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Apple Polyphenols Affect Protein Kinase C Activity and the Onset of Apoptosis in Human Colon Carcinoma Cells

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Polyphenol-rich apple extracts have been reported to suppress human colon cancer cell growth in vitro. The protein kinase C (PKC) is among the signaling elements known to play an important role in colon carcinogenesis. In the present study, we investigated whether apple polyphenols affect PKC activity and induce apoptosis in the human colon carcinoma cell line HT29. A polyphenol-rich apple juice extract (AE02) was shown to inhibit cytosolic PKC activity in a cell-free system. In contrast, incubation of HT29 cells for 1 or 3 h with AE02 up to 2 mg/mL did not affect the cytosolic PKC activity. After prolonged incubation (24 h), cytosolic PKC activity was modulated, albeit a u-shaped curve of effectiveness was observed, with an initial inhibitory effect followed by the recurrence and even induction of enzyme activity. Concomitantly, in the cytosol, a significant decrease of the protein levels of PKCα, PKCβ_{II}, and PKCγ together with a significant increase of a proapoptotic PKCδ fragment was observed. However, the effects on the protein levels of these PKC isoforms in the cytosol were not associated with translocation between the different cellular compartments but might instead result from the onset of apoptosis. Indeed, the treatment with AE02 was shown to induce apoptosis by the activation of caspase-3, DNA fragmentation, and cleavage of poly(ADP ribose) polymerase. So far, identified and available constituents of the apple extract did not contribute substantially to the observed effects on PKC and apoptosis induction. In summary, apple polyphenols were found to inhibit PKC activity in a cell-free system. However, our results indicate that within intact cells PKC does not represent the primary target of apple polyphenols but appears to be affected in the course of apoptosis induction.

KEYWORDS: Apple juice extract; phloretin; phloridzin; quercetin; caspase-3; DNA fragmentation; PARP cleavage

INTRODUCTION

Polyphenol-rich apple extracts have been reported to possess bioactive properties, suppressing human colon cancer cell growth in vitro (*1*−*4*). In a rat colon carcinogenesis model, a consumer relevant apple juice has been shown to decrease DNA damage, hyperproliferation, and aberrant crypt foci development induced by dimethylhydrazine treatment (*5*), indicating that apple polyphenols might be of interest in terms of chemoprevention. With respect to the underlying mechanism of action, several cellular effects of apple polyphenols have been previously reported. Polyphenol-rich apple extracts and selected apple polyphenols were found to reduce oxidative cell damage in human colon carcinoma cell lines in vitro (*6*). In previous studies, we have shown that apple polyphenols effectively inhibit the protein tyrosine kinase (PTK) activity of the epidermal growth factor receptor (EGFR), down regulating the activity of the subsequent mitogen-activated kinase (MAPK) cascade (*3*). Expression of the protein-tyrosine phosphatase DEP-1, a candidate tumor suppressor for colon carcinogenesis, is elevated upon treatment of colon epithelial cells with apple polyphenols (*7*). Furthermore, key elements of the Wnt pathway were found to be modulated (*8*−*10*). These data show that apple polyphenols modulate signaling cascades, which are crucial for the regulation of cell growth.

The protein kinase C (PKC) is among the signaling elements known to play an important role, for example, in colon cancer (*11*−*14*). Little is known so far about the impact of apple polyphenols on this family of serine/threonine kinases. The PKC family consists of 11 isoenzymes, which are divided into three classes: classical cPKCs (α, β, and γ), novel nPKCs (δ, ϵ, η, and θ), and atypical aPKCs (μ, ζ, and ι/λ), depending on homology, substrate specificity, and sensitivity to specific modulators (*14*, *15*). Several PKC isoenzymes are known to

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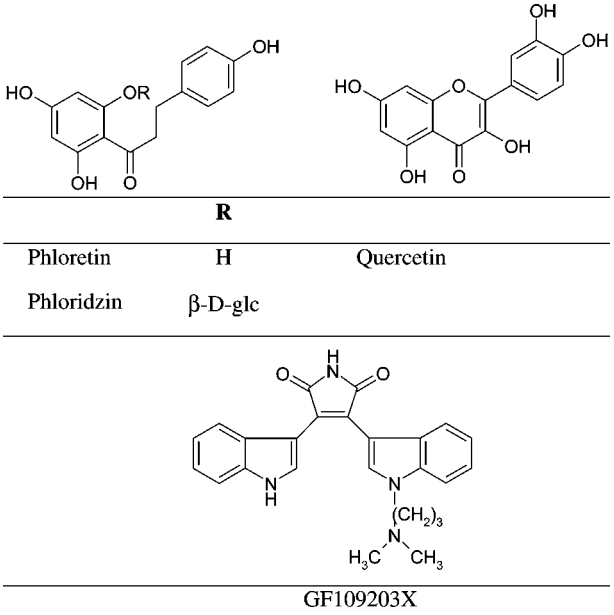

Figure 1. Structures of the dihydrochalcones PHL and PHD, the flavonol QUE, and the PKC inhibitor GF109203X.

play an important role in apoptotic processes. PKCα, β_{II}, ϵ, ζ, and ι/λ have been reported to suppress the onset of apoptosis (*14*, *16*, *17*). In contrast, PKCδ is involved in apoptosis progression, affecting Bax activation, cytochrome *c* release, and caspase activation (*17*, *18*). Mediated by caspase-3 cleavage, a catalytically active fragment (38 kDa) of PKCδ is generated (*18*−*20*).

In the present study, we investigated whether apple polyphenols affect PKC activity and induce apoptosis in human colon carcinoma cells (HT29) in vitro. For comparison, the apple characteristic dihydrochalcone phloridzin (PHD), the respective aglycon phloretin (PHL), and the flavonol quercetin (QUE) were included in the testing (**Figure 1**).

