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Nitric Oxide Levels in Human Preneoplastic Colonocytes Determine Their Susceptibility toward Antineoplastic Agents

UWE WENZEL, SABINE KUNTZ, and HANNELORE DANIEL

Department of Food and Nutrition, Molecular Nutrition Unit, Technical University of Munich, Freising-Weihenstephan, Germany

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ABSTRACT

The efficacy of antineoplastic compounds can depend heavily on the genetic background of the cells exposed to the drugs. This becomes evident by the fact that HT-29 human colon cancer cells but not primary murine nontransformed colonocytes are efficiently submitted to apoptosis by the flavonoid flavone. By determining caspase-3 activation, plasma membrane disintegration, and nuclear fragmentation, we show here that flavone also does not promote apoptosis in preneoplastic NCOL-1 colonocytes derived from a nontransformed human biopsy specimen. In clear contrast, the antitumor drug camptothecin potently induces apoptosis in NCOL-1 cells associated with a specific down-regulation of the antiapoptotic factor bcl-X_L at the mRNA and protein levels and with the activation of the mitochondrial apoptosis pathway. Confocal microscopy revealed an increased production of superoxide anion radicals in the mitochondria of NCOL-1 cells that preceded the apoptotic events. However, in the case of flavone, the mitochondrial oxygen radicals were effectively scavenged by physiological concentrations of nitric oxide (NO), whereas in the case of camptothecin, the available nitric oxide was rapidly scavenged by the production of large quantities of cytosolic superoxide anions. Increasing the levels of nitric oxide inside NCOL-1 cells by sodium nitroprusside prevented the apoptosis induction by camptothecin. Reducing the levels of nitric oxide by using the NO synthase inhibitor , N^{ω}-nitro-L-arginine methyl ester in NCOL-1 cells or using HT-29 cells that intrinsically have low NO levels enabled flavone to trigger the apoptosis pathway. In conclusion, our studies demonstrate that the intracellular levels of nitric oxide significantly change the apoptotic response to antineoplastic agents in colonic cells.

Apoptosis serves as the key mechanism to eradicate cells that have undergone DNA damage (White and McCubrey, 2001). The ability of tumor cells to resist apoptosis in response to death signals has become a central theme in the understanding of tumor development (Hanahan and Weinberg, 2000), and one of the major goals in cancer therapy is to restore the sensitivity of transformed cells toward apoptotic signals and the execution of apoptotic cell death (Nicholson, 1996a,b). However, several escape mechanisms have evolved that enable a tumor cell to survive (Pitti et al., 1998; Nakashima et al., 1999; Garrido and Algarra, 2001; Violette et al., 2002), stressing the importance of defining drug-gene interactions to design the best therapeutic concept possible. Indeed, because of genetic differences, the response to a compound can vary greatly between normal, preneoplastic, and transformed cells. For example, the green tea polyphenol epigallocatechin-3-gallate was shown to kill tumor cells much more efficiently than their nontransformed counterparts (Ahmad et al., 1997), and this specificity was associated with the inhibition of nuclear transcription factor κB (NF-κB) expression and the binding of NF-κB to DNA-*cis*-regulatory elements at much lower concentrations in the cancer cells than in the nontransformed cells (Ahmad et al., 2000).

We have shown formerly that the flavonoid flavone, occurring in cereal grains and in dill weed (Middleton and Kandaswami, 1993), induces apoptosis very efficiently in human colon cancer cells without affecting nontransformed primary murine colonocytes (Wenzel et al., 2000). Because the antitumor drug camptothecin potently promotes apoptosis in both cell types (Wenzel et al., 2000), it demonstrates that the apoptotic response of a cell varies depending not only on the genetic background but also on the apoptosis-inducing agent.

In the present study, we first investigated whether the selectivity of the apoptotic action of flavone in comparison to camptothecin could be seen also in NCOL-1 cells, a human colonic epithelial cell line derived from a nontransformed human biopsy specimen (Deveney et al., 1996). By using a semiquantitative RT-PCR approach and Western blotting, we assessed whether apoptosis-inducing activities of flavone and camptothecin were reflected at the transcript and pro-

ABBREVIATIONS: NF-κB, nuclear transcription factor κB; AMC, amino-4-methylcoumarin; CHAPS, 3-[(cholamidopropyl)-dimethyl-ammonium]-1-propane-sulfonate; COX-2, cyclooxygenase-2; DAF-2DA, 4,5-diamino-fluorescein diacetate; GAPDH, glyceraldehyd-3-phosphate dehydrogenase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; proxyl fluorescamine, 5-(2-carboxyphenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl)-3-phenyl-2-pyrrolin-4-one, potassium salt; RT-PCR, reverse transcriptase-polymerase chain reaction; SNP, sodium nitroprusside; SIN-1, 3-morpholinosydnonimine.

tein levels of apoptosis-relevant gene products. With the use of confocal microscopy, we also determined the role of oxygen radicals and nitric oxide (NO) in the apoptotic response of the cells to flavone and camptothecin.

Materials and Methods

Materials. Flavone, camptothecin, and the Hoechst dyes were purchased from Sigma Chemie (Deisenhofen, Germany). Media and supplements for cell culture were from Invitrogen (Karlsruhe, Germany). Cell-culture plates were from Renner (Dannstadt, Germany). Quadriperm wells were obtained from Merck Biosciences (Darmstadt, Germany). The fluorophores MitoTracker Red CMXRos, proxyl fluorescamine, and SYTOX-Green nucleic acid stain were from Bioprobe (Leiden, The Netherlands), MitoCapture was from BioCat GmbH (Heidelberg, Germany), and the fluorogenic caspase-3 substrate Ac-DEVD-AMC as well as DAF-2DA were obtained from Calbiochem (Bad Soden, Germany). All primary and secondary antibodies were purchased from Santa Cruz Biochemicals (Heidelberg, Germany). Polyvinylidene difluoride-blotting membranes were from Roth (Karlsruhe, Germany). All reagents for RNA preparation and RT-PCR were from MBI Fermentas (Heidelberg, Germany), and the primers were custom-synthesized by Eurogentec (Seraing, Belgium). Complete miniprotease inhibitor cocktail was purchased from Roche Diagnostics (Mannheim, Germany), and dye reagent for protein determination was from Bio-Rad (Munich, Germany).

Cell Culture. NCOL-1 cells (passage 50) were a kind gift of Professor Clifford W. Deveney and Dr. Michael J. Rutten (School of Medicine, Oregon Health & Science University, Portland, OR). Cells were cultured and passaged in Dulbecco's modified Eagle's medium/HEPES/glutamine supplemented with 10% fetal calf serum, modified Eagle's medium amino acids, bovine mammary epithelial vitamin solution, and 1 nM human recombinant epidermal growth factor. Antibiotics added to the media were 200 U/ml penicillin, 100 μg/ml streptomycin, 12.5 μg/ml gentamicin, and 1 μg/ml fungizone. The cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were passaged at preconfluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA.

