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Suppression of Tumour-promoting Factors in Fat-induced Colon Carcinogenesis by the Antioxidants Caroverine and Ubiquinone

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Abstract. *Fatty acid hydroperoxides are produced from unsaturated fatty acids in the presence of oxygen at elevated temperatures during food processing. Their effects on gene expression in colorectal tumour cells were studied using linoleic acid hydroperoxide (LOOH) as a model compound. Addition of LOOH to the medium of LT97 adenoma and SW480 carcinoma cells enhanced the production of hydrogen peroxide. Both cell lines were observed to increase VEGF factors based on mRNA. High consumption of dietary fat promotes colon carcinogenesis in the long term. While this effect is well known, the underlying mechanisms are not understood. An approach was made starting from the assumption that LOOH is present in dietary fats as a result of heating. LOOH undergoes homolytic cleavage in the presence of iron. Various radicals are formed on mixing LT97 or SW480 cells with LOOH. The expression of tumour-promoting factors was inhibited by caroverine and ubiquinone, which may be justified as active chemopreventive agents.*

Epidemiological investigations have shown that a diet of food rich in dietary oils causes colon cancer when taken over a long period of time (1-4). Aspirin was reported to partially suppress this type of cancer (5-8), as do ω-3 fatty acids (10-12) . Aspirin inhibits cyclooxygenase (9). Both cyclooxygenase and lipoxygenase produce reactive oxygen species (ROS). Therefore, we assumed that ROS play a role in the onset of colon carcinogenesis. Figure 1 illustrates our concept. Meat fried in dietary oils or fat produces organic hydroperoxides (LOOH) which, together with meat-derived ferrous iron, trigger the formation of highly dangerous alkoxyl-radicals ($RO^{\bullet}$). These alkoxyl-radicals initiate lipid peroxidation (LPO), giving rise to 4 hydroxynonenale and malondialdehyde, both geno- and cytotoxic metabolites. They both form premutagenic etheno-adducts with DNA. Figure 2 shows organic hydroperoxide formation on heating dietary oils. Dietary oils or fats, which contain polyunsturated fatty acids (PUFA), were exposed to 100˚C over a period of 44 hours. This was sufficient to transform PUFA to organic hydroperoxides. After 40 hours, there was a maximum of transformation to organic hydroperoxides, after which a decrease was observed due to the total oxidation of PUFA.

Our working hypothesis started from the fact that heating of dietary oils forms LOOH, which are supposed to trigger tumour-promoting factors *via* ROS. However, there are also other risk factors of colon carcinogenesis (Figure 3). Mutagenic aromatic amines are transformed to electrophilic metabolites *via* P_{450}. Bile acids (13, 14) and PGE_2 (15, 16) are known to induce VEGF and and diacylglycerole to stimulate growth (17). Protein kinase c induces c-fos (18), whereas COX and VEGF promoter regions contain AP-1 binding sites. PGE_2, derived from COX, stimulates growth and cell division *via* epithelial growth and tumour growth factors.

The adenoma and carcinoma cell lines used were incubated with organic hydroperoxides (Figure 4).

The mutated APC gene, which is located in chromosome 5, predisposes genetic lesions associated with adenomatous polyposis (19). Also ki-ras, which stimulates mitosis, promotes adenomatous polyposis cells to carcinoma cells, while DCC encodes a membrane-bound protein related to cellular adhesion molecules (20, 21). Finally, p53 mutations are common in colorectal carcinoma. P53 is induced by DNA damage and causes cell cycle arrest and apoptosis (22). Under the accumulation of these mutated genes, normal epithelial cells may be transformed to adenoma and then to cancer cells.

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Key Words: Tumour-promoting factors, fat, colon, carcinogenesis, caroverine, ubiquinone.

