The PI3K/Akt Pathway Is Involved in Procyanidin-Mediated Suppression of Human Colorectal Cancer Cell Growth

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Colorectal cancer (CRC) has the third highest incidence worldwide. Epidemiological studies showed that the consumption of fruit and vegetables containing procyanidins (PCA), polymers of flavan-3-ols, is associated with lower CRC risk. However, the molecular mechanisms supporting this positive association are unclear. This study investigated the capacity of PCA with different degrees of polymerization to reduce CRC cell growth, characterizing the underlying mechanisms. Compared to the monomer ((−)-epicatechin) and the trimer, the hexamer (Hex) was the most active at reducing CRC cell viability. Hex caused a concentration- (2.5–50 μM) and time- (24–72 h) dependent decrease in the viability of six human CRC cell lines in culture. Hex caused CRC apoptotic Caco-2 cell death within 24 h, as evidenced by caspase 3 and caspase 9 activation, DNA fragmentation, and changes in nuclear morphology/staining. Hex-induced apoptosis occurs through the mitochondrial pathway, as evidenced by an increased Bad mitochondrial translocation, and cytochrome c release from the mitochondria to the cytosol. Hex also arrested the Caco-2 cell cycle at G_2/M phase and upregulated genes involved in autophagy. Mechanistically, in Caco-2 cells Hex inhibited the PI3K/Akt signaling pathway, causing the downstream downregulation of proteins involved in the regulation of cell survival (Bad, GSK-3β). Accordingly, the Akt inhibitor MKK-2206 decreased Bad and GSK-3β phosphorylation. MKK-2206 decreased cell growth, having an additive effect with Hex. In conclusion, our results show that large PCA can inhibit CRC cell growth via the Akt kinase pathway, demonstrating a mechanism to explain the epidemiological evidence linking PCA-rich diets with lower CRC risk.
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Key words: apoptosis; proanthocyanidin; autophagy; cell cycle arrest

INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer among men and women and is the second leading cause of death in the United States [1]. Although the development of new therapeutic approaches and the implementation of early detection exams have reduced the incidence and improved the prognosis of CRC, still 50% of diagnosed patients die from metastasis [2]. Both genetic and environmental factors contribute to CRC development. In this regard, smoking, alcohol consumption, low physical activity, obesity, and diet constitute important environmental factors linked to increased CRC risk.

