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Early and Late Apoptosis Events in Human Transformed and Non-Transformed Colonocytes are Independent on Intracellular Acidification

Uwe Wenzel and Hannelore Daniel

Department of Food and Nutrition Science, Molecular Nutrition Unit, Technical University of Munich

Key Words

Intracellular pH • Flavonoids • Apoptosis • Caspase-3-like activity

Abstract

Intracellular acidification is discussed to play a pivotal role in the process of programmed cell death by providing an intracellular environment optimal for the activation of caspases and the execution of apoptosis. Using three human colon epithelial cell lines we tested whether effects on early and late apoptosis markers can be related to changes in intracellular pH (pH_i). Caspase-3-like activity and plasma membrane disintegration served as measures of early apoptosis whereas nuclear fragmentation served as indicator of late apoptosis events. The pH_i was measured using the pH-sensitive dye 2´, 7´-*bis* (2-carboxyethyl-5-(6)-carboxyfluorescein. Six flavonoids differing in apoptosis inducing activity were employed as tools. In HT-29 cells, quercetin and flavone proved to potently increase apoptosis without altering pH_i. In Caco-2 cells, quercetin and flavone increased early and late apoptosis parameters associated with a concomitant decline in pH_i. However, addition of imidazole prevented the acidification without altering the apoptotic response to the flavonoids. In NCOL-1 cells, only quercetin was able to induce apoptosis and changes observed correlate with the observed initial intracellular acidification rate. Here too, imidazole prevented the pH_i decline but failed to affect apoptosis execution. In conclusion, apoptosis in human colonocytes is not affected by alterations in pH_i.

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Introduction

Two major apoptosis pathways have been defined in mammalian cells. One involves extracellular signals transmitted via the tumour necrosis factor receptor 1, whereas the second, the mitochondrial pathway, is triggered by intrinsic pro-apoptotic stimuli such as DNA-damage and by extracellular and/or environmental stressors [1]. At the level of the effector caspase-3 both apoptosis pathways converge [1]. Increased acitivity of caspase-3 together with other effector caspases finally leads to the classical morphological changes observed in

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Uwe Wenzel
Department of Food and Nutrition Science, Molecular Nutrition Unit
Hochfeldweg 2, D-85350 Freising-Weihenstephan (Germany)
Tel. +49 (0) 8161/71-3997, Fax +49 (0) 8161/71-3999
E-Mail uwenzel@wzw.tum.de

cells undergoing apoptosis, characterized by membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation [2, 3]. However, plasma membrane disintegration, as an early marker of apoptosis [4] and fragmentation of DNA as a late apoptosis event [3] can also be observed in the absence of caspase-3 activation, depending on the cell type and the apoptosis initiating process [5, 6]. Since apoptosis is often preceded by intracellular acidification [7, 8] it has been suggested that a decline in pH_i may be either causal or permissive for a proper progression of the apoptotic cascade. However, in certain cell types a decline in pH_i was postulated to provide an optimal milieu for caspase activation but not to affect the execution phase of apoptosis [9] and in others maintainance of a pH_i above 7.2 did not prevent apoptosis [10].

To our knowledge, there are no reports that have addressed the role of intracellular acidification processes on apoptosis of colonocytes. These cells differ from other cell types as they are normally exposed to a luminal environment of varying pH that can be changed easily by dietary means [11, 12] and consequently these cells need effective mechanisms for maintaining pH_i. Once transformed and in the progression phase towards a solid tumor, cells are generally exposed to an acidic environment that results from the production of lactate and other acids [13]. However, the pH_i in tumor cells is close to that in normal cells or even sligthly higher [13-15]. The efficient maintenance of pH_i in tumor cells in spite of the acidic environment and their ability to resist apoptosis suggest that pH_i homeostasis and tumor development may be linked.

In the present study we used six structurally similar flavonoids as tools for assessing whether there is a link bewteen pH_i and apoptosis in HT-29 and Caco-2 human colon carcinoma cells and in the human preneoplastic colon epithelial cell line NCOL-1 [16]. The rationale for this approach was, that dispite similar structures and physicochemical characteristics, the compounds possess quite different potencies to initiate apoptosis in colonocytes, as previously shown [17]. In a first step, we assessed whether apoptosis induction by the flavonoids was dependent on caspase-3 activity. In a second step, we determined to which extent the compounds affect intracellular pH and whether there was a correlation between the measures of early and late apoptosis and the effects on pH_i. Finally, we measured early and late apoptosis parameters when a decline in intracellular acidification as caused by some flavonoids was prevented by imidazole.

Materials and Methods

Cell culture

All cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37^{0}C. Cells were passaged at preconfluent densities by the use of a solution containing 0.05 % trypsin and 0.5 mM EDTA.

HT-29 cells (passage 106) from American Type Culture Collection (Rockville, USA) were used between passage 150 and 200. Cells were cultured and passaged in RPMI-1640 supplemented with 10% FCS and 2 mM glutamine (all from Invitrogen, Karlsruhe, Germany). Antibiotics added to the media were 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Fresh medium was given every second day and on the day before the experiments were done.

Caco-2 cells (HTB 37, passage 31) were provided by American Type Culture Collection and were used between passages 40 and 65. Cells were cultured and passaged in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1% MEM non essential amino acids and 70 µg/ml gentamycin (all from Invitrogen) in a humidified incubator at 37°C under an atmosphere of 5% CO_2.

NCOL-1 cells (passage 50) were a kind gift of Prof. Clifford W. Deveney and Dr. Michael J. Rutten, School of Medicine, Oregon Health Sciences University, Oregon, USA. Cells were used between passages 50 and 80 and were cultured and passaged in DMEM/Hepes/glutamine supplemented with 10% FCS, MEM amino acids, BME vitamin solution and 1 nM human recombinant epidermal growth factor (all from Invitrogen). Antibiotics added to the media were 200 U/ml penicillin, 100 µg/ml streptomycin, 12.5 µg/ml gentamicin and 1 µg/ml fungizone.

Apoptosis assays

Caspase-3-like activity was measured as described previously [18], based on the method of Nicholson et al. [19]. In brief, colonocytes were seeded at a density of $5x10^5$ per well onto 6-well plates (Renner, Dannstadt, Germany) 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 minutes. Cytosolic extracts were prepared by adding 750 µl of a buffer containing 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 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 centrifugated at 100.000 x g at 4°C for 30 min and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-AMC (Calbiochem, Bad Soden, Germany) at a final concentration of 20 µM. Cleavage of the caspase-3 substrate was followed by determination of emission at 460 nm after excitation at 390 nm using a fluorescence multiwell-plate reader (Fluoroskan Ascent, Labsystems, Bornheim-Hersel, Germany).

