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The dietary antioxidants resveratrol and quercetin protect cells from exogenous pro-oxidative damage

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Abstract

In the colorectal epithelium oxidative stress is observed endogenously in premalignant adenoma cells or induced by nutritional factors like fatty acid hydroperoxides (LOOH). Bioactive phenols like resveratrol and quercetin can quench reactive oxygen species and protect from pro-oxidative damage. Our study used colorectal adenoma and carcinoma cell lines to assess antioxidant protective effects of resveratrol and quercetin. It demonstrated that both compounds efficiently protect from oxidative stress induced by LOOH. Effective concentrations (10 μM resveratrol and 1 μM quercetin) can easily be reached in the intestinal lumen after consumption of plant foods or food supplements. Both compounds prevent LOOH-induced formation of intracellular H_2O_2, stimulation of cyclooxygenase-2 and vascular endothelial growth factor. For reduction of endogenous H_2O_2 levels in colorectal tumor cells higher antioxidant-concentrations are needed in all cell lines. Quercetin (10 μM) alone even increased H_2O_2 in LT97 adenoma cells and stimulated VEGF production. Resveratrol and quercetin also induced 10–30% and 40–60% cell loss respectively by apoptosis. In summary, this indicates that resveratrol and quercetin have little protective capacity in absence of exogenous stress. They are however highly efficient in protecting against nutrition induced oxidative stress damage suggesting that this constitutes the major part of their tumor protective activity.

Keywords: Bioactive phenols; Resveratrol; Quercetin; Oxidative stress; Oxidized fatty acids; Colorectal cancer

1. Introduction

Oxidative stress plays a central role in colorectal carcinogenesis. It is caused by the inflammatory reactions that recruit phagocytes capable of oxidative burst into premalignant tissue (Coussens and Werb, 2002; Lewis and Pollard, 2006). In addition cyclooxygenases and lipoxygenases produce oxidized lipids that mediate vascularisation (Carmeliet and Jain, 2000; Nie and Honn, 2002), a process that is essential for premalignant colorectal polyps larger than 1 mm (Sonoshita et al., 2001; Seno et al., 2002). This is reflected by high levels of oxidative DNA-adducts in the polyps (Bartsch and Nair, 2000; Schmid et al., 2000). In addition exogenous oxidative stress comes from nutritional sources either through overnutrition (Hansel et al., 2004; Sies et al., 2005) or through the biological effects of oxidised fatty acids contained in the diet (Udilova et al., 2003; Jurek et al., 2005). The prototypic fatty acid hydroperoxide linoleic acid (LOOH) has been shown to induce H_2O_2 production in SW480 cells but not LT97 cells that already produced high concentrations of intracellular H_2O_2 without stimulation (Jurek et al., 2005). On the other hand, phytochemicals contained in fruits and vegetables may exert antioxidant effects that protect from the

Abbreviations: COX, cyclooxygenase; DHFC, 2′,7′-dichlorofluorescin; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; LH, linoleic acid; LOOH, linoleic acid hydroperoxide; MEM, minimal essential medium; PGE_2, prostaglandin E2; VEGF, vascular endithelial growth factor; $\Delta\psi_m$, mitochondrial membrane potential.

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pro-oxidative damage arising from endogenous and exogenous sources (Surh, 2003).

Resveratrol and quercetin are bioactive phenolic compounds contained in fruits and vegetables. Their consumption has been shown to have chemoprotective effects at various organ sites including the colon (Tessitore et al., 2000; Banerjee et al., 2002; Mouria et al., 2002). Resveratrol has gained prominence as it is supposed to be one of the mediators of the "french paradox" – the low incidence of heart disease and cancer in France in spite of high fat consumption (Renaud and de Lorgeril, 1992). Quercetin and its gylcosides (e.g. rutin) have been shown to protect rats from dimethylbenzanthracene induced mammary tumors (Pereira et al., 1996) and mice from azoxymethanol induced colon tumors (Deschner et al., 1991, 1993; Volate et al., 2005). On the other hand, there are conflicting results – e.g. some studies do not report any protective activity in min-mice (Mahmoud et al., 2000). One study even demonstrated an enhancement of azoxymethane induced colon-carcinogenesis in rats (Pereira et al., 1996). This indicates that context dependent indirect effects may play a role in chemoprevention by bioactive phenols. One possible indirect mechanism is the suppression of H_2O_2 production (Leiro et al., 2004; Martin et al., 2006) and scavenging cellular radicals (Martinez and Moreno, 2000) in other cell types. This work adresses the question whether resveratrol and quercetin can protect colorectal tumor cells from endogenous formation of H_2O_2 and from LOOH-induced damage.