MATERIALS AND METHODS

Chemicals. Chlorogenic acid, (−)-epicatechin (EC), procyanidins B1 and B2, and the QUE glycosides QUE-3-rhamnoside, QUE-3-galactoside, QUE-3-glucoside, and QUE-3-rutinoside were purchased from Extrasynthèse (Genay, France). Caffeic acid, PHL, and PHD were obtained from Roth (Karlsruhe, Germany). QUE and the specific PKC inhibitor 3-(N-[dimethylamino]propyl-3-indolyl)-4-(3-indolyl)maleimide (GF109203X) were received from Sigma (Taufkirchen, Germany). 4-Coumaroylquinic acid was a kind gift from Prof. Becker (Saarland University, Saarbruecken, Germany). Polyphenol-rich apple juice extract (AE02) was produced as reported previously (*1*, *3*, *6*). For all assays, the compound solutions were freshly prepared directly prior to the experiment, without the use of stored stock solutions. All compounds and mixtures were dissolved in dimethyl sulfoxide (DMSO) with a final concentration in the different test systems of maximum 1%.

Cell Culture. The human colon carcinoma cell line HT29 was cultivated in Dulbecco's modified Eagle's medium (DMEM) (with 4500 mg/L glucose, without sodium pyruvate), and the cell culture medium was supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. DMEM and the supplements were obtained from Invitrogen Life Technologies (Karlsruhe, Germany).

For the determination of cellular PKC activity, caspase-3 activity, DNA fragmentation and for Western blot analysis, 1.5×10^6 cells were seeded per Petri dish (56.7 cm^2) and allowed to grow for 48 h. Thereafter, the serum content of the culture medium was reduced to 1% for 24 h. The cells were incubated for 24 h, serum-free, with test compounds dissolved in DMSO (final DMSO concentration of 1%). Alternatively, apoptosis parameters were determined in nearly confluent cell cultures in six well plates treated with apple polyphenols (AE02) in the presence of 10% fetal calf serum for 24, 48, or 72 h.

Inhibition of Isolated PKC Activity. An aliquot of 5×10^6 cells was seeded per Petri dish (145 cm^2) and grown for 48 h. Before harvesting, the medium was removed and cells were washed twice with 5 mL of phosphate-buffered saline (PBS). Harvesting and lysate preparation were performed at 4 °C. HT29 cells were scraped in 0.3 mL of "swelling" buffer [30 mM Tris/HCl, pH 7.5, 10 mM potassium chloride, 5 mM magnesium acetate, and 5 mM EGTA; 0.31% (v/v) β-mercaptoethanol and 0.5% (v/v) protease inhibitor mix, 200 μM pepstatin and 200 μM leupeptin in 100 mM phenylmethylsulfonyl fluoride (PMSF), dissolved in isopropanol were freshly added to the "swelling" buffer]. Thereafter, the lysate was homogenized thoroughly. The cell lysate was shifted with the same volume of glycerol-containing "swelling" buffer (25% v/v) and subsequently centrifuged for 3 min (1000*g*, 4 °C). The cytosol-containing supernatant was obtained by a second centrifugation step for 30 min (100000*g*, 4 °C). The colorimetric procedure of Bradford (*21*) was applied to determine the content of protein using bovine serum albumin as a standard. The kinase activity was assayed by measuring the transfer of [γ-^{32}P]ATP (0.2 μCi) on a PKC substrate peptide using the PKC Biotrak Enzyme Assay (RPN 77, Amersham Biosciences, Piscataway, United States). Calcium buffer, PKC activators, PKC substrate peptide, stabilizers, [γ-^{32}P]ATP-solution, cell lysate, and test compounds were incubated for 15 min at 37 °C according to the manufacturer's protocol. Stop reagent (10 μL) was added, and 35 μL of the kinase reaction mix was spotted onto P81 phosphocellulose paper and air-dried. Filters were washed three times with 0.75% phosphoric acid and once with acetone and were allowed to air dry. The phosphorylated substrate was determined by liquid scintillation counting of the filters. The kinase activity was calculated as pmol ATP-turnover per minute and mg protein and plotted as test over control (%).

Western Blot Analysis. Cells were scraped two times in 0.2 mL of "swelling" buffer [30 mM Tris/HCl, pH 7.5, 10 mM potassium chloride, 5 mM magnesium acetate, and 5 mM EGTA; 0.31% (v/v) β-mercaptoethanol and 0.5% (v/v) protease inhibitor mix, 200 μM pepstatin and 200 μM leupeptin in 100 mM PMSF, dissolved in isopropanol were freshly added to the "swelling" buffer] at 4 °C. Thereafter, the lysate was homogenized thoroughly, supplemented with 400 μL of glycerol (25% v/v) containing "swelling" buffer, and subsequently centrifuged for 3 min (400*g*, 4 °C). The supernatant was centrifuged for 30 min (100000*g*, 4 °C), and a cell pellet containing the nuclear fraction was resuspended in 1 mL of "swelling" buffer added with 25% (v/v) glycerol and 0.1% (v/v) TritonX-100 and centrifuged for 3 min (9000*g*, 4 °C). This step was carried out twice. The cell pellet (nuclear fraction) was taken up in 0.2 mL of "sonication" buffer [10 mM Tris/HCl, pH 8.0, 100 mM sodium chloride, 2 mM magnesium acetate, 5 mM EGTA, and 45 mM β-mercaptoethanol and freshly added 1% (v/v) NP40, 14 μg/mL aprotinin, and 15 μg/mL leupetin] and sonicated three times (10 s, 38 kHz). The 100000*g* supernatant was used as the cytosolic fraction. The pellet (membrane fraction) was resuspended in "swelling" buffer/1% NP40 and sonicated three times (10 s, 38 kHz). Forty micrograms of total proteins of each fraction were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (10 or 12% polyacrylamide gel) and transferred onto a nitrocellulose membrane. Western Blot was performed using rabbit polyclonal antibodies against human PKCα (80 kDa), PKCβ_I (79 Da), PKCβ_{II} (82 kDa), PKCγ (80 kDa), PKCδ (78/38 kDa), PKCϵ (82 kDA), PKCθ (82 kDa), PKCλ/ι (74/65 kDa), and PKCζ (80 kDa) and a horseradish peroxidase conjugate anti-rabbit IgG (Cell Signaling, Frankfurt, Germany) as a secondary antibody. α-Tubulin (56 kDa) served as a loading control. All primary antibodies were purchased from Santa Cruz (Heidelberg, Germany). The respective chemoluminescent signals (LumiGLO, Cell Signaling Technology, United States) were analyzed using the LAS 3000 with the Image Analyzer software (AIDA 3.52) for quantification (Fuji, Raytest, Straubenhardt, Germany). Arbitrary light units were plotted as test over control (%).

