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ORIGINAL ARTICLE

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Differential modulation of cyclooxygenase-mediated prostaglandin production by the putative cancer chemopreventive flavonoids tricin, apigenin and quercetin

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Abstract *Objectives*: Diet-derived flavonoids possess cancer chemopreventive properties in preclinical models. The knowledge of the pharmacology of most flavonoids is insufficient to warrant their advancement to clinical evaluation. *Methods*: Here the three flavonoids tricin from rice bran, apigenin from leafy vegetables, and quercetin from onions and apples, were compared in terms of their ability to modulate cyclooxygenase- (COX-) catalyzed prostaglandin E-2 (PGE-2) generation. Specifically their effects on the following parameters were studied: (1) COX enzyme activity, (2) COX-2 expression in human-derived colon cancer cells HCA-7, which express COX-2 constitutively, (3) phorbol ester-mediated COX-2 induction in human colon epithelial cells (HCEC), and (4) PGE-2 levels in cellular incubations. *Results*: Tricin and quercetin inhibited enzyme activity in purified COX-1 and -2 preparations with IC_{50} values of near 1 (tricin) and 5 μM (quercetin). Apigenin at up to 25 μM did not affect COX enzyme activity. Flavonoids were incubated with cells for 6 or 24 h and COX-2 protein expression and PGE-2 levels were assessed by Western blot and competitive immunoassay, respectively. None of the agents affected constitutive COX-2 expression in HCA-7 cells. Apigenin, but not tricin or quercetin, down-regulated inducible COX-2 expression in HCEC cells on 6 h incubation. All three flavonoids reduced cellular levels of PGE-2 in the supernatant of HCA-7 cells at both time points and of HCEC cells at 6 h. *Conclusions*: The results demonstrate that these structurally similar flavonoids regulate COX-mediated PGE-2 production in different fashions. Their ability to attenuate prostanoid levels may contribute to their cancer chemopreventive efficacy.

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Introduction

Enhanced synthesis of prostanoids such as prostaglandin E-2 (PGE-2) generated by the catalytic activity of the enzyme cyclooxygenase-2 (COX-2) influences several processes linked to carcinogenesis, including cell proliferation, apoptosis, angiogenesis, and invasiveness [1]. COX-2, which is over-expressed in a variety of malignancies including those of the colorectum, has been strongly associated with the development and maintenance of the malignant phenotype. Therefore it is an attractive target for cancer chemopreventive agents [1]. The colorectal cancer preventive efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin has been intrinsically linked with their COX enzyme-inhibitory activity [2]. Recent clinical intervention studies using aspirin [3] and selective COX-2 inhibitors such as celecoxib [4] suggest that the chemoprevention of colorectal malignancies in humans may be a viable proposition. However the gastrointestinal toxicity of NSAIDs and the recent realization that long-term administration of selective COX-2 inhibitors can detrimentally affect the cardiovascular system with an increased risk of stroke or cardiac infarction [5] may eventually dampen the enthusiasm for their extensive use as cancer chemopreventive interventions. It is conceivable that diet-derived agents with a good safety record may be attractive alternatives to NSAIDs.

Flavonoids exemplified by the flavonol quercetin and the flavone apigenin (Fig. 1) are polyphenolic plant constituents whose major dietary sources are fruits and vegetables. In plants flavonoids occur almost exclusively as glycosides, which generate their respective aglycones on hydrolysis caused either at preparation of the foodstuffs for consumption or during food mastication. Epidemiological studies suggest that the consumption of fruits and

vegetables is associated with a decreased risk of colorectal cancer [6], and dietary flavonoids are thought to play an important role in delaying or preventing carcinogenesis [7]. Modulation of COX-2 is one of several mechanisms by which flavonoid aglycones are thought to interfere with carcinogenesis. Quercetin and apigenin at 5–50 μM down-regulated inducible COX-2 enzyme expression in macrophages [8, 9]. Tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone), which is closely related to apigenin (Fig. 1) and occurs in rice bran and other grass species, was recently shown to be a potent inhibitor of COX enzymes without marked effect on COX-2 expression in human-derived colon cells [10]. Flavonoids possess low systemic availability as a consequence, at least in part, of the propensity of their phenol functions to undergo avid metabolic conjugation reactions in the organism. Low systemic availability may render flavonoids potentially attractive as colorectal cancer chemopreventive agents. This notion is based on the possibility that flavonoids reach the systemic circulation only at sub-efficacious concentrations insufficient to exert pharmacological effects on organs remote from the site of absorption, whilst the un-absorbed fraction can elicit cancer chemopreventive activity in the colorectal tract. Mindful of these considerations we investigated quercetin, apigenin and tricin side-by-side in terms of their effects on COX-mediated PGE-2 production. We compared their abilities to inhibit COX enzyme activity, down-regulate COX-2 protein levels and decrease cellular generation of PGE-2. Effects on enzyme activity were measured in purified preparations of COX-1 and -2, influence on levels of COX-2 protein and PGE-2 was studied in two human colon-derived cell types, HCEC cells, which express COX-2 only after stimulation with a phorbol ester, and HCA-7 cells, which express COX-2 constitutively. Overall the work was designed to contribute to the knowledge base, which may ultimately help to select appropriate flavonoids for development as colorectal cancer chemopreventive agents.

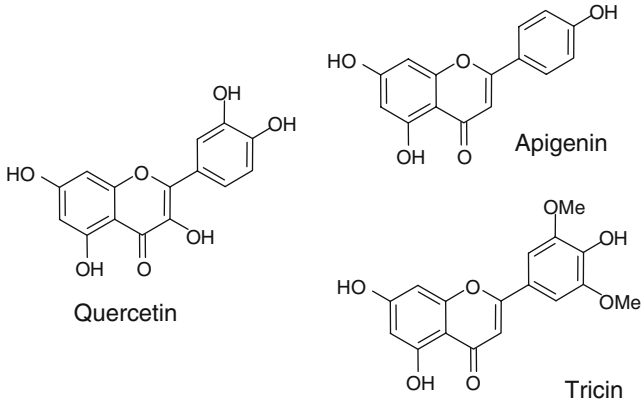

Fig. 1 Structures of tricin, apigenin and quercetin

Materials and methods

Reagents

Apigenin and quercetin were purchased from Apin Chemicals Ltd. (Abingdon UK). Tricin was custom-synthesized by Syncom (Groningen, The Netherlands) for the Chemoprevention Agent Development Research Group, Division of Cancer Prevention, at the US NCI. Flavonoid purity (>99%) was checked by HPLC analysis. Other reagents were purchased from Sigma Aldrich (Poole, UK). Flavonoids dissolved in DMSO were added to cell incubations.