2. Materials and methods

2.1. Cell lines

SW480 and HT29 human colon carcinoma cells were obtained from the American Type Culture Collection. The cell lines were kept under standard tissue culture conditions using minimal essential medium (MEM) containing 10% foetal calf serum (FCS). LT 97 human colon adenoma cell line was established in our laboratory (Richter et al., 2002). It was cultured in Ham F-12 medium containing 20% L-15 medium, 2% FCS, 2×10^{-10} M triiodotyronine, 2 μg/ml transferrin, 1 μg/ml hydrocortisone, 5×10^{-9} M Na-selenite, (basic Ham12) 10 μg/ml insulin, and 30 ng/ml epidermal growth factor (EGF) and had a doubling time of 96 h. VACO235 adenoma cells were a gift from James K.V. Willson (Ireland Cancer Center, Cleveland, OH) (Willson et al., 1987) and were cultured in MEM containing 2% FCS, 2×10^{-10} triiodothyronine, 1 μg/ml hydrocortisone, 10 μg/ml insulin, 2 μg/ml transferrin 5×10^{-9} selenite and 30 ng/ml EGF.

2.2. Cell treatment

Stock solutions of linoleic acid hydroperoxide (LOOH) were stored in liquid N_2 and diluted into medium containing 1 mg/ml BSA from ethanol stocks immediately before use. Resveratrol and quercetin were obtained from Sigma (St. Louis, MO) and stock solutions prepared in ethanol and stored at −20 °C.

2.3. Cell number

For determination of cell number neutral red (50 μg/ml) uptake from serum-free MEM during 2 h was measured. The dye is taken up into the lysosomes of viable cells from where it can be recovered with 1% acetic acid in 70% ethanol.

2.4. Determination of intracellular hydrogen peroxide

Cells were grown until 60–80% confluence, harvested by trypsinisation and suspended in HBSS containing 1% FCS at a density of 1×10^6 cells/ml. Cell suspensions were incubated with LOOH, antioxidant and 20 μM 2′,7′-dichlorofluorescin (DHFC) for 2 h at 37 °C. Intracellular H_2O_2 produces fluorescin from DHFC that can be analysed by FACS.

2.5. Production of VEGF

Cultures were grown in 24-well plates and exposed to control-, LOOH- or antioxidant-containing medium. Conditioned media were collected at the indicated time points, cleared by centrifugation and VEGF concentration determined by ELISA. ELISA kits for human VEGF were obtained from R&D Systems Europe (Abingdon, UK) and used according to the manufacturer's instructions.

2.6. Production of PGE_2

HT29 cells were left to secret PGE_2 for 24 h after addition of 10 μM arachidonic acid substrate into the medium. PGE_2 secreted during this period was determined using an indirect ELISA kit purchased from Caymen Chemicals (Ann Arbor, MI) according to the manufacturer's instructions.

2.7. Isolation of RNA and reverse transcriptase (RT)-PCR

Cells were exposed as described above and RNA isolation was performed using a standard Trizol-extraction protocol (Life Technologies, Gibco BRL). Purity and quantity of the RNA was determined using agarose gel electrophoresis and photometry. cDNA synthesis was performed on 2–5 μg of total RNA with random hexamer primers for 1 h at 42 °C using MMLV reverse transcriptase (Sigma, St. Louis, MO).