Changes in membrane permeability as another early apoptosis marker were assessed by incubating 3 x 10^4 cells on

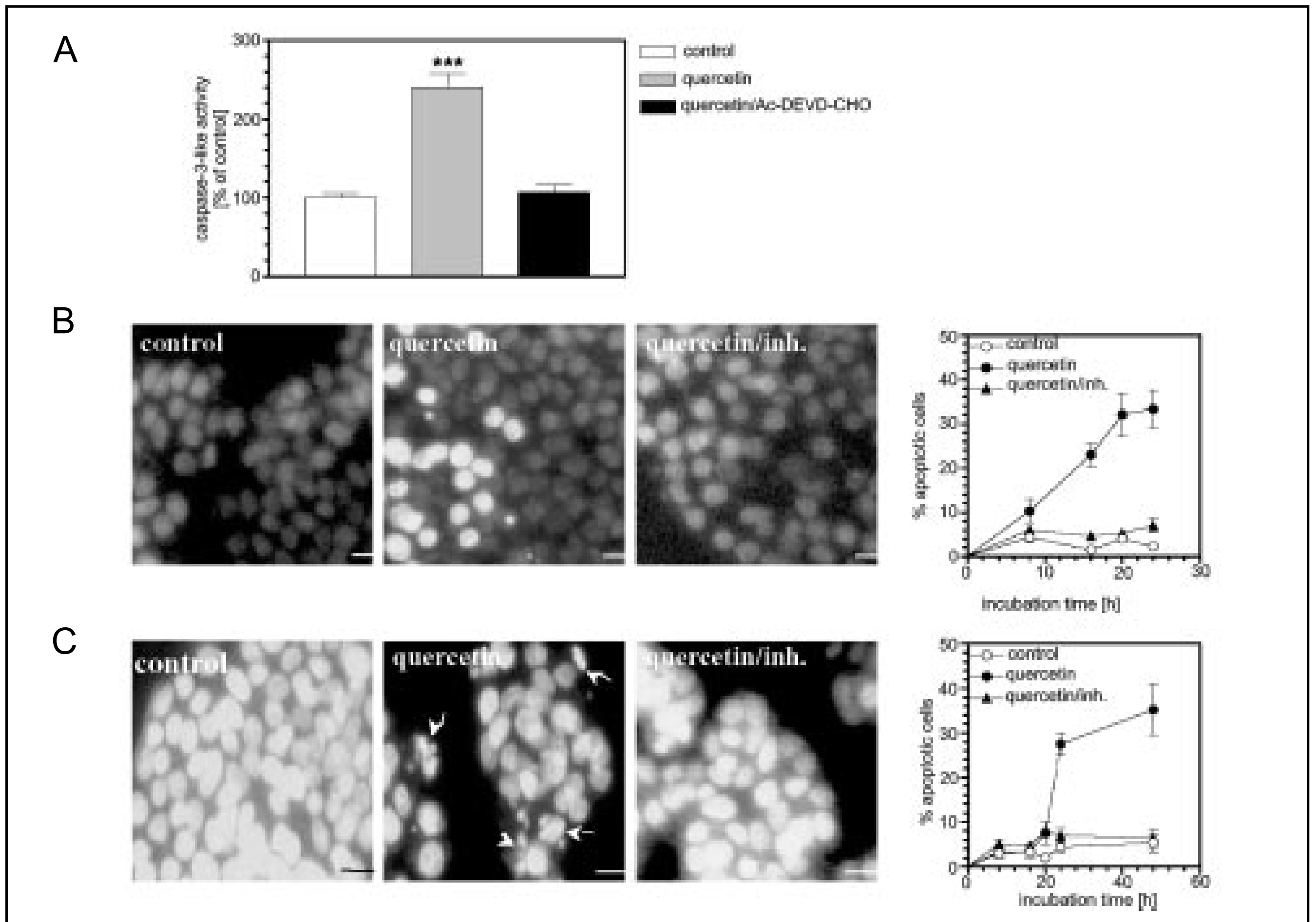


Fig. 1. Effects of quercetin on apoptosis markers in HT-29 cells. (A) Caspase-3-like activity was determined after 24 h in cells incubated with medium alone (control) or in addition with 150 µM quercetin or 150 µM quercetin and 10 µM of caspase-3 inhibitor Ac-DEVD-CHO. (B) Plasma membrane disintegration as another early apoptosis marker was assessed by Hoechst 33342 staining. The photographs show the uptake of Hoechst 33342 in control and quercetin or quercetin/Ac-DEVD-CHO (inh.) treated cells at 24 h. The percentage of apoptotic cells at different time points of incubation is shown in the right panel. Bars, 20 µm. (C) Nuclear fragmentation as a late marker of apoptosis was assessed in control cells and in cells treated with quercetin or quercetin plus Ac-DEVD-CHO (inh.). Colonocytes stained with Hoechst 33258 after 48 h incubation are shown in the photographs. Nuclear fragmentation is indicated by the arrows. In the right panel the percentage of apoptotic cells at different time points of incubation is shown. Bars, 20 µm.

24-well plates (Renner) with the test compounds or cell culture medium alone (control) for the times indicated. Cells were stained with 1 µg/ml Hoechst 33342 (Sigma, Deisenhofen, Germany) and rate of accumulation of the dye in early apoptotic cells [20] was detected using an inverted fluorescence microscope (Leica DMIL, Wetzlar, Germany) equipped with an bandpass excitation filter of 340-380 nm and a longpass emission filter of 425 nm.

Nuclear fragmentation as a late marker of apoptosis was determined by staining of DNA with Hoechst 33258 (Sigma). Cells (3 x 10^4) were incubated with the test compounds for the times indicated and thereafter washed with PBS, allowed to air-dry for 30 minutes, fixed with 2% paraformaldehyd and finally stained with 1 µg/ml Hoechst 33258. Visualization was done under the inverted fluorescence microscope.

pH_i-measurements

For pH_i-measurements 90% confluent cells were loaded with BCECF by preincubating the cells with 5 µM of the lipophilic acetoxymethyl-ester (BCECF-AM, Bioprobes, Leiden, The Netherlands) at 37°C for 45 min. Subsequently the monolayers were washed with a modified Krebs buffer containing (mM): NaCl 137, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.0, NaH_2PO_4 0.3, KH_2PO_4 0.3, glucose 10 and Hepes-Tris 10, pH 7.4. The buffers with or without substrates were changed by superfusion at the time points indicated in the graphs (left x-axis). For measuring pH_i-effects of flavonoids after incubation periods of 4 h or longer (right x-axis), cells were loaded with BCECF-AM for 45 min, washed with medium and then incubated with the flavonoids in medium. Prior to pH_i-measurements the medium was taken off from the cells and was reapplied directly after measurements.