Apoptosis Assays. Caspase-3–like activity was measured as described previously (Wenzel et al., 2000) with the use of the method described by Nicholson et al. (1995). Briefly, NCOL-1 cells were seeded at a density of 5×10^5 per well onto six-well plates and allowed to adhere for 24 h. Cells were then exposed for the times indicated to the test compounds. Subsequently, cells were trypsinized, cell numbers were determined, and then the cells were centrifuged at 2500*g* for 10 min. Cytosolic extracts were prepared by adding 750 μl of a buffer containing 2 mM EDTA, 0.1% CHAPS, 5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 20 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM HEPES/KOH, pH 7.4, to each pellet and homogenizing by 10 strokes. The homogenate was centrifuged at 100,000*g* at 4°C for 30 min, and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide substrate Ac-DEVD-AMC at a final concentration of 20 μM. Cleavage of the caspase-3 substrate was followed by the determination of emission at 460 nm after excitation at 390 nm using a multiwell plate reader (Fluoroskan Ascent; Labsystem, Bornheim-Hersel, Germany).

Changes in membrane permeability as an early apoptosis marker were assessed by incubating 3×10^4 NCOL-1 cells on 24-well plates with the test compounds or cell-culture medium alone (control) for the times indicated. Cells were stained with 1 μg/ml Hoechst 33342, and the rate of accumulation of the dye in early apoptotic cells (Elstein and Zucker, 1994) was detected using an inverted fluorescence microscope (DMIL; Leica, Wetzlar, Germany) equipped with an band-pass excitation filter of 340 to 380 nm and a long-pass emission filter of 425 nm.

Mitochondrial membrane potential was assessed using MitoCapture. Therefore, NCOL-1 cells were grown on glass slides placed into Quadriperm wells and incubated with or without effectors for the times indicated. Subsequently, the cells were stained with MitoCapture according to the manufacturer's instructions. In healthy cells, MitoCapture accumulates and aggregates in mitochondria, giving off a bright red fluorescence that was viewed under a TCS SP2 laser scanning confocal microscope (Leica) using an excitation of 543 nm and measuring emission at 590 to 650 nm. In apoptotic cells, MitoCapture cannot aggregate in the mitochondria because of the altered mitochondrial transmembrane potential, and thus it remains in the cytosol in its monomer form, fluorescing green, which was analyzed at excitation of 488 nm and emission of 500 to 530 nm.

Nuclear fragmentation as a late marker of apoptosis was determined by staining of DNA with Hoechst 33258. NCOL-1 cells (3×10^4) were incubated with the test compounds for the times indicated, and thereafter, cells were washed with phosphate-buffered saline, allowed to air dry for 30 min, and then were fixed with 2% *para*-formaldehyde before staining with 1 μg/ml Hoechst 33258 and visualization under the inverted fluorescence microscope.

Short-Term Cytotoxicity. Cytotoxicity was determined as described previously (Kuntz et al., 1999). Briefly, 5×10^4 adherent NCOL-1 cells were exposed for 3 h to the various test compounds. The percentage of viable cells in the cell population was determined by SYTOX fluorescence after cell lysis by 1% Triton X-100 (total cell counts) in relation to fluorescence measured before lysis of the cells (dead cells). Cell counts were determined from a calibration curve (Kuntz et al., 1999).

Semiquantitative RT-PCR. RNA from NCOL-1 cells was isolated at the times indicated in the figures according to the method described by Chomczynski and Sacchi (1987) with slight modifications (Wenzel et al., 2000). Reverse transcription was performed with 5 μg of isolated RNA. First-strand cDNA synthesis was accomplished with an oligo-$(dT)_{15}$ primer. Amplification of sequence-specific fragments was performed with 30 cycles (95°C denaturation for 1 min, 55°C hybridization for 2 min, 72°C extensions for 2 min) (Personal Cycler; Biometra, Göttingen, Germany). RT-PCR products were separated on a 1% agarose gel and visualized by ethidium bromide. The amount of first strand used to amplify specific sequences was derived from the linear range of amplification. The amplified glyceraldehyd-3-phosphate dehydrogenase (GAPDH) sequence was used as a con-

TABLE 1
Primers

GAPDH	5′-GACCACAGTCCATGCCATCACT-3′
	5′-TCCACCACCCTGTTGCTGTAG-3′
COX-2	5′-TAGGAATGTTCCACCCGCAGT-3′
	5′-GATATCATCTAGTCCGGAGCG-3′
NF-κB	5′-CTGAAGATGTGAAGCTGCAGC-3′
	5′-TCTGAGCACCTTTGGATGCAC-3′
cyclin B	5′-GAAGATCAACATGGCAGGCGC-3′
	5′-GTCACCAATTTCTGGAGGGTA-3′
cyclin E	5′-GAAGGAGCGGGACACCATGAA-3′
	5′-ACGCCACTTAAGGGCCTTCAT-3′
p16	5′-TGGACCTGGCTGAGGAGCT-3′
	5′-CAGTGTGACTCAAGAGAAGCC-3′
p21	5′-GGGATGTCCGTCAGAACCCAT-3′
	5′-TCTTGGAGAAGATCAGCCGGC-3′
p53	5′-CAGAAGCACCCAGGACTTCCA-3′
	5′-TGAAATCCTCCAGGGTGTGGG-3′
bak	5′-TTACCGCCATCAGCAGGAACA-3′
	5′-ATGGGACCATTGCCCAAGTTC-3′
bax	5′-ACCAGCTCTGAGCAGATCATG-3′
	5′-CTTCTTCCAGATGGTGAGCGA-3′
bcl-X_L	5′-ACTGAATCGGAGATGGAGACC-3′
	5′-AGGCTCTAGGTGGTCATTCAG-3′
c-myc	5′-AGCTTGTACCTGCAGGATCTG-3′
	5′-CAACTGTTCTCGTCGTTTCCG-3′
c-erbB2	5′-GGCTGGCTCCGATGTTTTGA-3′
	5′-CCGTAGGTGTCCCTTTGAAGG-3′
nm23	5′-ATGGCCAACTGTGAGCGTACC-3′
	5′-TCCAGTTCTGAGCACAGCTCG-3′

stitutively expressed control. The amplified products were photographed, and the intensity of the bands was analyzed by the SigmaGel software (SPSS Inc., Chicago, IL). No products were obtained for all genes without reverse transcription indicating the specificity of mRNA determination. A λ-DNA/EcoRI plus HindIII marker was used in all polymerase chain reaction experiments as a size control of the amplified products. Primers used are shown in Table 1.