Genes of interest were amplified from cDNA samples by standard PCR cycles of 1 min denaturation (94 °C), 30 s annealing at 55 °C and 1 min synthesis at 72 °C. Primersequences were as follows: GAPDH sense 5′-CGGGAAGCTTGTGATCAATGG-3′ and antisense 5′-GGCAGTGATGGCATGGACTG-3′; COX-2 sense 5′-TTCAAATGAGATTGTGGGAAAAT-3′ and antisense 5′-AGATCATCTCTGCCTGAGTA-3′; VEGF: sense 5′-CTGCTCTCTTGGGTGCACTG-3′ and antisense 5′-ATGTGACAAGCCAAGGCGGTG-3′. Amplification was done for 22 cycles for GAPDH and for 35 cycles for COX-2 and VEGF. Products were separated on 6% acrylamide gels using 0.5 μg/ml ethidium bromide for staining. GelDoc 2000 system and the program Quantity One 4.2.1. (Bio-Rad Laboratories, US) were used for quantification.

2.8. Statistics

All experiments were performed at least 3 times. If not otherwise indicated, data are expressed as mean ± SEM, and statistical differences were determined using ANOVA with significance considered as $p < 0.05$.

3. Results

3.1. Cell growth and viability

The effect of resveratrol and quercetin on cell viability and LOOH-induced cell loss was determined in LT97 colorectal adenoma cells and SW480 and HT29 colorectal carcinoma cells by neutral red uptake after 24 h of exposure. Resveratrol was only a weak growth inhibitor – concentrations up to 100 μM caused less than 50% cell loss in all cell lines except in SW480 cells (Fig. 1a). Quercetin was more active: ED50 concentrations were 41 μM for VACO235

