Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 496 (2004) 41–48

www.elsevier.com/locate/ejphar

Concentration-dependent differential effects of quercetin on rat aortic smooth muscle cells

Chun-Ming Shih[a,1], Heng Lin[a,1], Yu-Chih Liang[b], Wen-Sen Lee[c], Wei-Fung Bi[a], Shu-Hui Juan[c,*]

[a] *The Section of Cardiology, Department of Medicine, Taipei Medical University Hospital, 250 Wu-Hsing Street, Taipei 110, Taiwan*
[b] *Biomedical Technology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan*
[c] *Graduate Institute of Medical Sciences and Department of Physiology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan*

Received 27 May 2004; accepted 9 June 2004

Abstract

Quercetin is one of the most ubiquitous bioflavonoids in foods of plant origin. Although quercetin is generally considered to provide protection against oxidative injury and inflammation, recent studies have demonstrated that its cytoprotective effects occur within a narrow concentration range. We attempted to examine the concentration-dependent effect on proliferation and inflammation in the primary culture of rat aortic smooth muscle cells. We demonstrate that quercetin inhibited [^{3}H]thymidine incorporation into rat aortic smooth muscle cells only at concentrations $\leqq 50$ μM in a concentration-dependent manner. Nevertheless, quercetin, at concentrations $\geqq 100$ μM, reduced cell viability; this was further characterized as being due to apoptosis, which occurred through the proteolytic activation of pro-caspase-3. Additionally, the phosphorylation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK) substantially increased in rat aortic smooth muscle cells exposed to 100 μM quercetin, results which differ from observations by others and ourselves of cells exposed to $\leqq 50$ μM quercetin. Unlike P-JNK and P-p38, the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/ERK2) was not significantly affected by the concentration-dependent effects of quercetin. Surprisingly, the adverse effects of higher concentrations of quercetin could be ameliorated by adding the antioxidants, catalase, and *N*-acetylcysteine (NAC). Furthermore, the electrophoretic mobility shift assay (EMSA) showed that rat aortic smooth muscle cells exposed to quercetin at concentrations of $\leqq 50$ μM caused concentration-dependent inhibition of nuclear factor kappa B (NF-κB) activity, whereas concentrations of $\geqq 100$ μM resulted in increased NF-κB binding activity. We demonstrate for the first time that quercetin at low concentrations has antiproliferative and antiinflammatory effects, but at concentrations of $\geqq 100$ μM, is likely to induce the opposite effects on rat aortic smooth muscle cells.

Keywords: Aortic smooth muscle cell, rat; Quercetin; Mitogen-activated protein kinase; Extracellular signal-regulated kinase; c-Jun N-terminal kinase

1. Introduction

Atherosclerosis is a chronic pathological process of multiple oxidative insults. Although the underlying mechanism has not yet been fully determined, it is believed that oxidation and inflammation are two crucial events involved in the development of atherosclerosis. In response to injury and various stimuli, the activated vascular endothelium produces cytokines and growth factors, which promote the growth and migration of rat aortic smooth muscle cells, are key events in the formation of atherosclerotic lesions in humans.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the most ubiquitous bioflavonoids in foods of plant origin. In one estimate, humans normally consume approximately 1 g of flavonoids per day (Afanas'eva et al., 2001). Several studies have found that quercetin has antioxidative, free radical-scavenging, anticancer, and antiinflammatory activities (Chen et al., 2001a,b; Fisher-Dzoga et al., 1973; Shen et al., 2002). Additionally, quercetin inhibits the proliferation and migration of aortic smooth muscle cells, which coincides with the inhibition of p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation (Alcocer et al., 2002). These findings suggest new insights and a

* Corresponding author. Tel.: +886-2-27361661x3717; fax: +886-2-27361661x3716.
E-mail address: juansh@tmu.edu.tw (S.-H. Juan).
[1] The first 2 authors contribute equally to this work.

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.06.016

rationale for the potential use of quercetin in the prophylaxis of cardiovascular diseases.

In contrast, the biological effects of quercetin are matters of debate due to its inconsistent actions. For example, quercetin has been shown to be mutagenic in prokaryotes, as well as in in-vitro studies of mammalian cell lines (Aravindakshan et al., 1985). Additionally, quercetin causes cytotoxic effects on tumor cells in culture; however, 4-methylcatechol, its metabolite produced in the intestinal tract after ingestion, promotes rather than suppresses tumors in the rat stomach, despite its in-vitro cytotoxic activity (Morita et al., 2003). Furthermore, there are many reports describing how polyphenols act as pro-oxidants in the presence of metal ions (Nakayama, 1994). It has also been shown that quercetin itself has some intrinsic cytotoxicity at concentrations exceeding 75 μM in cultured renal tubular cells (Kuhlmann et al., 1998). Therefore, in view of the unavoidability of ingesting quercetin via a normal diet, there is a need to reevaluate the potential risk to humans due to quercetin ingestion.

The primordial role of inflammation in atherosclerosis is increasingly recognized by researchers. Nuclear factor kappa B (NF-κB) has been shown to orchestrate the transcription of a cassette of genes of great relevance to atherogenesis (Chen et al., 2002; Wilson et al., 2002; Yamamoto and Gaynor, 2001). In particular, it is a key regulator of atherogenic gene expression regulated by risk factors, including angiotensin II, thrombin, advanced glycated endproducts, etc. (Hattori et al., 1999; Kaur et al., 2003; Minami and Aird, 2001; Phillips and Kagiyama, 2002). However, the relationship between quercetin and the activation of NF-κB is also inconsistent. For instance, quercetin has been shown to exert inhibitory effects on the activation of NF-κB in murine fibroblast L-TK cells induced by interlukin-1 and hypoxia (Muraoka et al., 2002). On the contrary, it is reported that quercetin induces apoptosis and NF-κB in growing murine L1210 lymphocytic leukemic cells potentiated by TNF-alpha (Orzechowski et al., 2000).