For determination of poly(ADP ribose) polymerase (PARP) cleavage, appropriately treated cell cultures were washed with PBS and extracted with lysis buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1% NP40, 10% glycerol, and freshly added

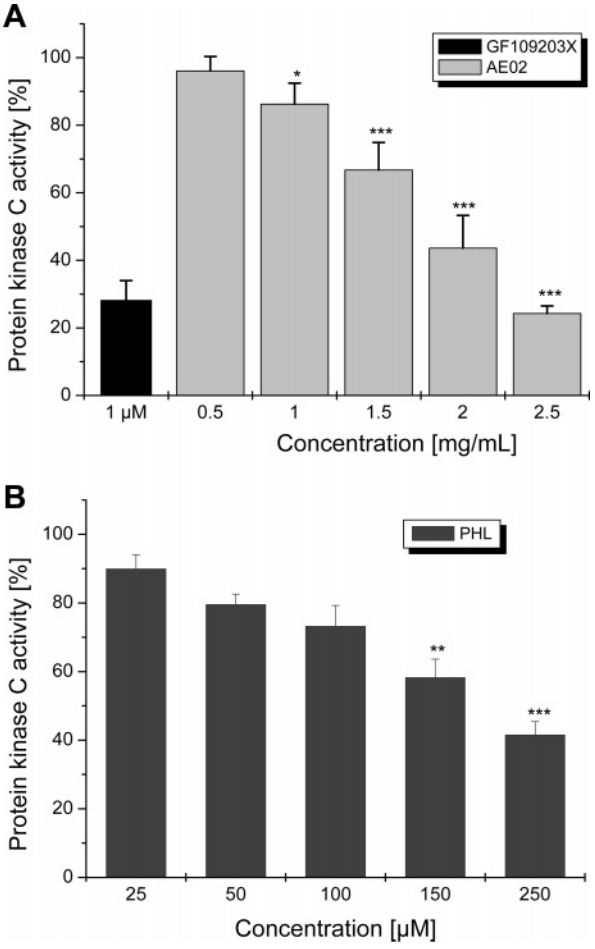

Figure 2. Inhibition of isolated cytosolic PKC activity determined as phosphorylation of a PKC substrate peptide (PKC Biotrak Enzyme Assay) by (**A**) specific PKC inhibitor GF109203X (1 μM) and apple juice extract (AE02) and (**B**) PHL. The data presented are the means ± SD of at least three independent experiments (* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.005$).

Table 1. Inhibition of Cytosolic PKC Activity from HT29 Cells by Apple Constituents, Determined as Phosphorylation of a PKC Specific Substrate Peptide (PKC Biotrak Enzyme Assay)

apple polyphenols	inhibition of PKC activity
(–)-EC	16 ± 2 % at 300 μM
procyanidin B1	*a*
procyanidin B2	*a*
PHD	*a*
caffeic acid	*a*
coumaroylquinic acid	*a*
chlorogenic acid	*a*
QUE-3-rhamnoside	22 ± 2% at 100 μM
QUE-3-glucoside	28 ± 8% at 100 μM
QUE-3-galactoside	*b*
QUE-3-rutinoside	*b*

[a] No inhibition up to 300 μM. [b] No inhibition up to 100 μM.

1 μg/mL leupeptin, 1 μg/mL pepstatin A, 200 KIE/mL aprotinin, 1 mM PMSF, 1 mM benzamidine, 0.1 mg/mL Pefabloc, and 1 mM sodium orthovanadate. Lysate aliquots were separated by SDS-PAGE with 10% gels. Immunoblots on PVDF membranes were developed with anti-PARP antibody (Cell Signaling).