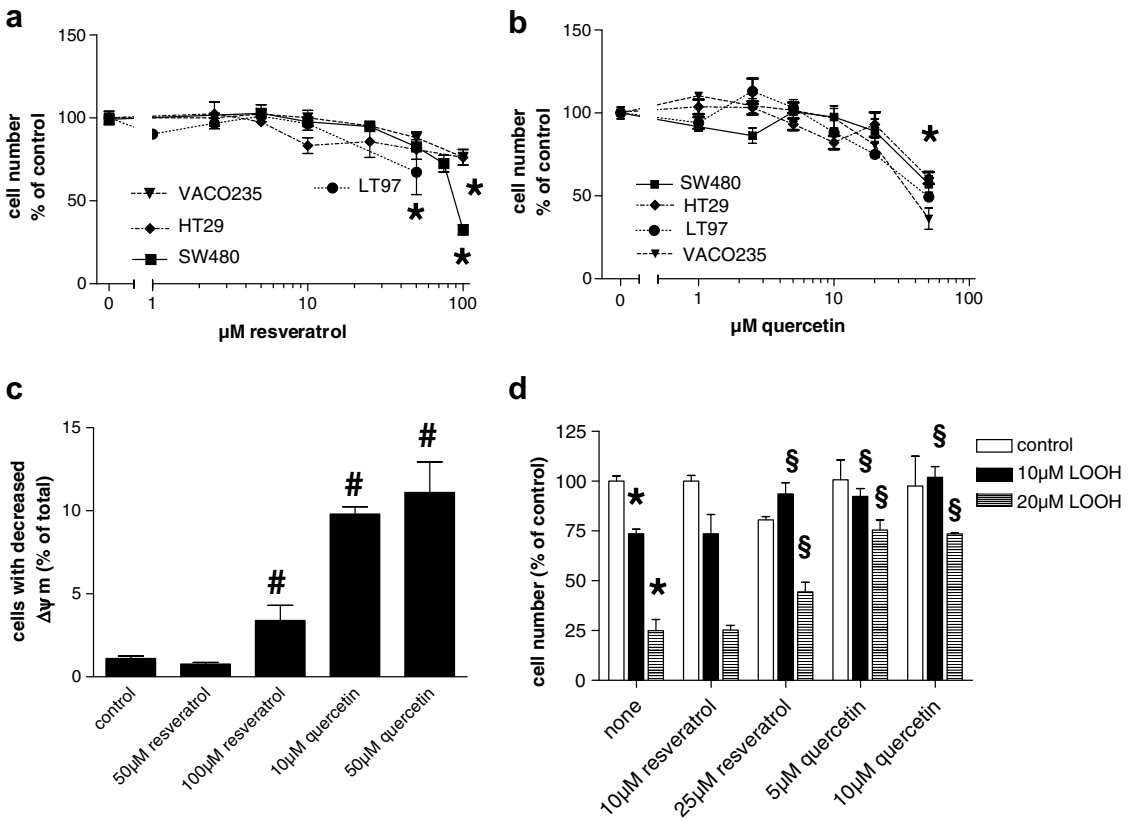

Fig. 1. Growth and survival of colorectal tumor cells. Resveratrol (a) and quercetin (b) were added to the culture medium of colorectal tumor cells at increasing concentrations and viability determined 48 h later by neutral red uptake. (c) HT29 carcinoma cells were incubated with resveratrol or quercetin for 48 h before they were harvested and $\Delta\psi_m$ determined by uptake of JC1 and FACS analysis as a determinant of apoptosis. (d) Resveratrol and quercetin were added to control cells as well as cultures exposed to LOOH and viability determined after 24 h to assess protective effects of the bioactive phenols. * decreased below control at $p \leqslant 0.005$, and increased above LOOH at $p \leqslant 0.005$.

and 48 μM for LT97 cells. In the carcinoma cell cultures (SW480 and HT29) 50 μM quercetin caused 40% cell loss after 24 h (Fig. 1b). For both resveratrol and quercetin cell loss was caused by apoptosis as shown by the loss of mitochondrial membrane potential ($\Delta\psi_m$) detected after 24 h of exposure to the antioxidant (Fig. 1c). For protection assays antioxidants were added to SW480 cultures together with 10 or 20 μM linoleic acid hydroperoxide (LOOH). The latter reduced cell number in a concentration dependent way to 75% and 25%, respectively. Both antioxidants protected cultures from this cell loss. 10 μM resveratrol was not protective, but 25 μM returned cell number in the 10 μM LOOH group to control and increased survival in the 20 μM LOOH group to 44%. Quercetin was even more effective: both 5 μM and 10 μM protected cultures exposed to 10 μM LOOH completely and reached 75% survival in the 20 μM LOOH group (Fig. 1d).

3.2. Production of H_2O_2

To assess protection from oxidative stress resveratrol and quercetin were added to the culture medium of colorectal tumor cells alone or together with 10 μM LOOH to induce H_2O_2 production. Resveratrol at concentrations of 10, 50 and 100 μM had little effect on endogenous H_2O_2 levels in either cells line. H_2O_2 induced fluorescence was still about 75% of the control even in the presence of 100 μM resveratrol (Fig. 2a). Quercetin (10 μM) were protective in the carcinoma cell lines only, but even increased H_2O_2 in LT97 adenoma cells. 50 μM of the flavonoid were necessary to reduce H_2O_2 production to 40%, 45% and 32% in SW480, HT29 and LT97 cells, respectively (Fig. 2b). Both resveratrol and quercetin were effective against LOOH-induced H_2O_2 production, however, which they inhibited in a concentration dependent manner (Fig. 2c). Again quercetin was the more effective compound – it reduced endogenous H_2O_2 levels at concentrations above 5 μM and reduced LOOH-induced H_2O_2 below control levels, while resveratrol blocked the LOOH effect reducing H_2O_2 production to control levels (Fig. 2c).

