q3G (μM)
(c)
MTT reduction (% of control)
120
90
60
30
0
H_2O_2
*
Control
H_2O_2
30
100
Q3M (μM)
(d)
LDH release (% of control)
250
200
150
100
50
0
H_2O_2
*
Control
H_2O_2
30
100
Q3M (μM)

Figure 10

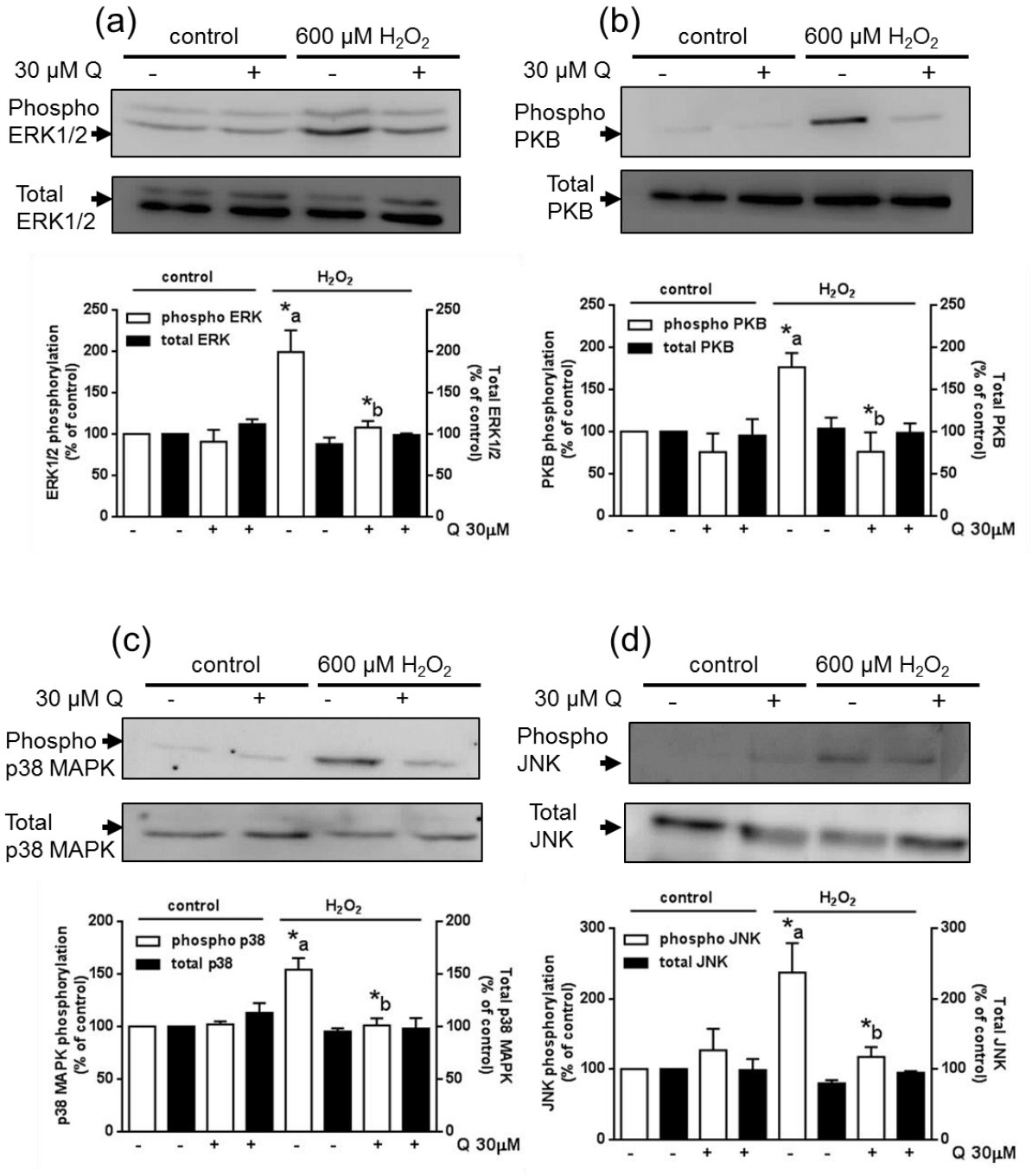

(a)
control
600 μM H_2O_2
30 μM Q
Phospho ERK1/2
Total ERK1/2
(b)
Phospho PKB
Total PKB
phospho ERK
total ERK
ERK1/2 phosphorylation (% of control)
Total ERK1/2 (% of control)
phospho PKB
total PKB
PKB phosphorylation (% of control)
Total PKB (% of control)
(c)
Phospho p38 MAPK
Total p38 MAPK
phospho p38
total p38
p38 MAPK phosphorylation (% of control)
Total p38 MAPK (% of control)
(d)
Phospho JNK
Total JNK
phospho JNK
total JNK
JNK phosphorylation (% of control)
Total JNK (% of control)
Q 30μM