Immunoblotting. NCOL-1 cells were incubated in 75-cm^2 flasks with or without effectors for 24 h and scraped off in 250 mM sucrose. Cells were pelleted subsequently by centrifugation for 10 min at 2500*g*. The pelleted cells were permeabilized through a syringe with a 24-gauge needle using 0.025% digitonin in a buffer containing 250 mM sucrose, 2.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, complete miniprotease inhibitor cocktail, and 20 mM HEPES/Tris, pH 7.2 (Hausmann et al., 2000). To allow 95 to 100% of the cells to be permeabilized, the cells were incubated for 10 min on ice by gentle agitation, and completeness of permeabilization was assessed by Trypan blue exclusion. Separation of organelles and cytosol was achieved by centrifugation at 13,000*g* for 2 min at 4°C. The supernatant (cytosol) was carefully removed, and the pellet, containing mitochondria, was solubilized in a buffer containing 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, protease inhibitor cocktail, and 50 mM Tris-HCl, pH 8.0 (Hausmann et al., 2000). Protein content was determined by the Bradford reaction (Bio-Rad). The homogenate, cytosol, and pellet fractions were resolved by SDS-polyacrylamide gel electrophoresis according to the method described by Schagger and von Jagow (1987) and were electroblotted onto polyvinylidene difluoride membranes. The control of protein transfer and identification of the molecular mass marker proteins was achieved by Ponceau Red staining. Thereafter, the blotting membranes were blocked for 1 h with Tris-buffered saline/Tween 20 and then incubated with the primary antibody (anti-NF-κB p65, sc-372; anti-bcl-X_L, sc-7195; anti-cytochrome *c*, sc-7159; anti-actin, sc-1615) for 1 h in a 1:1000 dilution. Bound antibodies were detected after 1-h incubation with horseradish peroxidase-conjugated secondary reagents (sc-2020 for anti-actin and sc-2004 for all other primary antibodies) and 3-amino-9-ethylcarbazole and H_2O_2 as substrates. Intensities of the bands were analyzed with use of the SigmaGel software.

Detection of Superoxide Radicals. NCOL-1 cells were loaded with 50 μM proxyl fluorescamine for 2 h in modified Krebs buffer

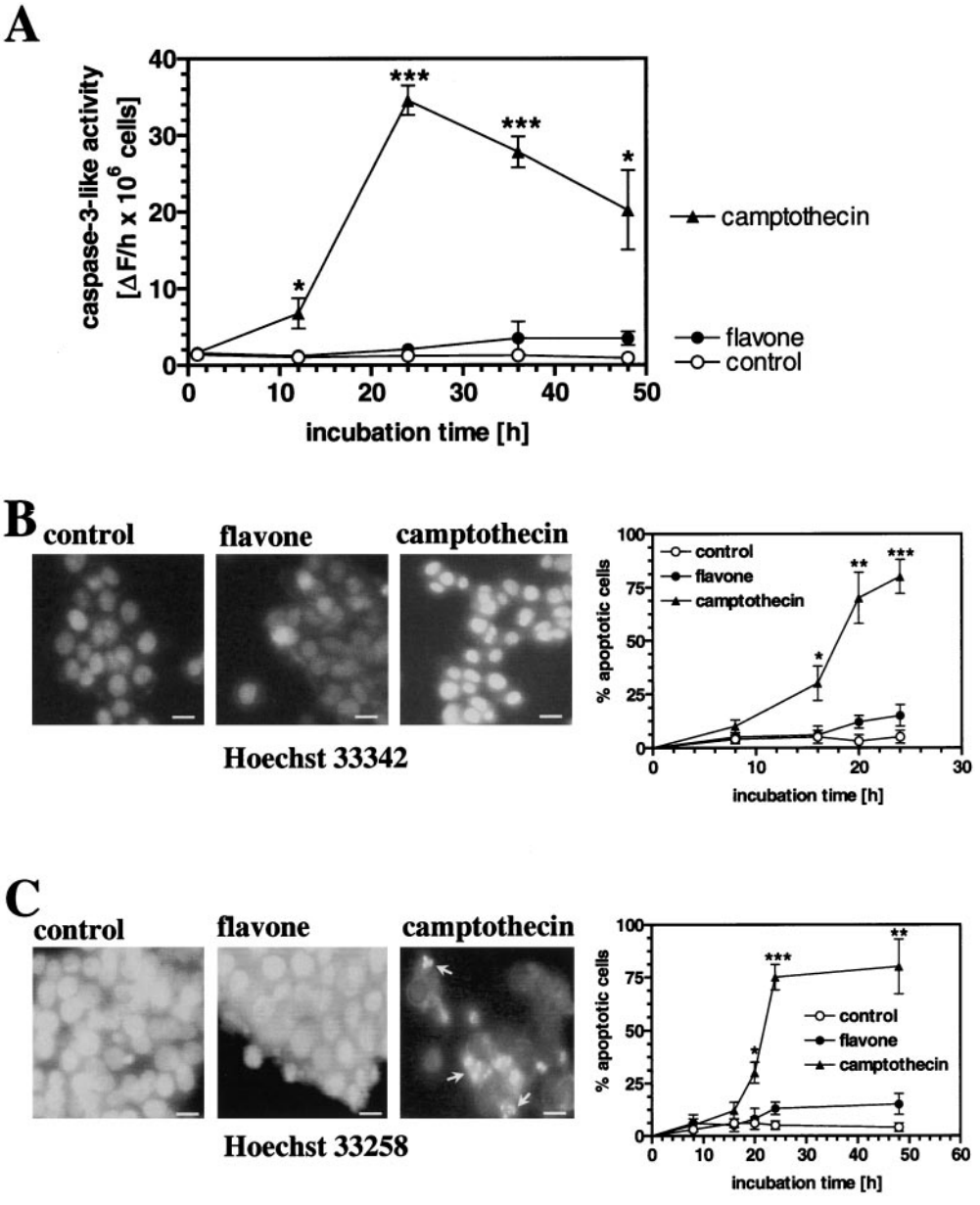

Fig. 1. Effects of flavone and camptothecin on apoptosis in NCOL-1 cells. A, caspase-3–like activity was measured in NCOL-1 cells incubated with medium alone (control) or in addition to 150 μM flavone or 25 μM camptothecin. ⋆, $P < 0.05$; ⋆⋆⋆, $P < 0.001$ versus the control. B, membrane disintegration in cells treated with medium alone (control) or in addition to 150 μM flavone or 25 μM camptothecin. Uptake of Hoechst 33342 as a marker of membrane disintegration is shown after 24 h. The percentage of apoptotic cells at different time points, as indicated by Hoechst 33342 staining of cells, is given at right. ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$; ⋆⋆⋆, $P < 0.001$ versus control cells. C, nuclear fragmentation (arrows) after 48 h was detected by Hoechst 33258 staining using an inverted fluorescence microscope. The percentage of cells displaying signs of chromatin condensation and DNA fragmentation is given at right at different time points of incubation. ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$; ⋆⋆⋆, $P < 0.001$ versus the control. Scale bars, 40 μm.

TABLE 2

Relative transcript levels of bcl- X_L and NF-κB in cells exposed to flavone or camptothecin

Transcript levels are given as the ratios of band intensities (area under the curve) of amplified cDNAs from target genes versus GAPDH (used as a constitutively expressed control). Before semiquantitative RT-PCR, cells were treated with medium alone (control) or with 150 μM flavone or 25 μM camptothecin for the times indicated.