Measurement of COX enzyme activity

Purified COX-2 (70% pure) and COX-1 (95% pure) enzyme preparations from sheep placenta and sheep seminal vesicles, respectively, were purchased from Alexis Biosciences (Lausen, Switzerland). COX activity was assessed in incubations with COX-1 or COX-2 enzyme using a "chemiluminescent enzyme activity kit" (Assay Designs, Ann Arbor, Michigan, USA), in which the COX peroxidase reaction is monitored with a co-substrate generating a chemiluminescent species on COX-catalyzed peroxidation. As stipulated by the manufacturer's instructions, the assay solution (tris buffer pH 7.3) contained hematin (0.02 μM) and COX-1 or COX-2 at a final activity of 60 units/ml. The mixture was incubated (5 min, room temperature), after which flavonoid (0.25–25 μM) was added, and the mixture was incubated for 2 h. Activity was measured after addition of arachidonic acid (50 μM) and the proprietary co-substrate. Light emission, which was recorded at 405 nm (15 s) using a BMG Fluostar Optima luminometer (BMG Labtech, Aylesbury, UK), was directly proportional to residual COX activity. In order to ascertain the specificity of the isoenzyme preparations and assay in our hands, enzymes were incubated with either the non-selective COX inhibitor ibuprofen or the COX-2 inhibitor NS398 (0.01 to 10 μM). Ibuprofen inhibited both isoenzymes similarly (e.g. at 10 μM COX-1 by 64%, COX-2 by 32%), whilst NS398 inhibited COX-2 (at 1 μM by 50%) but hardly COX-1 (at 1 μM by only 5%).

Cell culture

Human-derived malignant colorectal cell lines HT-29, HCA-7 cells (derived from a human mucinous adenocarcinoma of the colon [11]) and transformed, but non-malignant, human colon epithelial cells (HCEC) were provided by Prof C Paraskeva (Bristol University, Bristol, UK), Dr S. Kirkland (Imperial College, London, UK) and Dr A. Pfeifer (Nestec Ltd. Research Centre, Lausanne, Switzerland), respectively. Cells (from subculture 20–30) were seeded in 90 mm Petri dishes (Nunc, Fisher Scientific, Loughborough, UK) and grown in DMEM medium containing Glutamax I, glucose (4.5 g/l)

and 10% (v:v) foetal calf serum (Gibco, Paisley, UK). HCEC cells were cultured in dishes coated with Vitrogen 100 (10 µl/ml, Collagen Corp., Palo Alto, CA), human fibronectin (2.5 g/ml, Sigma) and bovine serum albumin (50 µg/ml, Gibco).

Effect of flavonoids on HCA-7 cell growth

Flavonoids were added to HCA-7 cell incubates 24 h after plating. Cells were counted at several time periods up to 7 days post addition of flavonoids using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, High Wycombe, UK). Control cultures were incubated with the vehicle only. The content of the flavonoid vehicle (DMSO) in the incubates did not exceed 0.1%, which on its own failed to affect cell growth. The IC_{50} value for apigenin given in the Results was calculated from the linear portion of the cell number-versus-flavonoid concentration curve at the 7 day time point. HCA-7 cell doubling time is approximately 36 h, so this time point allowed about 5 doublings to occur. The flavonoids were shown by HPLC analysis to be stable for this time period under cell culture conditions. For tricin and quercetin the highest concentrations used (40 µM for tricin and 80 µM for quercetin) only weakly reduced cell numbers thus confounding the delineation of a precise IC_{50} value.

Measurement of levels of COX-2 and microsomal PGE-2 synthetase proteins and of PGE-2

HCA-7 cells (2×10^6 per dish) were plated, left overnight and then incubated with flavonoids for 6 or 24 h. HCEC cells (0.6×10^6 cells per dish) were plated, left overnight, and pre-incubated for 1 h with flavonoids. Phorbol myristoyl acetate (PMA, dissolved in DMSO, 50 ng/ml), which induces COX-2 expression in these cells, was then added to the mixture. Six or 24 h later cells were harvested, and COX-2 was determined by Western blot analysis as previously described [12] using a polyclonal antibody against COX-2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Expression of microsomal (m) PGE synthases was assessed by Western blotting using a monoclonal anti-mouse antibody against m PGE synthase-1 and a polyclonal anti-rabbit antibody against m PGE synthase-2 (both from Cayman Chemical Comp., Ann Arbor, USA). Equal protein loading and transfer were monitored by probing for α-tubulin (Santa Cruz). Semi-quantitation including correction in relation to the respective α-tubulin band was by densitometric analysis using a Gene Gnome densitometer (Syngene Bio Imaging Systems, Frederick, MD). For the determination of PGE-2 secreted from cells into the supernatant, cells were incubated as described above (for the study of COX expression), except that in the case of the HCEC cells arachidonic acid (50 µM) was added to the medium 3 h after addition of PMA. PGE-2 was determined in aliquots (1 ml) of supernatant from incubates of cells with flavonoids for 6 or 24 h, by competitive immunoassay using a PGE-2 immunoassay kit (R&D Systems, Abingdon, UK) essentially as described before for curcuminoids [13]. In this assay PGE-2 competes with a fixed amount of alkaline phosphate-labelled PGE-2 for binding sites on a mouse monoclonal antibody coated onto the microplate. Following a wash step bound enzyme activity (as reflected by absorbance at 405 nm) was quantified on a FLUOstar OPTIMA plate reader (BMG Labtechnologies GmbH, Germany). The intensity of the colour was inversely proportional to the concentration of PGE-2 in the sample tested. PGE-2 values were corrected to cellular protein levels, thus allowing interpretation in terms of "cellular" PGE-2. In order to check for the possibility that flavonoids affect markedly levels of prostanoids other than PGE-2, pilot experiments were conducted using the Prostaglandin Screening EIA Kit (Cayman), which allows measurement of most prostaglandins produced by cells plus thromboxane B2. Results obtained with this kit were indistinguishable from those observed in the specific PGE-2 assay.