3.3. LOOH-induced VEGF production

Secretion of increased amounts of VEGF into the medium is one of the significant pro-tumorigenic effects of LOOH in colorectal tumor cells (Jurek et al., 2005). When resveratrol and quercetin were added to the medium of SW480 cells alone secretion of VEGF was not affected by

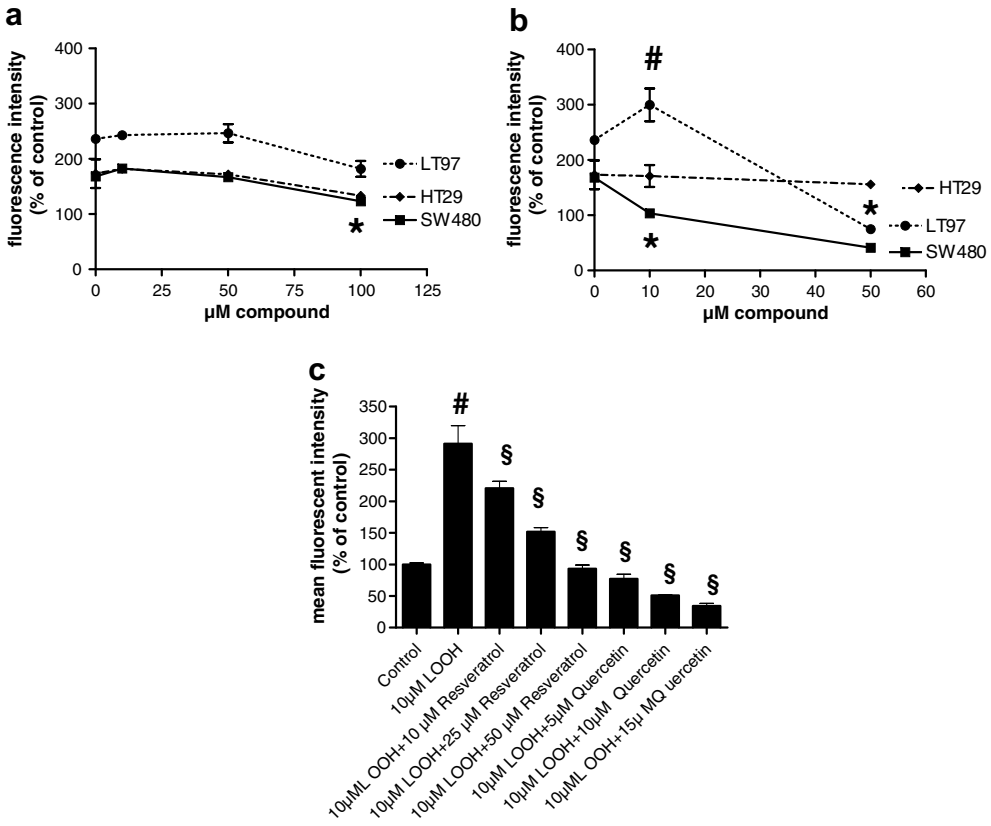

Fig. 2. Production of H_2O_2. Colorectal tumor cells were incubated with resveratrol (a) or quercetin (b) together with DHFC for 90 min. Intracellular H_2O_2 oxidised the reagent to fluorescin which was determined by FACS analysis. (c) The same assay was performed with SW480 exposed to LOOH to induce oxidative stress * decreased below control at $p \leqslant 0.005$, # increased above control at $p \leqslant 0.005$, § decreased below LOOH at $p \leqslant 0.005$.

resveratrol concentrations <100 µM. Quercetin (5 µM) on the other hand even increased VEGF production while 50 µM of the flavonoid had a strong inhibitory effect (Fig. 3a). In HT29 cells neither resveratrol nor quercetin had strong inhibitory effects on VEGF secretion (Fig. 3b). When antioxidants were added to the medium together with LOOH both had protective activity that exceeded their biological effects when given alone: resveratrol reduced VEGF production to control level in a dose dependent way and 5 µM and 10 µM quercetin reduced LOOH dependent growth factor secretion by 60% but did not abolish it completely (Fig. 4a).