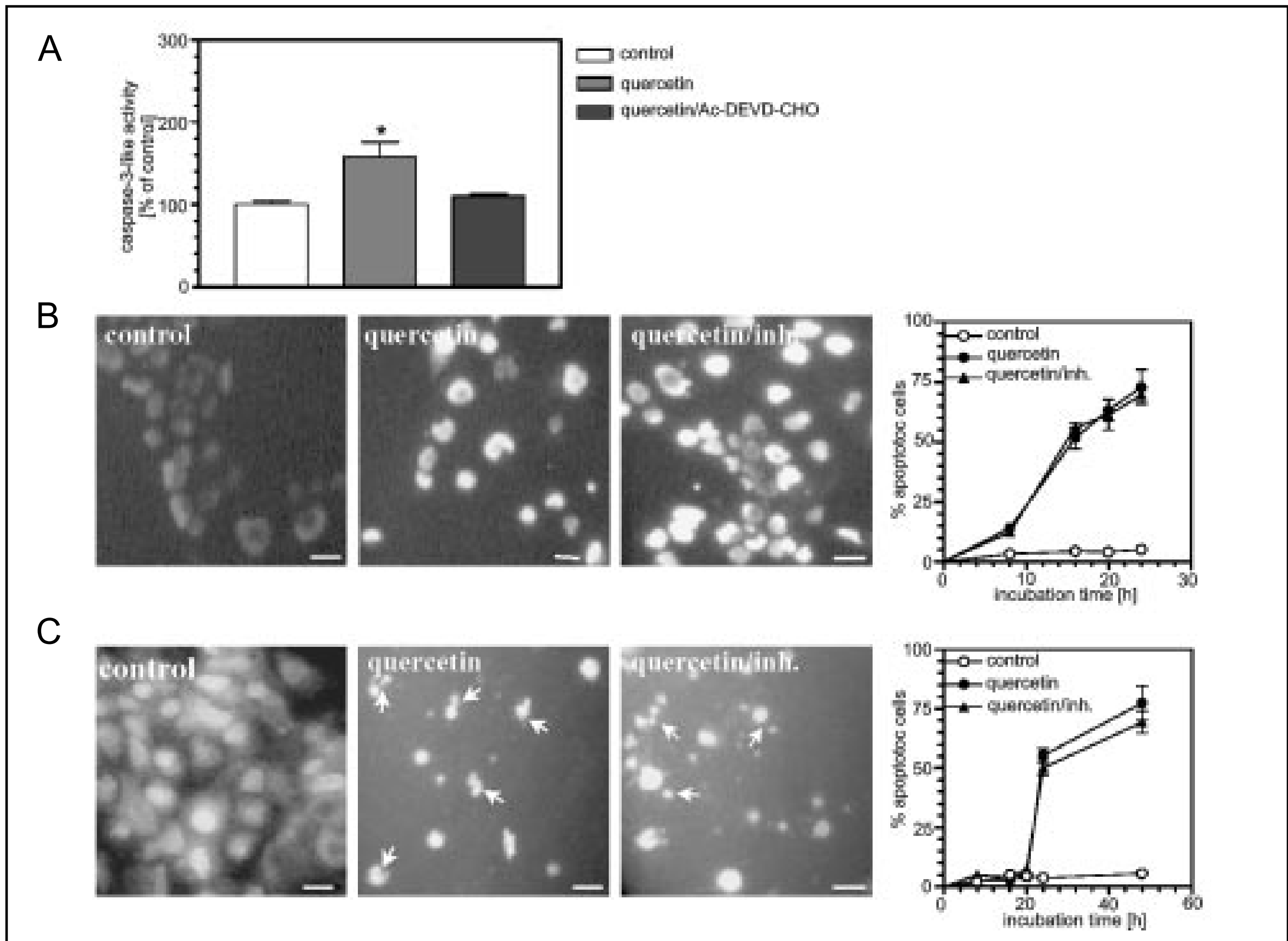


Fig. 2. Apoptosis markers as affected by quercetin in Caco-2 cells. (A) Caspase-3-like activity as measured after 24 h in control cells or cells treated with 150 µM quercetin or 150 µM quercetin and 10 µM of caspase-3 inhibitor Ac-DEVD-CHO. (B) Hoechst 33342 uptake into apoptotic cells with disintegrated plasma membrane. Uptake was visualized after 24 h in control and quercetin or quercetin/Ac-DEVD-CHO (inh.) treated cells. The percentage of cells staining positive for Hoechst 33342 uptake at different time points of incubation is shown in the right panel. Bars, 20 µm. (C) Colonocytes stained with Hoechst 33258 after 48 h incubation are shown in the photographs. Nuclear fragmentation is indicated by the arrows. The percentage of cells displaying signs of fragmentation at different time points of incubation is shown in the right panel. Bars, 20 µm.

This proceeding was chosen in order to simulate the conditions used for apoptosis measurements and moreover, to neglect influences on pH_i-measurements by BCECF diffusing out of the cells during these long periods. Intracellular H^+-activity was determined by measuring the intensity of emission at 538 nm after excitation of the fluorophore at 444 nm (isosbestic point) and 490 nm (pH-sensitive wavelength), respectively, using a microtiter plate reader (Fluoroskan Ascent, Labsystems, Bornheim-Hersel, Germany). The 444/490 fluorescence ratio was converted to pH_i from a calibration curve generated by estimation of the fluorescence ratio in buffers of different pH [21].

Calculations and statistics

All calculations were performed by using Prism 3.01 (Graph PAD, Los Angeles, CA). For each variable 4-8 independent experiments were carried out. Data are given as the mean ± S.E.M. Variance analysis between groups was performed by One-way ANOVA and significance of differences between control and treated cells were determined by a Dunnett´s test.

Results

Effects of six flavonoids on apoptosis parameters in the colonic cell lines HT-29, Caco-2 and NCOL-1

The effects of quercetin, flavone, 3-OH-flavone, 5-OH-flavone, 7-OH-flavone and 7,8-$(OH)_2$-flavone on