Numerous epidemiological and clinical studies support the concept that diet has a critical role in the risk of developing CRC. In this regard, high consumption of red and processed meats is associated with an increased risk of CRC [3], while diets rich in fruits, vegetables, and fiber can lower it [4–7]. Procyanidins (PCA) are flavan-3-ol oligomers which are present in large amounts, among others, in grapes, berries, cocoa, tea, and derived foods. Epidemiogical studies have shown a link between the consumption of PCA and a lower CRC risk [4,8,9]. Cocoa PCA consist of polymers of (+)-catechin or (−)-epicatechin (EC) subunits primarily linked by 4β→8 bonds (Figure 1). After ingestion, PCA are partially metabolized and can be found throughout the gastrointestinal tract [10,11]. Having anti-inflammatory and intestinal barrier protective effects, PCA could inhibit CRC development/progression through the modulation of pro-oncogenic signals [12,13]. In fact, apple procyanidins have shown anti-cancer effects in cell
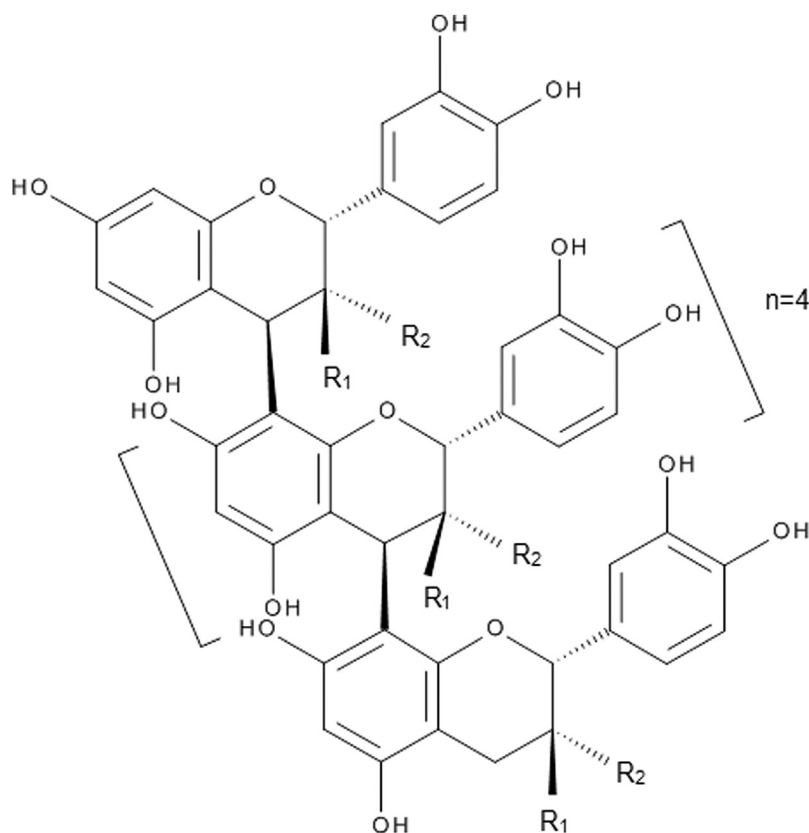
Figure 1. Chemical structure of a hexameric procyanidin constituted by subunits of (−)-epicatechin, linked by 4β→8 bonds, R1=H, R2=OH.

and animal models of CRC, and PCA enriched cocoa extracts promote CRC cell apoptosis and cell cycle arrest in G2/M phase [14].

The serine-threonine kinase Akt constitutes an important signaling pathway that modulates relevant cellular processes, including cell survival, proliferation, differentiation, and autophagy. Furthermore, deregulation of this signaling cascade is one of the most frequent alterations found in various types of cancers, including CRC [15–17]. Emerging evidence supports the PI3K/Akt pathway as a potential target for anticancer strategies [15,18–20]. Activation of PI3K causes the phosphorylation and activation of Akt via the phosphoinositide-dependent kinases (PDK1/2), leading to the translocation of Akt from the membrane to the cytosol and nucleus. Akt then activates downstream targets, including Bad and glycogen synthase kinase 3 (GSK-3β), which are involved in the promotion of pro-survival and proliferative events [21]. Akt is also associated to the regulation of cell survival via the modulation of autophagy [22].

We previously observed that the hexameric PCA (Hex), which consists of six subunits of EC/catechin, linked by 4β→8 bonds (Figure 1), inhibits deoxycholic acid-induced Akt activation in a model of

differentiated intestinal epithelial cells [23]. Although highly polymerized PCA, such as Hex, are not transported into the intestinal cells, they can regulate intracellular signaling cascades through their interactions with the cell membrane [24]. An anti CRC effect of cocoa PCA extracts was previously proposed [14], however the effects of isolated PCA and the molecular events underlying this action are still unknown. We hypothesized that highly polymerized PCA, although not absorbed at the gastrointestinal tract as the intact compounds, could exert local anti-CRC actions through the modulation of the Akt pathway. This work investigated the capacity of Hex derived from cocoa to promote apoptosis and inhibit the proliferation of human CRC cells, characterizing the involvement of the Akt pathway.

MATERIALS AND METHODS

Materials

Trimeric PCA, Hex, and the cocoa flavanol extract were prepared [25,26] and supplied by Mars Incorporated (Hackettstown, NJ). EC was purchased from Sigma Chem. Co. (St. Louis, MO). The grape seed extract was kindly donated by Kikkoman Corp. (Noda, Japan).