We speculated that the above-described discrepancies might be due to the effects of quercetin being tissue and concentration dependent. We proposed that quercetin might exert its biological effects like a double-edged sword, dependent on its concentrations. Therefore, we set out to examine the diverse effects of quercetin on rat aortic smooth muscle cells in a concentration-dependent manner, which might provide some important information on the amount of quercetin that should be consumed for protecting blood vessels, without resulting in any unexpected injuries.

2. Materials and methods

2.1. Agents

Quercetin, catalase, *N*-acetylcysteine (NAC), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dithiothreitol (DTT), HEPES, and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal antiphosphospecific extracellular signal-regulated kinase 1/2 (ERK1/ERK2), antiphosphospecific p38 MAPK, and antiphophospecific c-Jun N-terminal kinase (JNK) antibodies were purchased from New England Biolabs (Beverly, MA, USA). Anti-ERK1/ERK2, anti-p38 MAPK, and anti-JNK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Protein assay agents were purchased from Bio-Rad (Hercules, CA, USA). The gel-shift kit was from Panomics (Redwood City, CA, USA).

2.2. Isolation and primary culture of rat aortic smooth muscle cells

Rat arotic smooth muscle cells were isolated from the thoracic aorta of male prague–Dawley rats (200–250 g) using the explant technique (Fisher-Dzoga et al., 1973). Briefly, after removal of the endothelium and adventitia, the aortic explants were cultured in DMEM and supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 mM HEPES (pH 7.4). After 2 weeks, cells that had migrated out of the explants were removed by trypsinization and successively subcultured. The purity and identity of cells were examined by immunostaining using antibody specific against smooth muscle cell α-actin. Cells from passages 5 to 12 were used for the experiments.

2.3. MTT assay

The viability of cells was determined based on the activity of mitochondrial dehydrogenase to reduce MTT to formazan. Briefly, cells were plated in 24-well plates at a density of $(2-5)\times 10^4$cells/well treated with DMSO or varying concentrations of quercetin for 24 or 48 h. MTT was then added to each well, and incubation continued for 4 h in the culture. The formazan formed was dissolved by overnight incubation with 10 mM HCl containing 10% SDS, and then measured at a wavelength of 570 nm.

2.4. [^{3}H]Thymidine incorporation

As previously described (Jain et al., 1996; Lin et al., 2002), cells at a density of 1×10^4 cells/cm^3 were applied to 24-well plates in a growth medium (DMEM plus 10% fetal bovine serum). After the cells had grown to 70%–80% confluence, they were rendered quiescent by incubation for 72 h in DMEM containing 0.04% fetal bovine serum. The concentration of quercetin, as indicated, or dimethyl sulfoxide (DMSO) in 10% fetal bovine serum was added to the cells, and the mixture was allowed to incubate for 24 h. During the last 4 h of the incubation with or without quercetin, [^{3}H]thymidine was added at 1 μCi ml^{-1} (1 μCi = 37 kBq). The

incorporated [^{3}H]thymidine was extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

2.5. DNA ladder assay

Following DMSO or quercetin treatment, adherent and nonadherent cells were pooled and subjected to protease K digestion at 56 °C for 10 min. After spinning for 5 min at 300 × *g*, the supernatant was collected, and genomic DNA was extracted using a QIAmp DNA Mini Kit. DNA preparations were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

2.6. Western blots

Western blot analysis was carried out using the following antibodies: pro-caspase-3, α-tubulin, and total and phosphorylated MAPK subfamilies. To prepare whole-cell lysates, cells were washed twice with ice-cold phosphate-buffered saline (PBS), resuspended in ice-cold extraction buffer [10 mM Tris, pH 7.0; 140 mM NaCl; 5 mM DTT; 0.5% Nonidet P-40; 1 mM Na_3VO_4; 1 mM phenylmethylsulfonyl fluoride (PMSF); 1 μg/ml leupeptin; and 1 μg/ml aprotinin], incubated on ice for 30 min, and vortexed every 10 min, followed by centrifugation at 12,000 rpm for 30 min at 4 °C. Whole-cell lysates (80 μg) were electrophoresed on a 10% SDS-polyacrylamide gel and then transblotted onto a Hybond-P membrane (Pharmacia, Hong Kong). Membranes were blocked in PBS containing 0.1% Tween-20 and 5% skim milk at room temperature for 30 min. For the detection of pro-caspase-3, α-tubulin, and total and phosphorylated MAPK subfamilies, blots were incubated with the indicated antibodies (with dilutions used according to the manufacturer's instructions) in blocking buffer for 1 h at room temperature. After three washes with PBS containing 0.1% Tween-20, blots were incubated with alkaline phosphase/peroxidase-conjugated goat antirabbit immunoglobulin G (IgG)/antimouse IgG (1:2000) for 1 h at room temperature, followed by another washing. Expression of protein was detected by an enhanced chemiluminescence system.