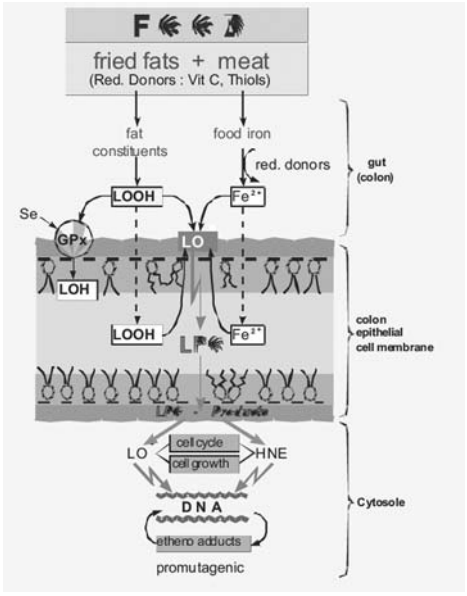

Figure 1. *Mechanism of fat-induced colon carcinogenesis.*

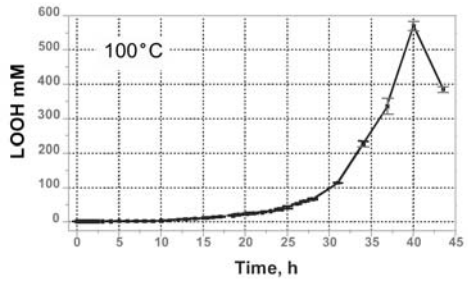

Figure 2. *Lipid hydroperoxide formation during heating of dietary oil.*

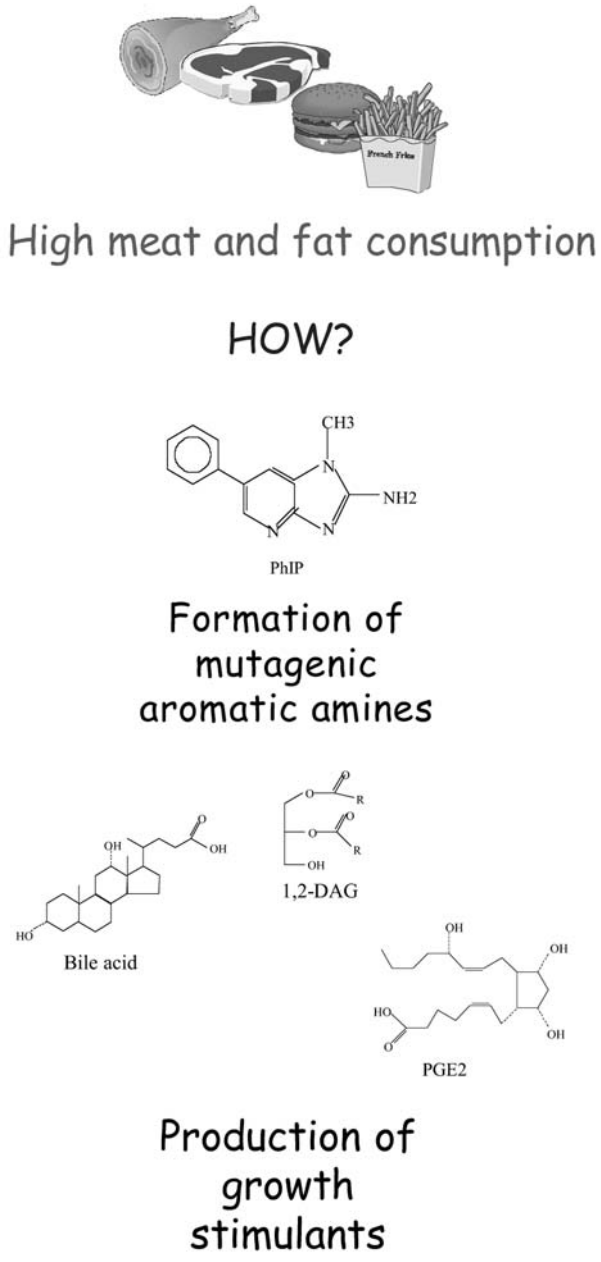

Figure 3. *Risk factors of colon carcinogenesis.*

Materials and Methods

Cell treatment. SW480 human colon carcinoma cells (American Type Culture Collection) were kept under standard tissue culture conditions using MEM containing 10% foetal calf serum (FCS). The LT 97 human colon adenoma cell line was cultured as described in (23).

LOOH was synthesized according to (24) and characterized as described earlier (25). Linoleic acid (LH) and LOOH were diluted into medium containing 1mg/mL BSA from ethanol stocks and dispersed by sonication for 3 x 5 sec immediately before use. The final concentration of ethanol in the medium was less then 0.05%. SC58560 and SC58236 were a generous gift from Searl (Skokie, IL, USA). Stock solutions were prepared in DMSO and stored at –20˚C. The cell number was determined by neutral red uptake, as described earlier (26).

HPLC analysis of UQ_6 and UQ_6H_2 in cell suspensions and supernatants. Ubiquinone-6 (2-((2E,6E,10E,14E,18E)-3,7,11,15,19,23-hexamethyl-tetracosa-2,6,10,14,18,22-hexaenyl)-5,6-dimethoxy-3-methyl-[1,4]benzoquinone) (UQ_6) was purchased from Sigma (St. Louis, MO, USA). SW480 cells were grown in 10 Pds dishes until 80% confluency, then treated with 1 μM UQ_6 in a serum-free MEM containing 1% BSA for 24 h. The supernatants were collected, the cells were trypsinized, washed and frozen at –80˚C, until HPLC analysis.