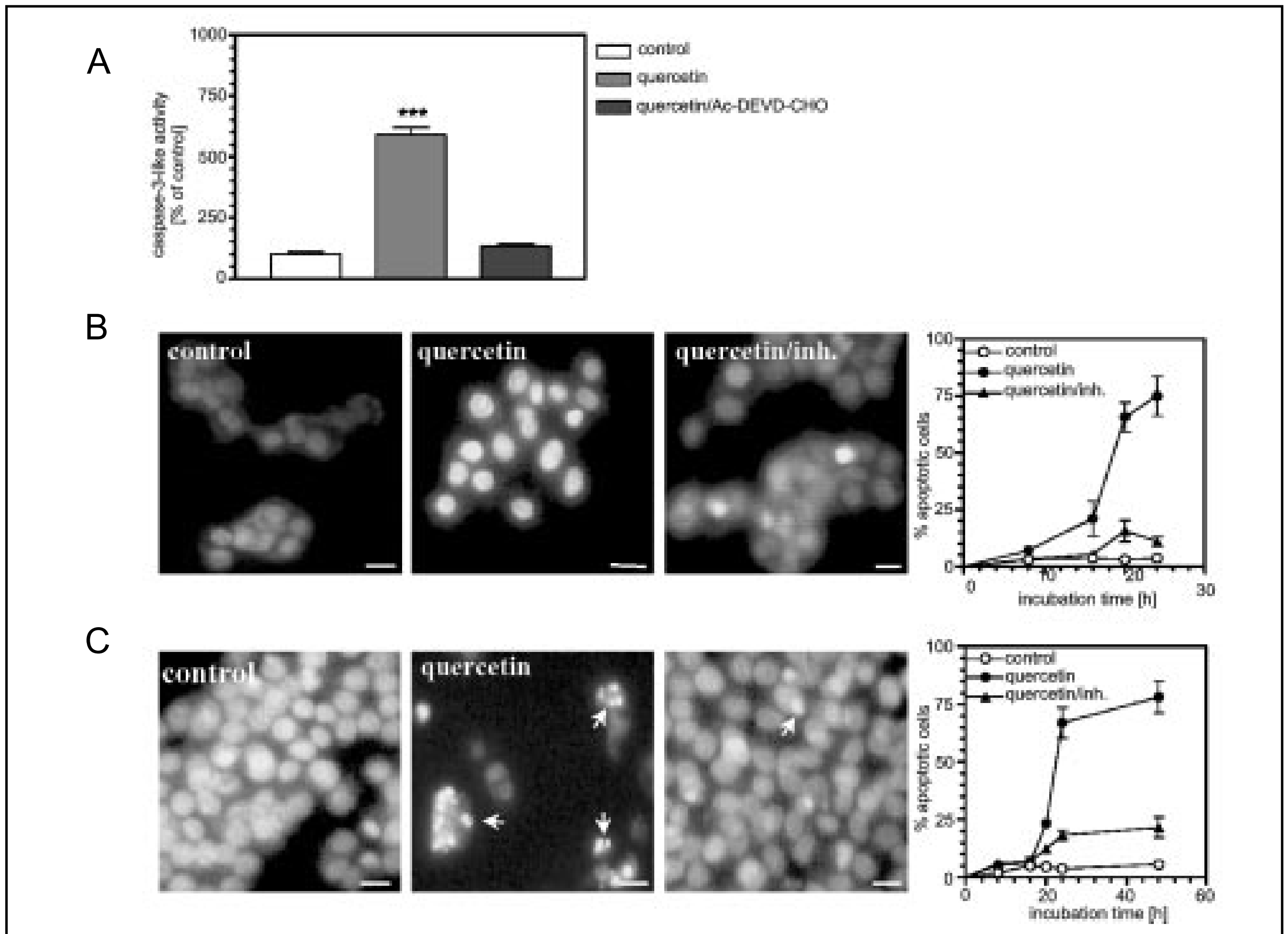


Fig. 3. Quercetin effects on apoptosis in NCOL-1 cells. (A) Caspase-3-like activity at 24 h incubation in control cells or cells treated with 150 µM quercetin or 150 µM quercetin plus 10 µM Ac-DEVD-CHO. *** $P < 0.001$ versus the control. (B) Hoechst 33342 uptake is shown in NCOL-1 incubated with quercetin or quercetin/Ac-DEVD-CHO (inh.) or medium alone (control). The percentage of Hoechst 33342 stained cells is shown in the right panel. Bars, 20 µm. (C) Hoechst 33258 staining after 48 h incubation revealed nuclear fragmentation (arrows). The percentage of cells with nuclear fragmentation at different time points of incubation is shown in the right panel. Bars, 20 µm.

caspase-3-like activity and plasma membrane disintegration as early apoptosis markers and on nuclear fragmentation as a late apoptosis marker were assessed after exposure of cells for 24 or 48 hours, respectively.

Quercetin at a concentration of 150 µM led to a 2.5 fold stimulation of caspase-3-like activity in HT-29 cells at 24 h ($P < 0.001$), that was completely blocked by the caspase-3 inhibitor Ac-DEVD-CHO (Fig. 1A). Ac-DEVD-CHO also prevented in quercetin-treated HT-29 cells the uptake of Hoechst 33342 that serves as the ultimate marker for early apoptosis (Fig. 1B). Finally, nuclear fragmentation as induced by quercetin in HT-29 cells was completely inhibited by the caspase-3 inhibitor (Fig. 1C) indicating that caspase-3 activity is also crucial for the execution of apoptosis in these cells.

In Caco-2 cells, caspase-3-like activity was only slightly increased by quercetin ($P < 0.05$) and this activation was again completely blocked by Ac-DEVD-CHO (Fig. 2A). However, Ac-DEVD-CHO was without effects on Hoechst 33342 uptake and nuclear fragmentation that was caused by quercetin in about 75% of the cells (Figs. 2B and 2C). These data suggest that apoptosis in Caco-2 cells can occur irrespective of caspase-3 activation.

In NCOL-1 colonocytes, caspase-3-like activity was increased 6-fold by quercetin ($P < 0.001$) (Fig. 3A) and this activation was associated with plasma membrane disintegration and nuclear fragmentation in around 75% of the cells (Figs. 3B and 3C). Similar to HT-29 cells, Ac-DEVD-CHO blocked caspase-3-like activity

Table 1. Effects of flavonoids on apoptosis markers in HT-29, Caco-2 and NCOL-1 cells. Caspase-3-like activity was determined by following the cleavage of Ac-DEVD-AMC in the cytosol of cells incubated for 24 h in the absence (control) or presence of 150 µM of the respective flavonoid. The percentage of apoptotic cells after 24 h of incubation with the flavonoids was determined by Hoechst 33342 staining. Cells in the late phase of apoptosis (after 48 h of exposure) show signs of nuclear fragmentation as visualized by Hoechst 33258 staining.

flavonoid	caspase-3-like activity at 24 h [% of control]	% apoptotic cells at 24 h	% apoptotic cells at 48 h
	HT-29		
quercetin	240.4 ± 17.3	33.3 ± 4.2	35.2 ± 5.7
flavone	662.0 ± 113.7	45.3 ± 4.4	44.4 ± 4.2
3-OH-flavone	137.2 ± 17.9	16.6 ± 5.2	15.5 ± 3.7
5-OH-flavone	169.0 ± 30.2	22.3 ± 5.0	20.9 ± 4.8
7-OH-flavone	165.3 ± 26.5	25.4 ± 5.2	28.5 ± 5.9
7,8-(OH)$_2$-flavone	229.0 ± 37.3	14.3 ± 3.6	12.3 ± 5.1
	Caco-2		
quercetin	158.2 ± 17.3	72.9 ± 7.2	77.4 ± 7.3
flavone	132.3 ± 14.9	73.6 ± 5.4	78.1 ± 5.9
3-OH-flavone	219.5 ± 15.0	23.2 ± 6.1	19.7 ± 6.3
5-OH-flavone	155.9 ± 22.1	35.3 ± 4.7	29.9 ± 8.2
7-OH-flavone	180.5 ± 30.7	7.3 ± 3.4	6.5 ± 3.2
7,8-(OH)$_2$-flavone	112.3 ± 17.6	14.7 ± 3.5	12.3 ± 4.0
	NCOL-1		
quercetin	530.5 ± 30.3	74.5 ± 5.5	78.2 ± 6.9
flavone	113.3 ± 5.4	15.1 ± 1.4	14.7 ± 3.3
3-OH-flavone	275.7 ± 25.9	26.9 ± 6.3	25.4 ± 7.1
5-OH-flavone	134.5 ± 15.2	15.1 ± 3.6	13.7 ± 7.4
7-OH-flavone	137.5 ± 17.5	15.4 ± 4.5	15.5 ± 5.3
7,8-(OH)$_2$-flavone	312.0 ± 34.6	30.2 ± 5.0	27.0 ± 6.1

(Fig. 3A) as well as other early and late apoptosis processes (Figs. 3B and C).