2.7. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed as previously described (Wung et al., 1997). To prepare nuclear protein extracts, cells in 10-cm^2 dishes after treatment with various concentrations of quercetin for 6 h, as indicated, were washed twice with ice-cold PBS and scraped off into 1 ml of PBS. After centrifugation of the cell suspension at 500 × *g* for 3 min, the supernatant was removed, and cell pellets were subjected to NE-PER™ nuclear extraction reagents (Pierce, Rockford, IL, USA), with the addition of 0.5 mg/ml benzamidine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 mM PMSF. The subsequence procedures of nuclear protein extraction followed the manufacturer's instructions. The fraction containing the nuclear protein was used for the assay or was stored at − 70 °C until use. The sequence of the oligonucleotides used was AGTTGAGGGGACTTTCCAGGC for NF-κB binding (Promega, Madison, WI, USA). The oligonucleotides were end-labeled with biotin. Extracted nuclear protein (10 μg) was incubated with 10 ng of biotein-labeled DNA at 15 °C for 30 min in 2 μl of 5 × binding buffer containing 1 μg of poly deoxyinosine-deoxycytidine (dI-dC). For competition with unlabeled oligonucleotides, a 100-fold molar excess of unlabeled oligonucleotides relative to the biotin-labeled probe was added to the binding assay. The mixtures were electrophoresed on 5% nondenaturing polyacrylamide gels and then transblotted onto a Hybond N$^+$ membrane (Pharmacia). Blots were incubated with blocking buffer, followed by additional streptavidin-horseradish peroxidase (HRP) conjugates. Blots were imaged by means of an enhanced chemiluminescence system.

2.8. Assay of intracellular reactive oxygen species

Intracellular reactive oxygen species production was measured by using the fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Eugene, OR, USA) with the ACAS interactive laser cytometer (Meridian Instruments, Okemos, MI, USA). Cells were preincubated with 10 μM DCF-DA in DMEM for 30 min at 37 °C before treatment. After exposure to the dye, the cells were rinsed with Tyrode's solution and examined by using the laser cytometer at 37 °C. Excitation of dichlorofluorescein (DCF) was achieved by using the 488-nm line of a 20-mW argon–ion laser. The emission above 515 nm was quantitated from two-dimensional scans generated by using a 1-μm laser beam and an X-Y scanning stage to obtain a fluorescence value from single cells. To provide a valid comparison, the same acquisition parameters were used for all observations. Quantification of the levels of DCF fluorescence was assessed on a relative scale from 0 to 4000 units. Baseline values from cells treated with DMSO were used as control. Values represent the mean ± S.D. of DCF fluorescence from 20 randomly selected cells for each experiment in the four investigations.

2.9. Statistical analysis

Values are expressed as the mean ± S.D. The significance of the difference between the control and each experimental test condition was analyzed by Student's *t*-test, and between two experimental groups was estimated by one-way analysis of variance. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity induced by quercetin at concentrations of ≧100 μM in rat aortic smooth muscle cells

Although quercetin is generally considered to provide protection against oxidative injury, a recent study

demonstrated that its cytoprotective effect occurs within a narrow range of concentrations (Kuhlmann et al., 1998). As shown in Fig. 1, cell growth, as assessed by the MTT assay for a period of 2 days, was markedly reduced in rat aortic smooth muscle cells exposed to quercetin in a concentration-dependent manner, particularly in cells exposed to quercetin at concentrations of ≧100 μM.

To further examine the specificity of a direct effect of quercetin on cellular proliferation, we performed [^{3}H]thymidine incorporation in rat aortic smooth muscle cells in the presence of various concentrations of this compound. The results on Fig. 1B show that quercetin in the presence 10 to 50 μM quercetin inhibited [^{3}H]thymidine incorporation into cells in a concentration-dependent manner. In contrast, quercetin at ≧100 μM exerted a mitogenic effect on cells due to the increased amounts of [^{3}H]thymidine incorporated into the cells.

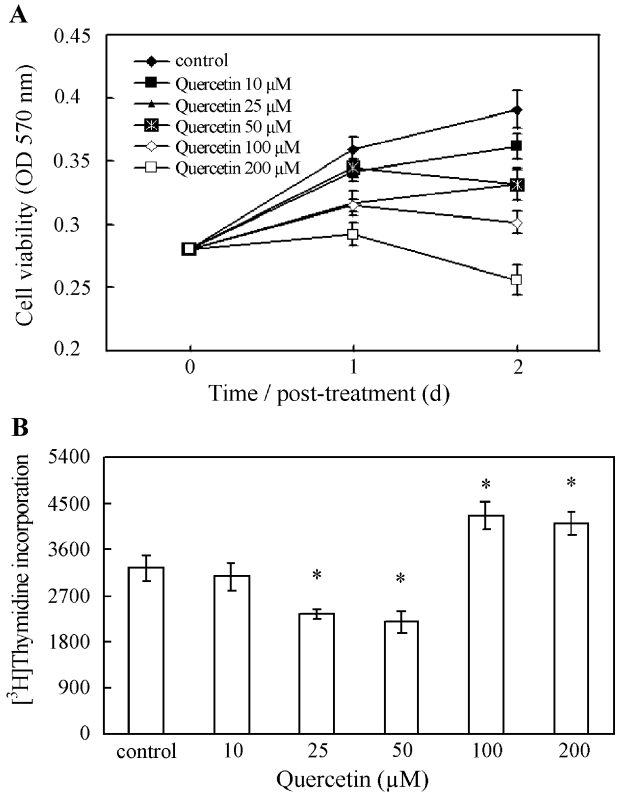

Fig. 1. Effect of quercetin on cell growth. (A) Effect of quercetin on cell viability. Cells plated at 5×10^5 cells/well in 24-well plates were treated with DMSO or quercetin at the indicated concentrations. Cell viability was determined by the MTT assay at given time points. Data shown are the mean ± S.D. of three independent experiments. (B) The differential growth pattern of cells in the presence of quercetin. To study the effect of quercetin on cell proliferation, [^{3}H]thymidine incorporation was investigated after cells release from quiescence by incubation in culture media supplemented with 10% fetal bovine serum and DMSO and indicated concentrations of quercetin in DMSO. Data shown are the mean ± S.D. of three independent experiments. A value of $*p < 0.05$ was considered statistically significant.