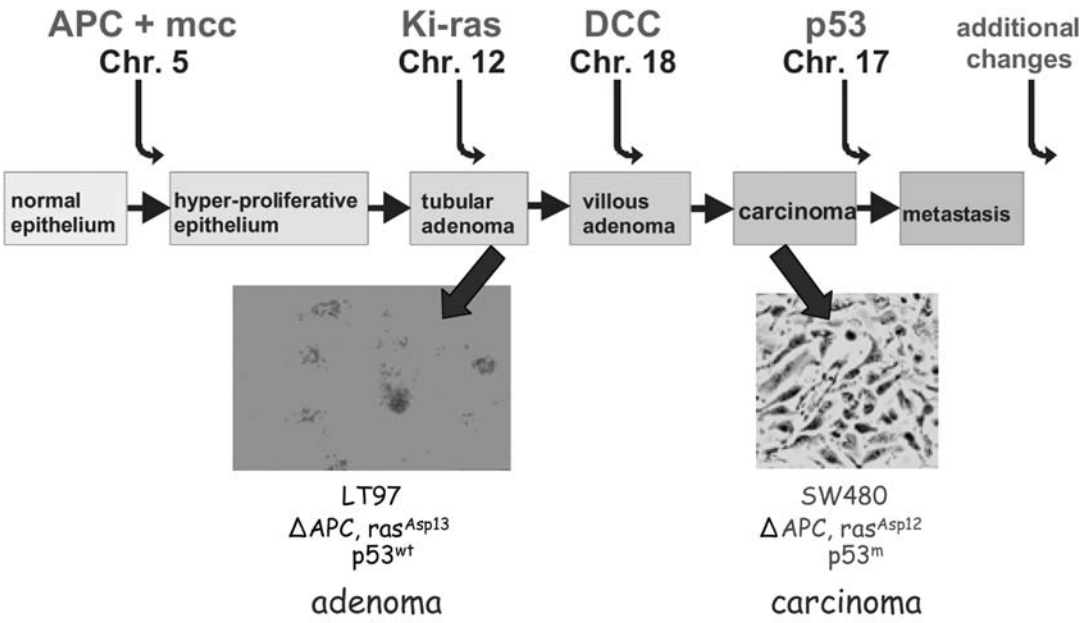

Figure 4. *Cell lines used.*

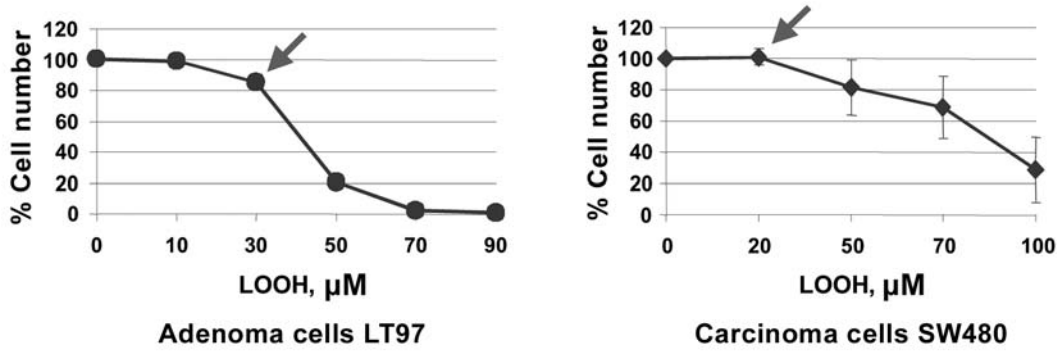

Figure 5. *LOOH is toxic to colon adenoma and carcinoma cells.*

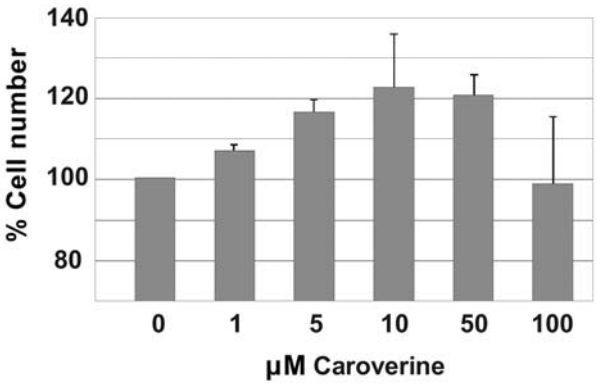

Figure 6. *Caroverine reduces cytotoxicity of LOOH.*

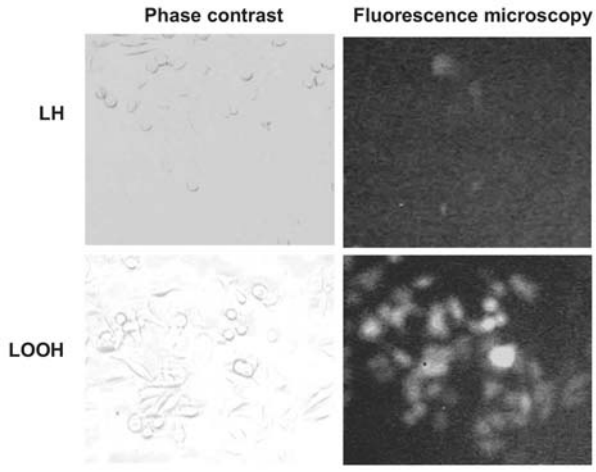

Figure 7. *LOOH induces intracellular H_2O_2 formation.*