Caspase-3 Activity. Before the cells were harvested, the medium was removed and the cells were washed twice with 1 mL of PBS. Thereafter, the cells were incubated with 1 mL of trypsin (3.6 U/mg) for 2 min at 37 °C. The cells were harvested, and the Petri dish was

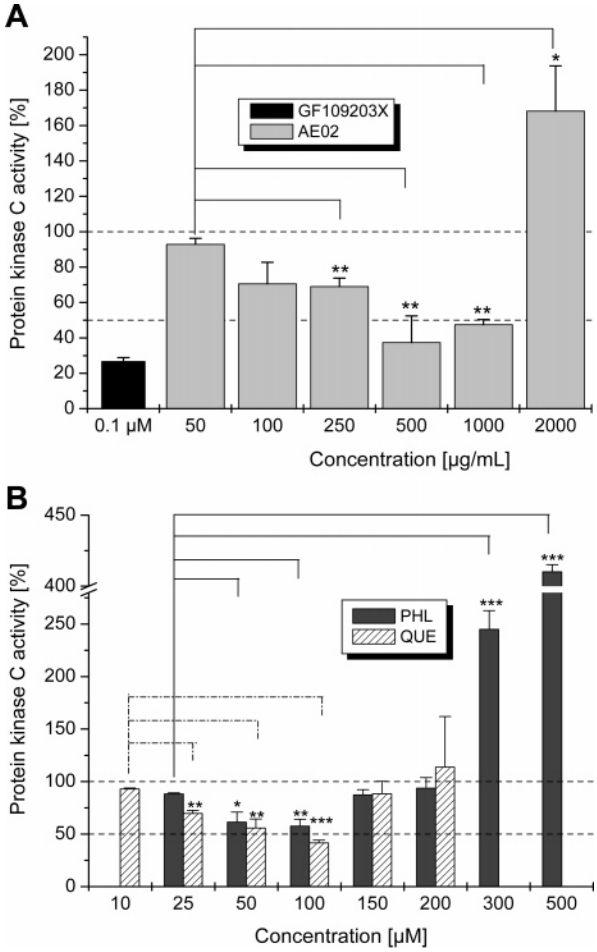

Figure 3. Inhibition of intracellular cytosolic PKC activity determined as phosphorylation of a PKC substrate peptide by (**A**) GF109203X (0.1 μM) and apple juice extract (AE02), (**B**) PHL, and QUE. The test compounds were used for treatment of HT29 cells for 24 h prior to the subsequent kinase assay. The data presented are the means ± SD of at least three independent experiments (* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.005$).

washed three times with serum-containing medium. The solutions of every step (see above) were transferred into the respective 15 mL PP tube and centrifuged for 10 min (200*g*, 20 °C). The cell pellet was carefully resuspended in 2 mL of PBS and centrifuged under the same conditions. Thereafter, the cell pellet was incubated in 150 μL of lysis buffer [50 mM HEPES (pH 7.4), 0.1 mM EDTA, 0.1% (v/v) Triton X-100, and 0.1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 μg/mL leupeptin, and 1 μg/mL pepstatin were freshly added to the lysis buffer] for 30 min at 4 °C and centrifuged (20000*g*, 10 min, 4 °C). The colorimetric procedure of Bradford (*21*) was used to determine the content of protein. Fifty microliters of the cell extract were incubated in assay buffer [50 mM HEPES (pH 7.4), 1 mM EDTA, 100 mM NaCl, 0.1% (w/v) CHAPS, 10% (v/v) glycerol, and 10 mM DTT] in the presence of 2 mM fluorogenic caspase substrate (Ac-DEVD-AFC, Biomol GmbH, Hamburg, Germany). Ac-DEVD-CHO (Biomol GMBH, Hamburg, Germany) was used as a specific capase-3 inhibitor. The reaction was performed at room temperature and was followed over 60 min in the fluorimeter (ex, 390 nm; em, 520 nm). The caspase-3 activity was calculated as the amount of AFC release that can be blocked by the selective caspase-3 inhibitor.

DNA Fragmentation. DNA fragmentation was determined by quantification of cytosolic oligonucleosome-bound DNA using the Cell death Detection ELISA^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany). HT29 cells were harvested with slight modifications as described previously (*22*). The cytosolic fraction (10 min, 200*g*,