3.2. DNA fragmentation and attenuated pro-caspase-3 in rat aortic smooth muscle cells treated at higher concentrations of quercetin

To characterize whether the cell death induced by quercetin at concentrations ≧100 μM results from apoptosis, we examined the integrity of chrosomal DNA of cells exposed to various concentrations of quercetin by electrophoretic analysis. Cell growth was arrested by maintaining cells in serum-deprived culture medium for 72 h, and the effect of various concentrations of quercetin on rat aortic smooth muscle cells in DMEM supplemented with 2% fetal bovine serum was examined. As shown in Fig. 2A, the appearance of DNA was observed in cells exposed to quercetin at concentrations of ≧100 μM. The evidence for apoptotic cell death was further confirmed by Western blot analysis of pro-caspase-3 in protein preparations. Results in Fig. 2B demonstrate attenuated amounts of pro-caspase-3 in cells treated with increasing concentrations of quercetin.

3.3. Concentration-dependent effects of quercetin on the MAPK pathway

Results shown in Fig. 1B suggest that quercetin caused a differential effect on rat aortic smooth muscle cells proliferation. Therefore, the concentration-dependent effects of quercetin on the signal transduction pathway in cells were investigated. We performed Western blot analysis of total and phosphorylated ERK1/ERK2, JNK, and p38MAPK exposed to a range of 10–100 μM quercetin. Cells were rendered quiescent for 72 h in DMEM medium containing 0.04% fetal bovine serum and were then treated with DMSO or indicated concentrations of quercetin for 5 or 10 min, respectively. Crude cell lysates were harvested and analyzed for total and phosphorylated MAPKs by Western blots. Phosphorylated ERK1/ERK2 (p44/p42) was detected in cells treated with DMSO (Fig. 3, lane 1), and a slight decline in the levels of these species was observed within 5 and 10 min in cells exposed to increasing concentrations of quercetin (Fig. 3, lanes 2–9). Conversely, the levels of phosphorylated p38 and JNK were increased in cells exposed to the high concentration of 100 μM quercetin for 10 min (Fig. 3, lane 9), as compared with cells incubated at concentrations of quercetin below 100 μM (Fig. 3, lanes 2–8). A discrepancy in the levels of phosphorylated p38 and JNK was not apparent in cells exposed to increasing concentrations of quercetin with 5 min of treatment (Fig. 3, lanes 2–5).

3.4. Concentration-dependent effects of quercetin on NF-κB DNA-binding activity in rat aortic smooth muscle cells

NF-κB plays an important role in the inducible expression of various cellular genes mostly related to inflammation. Quercetin has been shown to enhance or inhibit the activity of NF-κB in response to various stimuli in various tissues (Muraoka et al., 2002). Therefore, the discrepant

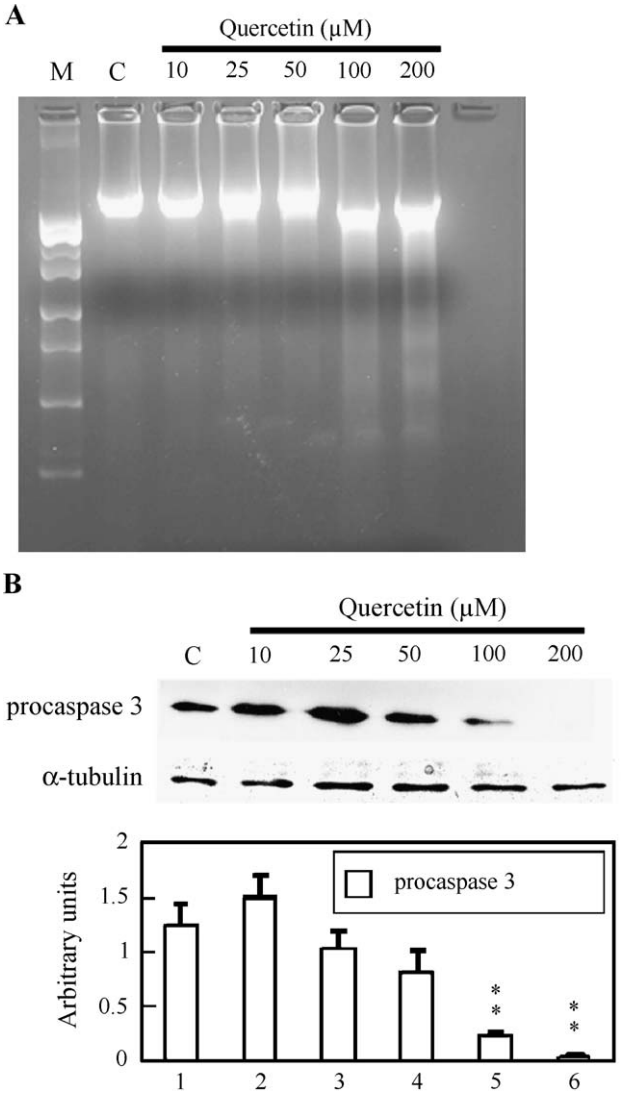

Fig. 2. Apoptosis in rat aortic smooth muscle cells exposed to ≧100 μM quercetin. (A) DNA fragmentation in rat aortic smooth muscle cells. Cells were exposed to the indicated concentrations of quercetin in DMEM supplemented with 2% fetal bovine serum for 2 days. Chromosomal DNA was then isolated 2 days after treatment with quercetin and analyzed by 2% agarose gel electrophoresis. (B) Western blot analysis of pro-caspase-3 in cells exposed to increasing concentrations of quercetin. Crude cell lysates were prepared, and the level of pro-caspase-3 was examined. α-Tubulin was used as an internal control. Bar charts in the lower part of Panel B show the band intensity of normalized pro-caspase-3 protein in terms of α-tubulin by densitometry (IS-1000 Digital Imaging System). Data were derived from three independent experiments and are presented as the mean ± S.D. $^{**}p < 0.01$ indicates significant differences from the DMSO-treated group.