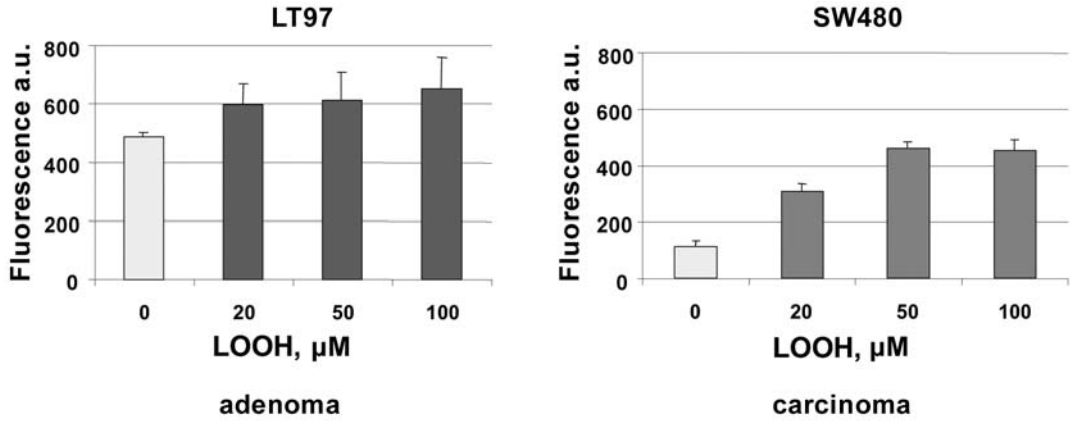

Figure 8. *LOOH at subtoxic concentrations causes intracellular H_2O_2 formation.*

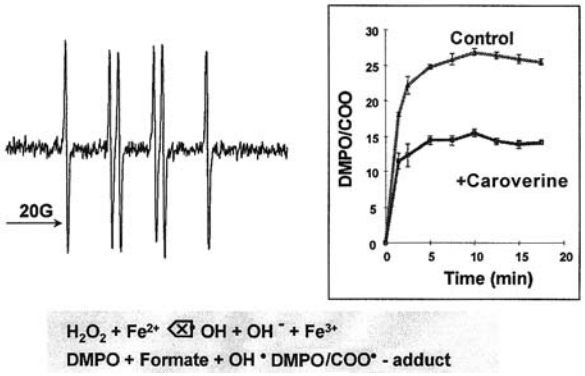

Figure 9. *Caroverine efficiently inhibits •OH radical generation from Fenton-Reaction.*

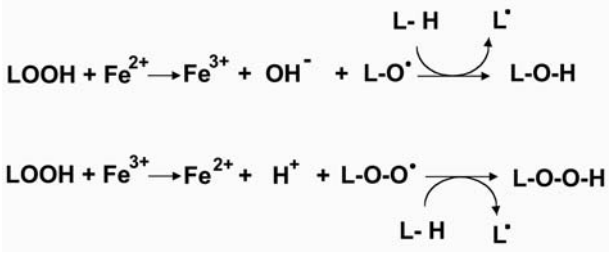

Figure 10. *Lipid hydroperoxides generate alkoxyl radicals in the presence of iron.*