Treatment	Time of Treatment			
	3 h	8 h	24 h	48 h
bcl-X_L				
Control	2.10 ± 0.31	2.30 ± 0.39	2.17 ± 0.34	2.25 ± 0.12
Flavone	2.31 ± 0.20	2.41 ± 0.32	1.99 ± 0.21	2.05 ± 0.33
Camptothecin	2.13 ± 0.19	1.14 ± 0.13*	0.85 ± 0.06*	0.80 ± 0.10**
NF-κB				
Control	1.54 ± 0.16	1.65 ± 0.22	1.86 ± 0.17	1.61 ± 0.16
Flavone	1.56 ± 0.25	1.36 ± 0.19	1.65 ± 0.15	1.84 ± 0.25
Camptothecin	1.53 ± 0.20	1.55 ± 0.23	0.92 ± 0.16*	0.85 ± 0.22*

*, $P < 0.05$; **, $P < 0.001$ when compared with controls ($n = 3$).

containing 200 μM cysteine to yield an increase in fluorescence emission because of the reduction of the fluorophore nitroxide to its corresponding hydroxylamine in the presence of superoxide (Pou et al., 1993). The cells were washed before the application of compounds, and the emission of proxyl fluorescamine was measured at 460 nm after excitation at 390 nm using the fluorescence plate reader. All runs containing camptothecin were corrected for the autofluorescence occurring when camptothecin is excited at 460 nm.

Production of superoxide radicals in mitochondria was visualized by laser scanning microscopy. Therefore, cells were grown on glass slides placed in Quadriperm wells and loaded with 50 μM proxyl fluorescamine for the last 2 h of incubation. For the staining of mitochondria, cells were loaded with 500 nM MitoTracker for the last 30 min of incubation. Superoxide radicals and mitochondria were detected after excitation with the UV laser or at 543 nm and at emissions of 440 to 480 and 590 to 650 nm, respectively.

Detection of NO. NCOL-1 cells were loaded with 10 μM DAF-2DA for 45 min in modified Krebs buffer. The cells were washed and the fluorescence of DAF-2 (excitation, 485 nm; emission, 538 nm) was followed in the presence or absence of effectors using the microtiter plate reader.

Calculations and Statistics. Variance analysis between groups was performed by one-way analysis of variance, and significance of differences between groups was determined by a nonpaired Student's *t* test (Prism; GraphPad Software Inc., San Diego, CA). For each variable, at least three independent experiments were carried out. Data are given as the mean ± S.E.M.

Results

Camptothecin but not Flavone Induces Apoptosis in NCOL-1 Cells. Flavone has been shown previously to induce apoptosis in HT-29 human colon cancer cells at least as potently as the classic antitumor drug camptothecin (Wenzel et al., 2000). Apoptosis induction was associated with a several-fold increased caspase-3–like activity, with plasma membrane disintegration and finally chromatin condensation as a late apoptosis event (Wenzel et al., 2000). When the effects of flavone and camptothecin were assessed in nontransformed NCOL-1 human colonocytes, camptothecin proved to be a very potent apoptosis inducer, whereas flavone failed to affect apoptosis (Fig. 1). These results were obtained at concentrations of 150 μM flavone and 25 μM camptothecin, which in both cases represents the highest concentration that does not induce necrosis as determined by SYTOX-Green nucleic acid stain exclusion (Kuntz et al., 1999). NCOL-1 cells turned out to be very sensitive to camptothecin-induced apoptosis, as indicated by an almost 30-fold activation of caspase-3–like activity after 24 h that resulted in almost 80% of the cells undergoing apoptosis (Fig. 1). In HT-29 cells, for comparison, camptothecin and flavone induce caspase-3–like activity only 7-fold, and only 40% of the cell population showed late signs of apoptosis (Wenzel et al., 2000).

Effects of Flavone and Camptothecin on Apoptosis-Relevant Transcript Levels in NCOL-1 Cells. To gain insights into the underlying mechanisms of the apoptosis induction by camptothecin in NCOL-1 cells and the selective triggering of apoptosis by flavone in transformed colonocytes only, we performed semiquantitative RT-PCR to determine changes in the transcript levels of 13 genes with relevance for apoptosis and/or cell-cycle control in colonocytes (Reed, 1996; Martinez et al., 1997; Zhan et al., 1999; Hellin et al., 2000; Greco et al., 2001; Sturm et al., 2001; Zvibel et al., 2001; Cao and Prescott, 2002). Relative mRNA levels of the following genes have been determined: *bcl-X_L*, *bax*, *bak*, *p16*, *p21*, *p53*, *COX-2*, *cyclin B*, *cyclin E*, *erb-B2*, *c-myc*, nm-23, and NF-κB. Special emphasis was put on those gene products that showed flavone-dependent alterations in HT-29 (Wenzel et al., 2000) but not in NCOL-1 cells but that changed expression in response to camptothecin in NCOL-1 cells.

Among the transcripts analyzed, neither bax, p16, p53, COX-2, erb-B2, c-myc, nor nm23 mRNAs showed any changes in response to camptothecin or flavone in NCOL-1 cells (data not shown). The mRNA levels of the proapoptotic *bak* were selectively increased after camptothecin treatment, but this increase in *bak* mRNA became significant only after 48 h of exposure (data not shown), a time point when almost all cells exposed to camptothecin already showed signs of the late apoptosis (Fig. 1C). This suggests that a direct involvement of bak in the initiation of apoptosis by camptothecin is very unlikely. An increase in the mRNA levels of the cyclin-dependent kinase inhibitor p21 and a concomitant decrease in transcript levels of the cell-cycle regulators cyclin B and cyclin E were seen after flavone as well as after camptothecin treatment. Thus, these gene products cannot be responsible for the induction of apoptosis in NCOL-1 cells by camptothecin. However, the growth-inhibitory potencies of flavone and camptothecin observed in NCOL-1 cells (data not shown) might involve the changes in mRNA levels of these gene products.

Both bcl-X_L and NF-κB have been shown to possess anti-apoptotic activities in colonocytes (Hirose et al., 1997; Wang et al., 1999). Previously, we demonstrated that both factors are efficiently down-regulated at the mRNA level in HT-29

cells in response to flavone (Wenzel et al., 2000). Similarly, in NCOL-1 cells, these mRNA levels are reduced by camptothecin at 8 and 24 h, respectively, whereas flavone did not affect bcl-X_L or NF-κB mRNA levels at all (Table 2). When the effects of camptothecin on the protein level were assessed at 24 h of incubation, a time point when a large number of cells already underwent late stages of apoptosis (Fig. 1C), bcl-X_L was significantly reduced by camptothecin, whereas the NF-κB protein levels remained unaltered compared with controls (Fig. 2). Flavone changed neither bcl-X_L nor NF-κB protein levels at 24 h of incubation (Fig. *2*).

Effects of Camptothecin and Flavone on the Mitochondrial Apoptosis Pathway. bcl-X_L serves as an antiapoptotic protein that remains constantly attached to mitochondria to prevent the release of cytochrome *c* by mechanisms that are still under debate (Hengartner, 2000). One important factor that is frequently associated with the down-regulation of bcl-X_L gene expression is reactive oxygen species. Their generation is suggested to precede the loss of mitochondrial membrane potential, the release of cytochrome *c*, and finally the activation of caspase-3 (Herrera et al., 2001).