effects of quercetin on NF-κB DNA binding activity might be tissue and stress dependent. We set out to examine whether the effect may also result from a concentration-dependent effect of quercetin in cell culture. Cells were cultured in the presence of quercetin, and nuclear proteins were extracted after 6 h. The mobility shift of biotin-labeled oligonucleotides harboring NF-κB recognition sequences was assayed by incubation with nuclear proteins. Fig. 4 depicts a representative gel shift assay for NF-κB oligonucleotides after 6 h of cell treatment, showing two protein–DNA complexes (κB-I and κB-II). Quercetin, at a concentration of 10–50 μM, caused a decline in the binding activity of κB-I in a concentration-dependent manner. This inhibition disappeared, and eventually, increased binding activity of κB-I was detected in aortic smooth muscle cells exposed to quercetin at ≧100 μM. A similar pattern of DNA binding activity in cells was observed in the κB-II complex with respect to the concentrations of quercetin used. The intensity of these complexes was reduced by an unlabeled NF-κB consensus oligonucleotides added in a 100-fold molar excess to the binding reaction (Fig. 4, lane 7).

3.5. Attenuation of quercetin-induced oxidative stress and cytotoxicity by catalase and NAC

We hypothesized that the increased phosphorylation of JNK and p38 and the apoptotic death induced by quercetin at concentrations ≧100 μM might be attributable to quer-

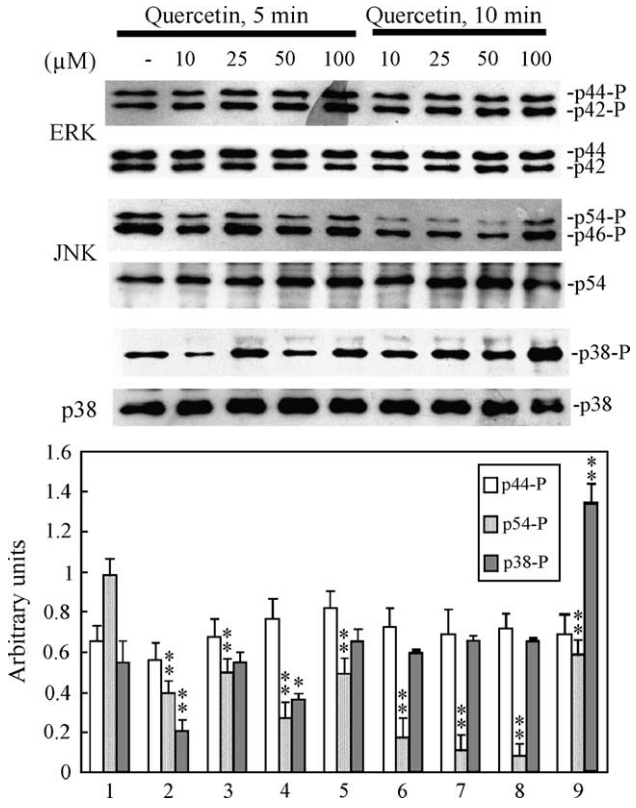

Fig. 3. Western blot analysis of MAPKs in rat aortic smooth muscle cells after exposure to various concentrations of quercetin. Cells were rendered quiescent for 72 h with 0.04% fetal bovine serum, followed by treatment with DMSO (lane 1) and indicated concentrations of quercetin for 5 and 10 min (lanes 2–5 and 6–9, respectively). Preparations of cell extracts, gel electrophoresis, and Western blots are described in Materials and methods. Individual MAPK was identified by its size (kDa), and (P) designates phosphorylated MAPK. Bar charts in the lower panel show the band intensity of normalized p44-P, p54-P, and p38-P proteins by densitometry. Data were derived from three independent experiments and are presented as the mean ± S.D. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicates significant differences from the DMSO-treated group.

cetin-induced oxidative stress. Fig. 5A showed the production of reactive oxygen species by quercetin at the concentration of ≥100 μM, which is analyzed the fluorescent product DCF, a peroxidative productive of DCF-DA. We also set out to determine the effect of the antioxidants, catalase and NAC, on the signal transduction pathway; it clearly showed that catalase at concentrations of 200 and 350 U/ml and NAC at concentrations of 5 to 10 mM were able to reverse the phosphorylation of JNK and p38 to the level of control cells (Fig. 5B). Consistently, catalase and NAC inhibited DNA fragmentation by 200 μM quercetin (Fig. 5C), suggesting that oxidative stress is indeed involved in the cytotoxicity induced by higher concentrations of quercetin.