The human CRC cells Caco-2, HCT-116, SW-480, HT-29, LoVo, and HCT-15 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell culture media and reagents were from Invitrogen Life Technologies (Carlbad, CA). Primary antibodies for Bcl-xL (sc-8392), Beclin-1 (sc-11427), cytochrome c (sc-7159), and LC3 (sc-292354) were from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies for p(Ser473)-Akt (#9271), p(Thr308)-Akt (#2965), Akt (#4691), p(Ser136)-Bad (#5286), Bad (#9239), Bcl-2 (#2876), ERK (#4695), p(Ser21/9)-GSK-3α/β (#9331), and GSK-3β (#9332) were from Cell Signaling Technology, Inc. (Danvers, MA).

The CellTiter-Glo® Luminiscent Cell Viability assay, Caspase-Glo® 3/7 assay, the oligonucleotides containing the consensus sequences for AP-1 and NF-κB, and the reagents for the EMSA assay were obtained from Promega (Madison, WI). The protease inhibitor cocktail and the Cell Death Detection ELISA Plus kit were from Roche Applied Science (Indianapolis, IN). MK-2206 was purchased from APExBio (Houston, Texas).

Cell Culture and Incubations

Caco-2 cells were cultured in Minimum Essential Medium supplemented with 20% (v/v) fetal bovine serum (FBS), HCT-116 and HT-29 cells in McCoy's 5A supplemented with 10% (v/v) FBS, and SW-480 and HCT-15 cells in RPMI medium supplemented with 10% (v/v) FBS. All media were supplemented with antibiotics (50 U/ml penicillin, and 50 μg/ml strepto-mycin). The media were replaced every 3 d. For Caco-2 cell differentiation, cells were cultured in growth culture medium for 21 days after reaching confluence. Differentiated Caco-2 cells were only used to evaluate the effects of the tested compounds on cell viability.

Cell Viability Assay

The effect of EC, trimers, Hex, cocoa, and grape seed extracts on CRC cell viability was determined with the CellTiter-Glo Luminiscent Cell Viability Assay, which measures cellular ATP levels. Briefly, cells (5,000 cells/well) were plated in 96-well plates and grown overnight. Cells were incubated in the absence or the presence of 2.5–100 μM EC, dimers, trimers, Hex or 17–170 μg/ml grape seed or cocoa extracts for 24–72 h. Cell viability was also measured in Caco-2 cells differentiated into intestinal epithelial cells using the same assay.

Clonogenic Assay

Caco-2 cells were seeded (500 cells/plate) in 30 mm plates and incubated for 24 h. Undifferentiated cells were subsequently incubated in the absence or presence of Hex (10–40 μM) for 24 h. The medium was removed and replaced by growth medium, and cells were allowed to grow for further 7 d. Colonies were visualized by staining with crystal violet. Briefly, after removing the medium and washing cells with warm Hanks solution, cells were fixed by incubation with 3.7% (w/v) paraformaldehyde in PBS for 15 min. The paraformal-dehyde solution was removed, and colonies stained by incubation for 30 min at room temperature in the presence of a solution of 0.05% (w/v) crystal violet in water. Plates were washed twice with ultrapure water, allowed to drain by inversion on a paper towel, and scanned for subsequent colony counting.