To determine whether this effect was exerted at the transcription level RNA was isolated from cultures exposed to LOOH alone or LOOH together with resveratrol and quercetin. The results did not show an inhibition of LOOH-induced VEGF mRNA by resveratrol, but 5 and 10 µM quercetin reduced VEGF mRNA to control levels (Fig. 4b).

In LT97 cells VEGF production was dependent on COX-2 activity (Jurek et al., 2005). We have therefore, also measured the effect of resveratrol on PGE_2 production in HT29 cells. The cultures were incubated for 24 h with the antioxidants and 20 µM arachidonic acid to provide substrate for the enzyme. Conditioned media were used to quantify secreted prostaglandin. As expected resveratrol inhibited PGE_2 production efficiently with an IC50 of 11 µM. 25 µM abolished PG production completely. Quercetin was less effective: above the IC50 concentration of 27 µM the effect leveled off and reached only 44% of control at the high concentration of 50 µM quercetin (Fig. 5a). Resveratrol also inhibited COX-2 expression in control cultures not exposed to LOOH. Production of prostaglandin E2 (PGE_2) could not be determined because LOOH interfered with the ELISA assay.

In LT97 cells exposed to LOOH or LOOH together with 20 µM resveratrol COX-2 mRNA was determined by standard RT-PCR. The antioxidant inhibited COX-2 expression in both control and LOOH exposed cultures (Fig. 5b). Quercetin did not affect COX-2 expression (data not shown).

4. Discussion

Both resveratrol and quercetin have been described as antioxidants protecting from oxidative stress related damage in several cell types (Martinez and Moreno, 2000; Leiro et al., 2004; Huang et al., 2006; Lee and Lee, 2006; Martin et al., 2006). They also have been shown to have chemoprotective activity against cancer at several organ sites (for review see Surh, 2003) inhibiting growth and inducing apoptosis in tumor cells (e.g. Richter et al., 1999; Surh

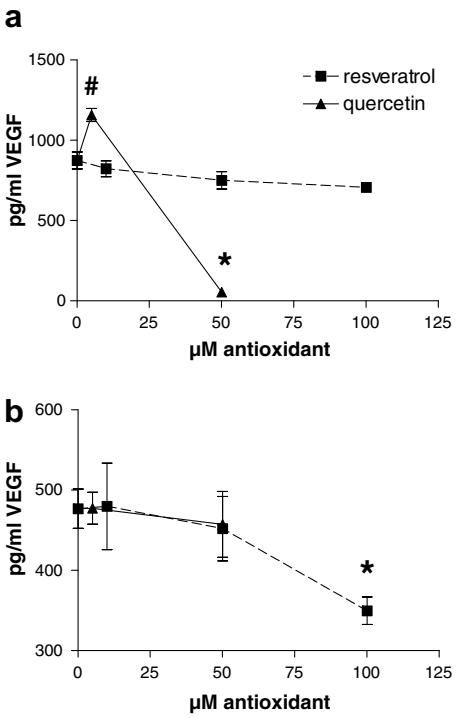

Fig. 3. VEGF secretion. Resveratrol and quercetin were added to the culture medidum of SW480 (a) and HT29 (b) colon carcinoma cells. Conditioned media were collected 24 h later and VEGF determined by ELISA. * decreased below control at $p \leqslant 0.005$.

et al., 1999; Tessitore et al., 2000; Joe et al., 2002; O'Prey et al., 2003; Volate et al., 2005; Sexton et al., 2006). We have used the adenoma cell lines LT97 and VACO235 and the carcinoma cell lines SW480 and HT29 as in vitro models to assess the cellular basis for the chemopreventive capacity of the compounds in colorectal tumor cells.