Statistical evaluation

Evaluation of significance of values as compared to the appropriate controls was performed by one-way ANOVA with subsequent Tukey's pairwise comparison.

Results

Effect of flavonoids on COX enzyme activity

Flavonoids were incubated with purified COX-1 and -2 enzymes, and enzyme activity was measured using a commercially available kit, which assesses COX peroxidase function. Both tricin and quercetin were good inhibitors of COX-1 and -2 enzymes, with IC_{50} values of near 1 µM for tricin and between 5 and 10 µM for quercetin (Fig. 2). Inhibition was not isoenzyme-selective. Apigenin did not affect COX enzyme activity at all at concentrations of up to 25 µM.

Effect of flavonoids on COX expression

The effect of flavonoids on COX-1 expression was studied in HT-29 cells. COX-2 expression was determined in HCA-7 colon cancer cells, which express COX-2 constitutively at high levels, and in HCEC cells, in which its expression was induced by exposure to PMA. It is important to note that arachidonate was added to incubations with HCEC cells to ensure swift generation of PGE-2, whilst arachidonate was not included in incubations of HCA-7 cells. Cells were incubated with flavonoids for 6 or 24 h and enzyme levels assessed by Western analysis. Expression of COX-2 in untreated (control) HCA-7 cells at the two time points was approximately similar (Fig. 3),

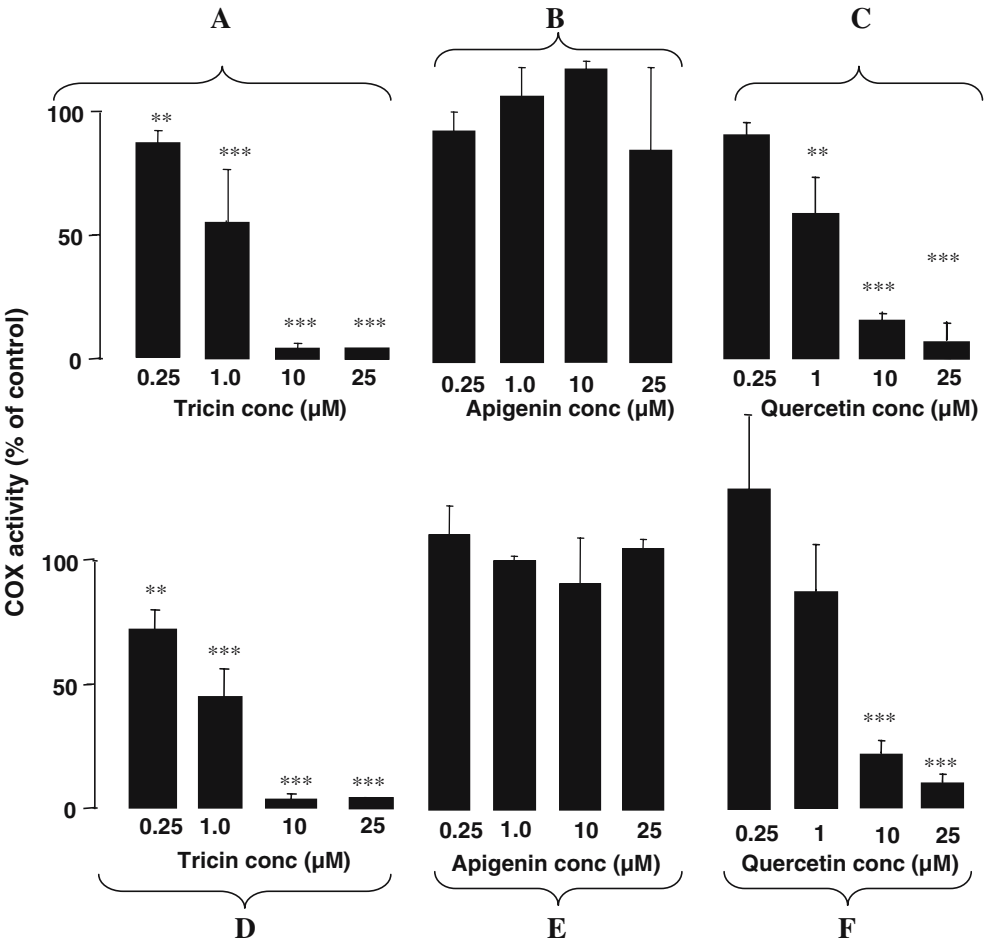

Fig. 2 Effect of tricin (**a**, **d**), apigenin (**b**, **e**) and quercetin (**c**, **f**) on activity of purified COX-1 (**a**, **b**, **c**) or COX-2 (**d**, **e**, **f**) enzyme. Measurement was by a kit, in which a co-substrate of the COX peroxidase reaction generates a chemiluminescent species, as per the vendor's protocol. Enzyme activity in the control incubates omitting flavonoid was taken as 100%. Results are the mean±SD (of $n=3$ for quercetin, $n=4$ for apigenin, $n=8$ for tricin), *asterisks* indicate that values were significantly different from controls (**$P<0.01$, ***$P<0.001$). For details of enzyme assay see Materials and methods

whereas in control HCEC cells the PMA-induced COX-2 protein band at 6 h was consistently stronger than that observed after 24 h (Fig. 4). Neither flavonoid affected levels of COX-1 protein in HT-29 cells (results not shown) or of COX-2 in HCA-7 cells (Fig. 3). In HCEC cells tricin hardly influenced inducible COX-2 protein expression after 6 h incubation (Fig. 4a), whilst it decreased COX-2 protein expression moderately after incubation for 24 h, even though the decrease was not linearly related to tricin concentration (Fig. 4d). Apigenin showed different effects in HCEC cells depending on incubation time (Fig. 4b, e). Incubation for 6 h caused a concentration-dependent decrease of COX-2 levels, at 40 μM down to 20% of control expression (Fig. 4b). Exposure of these cells for 24 h to apigenin at 5 or 10 μM failed to change COX-2 levels, whilst at 20 and 40 μM apigenin doubled COX-2 protein expression compared to control cells (Fig. 4e). Intriguingly, apigenin at 20 and 40 μM caused a similar overexpression of COX-2 after long-term incubation in HCA-7 cells, in which COX-2 protein was increased to 163±59 and 207±20% of controls, respectively, after 48 h and to 176±51 and 271±98%, respectively, after 72 h. Quercetin did not change COX-2 in HCEC cells after 6 h incubation (Fig. 4c), whereas it increased protein expression after 24 h, to almost 4-fold at 20 μM compared to protein expression in control cells (Fig. 4f).