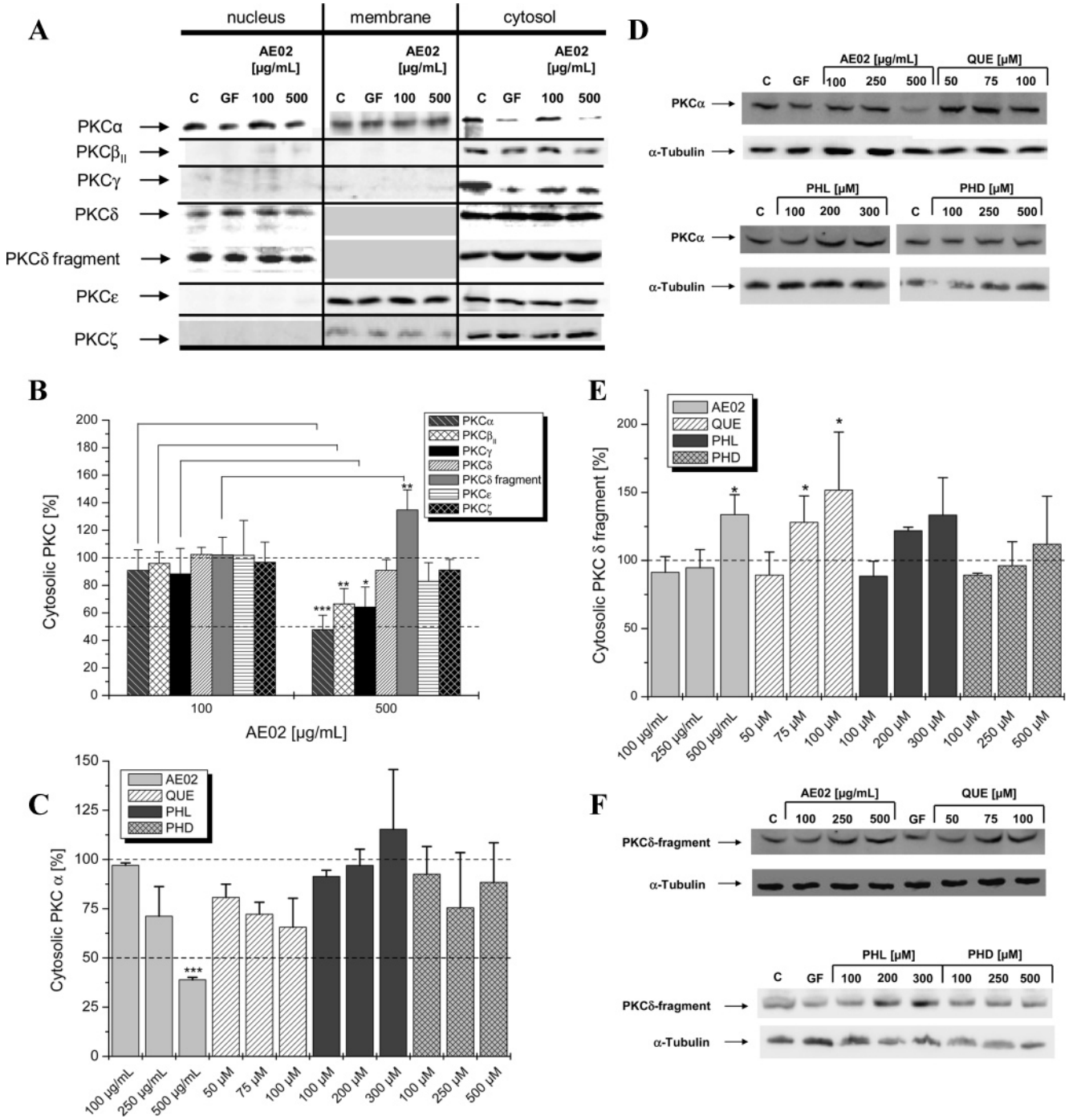

Figure 4. Western blot analysis of the protein levels of PKCα (80 kDa), PKCβ_{II} (82 kDa), PKCγ (80 kDa), PKCδ (78/38 kDa), PKCϵ (82 kDA), and PKCζ (80 kDa) in different cellular compartments of HT29 cells after 24 h of incubation with apple juice extract under serum-free conditions: (**A**) Representative Western blots and (**B**) data of the cytosolic protein levels of these isoenzymes. Western blot analysis of (**C**) PKCα protein levels (80 kDa) and (**E**) the 38 kDa PKCδ fragment in HT29 cells after 24 h of treatment with apple juice extract (AE02), QUE, PHL, and PHD. The data are plotted as test over control (%) with the control cells treated with 1% DMSO (solvent control). The data presented are the means ± SD of at least three independent experiments (* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.005$). (**D** and **F**) Western Blot of a representative experiment. C, solvent control; GF, GF109203X (0.1 μM); α-tubulin (56 kDa) was used as a loading control.

20 °C) of 15000 HT29 cells was used as an antigen source in the sandwich enzyme-linked immunosorbent assay (ELISA) with a biotin-labeled primary anti-histone antibody linked to a streptavidin-coated 96 well plate and a secondary anti-DNA antibody conjugated with a peroxidase. The DNA fragmentation was measured photometrical at 405 nm (BIOTEK Synergy HT, Bad Friedrichshall, Germany).

RESULTS

Inhibition of Isolated Cytosolic PKC Activity. The effect of polyphenol-rich apple juice extract (AE02) and PHL on isolated cytosolic PKC from HT29 cells was determined in a kinase assay system. The specific PKC inhibitor GF109203X (**Figure 1**) was used as a positive control (70% inhibition at 1

μM; **Figure 2A**). AE02 inhibited the PKC activity of a cytosolic preparation from HT29 cells in a concentration-dependent manner with an IC_{50} value of 1.88 ± 0.07 mg/mL (**Figure 2A**). Apple constituents identified and available so far showed marginal or no inhibitory properties (**Table 1**). The dihydrochalcone PHL diminished the kinase activity with an IC_{50} of 203 ± 19 μM (**Figure 1B**). The flavonol QUE inhibited PKC activity by about 40% at 25 μM (data not shown). However, because of limited solubility in the used kinase assay system, an IC_{50} value for QUE could not be determined.

Inhibition of Intracellular PKC Activity. To address the question whether the inhibitory effects of apple polyphenols on PKC activity in a cell-free system are also of relevance within intact cells, HT29 cells were incubated under serum-free conditions with the test compounds prior to the isolation of cytosolic PKC and the respective kinase assay. In contrast to the results in the cell-free system, AE02 did not affect the PKC activity of HT29 cells by incubation with up to 2 mg/mL for 1 or 3 h (data not shown). After 24 h of incubation with AE02, the cellular PKC activity was inhibited with an IC_{50} value of 403 ± 88 μg/mL (**Figure 3A**). The specific PKC inhibitor GF109203X (0.1 μM) decreased the intracellular PKC activity by about 80% (**Figure 3A**). In contrast, the apple characteristic dihydrochalcone glycoside PHD showed no significant effect on cellular cytosolic PKC activity in HT29 cells up to 500 μM (data not shown). The free aglycons PHL and QUE showed inhibitory effects on cellular PKC activity in a concentration-dependent manner (**Figure 3B**). Notably, the inhibition of PKC activity by AE02 achieved an apparent optimum at 500 μg/mL (**Figure 3A**). At higher concentrations, the recurrence and, at a concentration of 2 mg/mL, even a significant induction of the cytosolic PKC activity were determined (**Figure 3A**). Concomitantly, at concentrations >500 μg/mL, a substantial loss of viable cells was observed (data not shown). Comparable effects were detected by incubation with the aglycons PHL and QUE (**Figure 3B**). QUE significantly inhibited the intracellular PKC activity already at 50 μM. An apparent maximum of inhibition was achieved at 100 μM (IC_{50} value = 77 ± 15 μM). The recurrence of cellular PKC activity at concentrations ≥150 μM (**Figure 3B**) was associated with a substantial loss of viable cells (data not shown). However, up to 200 μM QUE, PKC activity was not significantly enhanced above the level of the solvent control (**Figure 3B**). Because of limited solubility, higher concentrations of QUE could not be applied in this test system. Treatment with PHL led to a significant inhibition of cellular PKC activity in HT29 cells but without reaching an IC_{50} value (45% inhibition at 100 μM) (**Figure 3B**). At concentrations ≥300 μM PHL, the cellular PKC activity was significantly enhanced as compared to the solvent control (**Figure 3B**). Also for PHL, the increase of PKC activity was accompanied by a substantial loss of viable cells (data not shown).