4. Discussion

In this study, we investigated the concentration-dependent effects of quercetin on rat aortic smooth muscle cells

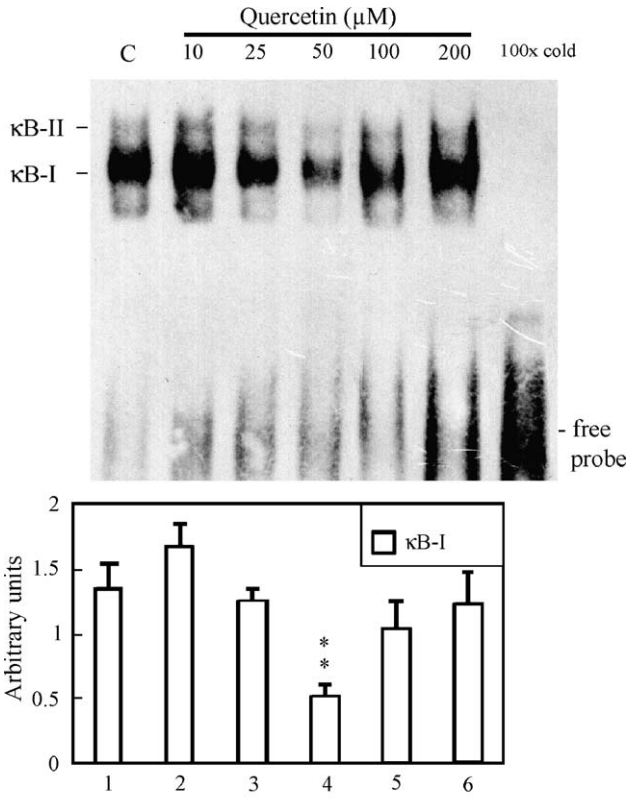

Fig. 4. Binding activity of NF-κB in rat aortic smooth muscle cells exposed to various concentrations of quercetin. Cells were cultured and treated with increasing concentrations of quercetin as indicated. Nuclear proteins were assayed for NF-κB binding activity by EMSA. 100× cold denotes a 100-fold molar excess of unlabeled oligonucleotides relative to the biotin-labeled probe; this was added to the binding assay for competition with the unlabeled oligonucleotides. Mobility of specific NF-κB complexes is indicated. Bar charts in the lower panel show the band intensity of κB-I by densitometry. Data were derived from four independent experiments and are presented as the mean ± S.D. $^{**}p<0.01$ indicates significant differences from the DMSO-treated group.

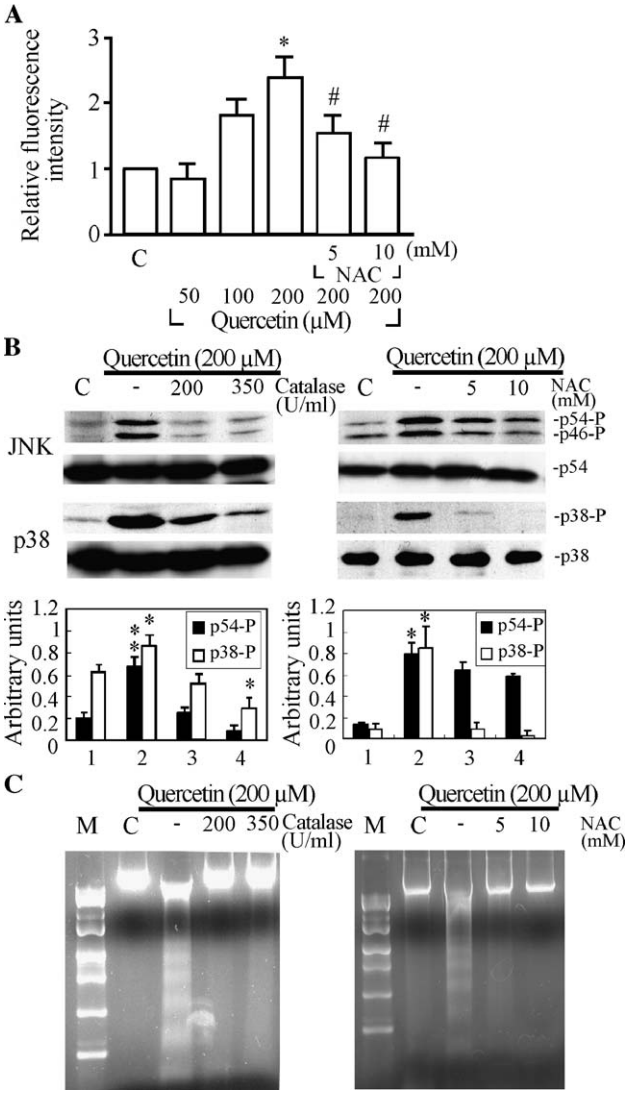

Fig. 5. Quercetin at high concentrations of ≧100 μM quercetin induced intracellular reactive oxygen species in rat aortic smooth muscle cells (A) and effect of antioxidants on the phosphorylation of JNK and p38 (B) and DNA fragmentation (C) in cells exposed to 200 μM quercetin. Cells were pretreated with 200 or 350 U/ml catalase and 5 or 10 mM NAC for 30 min prior to adding 200 μM quercetin, followed by incubation for 10 min in DMEM supplemented with 10% fetal bovine serum (B) and for 24 h in DMEM, supplemented with 2% fetal bovine serum (C), by the method described in Fig. 2. Bar charts in the lower part of Panel B show the band intensity of normalized p54-P and p38-P proteins by densitometry. Data were derived from three independent experiments and are presented as the mean ± S.D. $^{\#}p<0.05$ vs. quercetin alone, $^{*}p<0.05$ and $^{**}p<0.01$ vs. DMSO-treated group.

proliferation and viability. Alcocer et al. (2002) showed that quercetin in a range of concentrations (10–200 μM) inhibits the proliferation in human aortic smooth muscle cells in a concentration-dependent manner and arrests cells at the G0/G1 phase of the cell cycle. The results of our study confirmed those findings and showed that quercetin at a range of 10–200 μM decreased cell numbers in rat aortic smooth muscle cells and that quercetin at a range of 10–50 μM caused a gradual decrease in [^{3}H]thymidine incorpora-

tion (Fig. 1). In contrast, results of this study also showed that quercetin at concentrations of ≧100 μM increased cell proliferation in a [^{3}H]thymidine incorporation experiment and phosphorylation of stress- and mitogen-related protein kinases, including JNK and p38, and eventually led to cellular death.