Each sample, either 150 µl cell homogenate or 500 µl supernatant, were mixed with SDS (60 mM and 20 mM, respectively) prior to extraction with 1 ml ethanol/hexane (2:5) under argon. After phase separation, the organic phase was removed and evaporated to dryness. The residue was dissolved in ethanol and analyzed by HPLC on a Waters LC1 module equipped with an UV detector for measurement of oxidized ubiquinones at 275 nm. Reduced quinones and vitamin E were determined by an electrochemical detector (Shimadzu L-ECD-6A), which was set to a potential of +0.6 V. The column (Merck, Nova-Pak C18 3.9 x 150 mm) was eluted by 1 ml/min mobile phase consisting of $NaClO_4$ (50 mM) dissolved in a mixture of ethanol, methanol, acetonitrile and $HClO_4$ (400:300:300:1). The concentrations of UQ_6 and UQ_6H_2 were calculated using an external standard mixture of UQ_6/UQ_6H_2.

Determination of intracellular hydrogen peroxide. SW480 or LT97 cells were grown until 60-80% confluence, exposed to 100 µM LH or LOOH together with 20 µM 2',7'-dichlorofluorescein diacetate (DHFC) in Hanks Balanced Saline Solution (HBSS) with Ca^{2+}, Mg^{2+} and 0.3% FCS. After 2 h, the cells were washed, fixed with 3% formalin and examined by fluorescence microscopy.

For quantification of H_20_2 induction, 10^5 cells/ml cells were incubated in HBSS with 20 µM DHFC for 2 h at 37˚C. A Coulter Epics XL flow cytometer with EXPO32 software was used to analyse fluorescin formation.

Production of VEGF. Cultures were grown in 6-well plates. Conditioned media were collected at the indicated time-points, cleared by centrifugation and the VEGF concentration determined by ELISA (R&D Systems Europe, Abingdon, UK).

Isolation of RNA and reverse transcriptase (RT)-PCR. RNA isolation was performed using a standard Trizol-extraction protocol (Life Technologies, Gibco BRL). The 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 oligo-dT primers for 1 h at 42˚C, using MMLV reverse transcriptase (Sigma). Genes of interest were amplified from cDNA samples by standard PCR cycles, as described earlier (26). Real-time PCR reactions were performed using the Assays on demand (Applied Biosystems, USA), as described earlier (26).

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 $p<0.05$.

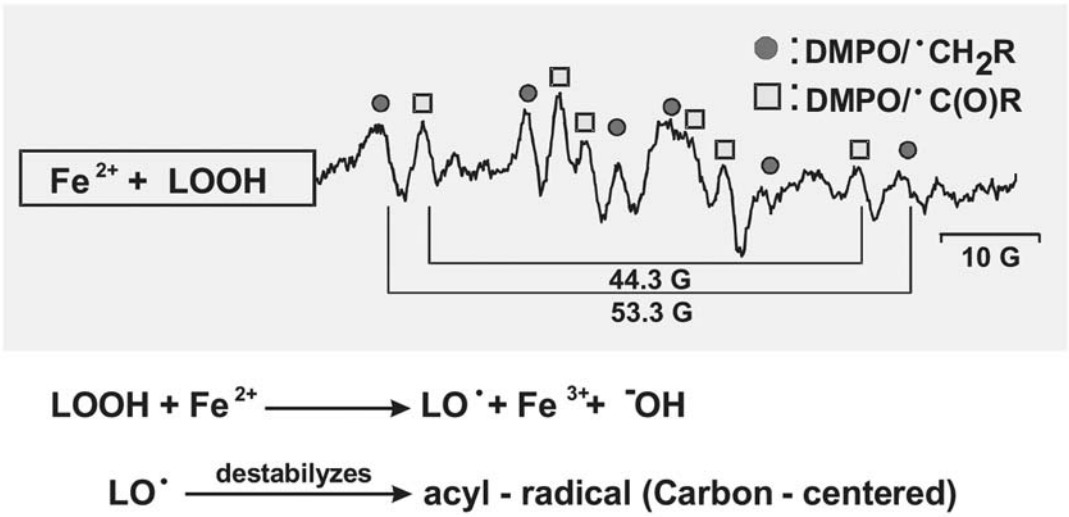

Figure 11. *Transition metals induce reductive homolytic cleavage of organic hydroperoxides giving rise to the formation of alkoxyl radicals.*