Modulation of PKC Protein Levels. We further addressed the question of whether the modulation of PKC kinase activity by apple polyphenols in HT29 cells is associated with the translocation of PKC isoenzymes between the different cellular compartments. In the cytosol of HT29 cells, the PKC isoenzymes α, β_{II}, γ, δ, ϵ, and ζ were detected by Western Blot analysis (**Figure 4A**), whereas in the membrane compartment, only PKC α, ϵ, and ζ were observed. In the nucleus only, the PKC α and δ isoforms were found. Additionally, marginal amounts of PKCβ_I, PKCλ/ι, and PKCθ were detected in the cytosol (data not shown).

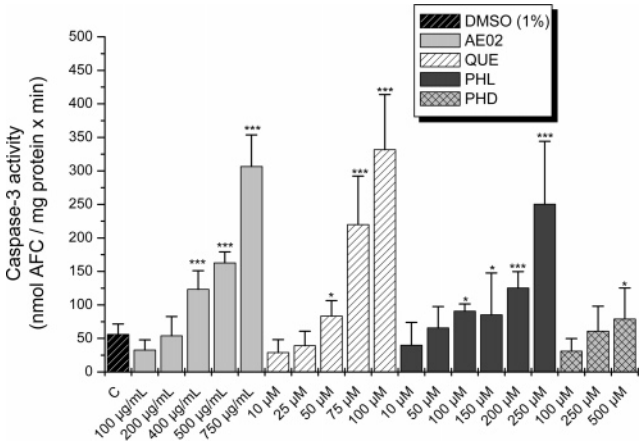

Figure 5. Induction of caspase-3 activity by apple juice extract (AE02), QUE, PHL, and PHD. HT29 cells were incubated for 24 h with the respective compound in serum-free medium. Caspase-3 activation was determined as release of the fluorescent AFC from the peptide substrate DEVD-AFC. Caspase 3 activity was calculated as the amount of AFC release, which can be blocked by the selective caspase-3 inhibitor N-acetyl-DEVD-aldehyde (DEVD-CHO). The data presented are the means ± SD of at least three independent experiments each performed in duplicate (* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.005$). C = DMSO, solvent control (1%).

Incubation of HT29 cells with a 100 nM concentration of the selective PKC inhibitor GF109203X for 24 h induced a slight decrease of the classical PKC isoforms α, β_{II}, and γ in the cytosol without any indication of translocation (**Figure 4A**). An increase in the concentration of GF109203X up to 1 μM led to a substantial loss in cell viability, impeding further testing (data not shown).

Incubation of HT29 cells with AE02 (500 μg/mL) for 24 h under serum-free conditions significantly reduced the protein content of cytosolic PKCα, β_{II}, and γ (**Figure 4B**), without any indication of translocation into other cellular compartments (**Figure 4A**). The protein levels of PKCδ, ϵ, and ζ were only marginally affected in the respective compartments (**Figure 4A**,**B**). In contrast, the protein level of the 38 kDa PKCδ fragment was found to be significantly increased in the cytosol (**Figure 4B**). In the nucleus, a slight but not significant decrease of PKCα and the 38 kDa PKCδ fragment was observed (**Figure 4A**).

In contrast to AE02, QUE, PHL, and PHD did not significantly affect the protein level of the cytosolic PKCα (**Figure 4C**,**D**). However, the treatment with QUE enhanced the amount of the 38 kDa PKCδ fragment in the cytosol, whereas PHL and PHD mediated a slight but not significant increase yet only in high concentrations (**Figure 4E**,**F**).

Induction of Caspase-3 Activity. Activation of caspase-3, a cysteine-dependent aspartase, is a characteristic element of the apoptotic process. Caspase-3 activity was measured using the caspase substrate DEVD-AFC. The active caspase cleaves the substrate, releasing the fluorescent AFC (*22*).

Serum-free incubation (24 h) of HT29 cells with apple juice extract (AE02) in concentrations ≥400 μg/mL resulted in a significant induction of caspase-3 activity (**Figure 5**). Both tested aglycons QUE (≥50 μM) and PHL (≥100 μM) also activated caspase-3 (**Figure 5**). The dihydrochalcone glycoside PHD showed a slight but significant increase of caspase-3 activity at high concentrations (≥500 μM) (**Figure 5**).