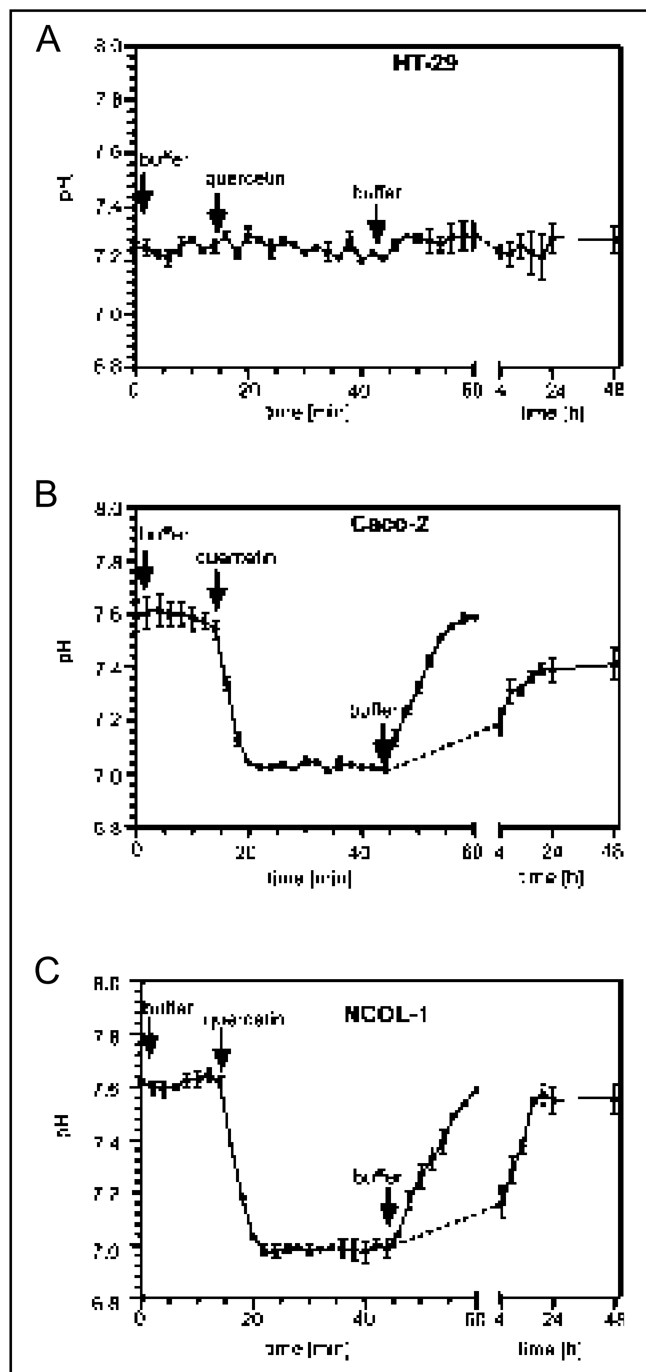
The effects of the six flavonoids tested on the parameters of early and late apoptosis are summarized in Table 1. It becomes evident, that elevated caspase-3-like activity in HT-29 and NCOL-1 cells is always asscociated with increased plasma membrane disintegration and enhanced nuclear fragmentation, whereas in Caco-2 cells it is not (Table 1). Among the flavonoids tested, in HT-29 and Caco-2 cells the strongest apoptosis inducers found were quercetin and flavone whereas in the NCOL-1 cell line quercetin revealed to be by far the most potent pro-apoptotic compound (Table 1).

Association of the flavonoids´ effects on pH_i with changes in early and late apoptosis markers

Quercetin did not alter pH_i in HT-29 cells, neither after exposure for 24 h nor at 48 h (Fig. 4A), although plasma membrane disintegration and nuclear fragmentation were clearly visible (Figs. 1B and 1C). Exposure of Caco-2 and NCOL-1 cells to quercetin was associated with a rapid decline in pH_i that slowly recovered over 48 h (Figs. 4B and 4C). The effects of all six flavonoids on pH_i in the three cell lines either in the initial phase of incubation (10 min) or after 24 h or 48 h are summarized in Table 2. To assess whether the flavonoids´ effects on pH_i are related to early apoptosis events a correlation was made between initial pH_i effects (at 10 min) of incubation (Fig. 5, left panels) or at 24 h (Fig. 5, mid panels) with uptake of Hoechst 33342. The association bewteen pH_i changes and late apoptosis as given by nuclear fragmentation assessed at 48 h is shown in Fig. 5 (right panels).

In HT-29 cells, there was essentially no correlation between the effects on pH_i and early or late apoptosis markers (Fig. 5A). In general, flavonoids that caused the most pronounced decrease in pH_i proved to be the least effective apoptosis inducers (Fig. 5A). In Caco-2 cells, more pronounced pH_i changes were observed and the

Fig. 4. Effects of quercetin on pH_i in HT-29 (A), Caco-2 (B) and NCOL-1 (C) colonocytes. For measurements of immediate effects on pH_i, cells were loaded with BCECF-AM, washed free of medium with buffer pH 7.4 and then pH_i was recorded on-line over 15 min in the same buffer. Subsequently, cells were superfused with 150 µM quercetin at pH 7.4 for 30 min before the cells were allowed to recover pH_i by superfusion with buffer pH 7.4 for 15 min. For determination of long-term effects on pH_i cells were loaded with BCECF and then incubated with 150 µM quercetin or with medium alone (control) for 48 h. At the time points indicated on the right x-axis the medium was taken off, pH_i was determined and then the medium with or without quercetin was reapplied. The dotted line represents the pH_i characteristics if quercetin was not washed out by buffer pH 7.4.



acidification could indeed promote apoptosis execution induced by quercetin and flavone (Fig. 5B). However, acidification alone seems not efficient to induce apoptosis since 7,8-$(OH)_2$-flavone caused a large pH_i decline, especially at 10 min of incubation, but had only modest effects on the apoptosis markers (Fig. 5B). In NCOL-1 cells initial acidification at 10 min correlates well with the uptake of Hoechst 33342 (r^2=0.976) but pH_i at later time points was not found to be related to apoptosis parameters (Fig. 5C).

Prevention of acidification does not prevent the excecution of apoptosis

Since the sustained low pH_i as observed in Caco-2 cells by treatment with quercetin as well as the initial pH_i decline seen in NCOL-1 cells by quercetin could provide a permissive milieu for apoptosis initiation and excecution (Figs. 5B and 5C), we tested whether the application of the base imidazole affects the pH_i response to quercetin and in turn alters the early and late apoptosis markers response. Imidazole completely prevented the pH_i decline caused by quercetin in Caco-2 cells (Fig. 6A) but did not influence the effects of quercetin on plasma membrane disintegration (Fig. 6B) or nuclear fragmentation (Fig. 6C). In NCOL-1 cells, in which apoptosis is obviously dependent on caspase-3 activity, imidazole again completely blocked the pH_i decline caused by quercetin (Fig. 7A) but did not display any effects on apoptosis parameters (Figs. 7B-7D). Imidazole alone did also not affect the apoptosis parameters in Caco-2 and NCOL-1 cells (data not shown).