Western Blot Analysis

For the preparation of total cell extracts, after the corresponding treatments, Undifferentiated Caco-2 cells were rinsed with PBS, scrapped and centrifuged. The pellet was rinsed with PBS, and suspended in 200 μl of 50 mM Hepes (pH 7.4), 125 mM KCl which contained protease and phosphatase inhibitors, and 2% (v/v) Igepal. The final concentration of the protease inhibitors was 0.5 mM PMSF, 1 mg/L leu-peptin, 1 mg/L pepstatin, 1.5 mg/L aprotinin, and 2 mg/L bestatin. Samples were incubated at 4°C for 30 min, and centrifuged at 15,000g for 30 min. The supernatant was decanted and protein concentration was measured [27]. Aliquots of total cell fractions containing 30–50 μg protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. Colored (Bio-Rad Laboratories, Hercules, CA) and biotinylated (Cell Signaling Technologies, Danvers, MA) molecular weight standards were ran simultaneously. Membranes were blotted for 2 h in 5% (w/v) non-fat milk, incubated overnight in the presence of the corresponding antibodies (1:500–1:1,000 dilution) in 5% (w/v) bovine serum albumin in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.6), containing 0.1% (v/v) Tween-20. After incubation for 90 min at room temperature in the presence of the secondary anti-body (HRP-conjugated) (1:10 000 dilution), the con-jugates were visualized by chemiluminescence detection in a Phosphoimager 840 (Amersham Phar-macia Biotech. Inc., Piscataway, NJ).

Evaluation of Apoptosis

Apoptosis was evaluated by measuring caspase 3/7 and caspase 9 activities, cytoplasmic mono, and oligonucleosomes, cell morphology by fluorescence microscopy, and Annexin V staining. Caspase 3/7 and caspase 9 activities were measured using Caspase Glo assays (Promega, Madison, WI) following the manu-facturer's protocol. Mono- and oligonucleosomes were determined using the Cell Death Detection ELISA Plus (Roche Applied Science, Indianapolis, IN) which quantifies cytoplasmic-histone DNA frag-ments. The assay was done following the manufac-turer's protocol. Values ($Abs_{405 nm}$—$Abs_{490 nm}$) were referred to those obtained for control untreated cells.

Fluorescence Microscopy for Hoechst and Propidium Iodide (PI) Staining

Undifferentiated Caco-2 cells were seeded (300,000 cells/35 mm^2 plate) and incubated in the

absence or presence of Hex (10–40 μM) for 24 h. The Hoechst 33342 reagent (0.5 μg/ml) was added to the media and cells incubated in the dark for 15 min at 37°C. Cells were subsequently added with PI (5 μg/ml) and incubated for further 5 min. The media was discarded and cells washed with cold PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature. Cells were finally washed with PBS, added with 2 ml PBS and visualized by fluorescence microscopy (Leica DMI3000B).

Determination of Apoptotic Cell Death By Annexin V and Propidium Iodide Dual Staining

Undifferentiated Caco-2 cells were treated with Hex 20–40 μM for 48 h and apoptotic cells measured by FACS as previously described [28]. Briefly, all cells (attached and suspended) were collected and stained with annexin V-FITC (100× dilution) and propidium iodide (PI) (0.5 μg/ml) for 15 min. Annexin V-FITC and PI fluorescence intensities were analyzed using a BD Biosciences FACSCalibur (San Jose, CA). Annexin V(+)/PI(−) cells are early apoptotic cells; Annexin V(+)/PI(+) cells are late apoptotic cells; while Annexin V(−)/PI(+) cells are necrotic cells.

Evaluation of Cell Cycle Progression

Following Hex treatment, floating and adherent cells (harvested by trypsinization) were combined, washed with PBS, and fixed in ice-cold 70% (v/v) ethanol for 30 min. After fixation, cells were washed again with PBS and stained for 30 min at room temperature with PI containing 4 KU/ml RNase type IIA. Nuclear PI fluorescence was measured by flow cytometry; determining for each sample at least 10,000 events. The percentage of cells in G_0/G_1, S, and G_2/M was determined from DNA content histograms.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear fractions were isolated as previously described [29]. For the EMSA, the oligonucleotides containing the consensus sequence for AP-1 or NF-κB were end-labelled with [γ-^{32}P] ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. Samples were incubated with the labelled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 1× binding buffer [5× binding buffer: 50 mM Tris-HCl buffer, pH 7.5, containing 20% (v/v) glycerol, 5 mM MgCl$_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 0.25 mg/ml poly(dI-dC)]. The products were separated by electrophoresis in a 6% (w/v) non-denaturing polyacrilamide gel using 0.5× TBE (Tris/borate 45 mM, EDTA 1 mM) as the running buffer. The gels were dried and the radioactivity quantified in a Phosphoimager 840.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC). Fisher least significance difference test was used to examine differences between group means. A P value <0.05 was considered statistically significant. Data are shown as mean ± SEM.