Stiko et al. (1996) indicated that a low level of oxidative stress may induce proliferative events, and that with an increased intensity of stress, cell growth gradually ceases, and subsequent cellular death occurs. In agreement with those findings, results of our study showed that quercetin at concentrations of ≧100 μM had a mitogenic effect on rat aortic smooth muscle cells and caused increased DNA synthesis (Fig. 1B), resulting in cellular death (Fig. 1A). Fig. 5A showed the direct evidence of quercetin-induced intracellular reactive oxygen species production at high concentrations of ≧100 μM, which can be reversed by adding NAC. Furthermore, the results of our study also showed that this cellular damage was further characterized as apoptotic cell death, with evidence of DNA fragmentation (Fig. 2A) and attenuated levels of pro-caspase-3 (Fig. 2B). Likewise, these effects could be inhibited by adding antioxidants, including catalase and NAC (Fig. 5B). Based on all of these findings, we suggest that reactive oxygen species are involved in quercetin-induced cytotoxicity and that cells exposed to quercetin at concentrations exceeding 50 μM experienced increased oxidative cell injury, which induced apoptosis through the proteolytic cleavage of pro-caspase-3.

To our knowledge, this is the first study to show that quercetin exerts its antiproliferation/proliferation and anti-oxidant/pro-oxidant effects on rat aortic smooth muscle cells in a concentration-dependent manner. But the mechanism for generating reactive oxygen species and downstream signaling remains to be elucidated. The activation of the NAD(P)H enzymatic system is regarded as the most important source of the primordial oxygen radical, superoxide, in the vessel wall, and Rac1GTPase plays a pivotal role in this activation (Bhunia et al., 2002). Therefore, further research is needed to investigate how genes involved in the generation of reactive oxygen species are transactivated by a high concentration of quercetin to induce oxidative stress in rat aortic smooth muscle cells.

Intracellular oxygen radicals regulate gene transcription. NF-κB has been identified as a peroxide-activated transcription factor in eukaryotic cells, and its DNA binding and transactivation are strongly activated by H_2O_2 (Meyer et al., 1993). After activation, this factor induces transcription of a number of genes involved in inflammatory and immune responses (Collart et al., 1990). Interestingly, the findings in our study support these notions by showing that quercetin at higher concentrations resulted in oxidative stress and enhanced the binding activity of NF-κB (Fig. 4), whereas this compound at lower concentrations, as an antioxidant, inhibited its activity in a concentration-dependent manner. The findings of our study once again demonstrate that quercetin exerts an antioxidant/prooxidant effect on rat aortic smooth muscle cells dependent on the concentrations of quercetin used.

To sum up, we suggest that the inhibitory effect of quercetin on rat aortic smooth muscle cells growth in our model is related to quercetin-induced oxidative stress at higher concentrations. Concentrations of quercetin of ≧100 μM are cytotoxic to cells, and the cytotoxicity is related to the generation of hydrogen peroxide and reactive oxygen species under the primary culture conditions. This conclusion is supported by the findings that the addition of catalase and NAC can neutralize the cytotoxic pro-oxidant action of quercetin. Results of our findings are confined to in-vitro study, but cells used in this study directly outgrew from the thoracic aorta of rat, which might approximately reflect in-vivo condition. The problem associated with the uncommon application of quercetin to the treatment of human diseases is due to its poor absorptive efficacy and higher effective doses required, although quercetin has been extensively studied to have beneficial effects on humans. Therefore, before quercetin enters trials for cardiovascular diseases, the issue of the bioavailable concentration after quercetin ingestion in humans needs to be further investigated; this will provide more information on the optimal amount of quercetin that should be consumed to protect blood vessels.

Acknowledgements

This study was supported by grants from the National Science Council, Taiwan (92-2320-B-038-015), Taipei Medical University (TMU90-Y05-A151), and Shin-Kong Wu Ho-Su Memorial Hospital (SKH-TMU-92-13).

References

Afanas'eva, I.B., Ostrakhovitch, E.A., Mikhal'chik, E.V., Ibragimova G.A., Korkina, L.G., 2001. Enhancement of antioxidant and anti-inflammatory activities of bioflavonoid rutin by complexation with transition metals. Biochem. Pharmacol. 61, 677–684.

Alcocer, F., Whitley, D., Salazar-Gonzalez, J.F., Jordan, W.D., Sellers, M.T., Eckhoff, D.E., Suzuki, K., Macrae, C., Bland, K.I., 2002. Quercetin inhibits human vascular smooth muscle cell proliferation and migration. Surgery 131, 198–204.

Aravindakshan, M., Chauhan, P.S., Sundaram, K., 1985. Studies on germinal effects of quercetin, a naturally occurring flavonoid. Mutat. Res. 144, 99–106.

Bhunia, A.K., Schwarzmann, G., Chatterjee, S., 2002. GD3 recruits reactive oxygen species to induce cell proliferation and apoptosis in human aortic smooth muscle cells. J. Biol. Chem. 277, 16396–16402.