Discussion

Regulation of apoptosis is a central theme in cancer development as well as in cancer therapy since apoptosis is impaired during carcinogenesis [22, 23]. One of the major goals in cancer therapy, therefore, is to restore the sensitivity of transformed cells towards apoptotic signals

Table 2. Effects of flavonoids on pH_i in HT-29, Caco-2 and NCOL-1 cells. Intracellular pH in the colonic cell lines was determined using the pH-sensitive dye BCECF. For measurements of immediate effects on pH_i, cells were washed free of medium with buffer pH 7.4 and pH_i was recorded over 15 min in the same buffer. Subsequently, cells were superfused with 150 µM of the respective flavonoid at pH 7.4 for 30 min and the initial decline in pH_i (ΔpH_i/10 min) was calculated. For determination of the effects on pH_i at 24 h and 48 h cells were loaded with BCECF and then incubated for 24 h or 48 h with 150 µM of the respective flavonoid or with medium alone (control). ΔpH_i/24 h and ΔpH_i/48 h in flavonoid treated cells was determined as described in the Methods section and is expressed versus the control.

flavonoid	ΔpH_i/10 min	ΔpH_i/24 h	ΔpH_i/48 h
	HT-29		
quercetin	0.01 ± 0.04	0.04 ± 0.05	0.03 ± 0.05
flavone	0.07 ± 0.01	0.05 ± 0.05	0.01 ± 0.02
3-OH-flavone	0.01 ± 0.03	0.45 ± 0.03	0.63 ± 0.03
5-OH-flavone	0.01 ± 0.03	0.50 ± 0.10	0.67 ± 0.10
7-OH-flavone	0.01 ± 0.04	0.41 ± 0.03	0.46 ± 0.04
7,8-(OH)$_2$-flavone	0.23 ± 0.06	0.20 ± 0.12	0.22 ± 0.14
	Caco-2		
quercetin	0.61 ± 0.03	0.20 ± 0.05	0.15 ± 0.06
flavone	0.38 ± 0.04	0.32 ± 0.06	0.30 ± 0.04
3-OH-flavone	0.19 ± 0.03	0.13 ± 0.03	0.10 ± 0.03
6-OH-flavone	0.17 ± 0.02	0.12 ± 0.04	0.17 ± 0.03
7-OH-flavone	0.18 ± 0.04	0.11 ± 0.05	0.07 ± 0.01
7,8-(OH)$_2$-flavone	0.65 ± 0.02	0.22 ± 0.10	0.15 ± 0.02
	NCOL-1		
quercetin	0.53 ± 0.03	0.07 ± 0.05	0.06 ± 0.06
flavone	0.12 ± 0.01	0.01 ± 0.07	0.07 ± 0.03
3-OH-flavone	0.27 ± 0.02	0.25 ± 0.04	0.26 ± 0.04
6-OH-flavone	0.10 ± 0.01	0.10 ± 0.04	0.14 ± 0.05
7-OH-flavone	0.15 ± 0.01	0.16 ± 0.05	0.24 ± 0.06
7,8-(OH)$_2$-flavone	0.24 ± 0.01	0.27 ± 0.07	0.41 ± 0.06

and the execution of apoptotic cell death [24, 25]. Consequently, it is of special importance to define the factors that contribute to the control of apoptosis. Intracellular acidification was suggested to play a pivotal role in the process of apoptosis, either by directly triggering the apoptosis pathway [7] or by providing a permissive milieu for the enzymatic processes that control programmed cell death [8]. Especially the activation of caspases is regarded as a pH-dependent process [26]. In a cytotoxic T-lymphocyte cell line it was observed that intracellular acidification always occured during apoptosis, but maintainig the pH_i above 7.2 did not prevent apoptosis [10]. It is questionable therefore whether acidification of the cytosol is in general required for enabling apoptosis to occur. Moreover, intracellular acidification might affect different apoptosis events differently and this again may depend on the cell type. This is emphasized by results obtained in a leukemia cell line [8] suggesting that acidification is an effector mechanism for drug induced apoptosis, whereas in another leukemia cell line acidification was demonstrated to affect activity of the caspases but failed to alter the execution phase of apoptosis [9].

So far, the interdependence of cytosolic acidification and apoptosis has only been assessed in transformed blood cell lines. In the present study, we investigated the influence of intracellular acidification on apoptosis in the human colon epithelial cell lines HT-29, Caco-2 and NCOL-1. As effector tools, six flavonoids were applied to the cells that differ only in the position and number of hydroxyl groups attached to the phenylchromane ring but that displayed markedly different effects in induction of apoptosis in the three cell lines. In HT-29 human colon carcinoma cells, quercetin and flavone proved to be the strongest inducers of apoptosis as indicated by strong caspase-3 activation, plasma membrane disintegration and finally nuclear fragmentation (Table 1). However, both flavonoids failed to significantly affect pH_i in all phases of analysis (Table 2) indicating that acidification is not a prerequisite or a modulator of early or late apoptosis