Effects of flavonoids on cellular PGE-2 levels

In analogy to the experimental design described for the study of the effects of flavonoids on COX enzyme protein levels, their influence on constitutive and inducible PGE-2 production by cells (as reflected by PGE-2 levels in the supernatant) was determined in HCA-7 and PMA-exposed HCEC cells, using the same incubation times of 6 and 24 h. PGE-2 levels in control cells after 6 and 24 h were as follows (expressed as ng PGE-2 per mg cellular protein): In HCA-7 cells 545±104 and 233±24, in HCEC cells 113±20 and 753±212, respectively. After incubation for 6 h tricin at concentrations of ≥5 μM decreased cellular PGE-2 to about 60–50% of control values in HCA-7 cells (Fig. 5a) and to about 80–60% of control in HCEC cells (Fig. 6a). This reduction was not linear with tricin concentration. Apigenin decreased intracellular PGE-2 levels in both cell types in a concentration-dependent fashion (Figs. 5b, e, 6b, e). The decrease was particularly marked in HCEC cells, in which 20 or 40 μM apigenin removed measurable PGE-2 almost completely (Fig. 6b, e). Quercetin decreased the PGE-2 content in HCA-7 cells with an IC_{50} of less than 5 μM (Fig. 5c, f). Its effect on PGE-2 in HCEC cells was time-dependent. Incubation for 6 h caused a concentration-dependent decrease in PGE-2 (Fig. 6c), whilst incubation with 20 or

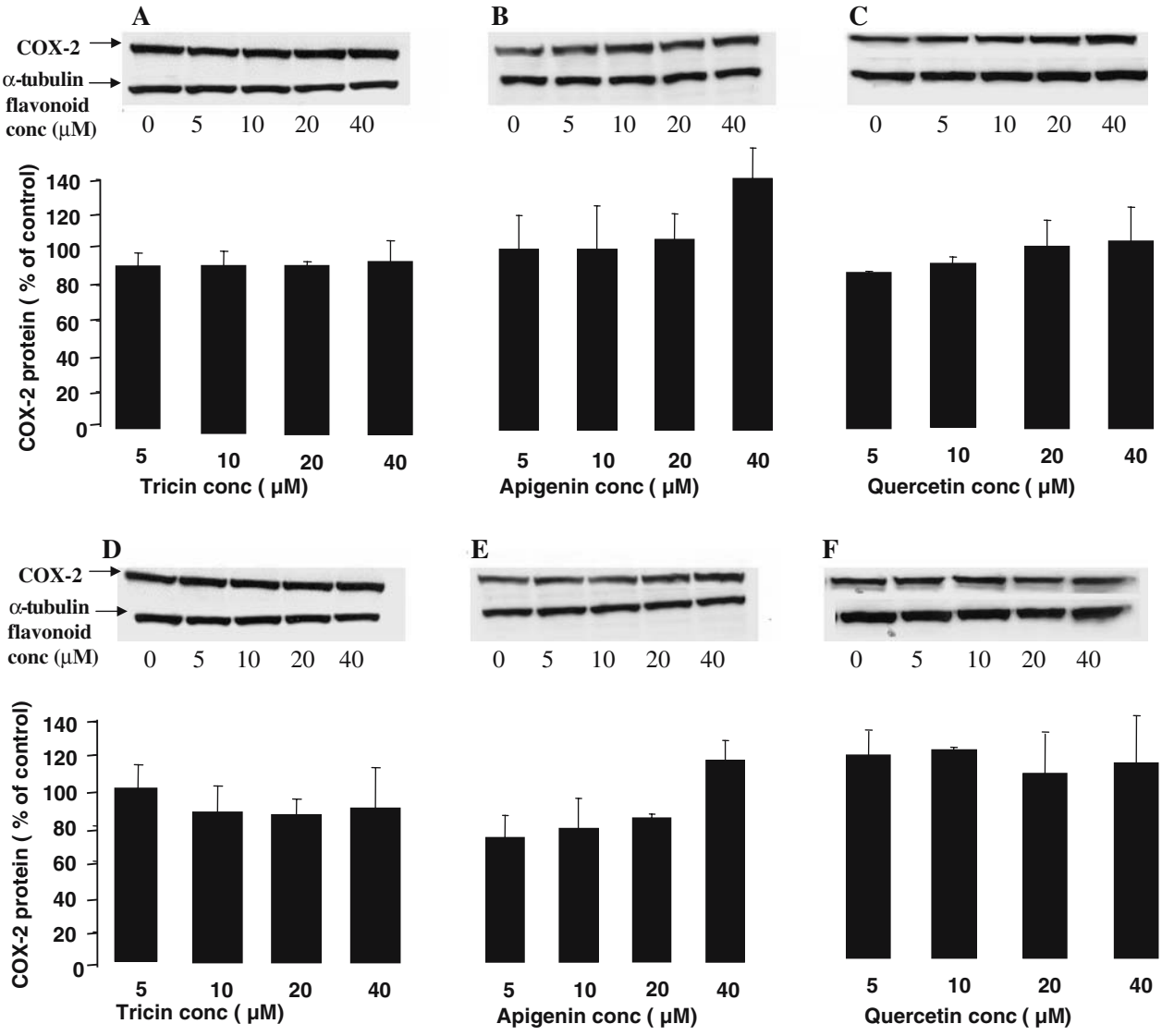

Fig. 3 Effect of tricin (**a**, **d**),apigenin (**b**, **e**) and quercetin (**c**, **f**) on COX-2 levels in HCA-7 colon cancer cells after incubation for 6 (**a**, **b**, **c**) or 24 h (**d**, **e**, **f**). Protein levels were measured by Western blotting. The top band is COX-2, the bottom one α-tubulin, which was checked for to monitor protein loading. Semi-quantitation was by densitometry. Blots are from one representative experiment; *bars* represent band densities in percentage of control and are the mean±SD of 3–5 separate experiments. For details of culture conditions and Western analysis see Materials and methods

40 μM quercetin for 24 h elevated PGE-2 up to approximately 5-fold over levels observed in control cells (Fig. 6f).