RESULTS

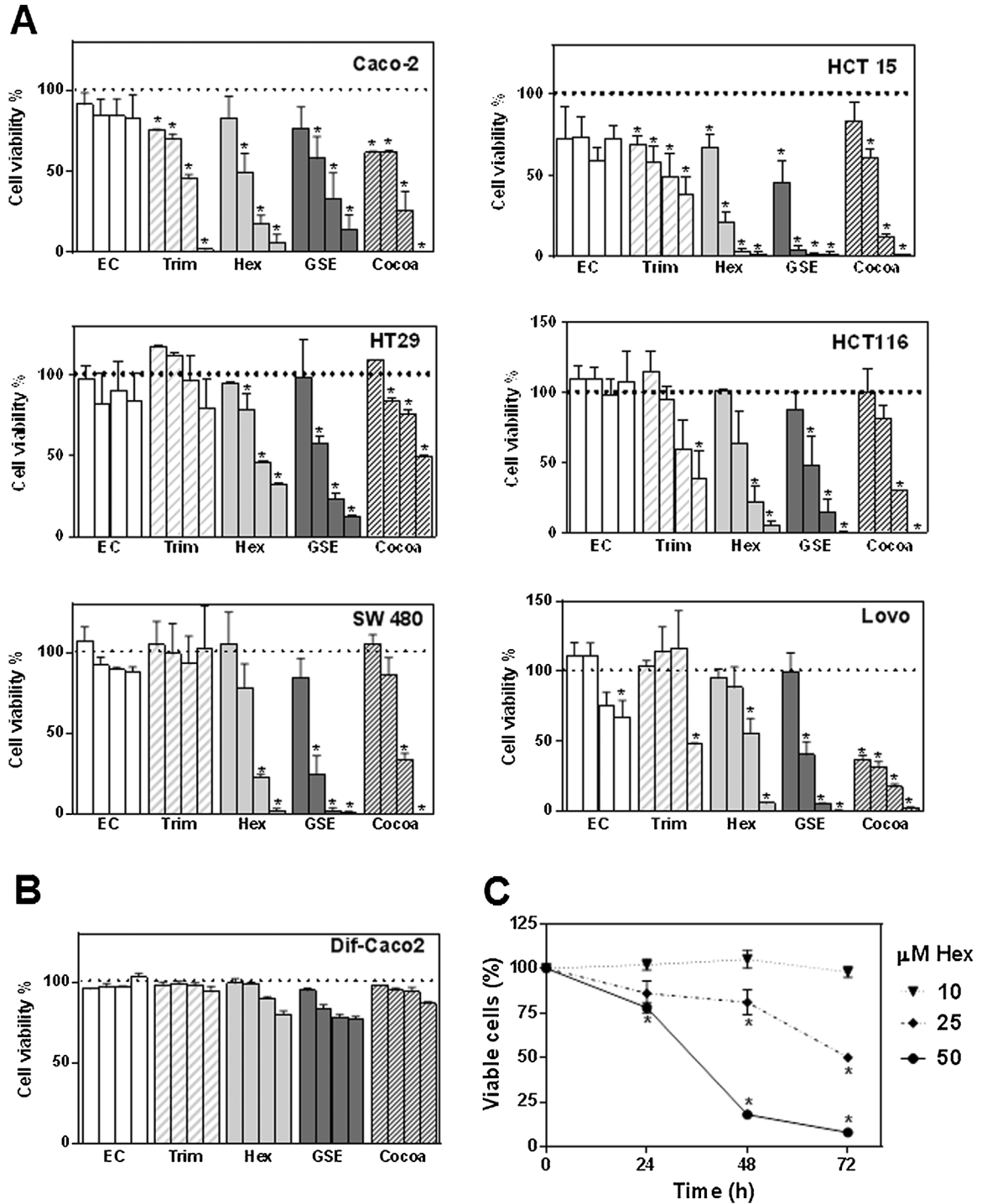### Hex Reduces CRC Cell Viability in a Concentration- and Time-Dependent Manner