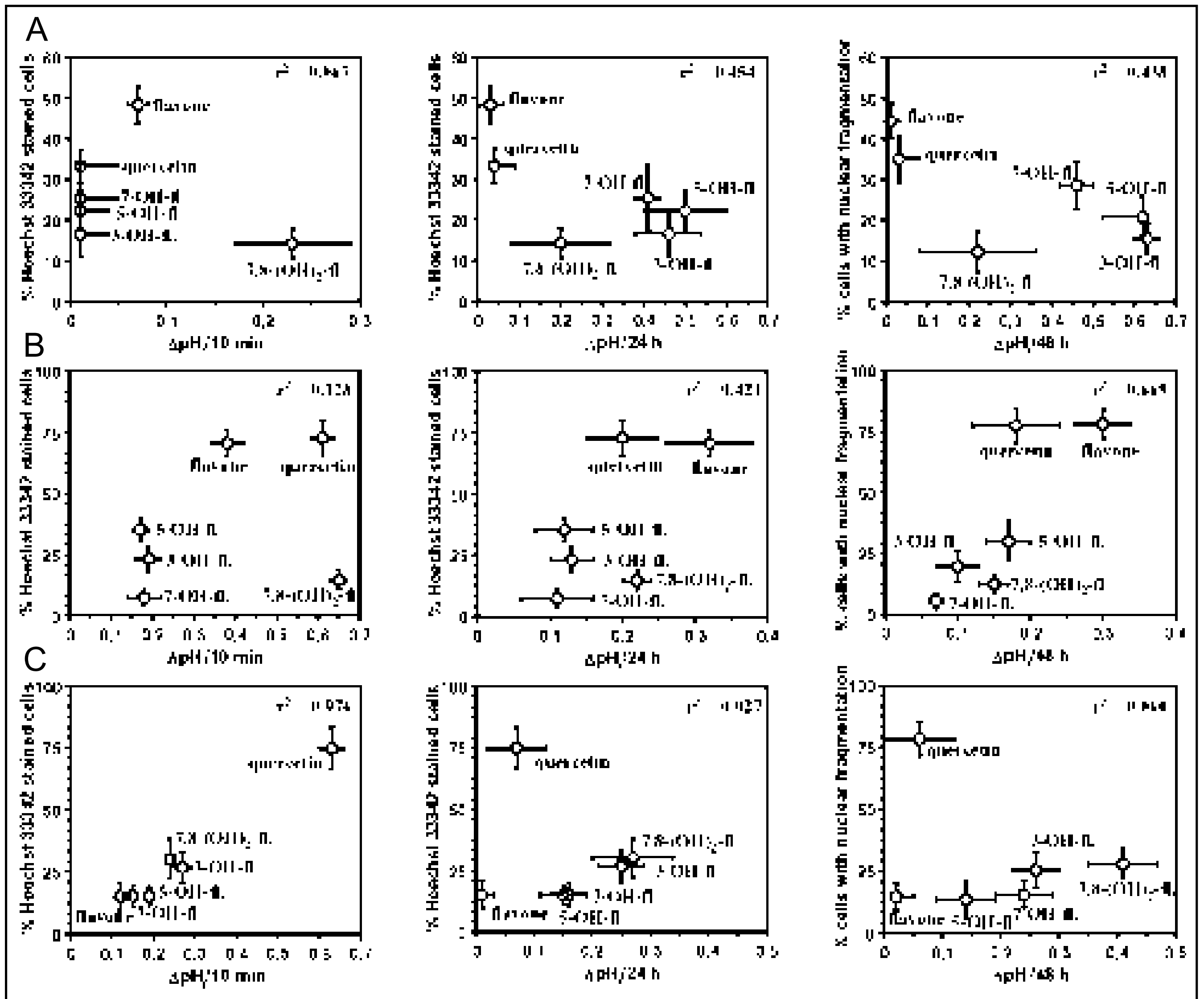


Fig. 5. Correlation of acidification rates and parameters of early and late apoptosis phases in HT-29 cells (A), Caco-2 cells (B) and NCOL-1 cells (C). Left and central panels show the effects of the flavonoids on Hoechst 33342 uptake at 24 h as a function of pH_i changes induced within the first 10 min (left side panel) or after 24 h (mid panel) under flavonoid action. The right panels show the percentage of cells displaying signs of the late apoptosis in relation to pH_i changes when cells were exposed to the flavonoids for 48 h.

parameters in HT-29 cells. On the contrary, 7,8-$(OH)_2$-flavone decreased pH_i initially and over 24 h by around 0.2 pH units but did not cause apoptosis, suggesting that an intracellular acidification in HT-29 cells does not induce apoptosis per se (Tables 1 and 2). This is also evidenced by the findings with 3-OH-, 5-OH- and 7-OH-flavone, which all reduced pH_i in HT-29 cells after 24 h strongly but were less effective in activation of caspase-3 and the other apoptosis parameters measured than were quercetin or flavone (Tables 1 and 2).

In Caco-2 human colon carcinoma cells, quercetin and flavone again displayed the strongest apoptosis inducing activities (Fig. 2). Although both flavonoids decreased pH_i initially and showed a sustained effect even after 24 h and 48 h (Table 2, Fig. 5B), intracellular acidification per se cannot contribute to the apoptotic effects of the flavonoids in this cell line for the following reasons. Firstly, apoptosis execution occurs caspase-3 independent in Caco-2 cells (Fig. 2) and consequently providing a permissive milieu for the activation of caspases