Effects of apigenin on cellular expression of m PGE synthase-1 and -2

The possibility was considered that the marked decrease in cellular PGE-2 seen after incubation with apigenin was the consequence, at least in part, of down-regulation of the expression of m PGE synthase-1 or -2. These enzymes catalyze the isomerization of PGH-2, the product of COX-catalyzed arachidonate metabolism, to PGE-2 [14]. When HCEC or HCA-7 cells were incubated with apigenin or the other two flavonoids at 40 μM for 6 or 24 h, expression of m PGE synthase enzymes was not affected (Fig. 7).

Effects of flavonoids on cell growth

We wished to relate the observed consequences of exposure to flavonoids for the COX system with their effects on HCA-7 cell proliferation. After incubation of cells with flavonoids for 7 days the IC_{50} values for growth inhibition were established to be ~40 μM for tricin, 18±1 μM for apigenin and >80 μM for quercetin (mean±SD of $n=3$, each performed in duplicate).

Discussion

The results presented here illustrate that in spite of their structural similarity the three flavonoids investigated

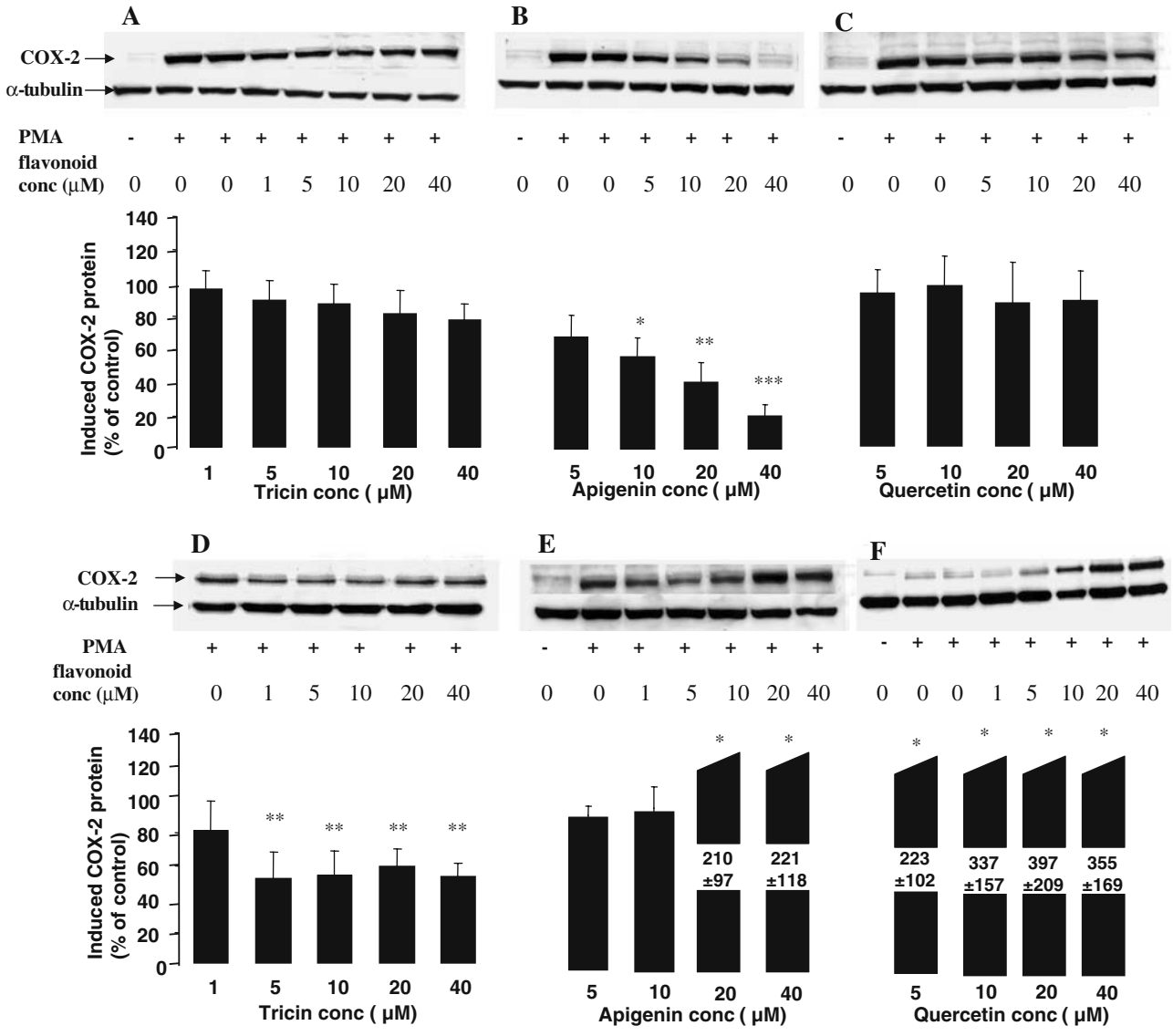

Fig. 4 Effect of tricin (**a**, **d**),apigenin (**b**, **e**) and quercetin (**c**, **f**) on induced COX-2 levels in HCEC cancer cells after incubation for 6 (**a**, **b**, **c**) or 24 h (**d**, **e**, **f**). COX-2 expression was induced by exposure to PMA. Protein levels were measured by Western blotting. The top band is COX-2, the bottom one α-tubulin, which was checked for to monitor protein loading. In **a**, **b**, **c** and **f** the second and third lanes represent COX-2 expression in the presence of PMA without or with flavonoid vehicle (DMSO), respectively. Semi-quantitation was by densitometry. Blots are from one representative experiment; the *bars* represent band densities in percentage of control and are the mean±SD of 3–5 separate experiments. Densitometry values for quercetin and apigenin at 20 and 40 μM are cut off, as they considerably exceed 100%, and values are given within the bars. *Asterisks* indicate that densitometry values were significantly different from controls (*$P<0.05$, **$P<0.01$, ***$P<0.001$). For details of culture conditions and Western analysis see Materials and methods