Figure 11

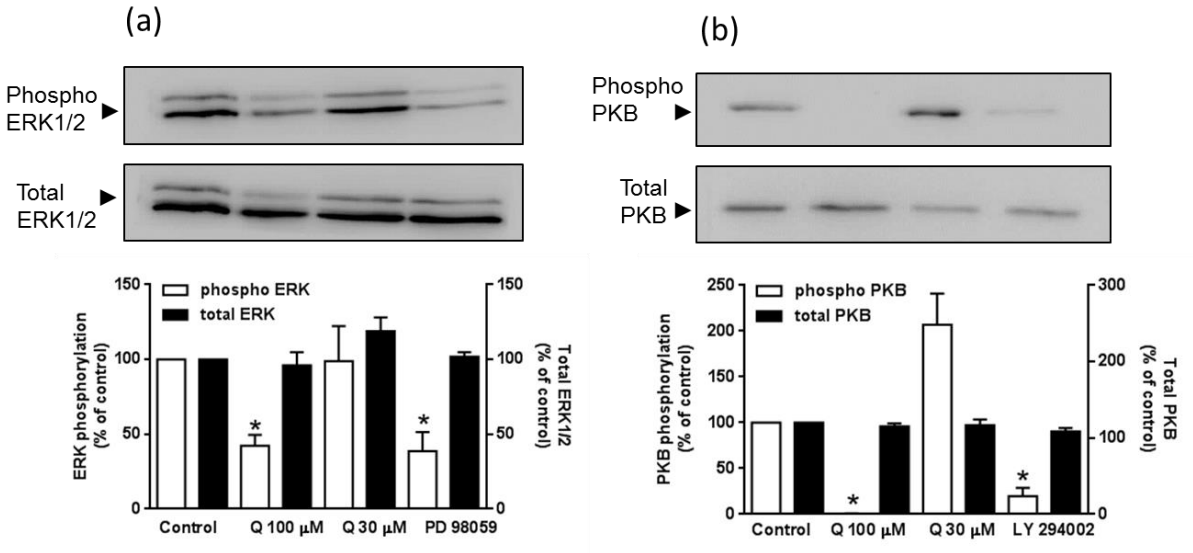
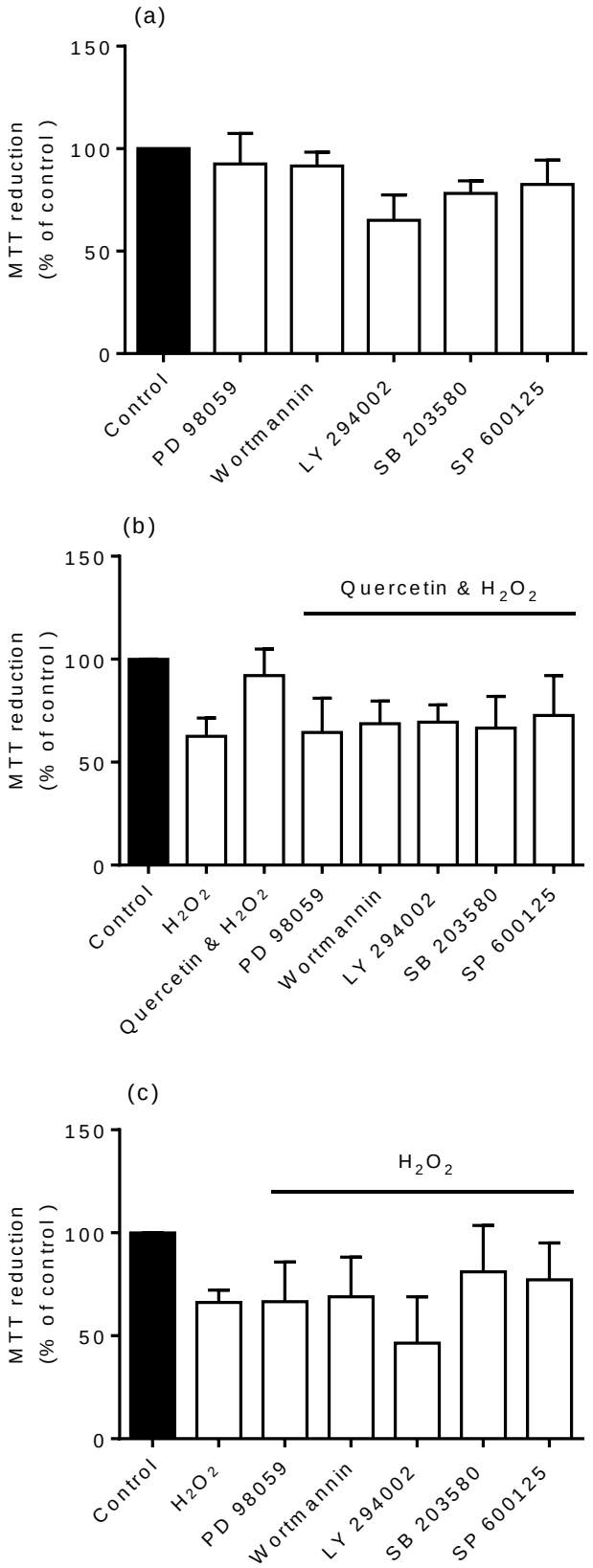

(a)
Phospho ERK1/2
Total ERK1/2
phospho ERK
total ERK
ERK phosphorylation (% of control)
Total ERK1/2 (% of control)
150
100
50
0
Control
Q 100 µM
Q 30 µM
PD 98059
(b)
Phospho PKB
Total PKB
phospho PKB
total PKB
PKB phosphorylation (% of control)
Total PKB (% of control)
250
200
150
100
50
0
300
Control
Q 100 µM
Q 30 µM
LY 294002

Figure 12


(a)
MTT reduction (% of control)
150
100
50
0
Control
PD 98059
Wortmannin
LY 294002
SB 203580
SP 600125
(b)
Quercetin & H_2O_2
Control
H_2O_2
Quercetin & H_2O_2
PD 98059
Wortmannin
LY 294002
SB 203580
SP 600125
(c)
H_2O_2