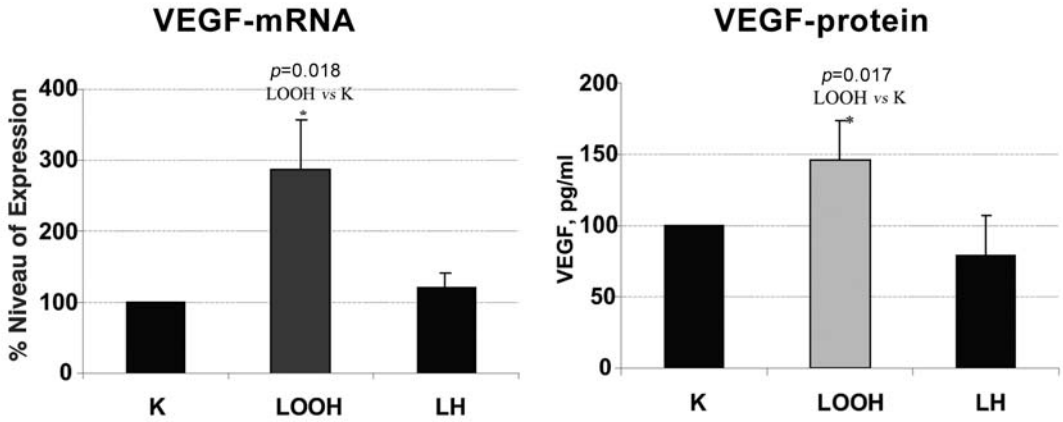

Figure 12. *LOOH stimulates expression of VEGF in human colon carcinoma cells (SW480).*

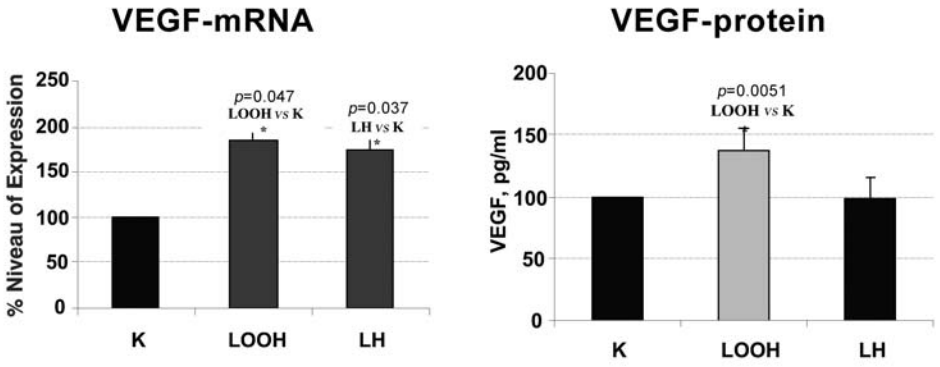

Figure 13. *LOOH stimulates expression of VEGF in human colon adenoma cells (LT97).*

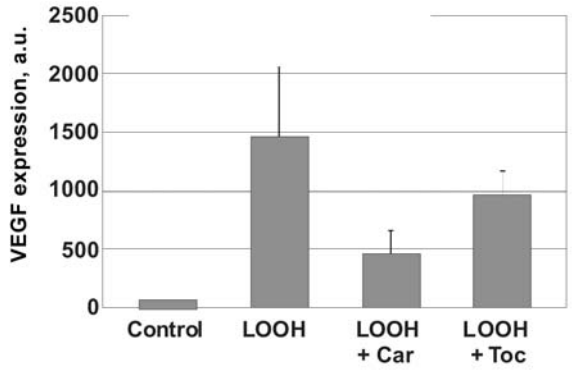

Figure 14. *Caroverine inhibits LOOH-induced expression of VEGF.*

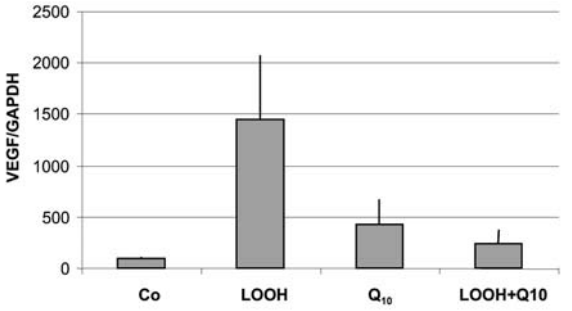

Figure 15. *Q_{10} inhibits LOOH-induced VEGF expression in SW480 carcinoma cells.*