here regulate the COX-catalyzed generation of prostanoids from arachidonate at different levels. Whilst tricin was a good COX enzyme inhibitor, it down-regulated inducible COX-2 protein expression only very weakly and decreased cellular PGE-2 levels moderately. Apigenin lacked COX enzyme-inhibitory activity in the cell-free system, and it down-regulated inducible COX-2 expression within the 6 h incubation period, whilst elevating it after 24 h. Apigenin reduced cellular PGE-2 moderately in HCA-7 cells and dramatically in HCEC cells. Quercetin significantly inhibited COX enzyme activity and elevated inducible COX-2 expression after long-term incubation. It also decreased PGE-2 levels, strongly in HCA-7 cells, but only ephemerally in HCEC cells. Intriguingly, the modulation of COX enzyme expression by apigenin and quercetin was observed only in the cells, in which protein expression is inducible (HCEC), but was not seen in the cells, which express COX-2 constitutively (HCA-7). Fig. 8 summarizes in schematic fashion the multiple effects of the three flavonoids on COX-mediated prostaglandin production described here and by others previously. Apigenin and quercetin have been reported before to down-regulate COX-2 expression in macrophages [8, 9]. Quercetin inhibited COX activity derived from guinea pigs, whilst apigenin did not [15]. Furthermore quercetin inhibited

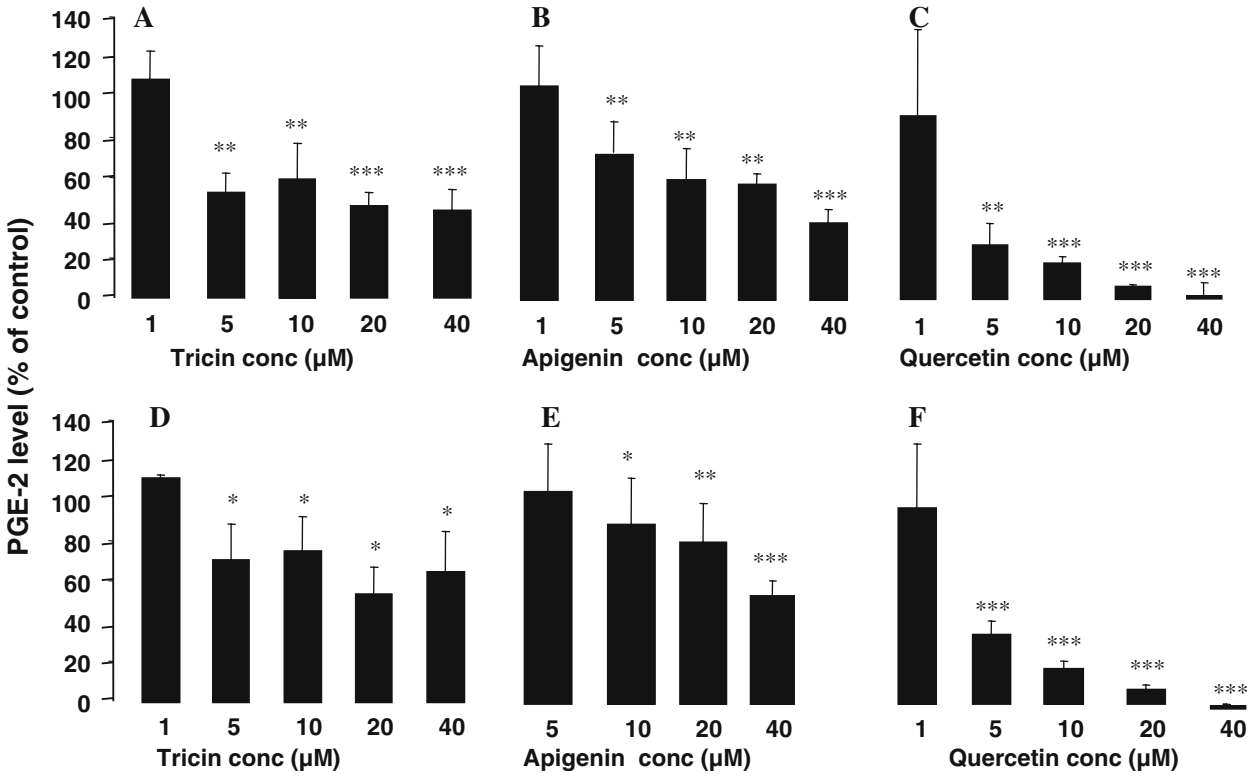

Fig. 5 Effect of tricin (**a**, **d**), apigenin (**b**, **e**) and quercetin (**c**, **f**) on PGE-2 levels in HCA-7 cells colon cancer after incubation for 6 (**a**, **b**, **c**) or 24 h (**d**, **e**, **f**). PGE-2 was measured by enzyme immunoassay. Control cells (not exposed to flavonoids) contained 545±104 and 233±24 ng PGE-2/mg cellular protein, at 6 and 24 h, respectively (=100%). Results are the mean±SD of 3–5 separate experiments, each conducted in duplicate. *Asterisks* indicate that values were significantly different from controls (*$P<0.05$, **$P<0.01$, ***$P<0.001$). For details of culture conditions and PGE-2 assay see Materials and methods