The bioactive phenols had moderate growth inhibitory effects mediated through the induction of apoptosis in our cell lines with resveratrol being the weaker agent. For SW480 carcinoma cells a concentration of 100 µM resveratrol was needed to obtain a 50% growth reduction after 24 h. All other cell lines were even less affected. Growth inihibition by quercetin was similar in all cell lines used with IC50 concentrations of 48 µM and 41 µM, respectively in the adenoma cell lines LT97 and VACO235 and above 50 µM in the carcinoma cell lines. Induction of apoptosis was also reported for apple-extracts rich in polyphenols and may be caused by the high quercetin content of such juices (Veeriah et al., 2007). For resveratrol the effective concentrations to induce cell loss in our study as well as others (Joe et al., 2002) were rather higher. This is in agreement with the low efficiency of synthetic COX-inhibitors as direct growth inhibitors (Richter et al., 2001). On the other hand, HL60 cells were more sensitive: inhibition of cell growth was observed around 10 µM resveratrol and synergistic effects with chemotherapeutic drugs were observed (Horvath et al., 2005).

The high concentrations of resveratrol or quercetin needed to directly affect colorectal tumor cell viability cannot be achieved in serum or tissues, but they can be reached in the gut lumen after fruit and vegetable consumption. Longer exposure times will further enhance the effects. Especially quercetin should be able to inhibit growth and induce apoptosis in colorectal tumor cells at the gut lining. Still effective concentrations for both resveratrol and quercetin are too high to assume that induction of apoptosis is the only and solely sufficient mechanism of chemoprevention.

Exposure of colorectal epithelium to oxidized fatty acids causes oxidative stress and the expression of the

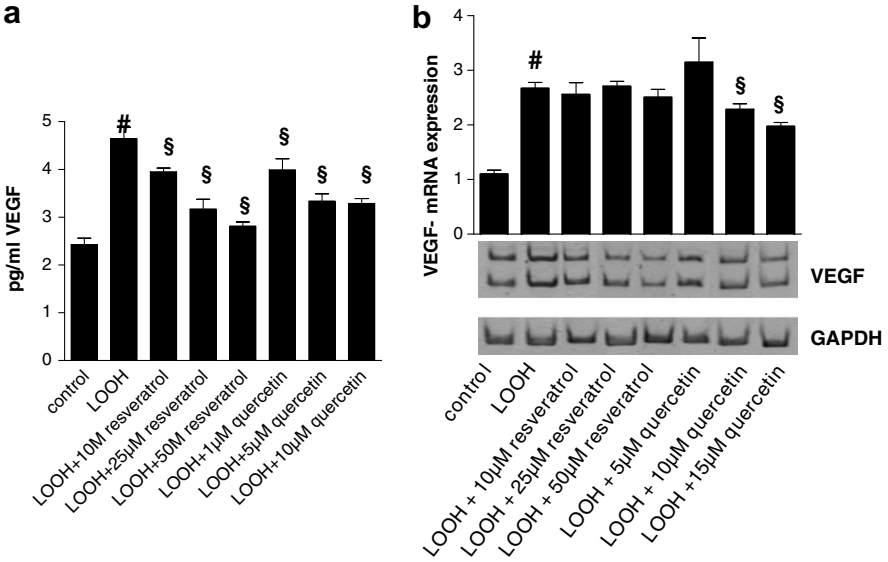

Fig. 4. LOOH-induced VEGF secretion. Resveratrol and quercetin were added to the culture medium of SW480 together with 10 µM LOOH. (a) Conditioned media were collected 24 h later and VEGF determined by ELISA. (b) RNA was isolated and VEGF expression determine by standard RT-PCR. # increased above control at $p \leqslant 0.005$, § decreased below LOOH at $p \leqslant 0.005$.