DNA Fragmentation and PARP Cleavage. DNA fragmentation was determined by quantification of cytosolic oligonu-

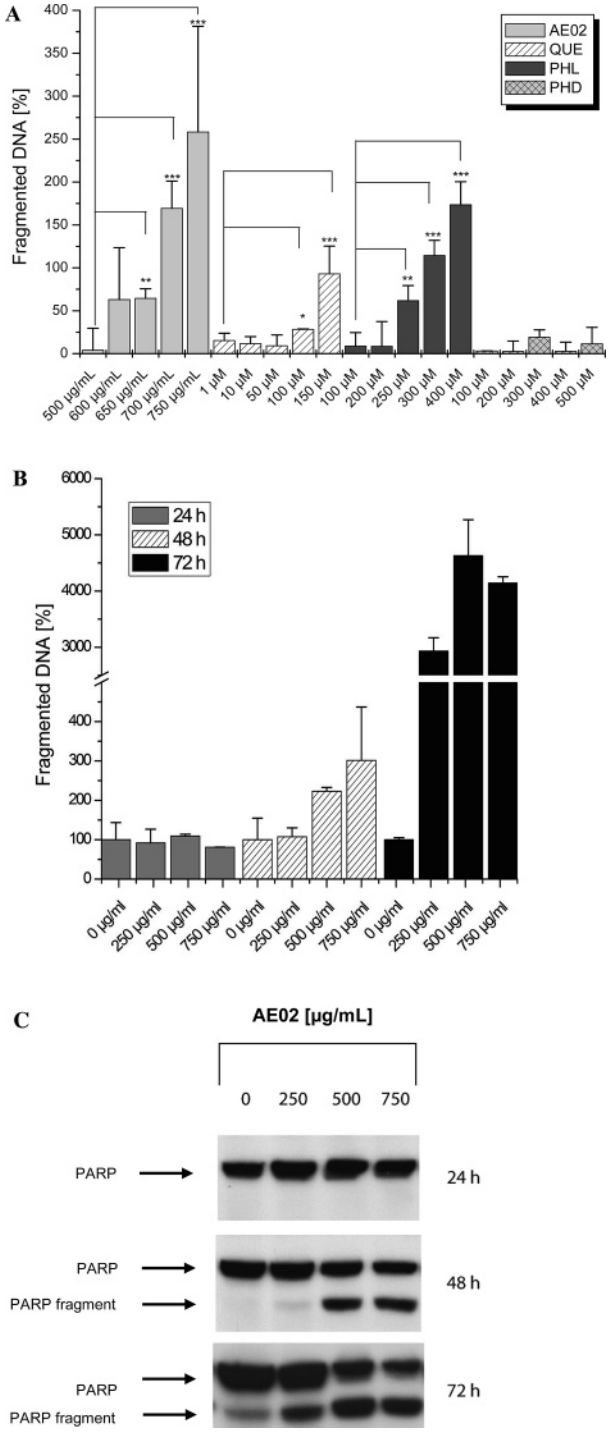

Figure 6. Induction of DNA fragmentation and PARP cleavage by apple juice extract (AE02), QUE, PHL, and PHD. (**A**) HT29 cells were incubated for 24 h with the respective compound in serum-free medium, and the DNA fragmentation was measured by cell death detection ELISA^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany). The values presented are the means ± SE of three independent experiments, each performed in triplicate (* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.005$). (**B**) HT29 cells were incubated in medium containing 10% FCS for the indicated time periods with apple juice extract (AE02), and DNA fragmentation was measured as in part **A**. The values are means of duplicate determinations and are representative for three experiments with consistent results. (**C**) Cell lysates of apple polyphenol extract (AE02)-treated HT29 cells were subjected to SDS-PAGE and immunoblotting with anti-PARP antibodies. The antibody detects intact PARP (116 kDa) and a cleavage fragment (89 kDa) arising upon apoptosis. The shown experiment is representative for three with consistent results.

cleosome-bound DNA by ELISA. After 24 h of incubation, AE02 was found to significantly induce DNA fragmentation in HT29 cells at concentrations ≥650 µg/mL (**Figure 6A**). A significant increase in DNA fragmentation was also mediated by treatment with QUE (≥100 µM) and PHL (≥250 µM) (**Figure 6A**). PHD was ineffective up to 500 µM (**Figure 6A**). In the presence of serum, 24 h of treatment with up to 750 µg/mL AE02 did not result in any detectable DNA fragmentation (**Figure 6B**). Upon longer treatment, for 48 and 72 h, DNA fragmentation was induced in a concentration-dependent manner. Notably, treatment with 500 and 750 µg/mL for 72 h led obviously to a strongly elevated level of apoptosis. Consistent with DNA fragmentation, cleavage of PARP, a common indicator of apoptosis, was also induced by apple polyphenol treatment (**Figure 6C**).

DISCUSSION

In the present study, a polyphenol-rich apple juice extract (AE02) was shown to inhibit cytosolic PKC activity in a cell-free system (**Figure 2A**). From the constituents of AE02 identified so far, only (−)-EC and the QUE-glycosides QUE-3-rhamnoside and QUE-3-glucoside showed marginal PKC inhibitory properties (**Table 1**). The apple characteristic dihydrochalcone glycoside PHD was found to be inactive against PKC in cell-free systems and can therefore be excluded to contribute to the PKC inhibitory properties of the extract. In contrast to the glycoside, the free aglycon PHL exhibited inhibitory properties (**Figure 2B**). PHL has already been reported to target PKC (*23*). However, the free aglycon has not been identified in the extract so far.

QUE is known to possess potent PKC inhibitory properties in human colon carcinoma cells (*24*−*27*). However, the free aglycon appears to be not present in the apple extract AE02 (*1*, *3*, *6*). In contrast, QUE-glycosides, constituents of AE02, were found to be inactive or at least less active than the respective aglycon. Therefore, it could be assumed that the apple polyphenols available and identified so far only marginally contribute to the PKC inhibitory properties of the original apple juice extract in the cell-free system.