COX-catalyzed PGE-2 generation, but not COX-2 protein expression in A549 human lung carcinoma cells [16]. Some of the effects of the flavonoids on the different components of the COX system described here are consistent with each other, whereas others seem to oppose each other. The inhibition of COX-2 enzyme activity by tricin can explain its ability to decrease cellular PGE-2 levels. For apigenin, the amelioration of induced COX-2 expression after 6 h incubation is consistent with the observed reduction of cellular PGE-2 in this time frame. In contrast, the increased level of COX-2 protein in HCEC cells after longer term incubation with apigenin compared to unexposed cells did not translate into increased cellular PGE-2 levels over those seen in control cells. For quercetin the ability to inhibit COX-2 enzyme activity is congruent with its reduction of cellular PGE-2 levels on 6 h incubation, and its ability to increase COX-2 protein levels after exposure for 24 h may be related to the quercetin-induced cellular increase of PGE-2 in that time scale. Some of the discrepancies observed here hint at the possibility that mechanisms of regulation other than those involving COX are likely to exist by which these agents affect arachidonic acid metabolism and PGE-2 levels. The potent inhibition of COX activity by tricin suggests that it should strongly reduce intracellular PGE-2 levels. Yet tricin ameliorated intracellular PGE-2 only modestly, and this decrease was not linearly related with tricin concentration. In order to explain this observation, we considered the possibility that the presence of tricin buttresses the chemical stability of PGE-2, thus counteracting to some extent the corollary of its inhibitory effect on COX-mediated PGE-2 generation. However this contention was not consistent with the finding that tricin, when incubated with preformed PGE-2, failed to affect the rate of PGE-2 decay (result not shown). The marked decrease in cellular PGE-2, which apigenin caused, was not the consequence of down-regulation of the expression of m PGE synthase-1 or -2. It remains conceivable that apigenin interfered with the activity of these enzymes, which could not be studied. Apigenin has been shown to inhibit the liberation of arachidonic acid from precursor phospholipids via interference with extracellular regulated kinases (ERK) 1/2 and mitogen-activated protein kinase (MAPK)-associated signal transduction pathways [17, 18]. Suppression of arachidonic acid liberation may have contributed to the decrease in intracellular PGE-2 levels observed here for apigenin in HCA-7 cells, even though in a preliminary experiment using these cells we did not detect inhibition of ERK 1/2 by apigenin (result not shown). Inhibition of arachidonic acid release would not explain the apigenin-mediated decrease of PGE-2 in HCEC cells, in which preformed arachidonate was included in the incubation. Another potential explanation for the discrepancy

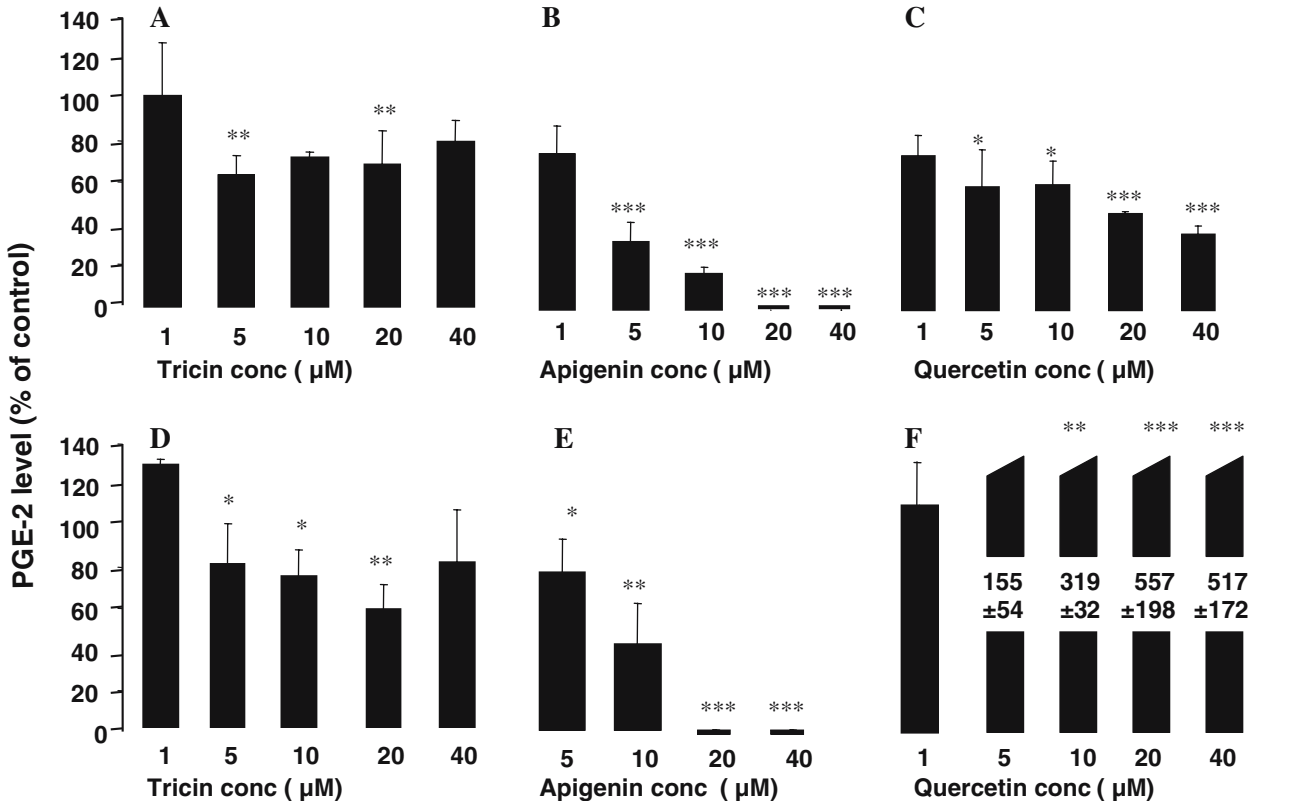

Fig. 6 Effect of tricin (**a**, **d**), apigenin (**b**, **e**) and quercetin (**c**, **f**) on induced PGE-2 levels in HCEC colon cells after incubation for 6 (**a**, **b**, **c**) or 24 h (**d**, **e**, **f**). COX-2 expression was induced by exposure to PMA. PGE-2 was measured by enzyme immunoassay. Control cells (not exposed to flavonoids) contained 113±20 and 753±212 ng PGE-2/mg cellular protein, at 6 and 24 h, respectively (=100%). Values for quercetin after 24 h incubation are cut off, as they considerably exceed 100%, and values are given within the bars. Results are the mean±SD of 3–5 separate experiments, each conducted in duplicate. *Asterisks* indicate that values were significantly different from controls (*$P<0.05$, **$P<0.01$, ***$P<0.001$). For details of culture conditions and PGE-2 assay see Materials and methods