We observed that the down-regulation of bcl-X_L in camptothecin-treated NCOL-1 cells was associated with the release of cytochrome *c* into the cytosol (Fig. 3A) and the dissipation of the mitochondrial inner membrane potential (Fig. 3B), which preceded the cytochrome *c* release by approximately 4 h (data not shown).

By confocal microscopy, we observed an early increase of superoxide anion production in the mitochondria of NCOL-1 cells only 6 h after exposure to camptothecin, whereas flavone-treated cells did not respond (Fig. 4A). Because camptothecin, in contrast to flavone, is a potent producer of superoxide anions in the cytosol (Fig. 4B), the activation of the

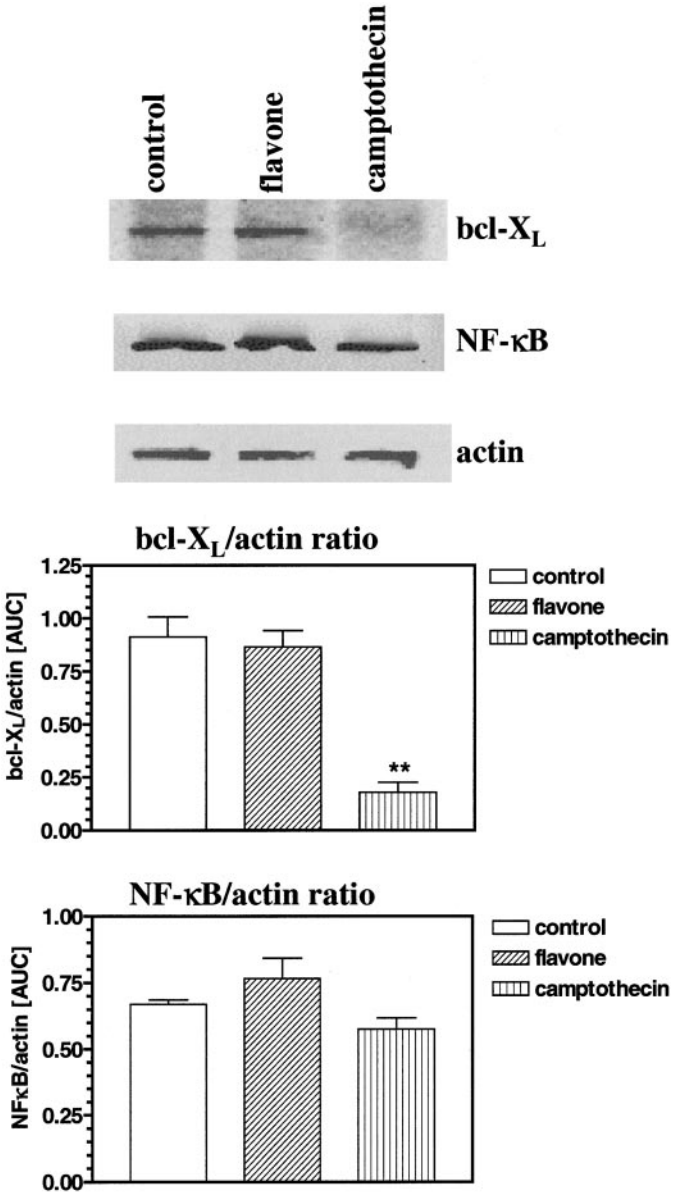

Fig. 2. Expression of bcl-X_L and NF-κB in NCOL-1 cells at the protein level. The cells were incubated for 24 h with either medium alone (control), 150 μM flavone, or 25 μM camptothecin. Top, immunochemically detected proteins in the homogenate; middle and bottom, ratio of bcl-X_L or NF-κB to actin, used as a constitutively expressed control. $\star\star$, $P < 0.01$ versus the control ($n = 3$).

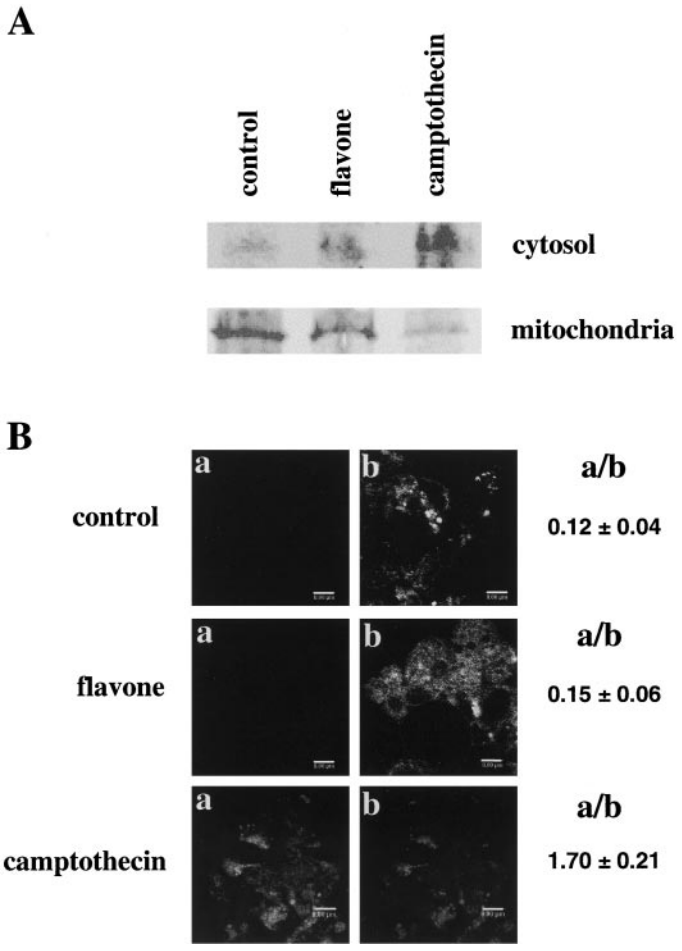

Fig. 3. Cytochrome *c* release and dissipation of the inner mitochondrial membrane potential are induced by camptothecin but not flavone. Cells were incubated with medium alone (control), with 150 μM flavone, or with 25 μM camptothecin. A, cytochrome *c* in the organelle fraction (containing mitochondria) and in the cytosol was detected by Western blotting after 24 h. B, dissipation of the mitochondrial membrane potential in apoptotic cells was indicated after 20 h by green fluorescence emitted with the use of MitoCapture (BioCat GmbH, Heidelburg, Germany) in the cytosol (a), whereas in cells not undergoing programmed cell death, MitoCapture fluoresces red in the mitochondria (b). The ratio of green-to-red fluorescence (a/b) was determined at the area of complete cells. Scale bars, 8 μm.

mitochondrial apoptosis pathway could be a direct consequence of superoxide anions entering the mitochondria. We have validated this hypothesis by analyzing the effects of menadion on apoptosis induction. Menadion is a classic producer of superoxide anions in the cytosol, but in contrast to camptothecin, menadion inhibited caspase-3–like activity in NCOL-1 instead of increasing it (data not shown). It therefore seems that other mitochondrial mechanisms that lead to the one-electron reduction of O_2 are the cause of apoptosis triggering by camptothecin, and those are not directly dependent on superoxide anions released into the cytosol.