Chen, Y.C., Shen, S.C., Chen, L.G., Lee, T.J., Yang, L.L., 2001a. Wogonin, baicalin, and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide. Biochem. Pharmacol. 61, 1417–1427.

Chen, Y.C., Shen, S.C., Lee, W.R., Hou, W.C., Yang, L.L., Lee, T.J., 2001b. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide

induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. J. Cell. Biochem. 82, 537–548.

Chen, H., Li, D., Mehta, J.L., 2002. Modulation of matrix metalloproteinase-1, its tissue inhibitor, and nuclear factor-kappa B by losartan in hypercholesterolemic rabbits. J. Cardiovasc. Pharmacol. 39, 332–339.

Collart, M.A., Baeuerle, P., Vassalli, P., 1990. Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. Mol. Cell. Biol. 10, 1498–1506.

Fisher-Dzoga, K., Jones, R.M., Vesselinovitch, D., Wissler, R.W., 1973. Ultrastructural and immunohistochemical studies of primary cultures of aortic medial cells. Exp. Mol. Pathol. 18, 162–176.

Hattori, Y., Banba, N., Gross, S.S., Kasai, K., 1999. Glycated serum albumin-induced nitric oxide production in vascular smooth muscle cells by nuclear factor kappaB-dependent transcriptional activation of inducible nitric oxide synthase. Biochem. Biophys. Res. Commun. 259, 128–132.

Jain, M., He, Q., Lee, W.S., Kashiki, S., Foster, L.C., Tsai, J.C., Lee, M.E., Haber, E., 1996. Role of CD44 in the reaction of vascular smooth muscle cells to arterial wall injury. J. Clin. Invest. 97, 596–603.

Kaur, J., Woodman, R.C., Kubes, P., 2003. P38 MAPK: critical molecule in thrombin-induced NF-kappa B-dependent leukocyte recruitment. Am. J. Physiol., Heart Circ. Physiol. 284, H1095–H1103.

Kuhlmann, M.K., Horsch, E., Burkhardt, G., Wagner, M., Kohler, H., 1998. Reduction of cisplatin toxicity in cultured renal tubular cells by the bioflavonoid quercetin. Arch. Toxicol. 72, 536–540.

Lin, S.Y., Liu, J.D., Chang, H.C., Yeh, S.D., Lin, C.H., Lee, W.S., 2002. Magnolol suppresses proliferation of cultured human colon and liver cancer cells by inhibiting DNA synthesis and activating apoptosis. J. Cell. Biochem. 84, 532–544.

Meyer, M., Schreck, R., Baeuerle, P.A., 1993. H_2O_2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO J. 12, 2005–2015.

Minami, T., Aird, W.C., 2001. Thrombin stimulation of the vascular cell adhesion molecule-1 promoter in endothelial cells is mediated by tandem nuclear factor-kappa B and GATA motifs. J. Biol. Chem. 276, 47632–47641.

Morita, K., Arimochi, H., Ohnishi, Y., 2003. In vitro cytotoxicity of 4-methylcatechol in murine tumor cells: induction of apoptotic cell death by extracellular pro-oxidant action. J. Pharmacol. Exp. Ther. 306, 317–323.

Muraoka, K., Shimizu, K., Sun, X., Tani, T., Izumi, R., Miwa, K., Yamamoto, K., 2002. Flavonoids exert diverse inhibitory effects on the activation of NF-kappaB. Transplant. Proc. 34, 1335–1340.

Nakayama, T., 1994. Suppression of hydroperoxide-induced cytotoxicity by polyphenols. Cancer Res. 54, 1991s–1993s.

Orzechowski, A., Grzelkowska, K., Zimowska, W., Skierski, J., Ploszaj, T., Bachanek, K., Motyl, T., Karlik, W., Filipecki, M., 2000. Induction of apoptosis and NF-kappaB by quercetin in growing murine L1210 lymphocytic leukaemic cells potentiated by TNF-alpha. Reprod. Nutr. Dev. 40, 441–465.

Phillips, M.I., Kagiyama, S., 2002. Angiotensin II as a pro-inflammatory mediator. Curr. Opin. Investig. Drugs 3, 569–577.

Shen, S.C., Lee, W.R., Lin, H.Y., Huang, H.C., Ko, C.H., Yang, L.L., Chen, Y.C., 2002. In vitro and in vivo inhibitory activities of rutin, wogonin, and quercetin on lipopolysaccharide-induced nitric oxide and prostaglandin E(2) production. Eur. J. Pharmacol. 446, 187–194.

Stiko, A., Regnstrom, J., Shah, P.K., Cercek, B., Nilsson, J., 1996. Active oxygen species and lysophosphatidylcholine are involved in oxidized low density lipoprotein activation of smooth muscle cell DNA synthesis. Arterioscler. Thromb. Vasc. Biol. 16, 194–200.

Wilson, S.H., Best, P.J., Edwards, W.D. , Holmes Jr., D.R., Carlson, P.J., Celermajer, D.S., Lerman, A. 2002. Nuclear factor-kappaB immunoreactivity is present in human coronary plaque and enhanced in patients with unstable angina pectoris. Atherosclerosis 160, 147–153.

Wung, B.S., Cheng, J.J., Hsieh, H.J., Shyy, Y.J., Wang, D.L., 1997. Cyclic strain-induced monocyte chemotactic protein-1 gene expression in endothelial cells involves reactive oxygen species activation of activator protein 1. Circ. Res. 81, 1–7.

Yamamoto, Y., Gaynor, R.B., 2001. Role of the NF-kappaB pathway in the pathogenesis of human disease states. Curr. Mol. Med. 1, 287–296.