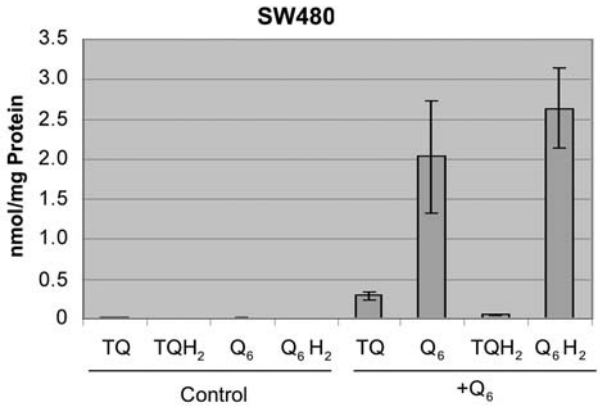

Figure 16. *Quinones are reduced by cellular enzymes.*

Results

Addition of LOOH to adenoma or carcinoma cells caused a decrease of cell numbers (Figure 5). Carcinoma cells were more resistant than adenoma cells.This may indicate that carcinoma cells possess more antioxidants. The control experiments were performed with unoxidized LH and cell numbers were unaffected. Caroverine reduced the cytotoxicity of LOOH (Figure 6).

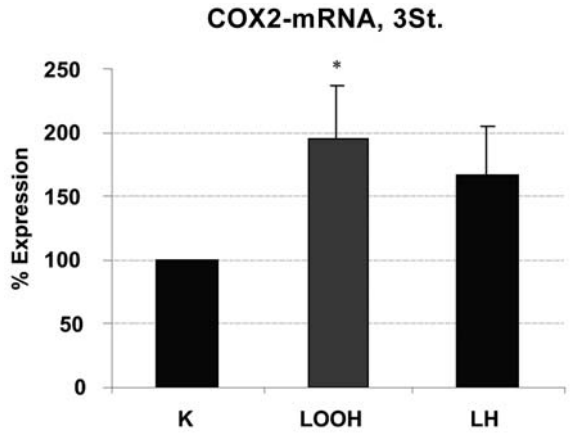

Figure 17. *LOOH stimulates COX-2 expression.*

To understand the molecular basis of LOOH-derived cell death, H_2O_2 formation was determined using the DCF-technique (Figures 7, 8). The controls which were affected with non-oxidized LH did not produce H_2O_2, while adenoma and carcinoma cells produced H_2O_2 when in contact with LOOH. The lesser formation of H_2O_2 in carcinoma cells supports our assumption that these cells produce antioxidants scavenging H_2O_2. H_2O_2 formation in cells leads to $^{\bullet}OH$ radical generation. Caroverine efficiently inhibited $^{\bullet}OH$ radical formation, the latter being toxic to all biomolecules. In adenoma cells, H_2O_2 production was clearly higher than in carcinoma cells (Figure 8).

As can be seen from the equation (Figure 9), H_2O_2 forms $^{\bullet}OH$ radicals in the presence of ferrous iron. EPR spectra in the presence of formate form $COO^{\bullet}$ adducts, which give a characteristic sextet with DMPO. When caroverine was added, $^{\bullet}OH$ radical formation decreased by almost 50 % (Figure 9). Analogously, organic peroxides form alkoxyl radicals in the presence of ferrous iron (Figure 10), starting a chain reaction in lipid membranes. Figure 11 shows the respective EPR spectrum of the stable metabolite of $RO^{\bullet}$, the acyl radical.

LOOH was found to induce VEGF in human carcinoma cells (Figure 12), determined on the basis of mRNA, and confirmed on the protein level. LOOH also stimulated the expression of VEGF in human colon adenoma cells (Figure 13), both at the level of mRNA and protein. Caroverine efficiently inhibited LOOH-induced mRNA expression of VEGF, while vitamin E was less effective (Figure 14).

Ubiquinone also had a strong suppressive effect on LOOH-induced mRNA VEGF expression in colon carcinoma cells, even though fully oxidized ubiquinone had been added to these cells (Figure 15).