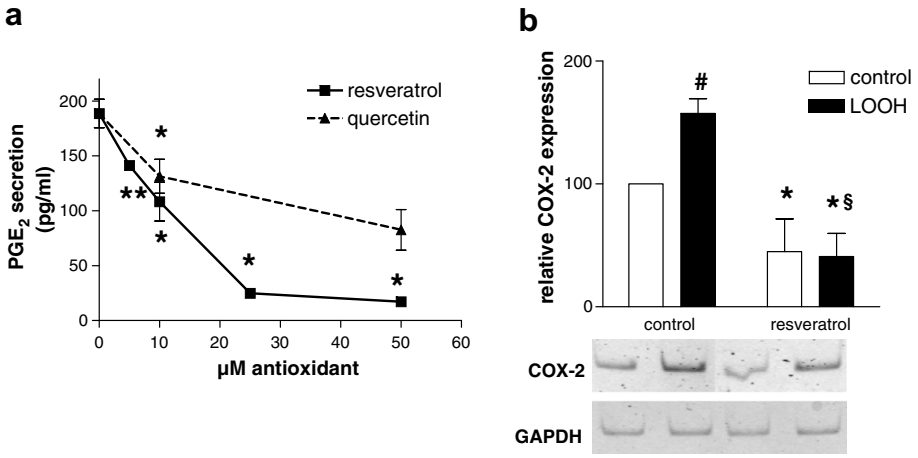

Fig. 5. Activity and expression of COX-2. (a) Resveratrol was added to the culture medium of HT29 cells together with 20 μM arachidonic acid. Conditioned media were collected 24 h later and PGE_2 determined by ELISA. (b) Resveratrol was added to the culture medium of LT97 together with 20 μM LOOH and RNA isolated 3 h later. Expression of COX-2 was determined by standard RT-PCR. * decreased below control at $p \leqslant 0.005$, # increased above control at $p \leqslant 0.005$, § decreased below LOOH at $p \leqslant 0.005$.

pro-tumorigenic gene products VEGF and COX-2 (Udilova et al., 2003; Jurek et al., 2005). Reduction of reactive oxygen species formation should also protect from the expression of oxidative stress induced genes. Actually the prevention of oxidative stress induced DNA-damage by several polyphenols including flavonoids has already been shown in NIH3T3 and HT29 cells (Pool-Zobel et al., 2000). Our results demonstrate that the protective effect can also be observed at the level of viability and gene expression. Both antioxidants were effective to inhibit LOOH-induced H_2O_2 production and prevent LOOH-induced damage at concentrations well below those needed for growth inhibition. Already 10 μM resveratrol inhibited LOOH-induced H_2O_2 production by 37% and 5 μM quercetin even reduced H_2O_2 below the control level. VEGF production that is a mechanistically crucial effect of LOOH (Jurek et al., 2005) was suppressed by 10 μM resveratrol and 1 μM quercetin, while neither resveratrol nor quercetin alone had strong inhibitory effects at these concentrations. As one of the inducers of VEGF expression is PGE_2 (Masferrer et al., 1999; Jurek et al., 2005), inhibition of COX-2 activity by both compounds may contribute to this effect. For the action of resveratrol this seems to be especially important, as the compound is an efficient inhibitor of COX activity (e.g. Martinez and Moreno, 2000; Murias et al., 2004). In our experiments resveratrol was less effective than quercetin in quenching intracellular H_2O_2 but a strong inhibitor of COX-2 both at the level of activity and on the level of gene expression indicating that prevention of PGE_2 production is a central mechanism of action. Alternatively inhibition of lipoxygenase enzymes that also produce angiogenic mediators (Nie and Honn, 2004) may play a role, as both compounds have recently been shown to inhibit LOX-activity in colorectal tumor cells (Bednar et al., 2007).

Both resveratrol and quercetin were less effective protecting against endogenous oxidative stress in the absence of LOOH. Quercetin reduced intracellular H_2O_2 in SW480 carcinoma cells and LT97 adenoma cells, but only at 50 μM. Quercetin at concentrations of 5–10 μM even stimulated H_2O_2 production in the cells and VEGF secretion in some instances.

Our results indicate that resveratrol and quercetin are effective against nutrition induced oxidative stress damage in the colorectal epithelium, but may not be useful to protect from endogenous oxidative stress. In addition, a compound like quercetin may be protective in a situation of oxidative stress but induce damage when this stress is absent. This may well explain contradictory effects of antioxidants reported in different studies of colon carcinogenesis (e.g. Pereira et al., 1996; Mahmoud et al., 2000).

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