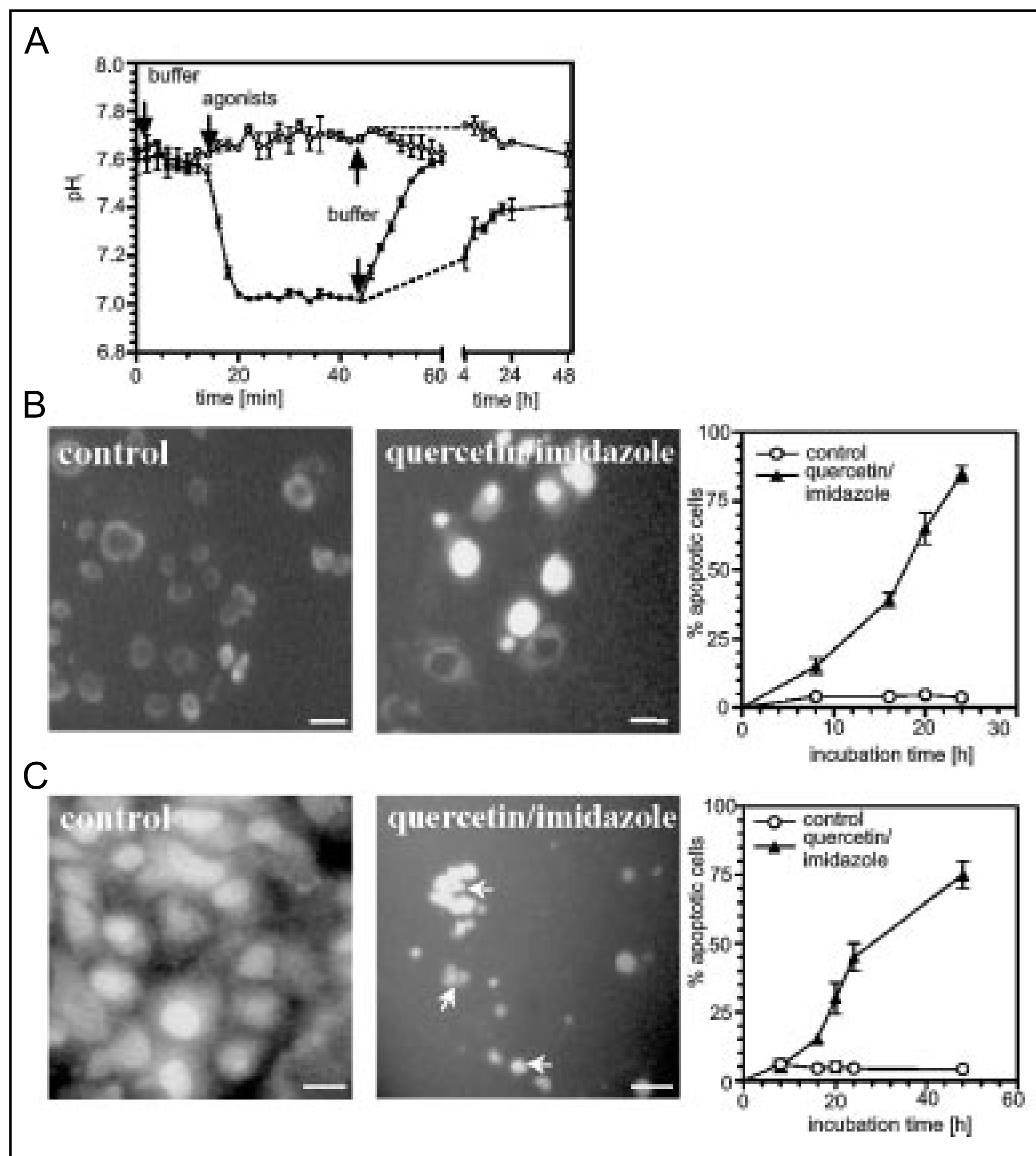


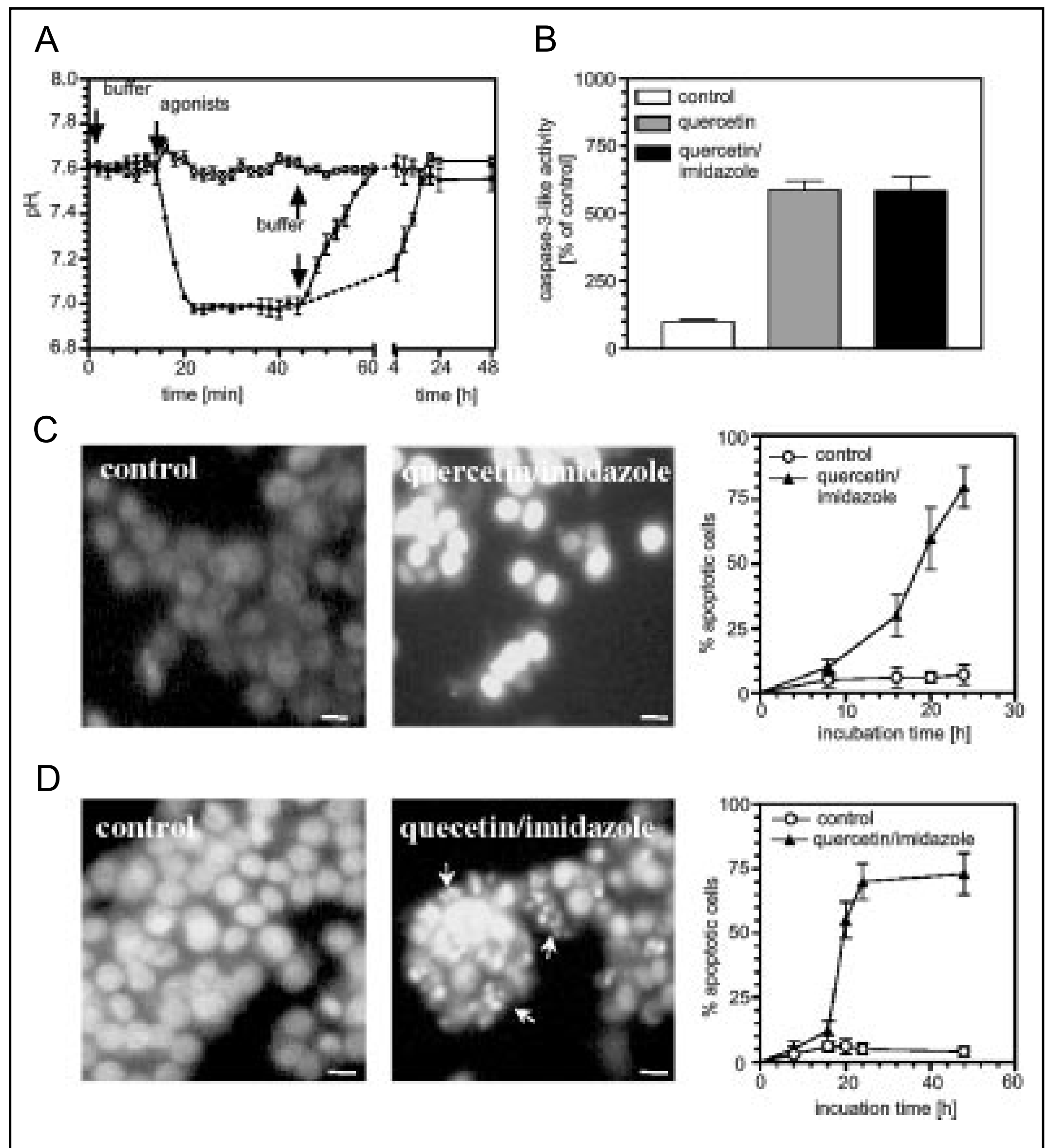
Fig. 6. Effects of imidazole on pH_i and apoptosis by quercetin treatment of Caco-2 cells. (A) pH_i was determined in Caco-2 cells perfused for 15 min with buffer pH 7.4. Thereafter 150 µM quercetin (●) or 150 µM quercetin plus 10 mM of the base imidazole (○) were applied before the agonists were washed out by buffer pH 7.4. The dotted lines represent the pH_i characteristics if the agonists were not washed out by buffer pH 7.4. (B) Plasma membrane disintegration in the absence (control) or presence of 150 µM quercetin/10 mM imidazole was assessed by the uptake of Hoechst 33342 at the time points indicated in the right panel. Uptake of the dye into apoptotic cell at 24 h is shown in the photographs. Bars, 20 µm. (C) The percentage of Caco-2 cells showing nuclear fragmentation at various time points of incubation is shown in the right panel. The photographs show cells with DNA stained by Hoechst 33258 at 48 h. Bars, 20 µm.

by a low pH_i has no impact on apoptosis. Secondly, the base imidazole was without any effect on apoptosis induced by quercetin, although the pH_i decline caused by this flavonoid was completely prevented (Fig. 6). Thirdly, the effects of 7,8-$(OH)_2$-flavone on pH_i in Caco-2 cells were almost identical to those caused by quercetin, but 7,8-$(OH)_2$-flavone was a very weak apoptosis inducer in this cell line (Fig. 5).

In the preneoplastic cell line NCOL-1, in which apoptosis occurs in a caspase-3 dependent manner, quercetin proved to be by far the strongest apoptosis inducing compound (Table 1). Its effects on apoptosis were associated with a rapid pH_i decline of 0.6 units (Table 2). Although it could be suggested from this, that acidification is a trigger of apoptosis in NCOL-1 cells, preventing the decline in pH_i by imidazole had no effect on the apoptosis execution induced by quercetin (Fig. 7), indicating that pH_i also in NCOL-1 cells does not affect apoptosis.

In summary, our studies show that early and late events of the apoptosis pathway are not affected by changes of pH_i in human colonic epithelial cells. Moreover,

Fig. 7. Imidazole prevents the pH_i decline by quercetin but does not prevent apoptosis in NCOL-1 cells. (A) pH_i was recorded on-line in the presence of agonists (150 µM quercetin, 150 µM quercetin plus 10 mM imidazole) at pH 7.4 as described in Fig. 6B. (B) Caspase-3-like activity in NCOL-1 cells treated for 24 h with medium alone (control) or in addition with 150 µM quercetin or 150 µM quercetin/ 10 mM imidazole. (C) Hoechst 33342 uptake at 24 h is shown in the left panels, the percentage of cells that have taken up the dye at various time points is shown in the right panel. (D) Nuclear fragmentation as affected by quercetin/imidazole at 48 h is shown in the photographs, whereas the right panel shows the percentage of cells with nuclear fragmentations at different time points.



a cytosolic acidification alone is not sufficient to initiate apoptosis in transformed or non-transformed colonic cell lines.

Abbreviations

Ac-DEVD-AMC (acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methyl-coumarine); Ac-DEVD-CHO (acetyl-aspartyl-glutamyl-valyl-aspartyl-aldehyde); BCECF-AM, 2´, 7´-*bis* (2-carboxyethyl-5-(6)-carboxyfluorescein acetoxy-methylester); DMEM (Dulbecco´s modified Eagle medium); Hepes; N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic-acid; Mes (2-(N-morpholino) ethane-sulfonic-acid); pH_i (intracellular pH).

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