NO Inhibits the Mitochondrial Apoptosis Pathway by Scavenging O_2^-. NO is an endogenously generated radical that reacts very fast with O_2^- to form peroxynitrite (Squadrito and Pryor, 1998). In this regard, NO functions as a potent antioxidant (Chiueh, 1999) with the capability to scavenge O_2^- produced in the cytosol or mitochondria. When analyzing the NO levels in NCOL-1 cells in response to camptothecin, we observed a decline in endogenous free NO concentration (Fig. 5A), which obviously prevents a quenching of O_2^- later on in the mitochondria (Fig. 4A). When we applied the NO donor SNP, a sustained, increased NO level in camptothecin-treated NCOL-1 cells resulted (Fig. 5A), and this efficiently prevented the camptothecin-induced generation of mitochondrial O_2^- (Fig. 5B). Moreover, when NO levels were reduced by the NO-synthase inhibitor L-NAME (Fig. 5A), even in flavone-treated cells, an increased production of O_2^- in mitochondria was seen after 6 h of exposure (Fig. 5B). Flavone alone did not affect the NO level (Fig. 5A). That the NO level inside NCOL-1 cells is strictly related to the mitochondrial apoptosis pathway was also confirmed by measuring mitochondrial membrane potential (Fig. 6A), levels of bcl-X_L (Fig. 6B), and the release of cytochrome *c* (Fig. 6C). All of these parameters indicated that camptothecin as well as flavone can activate the mitochondrial apoptosis pathway, but NO levels inside the cells seem to select whether apoptosis is executed or not. When NO levels decline, as observed in the presence of camptothecin or in the presence of a combination of flavone and L-NAME, apoptosis can occur. When NO levels are maintained or increased, as in the case of flavone or the combination of camptothecin with SNP, the mitochondrial apoptosis pathway is efficiently blocked. These effects of the NO level on the mitochondrial apoptosis parameters were completely mirrored by its actions on the early or late stages of apoptosis (Fig. 7).

The selectivity of NO action on apoptosis in NCOL-1 cells is

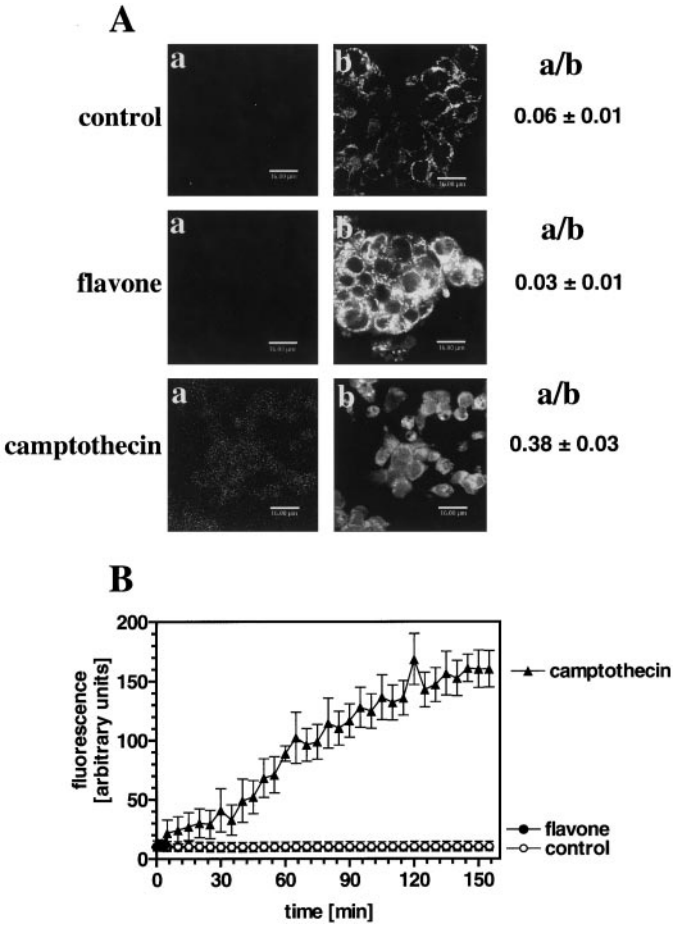

Fig. 4. Camptothecin releases O_2^- in mitochondria and in the cytosol. Cells were incubated with medium alone (control), with 150 μM flavone, or with 25 μM camptothecin. A, during the last period of a 6-h incubation, cells were loaded with MitoTracker (detection of mitochondria, as shown in b) in combination with proxyl fluorescamine (detection of O_2^-, as shown in a). The fluorescence ratio of a/b was determined at the area of mitochondria. Scale bars, 16 μm. B, during the first 160 min of incubation, the generation of O_2^- was monitored using the microtiter plate reader and proxyl fluorescamine as a probe.

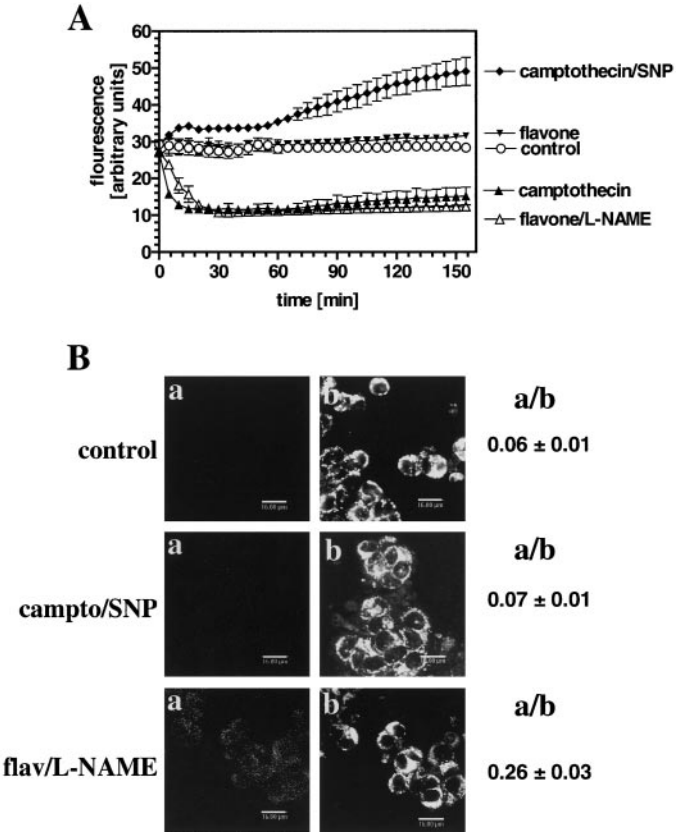

Fig. 5. NO scavenges mitochondrial O_2^-. A, NO levels in NCOL-1 cells were monitored for the initial 160 min of incubation with medium alone (control) or containing either 25 μM camptothecin, 25 μM camptothecin plus 250 μM SNP, 150 μM flavone, or 150 μM flavone/1 mM L-NAME using DAF-2DA as described under *Materials and Methods*. B, generation of O_2^- in mitochondria was measured by confocal laser-scanning microscopy after loading the cells with proxyl fluorescamine and MitoTracker for the last period of 6-h incubation with medium alone (control), 25 μM camptothecin/250 μM SNP (campto/SNP), or 150 μM flavone/1 mM L-NAME (flav/ L-NAME). Scale bars, 16 μm.

further stressed by the fact that high doses of flavone (150, 250, and 500 μM) do not change NO levels (Fig. 8C) but clearly cause concentration-dependent cell death (Fig. 8A) in the absence of an activation of caspase-3 (Fig. 8B). When using camptothecin at higher concentrations (25, 50, and 150 μM), NO levels are reduced to a similar extent (Fig. 8C), caspase-3–like activity is increased to similar levels at all concentrations, whereas cell death is strictly dose-dependent (Fig. 8A). When caspase-3–like activity was measured after 3 h of exposure to the test compounds, no changes over that seen in control cells was observed (data not shown), which strongly suggests that the simultaneously observed cytotoxicity of high doses of flavone or camptothecin are not linked to apoptosis.