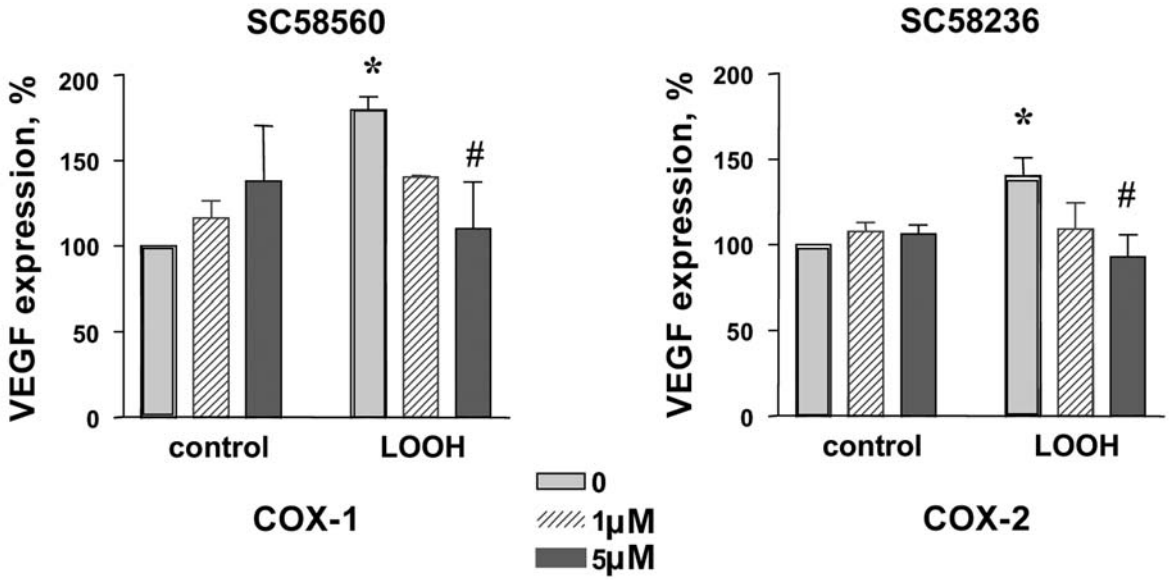

Figure 18. *COX-1 and COX-2 inhibitors prevent LOOH-induced VEGF expression.*

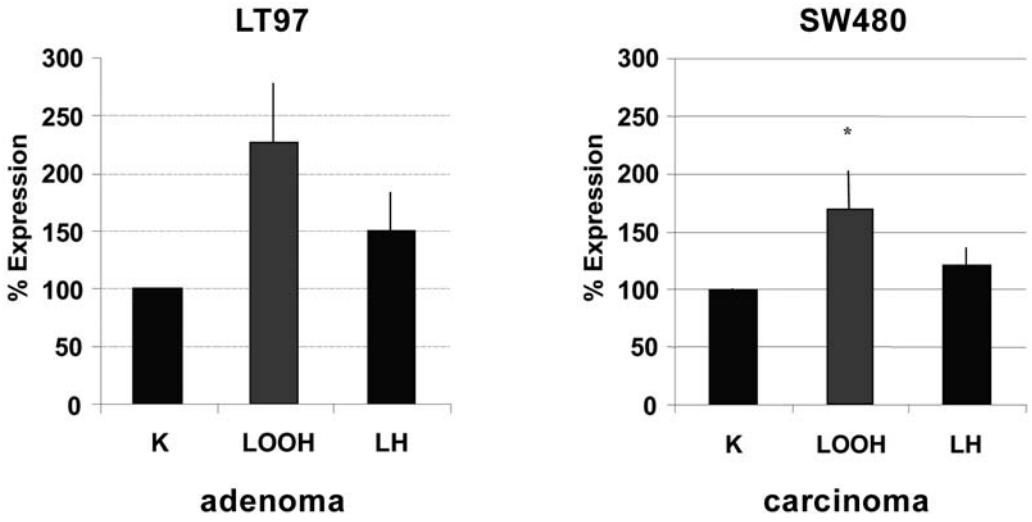

Figure 19. *LOOH stimulates c-fos expression in colon adenoma and carcinoma cells.*

However, oxidized ubiquinone was totally reduced to ubiquinol by cellular enzymes (Figure 16). In addition, we found that LOOH induced the expression of COX-2 (Figure 17), while COX-1 and COX-2 inhibitors prevented LOOH-derived VEGF mRNA expression (Figure 18). It was also found that LOOH induced the expression of c-fos in adenoma and carcinoma cells (Figure 19).

C-fos together with c-jun forms AP_1, which controls VEGF and COX-2 transcription. From the latter arises elevated levels of PGE_2 in the tumor tissues. PGE_2 activates growth and division of colon epithelial cells *via* epithelial growth factors (27) and tumour growth factors (28).

Conclusion

Tumour-associated genes are activated *via* organic hydroperoxide, which generates ROS. Growth factors are expressed which stimulate tumour growth and metastasis. Caroverine and ubiquinone suppress the growth factor VEGF, thus preventing angiogenesis which stimulates tumour growth. Also COX-1 and COX-2 inhibitors prevent VEGF expression.

According to our hypothesis, starting from the assumption that ROS are involved in the onset of colon carcinogenesis, we observed a clear decrease of both H_2O_2

production in carcinoma cells and the sensitivity of carcinoma cells to LOOH.

Since caroverine was shown to effectively scavenge •OH radicals, we conclude that ROS plays a role in the transformation of normal epithelial colon cells *via* adenoma to carcinoma cells.

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