We furthermore investigated whether the observed PKC inhibitory properties of apple polyphenols are of relevance within intact cells. In contrast to the results in the cell-free system, incubation of HT29 cells for 1 or 3 h with AE02 did not affect the cytosolic PKC activity. After prolonged incubation (24 h), cytosolic PKC activity was affected, albeit a u-shaped curve of effectiveness was observed (**Figure 3A**). Inhibition of PKC activity in SW620 colon carcinoma cells by an apple juice extract has already been reported (*2*). In HT29 cells, a u-shaped curve of effectiveness was not only observed for AE02, but also for the aglycons PHL and QUE (**Figure 3B**), with an initial inhibitory effect followed by the recurrence of enzyme activity.

The lack of effectiveness of AE02 in short-time incubations and the potent PKC inhibition observed after 24 h of incubation, exceeding by far the inhibitory potency of the extract in the cell-free system, suggests that PKC is not the primary target of apple polyphenols in intact cells. The observed inhibition of PKC activity after 24 h of incubation might result from effects on upstream signaling elements. AE02 has already been identified as a potent inhibitor of the PTK activity of the EGFR, suppressing the activity of the subsequent MAPK cascade in HT29 cells (*3*). After 5 h of incubation, a substantial decrease in the phosphorylation of the extracellular regulated kinases ERK1 and ERK2 in HT29 cells was already demonstrated at a concentration of 0.1 µg/mL AE02, thus exceeding by several

orders of magnitude the inhibitory concentrations in the PKC assay. Furthermore, we have shown earlier that in HT29 cells, AE02 inhibits the activity of glycogen synthase kinase 3β, which might subsequently affect PKC activity (*9*).

A direct activation of PKC activity by apple extract constituents can be excluded, considering the inhibitory effect of AE02 in the cell-free system. However, an increase in PKC activity has been reported to be associated with the induction of apoptosis (*14*, *17*, *18*). Several PKC isoenzymes have been shown to be involved in apoptotic processes (*17*−*20*). PKCα has been linked to an increase in cell proliferation whereas PKCδ has been reported to play an important role in apoptosis (*14*, *19*, *28*). Albeit the contribution of classical PKCα on cell proliferation is controversially discussed, in colon cancer, antiapoptotic processes seem to be prior (*14*, *29*, *30*). The mechanisms of antiapoptotic properties of PKCα are not yet identified, but phosphorylation of the antiapoptotic protein Bcl-2 as well as the activation of Raf-1 and the subsequent mitogen-activated signaling cascade are discussed (*31*). The novel PKC isoform PKCδ is ubiquitously expressed. Signals involved in the onset of apoptosis like the initiation of the death receptor CD95 or topoisomerase II poisoning have been reported to activate PKCδ (*19*, *32*). During apoptosis, a catalytically active fragment (38 kDa) of PKCδ is generated mediated by caspase-3 cleavage (*17*−*20*). Additionally, it has been shown that a decrease of PKCδ expression is associated with an increase of cell proliferation in vitro (*33*). Therefore, we addressed the question whether apple polyphenols modulate the protein level of PKC isoenzymes. The pattern of PKC isoenzyme expression in the different cellular compartments of HT29 cells, determined by Western blot analysis, is in line with earlier results of (*34*). At PKC inhibitory concentrations of AE02, a significant decrease of cytosolic PKCα, PKCβ_{II}, and PKCγ levels and a significant increase in the amount of the PKCδ fragment were observed, without indication of translocation between the different cellular compartments.

Prompted by the modulation of PKC isoform protein levels, we investigated whether the apple juice treatment is associated with the onset of apoptosis. Activation of caspase-3, a cysteine-dependent aspartase, is a characteristic early event of apoptosis whereas DNA fragmentation is linked to late apoptotic stages. In line with the results of ref *2*, we found a significant induction of caspase-3 activity (**Figure 5**) and DNA fragmentation (**Figure 6A**) in HT29 cells resulting from the treatment with AE02 after 24 h of incubation. The incubation of HT29 cells with QUE led to a significant increase of caspase-3 activity as previously described (*35*). However, QUE itself appears to be not present as the free aglycon in the apple extract AE02, whereas several QUE-glycosides have been identified (*1*, *3*, *6*). In different test systems, these QUE-glycosides as well as yet identified human QUE metabolites were found to be inactive or at least less active as compared to the respective aglycon (*1*, *3*, *36*, *37*). Therefore, it could be assumed that the QUE-glycosides only marginally contribute to the effective activation of caspase-3 by AE02 in HT29 cells.

In accordance with the results in the caspase-3 assay, AE02 as well as the aglycons QUE and PHL were found to induce enhanced DNA fragmentation. The concentrations resulting in significant induction of DNA fragmentation were higher as compared to respective effects in the caspase-3 assay, which might be due to the fact that DNA fragmentation represents a late apoptotic event. Prolonged treatment with apple polyphenols for 48 or 72 h led to the induction of DNA fragmentation even in the presence of serum. Consistent with these data, apoptosis induction by AE02 could also be detected using PARP cleavage as a marker, when HT29 cells were treated for a prolonged time in the presence of serum (**Figure 6B**,**C**).

In summary, apple polyphenols were found to inhibit PKC activity in a cell-free system. However, our results indicate that within intact cells PKC does not represent the primary target of apple polyphenols but appears to be affected in the course of apoptosis induction. The onset of apoptosis in HT29 cells resulting from the treatment with the apple extract AE02 was shown by the activation of caspase-3, DNA fragmentation, and PARP cleavage. Apoptosis induction in transformed colon epithelial cells may be an aspect of the presumed cancer preventive activity of apple polyphenols and may contribute to its previously observed cytostatic effects (*1*). So far, identified and available constituents of the apple extract have not contributed substantially to the observed effects on PKC and apoptosis induction.

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