Discussion

The resistance of genetically transformed cells toward death signals is regarded as one of the major parameters that allow tumor development (Hanahan and Weinberg, 2000). Several escape mechanisms are described which enable a tumor cell to survive, and those are derived from genetic differences between the parent nontransformed cells and the malignant cancer cell (Pitti et al., 1998; Nakashima et al., 1999; Garrido and Algarra, 2001; Violette et al., 2002). The identification of molecular markers that determine whether apoptosis in a cell occurs or not is consequently crucial for the development of strategies that specifically target cancer cells. In previous studies, we collected evidence that the flavonoid flavone is capable of submitting selectively tumor cells to apoptosis and of overcoming the escape mechanisms. It efficiently induces apoptosis in HT-29 human colon cancer cells but not in primary murine nontransformed colonocytes (Wenzel et al., 2000). This selectivity was not found for camptothecin, a classic apoptosis-inducing antitumor drug. In search of the underlying mechanisms for the selectivity of flavone, we compared its effects with those of camptothecin in a human nontransformed colonocyte line derived from a human biopsy specimen (Deveney et al., 1996). Although NCOL-1 cells were found not to form colonies in a soft-agar assay, suggesting that the long-term culturing did not cause these cells to become transformed (Deveney et al., 1996), they

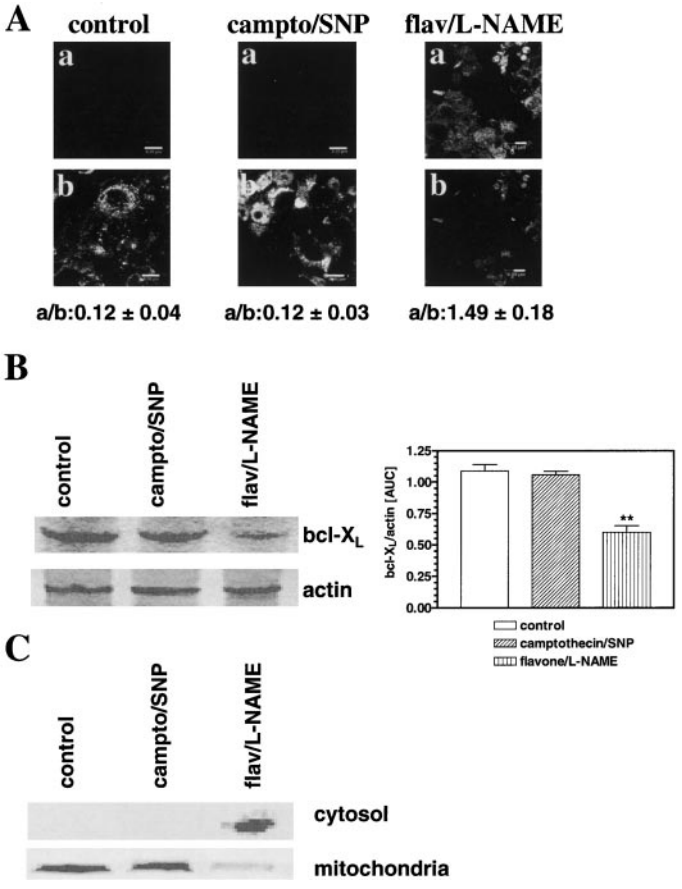

Fig. 6. NO levels inside NCOL-1 cells affect the mitochondrial apoptosis pathway. NCOL-1 cells were incubated with medium alone (control) or in addition to either 25 μM camptothecin/250 μM SNP (campto/SNP) or 150 μM flavone/1 mM L-NAME (flav/ L-NAME). A, dissipation of the mitochondrial membrane potential in apoptotic cells was indicated after 20 h by green fluorescence emitted with the use of MitoCapture in the cytosol (a), whereas in mitochondria with intact membrane potential, MitoCapture fluoresces red (b). The ratio of green-to-red fluorescence (a/b) was determined at the area of complete cells. B, Western blotting of bcl-X$_L$ after 24-h incubation. Left, immunochemically detected proteins in the homogenates. Right, ratio of bcl-X$_L$ to actin. ⋆⋆, $P < 0.01$ ($n = 3$). C, cytochrome *c* in the mitochondrial and cytosolic fraction, as assessed by Western blotting.

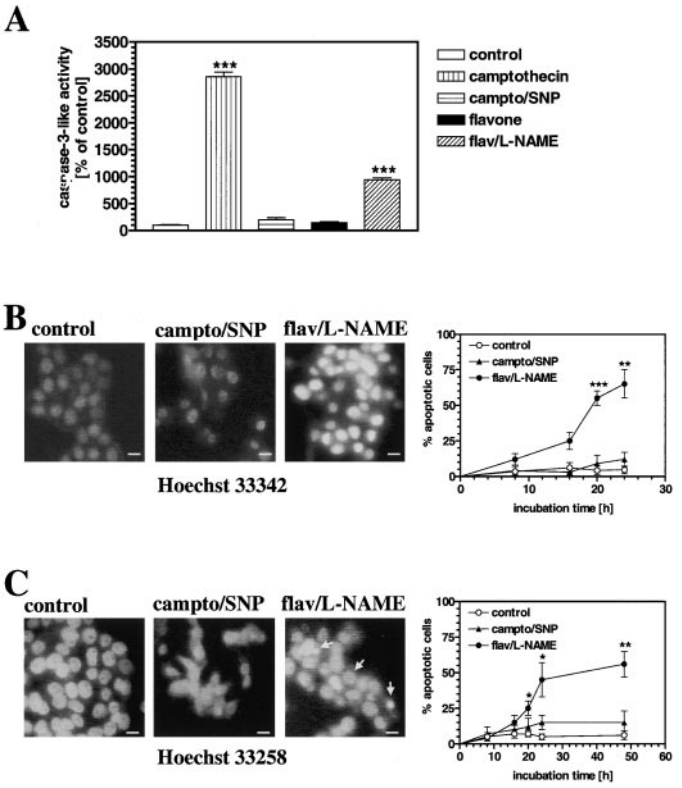

Fig. 7. NO levels in NCOL-1 cells affect early and late stages of apoptosis. A, caspase-3–like activity was measured after 24 h of incubation with medium alone (control) or in addition to 25 μM camptothecin, 25 μM camptothecin plus 250 μM SNP (campto/SNP), 150 μM flavone, or 150 μM flavone plus 1 mM L-NAME (flav/ L-NAME). ⋆⋆⋆, $P < 0.001$ versus the control. B, membrane disintegration was assessed in cells incubated with medium alone (control) or in addition to 25 μM camptothecin/250 μM SNP (campto/SNP) or 150 μM flavone/1 mM L-NAME (flav/ L-NAME) by measuring the uptake of Hoechst 33342 after 24 h. The percentage of apoptotic cells at different time points is indicated in the right panel. ⋆⋆, $P < 0.01$; ⋆⋆⋆, $P < 0.001$ versus control cells. C, nuclear fragmentation (arrows) after 48 h was detected by Hoechst 33258 staining with the percentage of apoptotic cells given in the right panel. ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$ versus the control. Scale bars, 30 μm.

should be regarded as preneoplastic because of their growth-dependent selection.