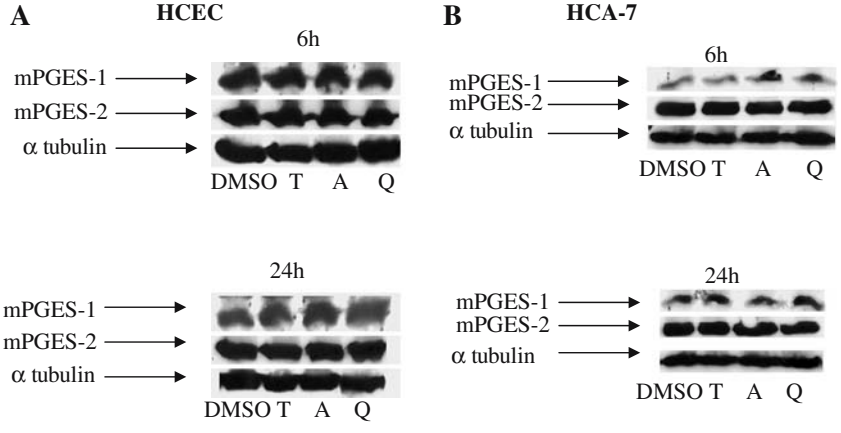

Fig. 7 Effect of tricin (T), apigenin (A) and quercetin (Q) on expression of m PGE synthase (mPGES) -1 or -2 in HCEC (**a**) and HCA-7 cells (**b**) after incubation for 6 or 24 h with 40 μM flavonoid. Protein levels were measured by Western blotting . Expression of α-tubulin was checked for to monitor protein loading. "DMSO" indicates protein expression in control cells. Results are representative of three separate experiments. For details of culture conditions and Western analysis see Materials and methods

between the observed effects of apigenin on PGE-2 levels on the one hand and COX-2 expression on the other is the possibility that apigenin may have reduced cellular PGE-2 levels by induction or stimulation of the PGE-2 metabolizing enzyme 15-hydroxyprostaglandin dehydrogenase, which has been reported to be expressed by HCA-7 cells and to undergo induction by indomethacin in certain cells [19].

How may the different abilities of the three flavonoids investigated here to regulate the COX system impact on their cancer chemopreventive efficacy? Apigenin was a more potent inhibitor of HCA-7 cell growth than quercetin, yet both agents decreased PGE-2 levels in these cells, quercetin more effectively than apigenin. Thus one may tentatively infer that diminution of intracellular prostanoid levels is probably not a primary mechanism by

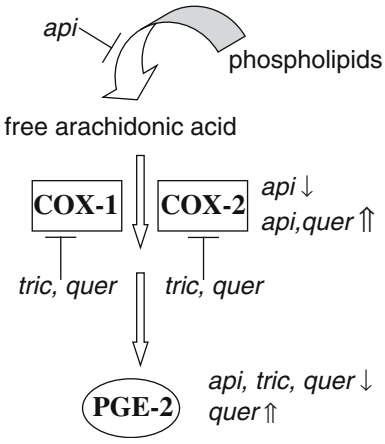

Fig. 8 Schematic summary of effects of tricin, apigenin and quercetin on COX-mediated production of PGE-2 from arachidonic acid, as shown in this paper and by others. *Api* apigenin, *quer* quercetin, *tric* tricin, ⊥ inhibition of enzyme activity, ↓ decrease within 6 h incubation, ⇑ increase within 24 h incubation. Inhibition of arachidonic acid liberation via interference with ERK1/2 has been previously demonstrated by others [17, 18]

which flavonoids attenuate proliferation of these cells. It is not clear how the COX modulation outlines here may relate to the efficacy of these flavonoids in the Apc^{Min} mouse, a model of human colorectal malignancies characterized by mutated and inactivated APC. Consumption of tricin, which we characterized here and previously [10] as a good COX enzyme inhibitor, delayed gastrointestinal carcinogenesis in this model [10], but quercetin, also a COX enzyme inhibitor, has been reported to be without effect in this model [20]. The ability of apigenin to affect intestinal carcinogenesis in Apc^{Min} mice remains to be investigated.

Mechanism-guided combination of different cancer chemopreventive agents is a rational strategy in experimental cancer chemoprevention. The results presented here hint at the possibility that, given more comprehensive insight into the mechanisms of flavonoids, it may be possible in the future to rationally select a combination of flavonoids or their dietary sources with the aim to subvert cellular prostanoid levels in a variety of mechanistically complementary ways. It is important to bear in mind that flavonoids engage several mechanisms of action in addition to COX modulation. Among the mechanisms, which are thought to contribute to the ability of flavonoids to prevent cancer, are anti-oxidation, induction of apoptosis, and suppression of oncogenic signalling, to name but a few. It is impossible to gauge the functional significance of individual mechanistic components vis-a-vis each other for the chemopreventive activity of a flavonoid. The cocktail of mechanisms responsible for the efficacy of a flavonoid in a particular chemoprevention paradigm is likely to be unique to the agent and to the model. A similar situation applies to NSAIDs and COX-2 inhibitors, which are now known to engage mechanisms germane to cancer chemoprevention in addition to COX inhibition [21]. As is the case for flavonoids, the relative importance of these mechanisms versus COX inhibition for the overall chemopreventive activity of NSAIDs remains unresolved.

The rational design of exploratory studies of flavonoids in animals or of clinical pilot studies requires inclusion of suitable mechanism-based pharmacodynamic markers of pharmacological activity. The results presented here indicate that measurement of PGE-2 in colorectal tissue, in which flavonoids are thought to prevent malignancies, might be a pharmacodynamic marker candidate worthy of further exploration for apigenin, and perhaps also for tricin and quercetin. Safety considerations may render flavonoids attractive alternatives to NSAIDs for the prevention of malignancies. Mechanistic data such as that described here together with more results of their efficacy in suitable rodent models will ultimately help chose selected flavonoids for clinical development.

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