We observed that flavone did not induce apoptosis in NCOL-1 cells. Camptothecin, in contrast, was a very potent apoptosis inducer in NCOL-1 cells, as evidenced by its effects on caspase-3–like activity, on plasma membrane disintegration and nuclear fragmentation.

Taken from a screening of the transcript levels of 13 apoptosis-relevant genes in NCOL-1 cells, only the antiapoptotic factors bcl-X_L and NF-κB showed a down-regulation by camptothecin but not flavone treatment. In HT-29 colon cancer cells, however, flavone also reduced the expression of bcl-X_L and NF-κB mRNAs (Wenzel et al., 2000). At the protein level, bcl-X_L but not NF-κB was down-regulated by camptothecin, and this could be observed when the majority of NCOL-1 cells showed signs of late apoptosis. These data demonstrate the prominent role of bcl-X_L in the regulation of apoptosis in colonocytes and consequently in the development of colonic cancers (Maurer et al., 1998).

Because reactive oxygen species produced in mitochondria were found to be responsible for the down-regulation of bcl-X_L in hepatocytes followed by a loss of the mitochondrial membrane potential, the release of cytochrome *c,* and finally the activation of the effector caspase-3 (Herrera et al., 2001), we concluded that flavone and camptothecin could differ in their ability to generate mitochondrial superoxide anions in NCOL-1 cells. Indeed, only camptothecin initiated the generation of O_2^- in the mitochondria of NCOL-1 cells followed by dissipation of the mitochondrial membrane potential, release of cytochrome *c*, caspase-3 activation, plasma membrane disintegration, and finally nuclear fragmentation. Because HT-29 cells do not generate detectable amounts of NO endogenously (Ambs et al., 1998b) but respond to flavone with apoptosis, we suggested that NO could act as an antioxidant in NCOL-1 cells and interfere with the mitochondrial apoptosis pathways. Here we show that camptothecin produces cytosolic O_2^- and thereby scavenges free NO with a concomitant decline of cellular NO levels that could allow apoptosis to occur. When NO levels in camptothecin-treated NCOL-1 cells were increased by the coadministration of the NO donor SNP, all proapoptotic processes induced by camptothecin were blocked. When NO levels were reduced in NCOL-1 cells by using the NO-synthase inhibitor L-NAME, flavone was enabled to induce apoptosis via the mitochondrial pathway. The product of the reaction between NO and O_2^- is peroxynitrite, which is known to induce apoptosis in various cell types as well (Ambs et al., 1998a). To elucidate whether peroxynitrite contributes to camptothecin-mediated apoptosis in NCOL-1 cells, we applied 100 μM SIN-1, a peroxynitrite generator. Neither SIN-1 alone nor in combination with camptothecin affected caspase-3–like activity or other indicators of apoptosis, but SIN-1 possessed necrotic effects, which were not seen under any of the experimental conditions with camptothecin or flavone (data not shown). This indicates that the amount of peroxynitrite formed has an impact on its effects as well.

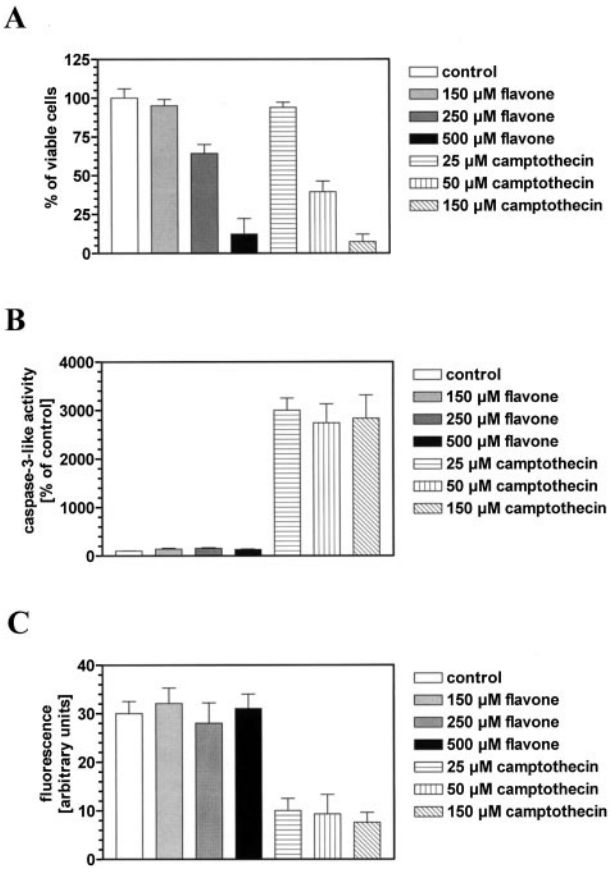

Fig. 8. Dose-dependence of the effects of flavone and camptothecin on cytotoxicity, caspase-3–like activity, and NO levels. A, cytotoxicity was determined by SYTOX exclusion after 3 h of exposure of cells to the test compounds, and a count of >90% of viable cells was considered to be nontoxic. B, caspase-3–like activity was determined at 24 h of incubation from the cleavage rate of Ac-DEVD-AMC. C, NO levels were measured using the NO-sensitive dye DAF2-DA.

In conclusion, our comparative analysis of flavone and camptothecin effects in NCOL-1 and HT-29 cells demonstrates that superoxide anions produced in mitochondria regulate apoptosis in colonocytes by affecting the expression level of the antiapoptotic factor bcl-X_L. By its ability to scavenge O_2^-, the cellular concentration of NO decisively controls the occurrence of apoptosis. In HT-29 cells, the intrinsic low level of NO allows flavone to efficiently induce apoptosis, and this is prevented when cellular NO levels are increased (Wenzel et al., 2003). Moreover, our studies demonstrate that small genetic differences between cells can have a great impact on the therapeutic responsiveness to drug exposure, stressing the importance of investigating drug-gene interactions.

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Address correspondence to: Dr. Uwe Wenzel, Department of Food and Nutrition, Molecular Nutrition Unit, Hochfeldweg 2, D-85350 Freising-Weihenstephan, Germany. E-mail: uwenzel@wzw.tum.de