We initially evaluated whether EC, and cocoa PCA trimer, and hexamer (Hex) affect cell viability in six human CRC cell lines. These effects were also compared to those of a total cocoa flavanol extract containing monomers (EC and catechin) and PCA from 2 to more than 10 subunits, and of a GSE, which also contain epicatechin-gallate, and epigallocatechin as PCA subunits. Cells were incubated in the absence or presence of 2.5–100 μM EC, trimers and Hex for 72 h (Figure 2A and B) or with Hex for 24–72 h (Figure 2C). After 72 h of incubation, the Hex was more potent than the monomer or trimer in decreasing CRC cell growth. At the concentrations (17.4–174 μg/ml) used for Hex, the effects of the cocoa and GSE extracts were significantly different between them. Hex decreased the cell viability of the six tested human CRC cell lines in a concentration (2.5–50 μM)-dependent manner. To note, of the six CRC cell lines studied, LoVo cells, which was the only grade IV adenocarcinoma cell line, appeared to be the most resistant to the Hex treatment, needing higher concentration of Hex to achieve a reduction in cell viability. The IC$_{50}$ for the capacity of Hex to decrease Caco-2 cell viability after 72 h incubation was 20 μM. Importantly, Hex did not affect the viability of Caco-2 cells differentiated into intestinal epithelial cells (Figure 2B). In addition, Hex (10–40 μM) caused a concentration-dependent decrease in Caco-2 cell colony formation (Figure 3A). Given the higher sensitivity of Caco-2 cells to Hex, we performed the subsequent mechanistic studies using this undifferentiated CRC cell line.

Hex Induces Cell Cycle Arrest in G_2/M

We next evaluated the effects of Hex on Caco-2 cell cycle progression using PI staining and subsequent flow cytometry analysis. Cells were incubated for 24, 48, and 72 h in the absence or presence of Hex (10–30 μM) (Figure 3B and C). While no significant changes were observed at 24 and 48 h (data not shown), the percentage of cells in G_2/M after 72 h incubation was significantly higher in the cells treated with 30 μM Hex, indicating that Hex arrested the cell cycle at G_2/M phase.

Hex Induces Apoptotic Cell Death and Increased Expression of Autophagy Markers

The capacity of Hex to promote apoptotic cell death was investigated by evaluating changes in cell nuclear morphology (chromatin condensation),

Figure 2. Effects of EC, PCA, and PCA-rich extracts (grape seed (GSE) and cocoa (Cocoa) extracts on CRC cell viability. Human CRC cells were incubated in the absence or presence of 10, 25, 50, and 100 μM (−)-epicatechin (EC), trimer (Trim), hexamer (Hex), and 17.4, 43.5, 87, and 174 μg/ml GSE and cocoa extracts for 72 h (A,B) or with 10–50 μM Hex for 24, 48, and 72 h in Caco-2 cells (C). Cell viability was measured as described in Methods. Values are shown as means ± SEM of at least three independent experiments. Dif-Caco-2: differentiated Caco-2 cells.

DNA fragmentation, and phosphatidylserine externalization. After 24 h incubation. Hex (10–40 μM) triggered apoptotic cell death as evidenced by an increase in cells with rounded nuclei and bright condensed chromatin, with little or no sign of necrosis (Figure 4A). After 24 h, Hex also induced DNA fragmentation in a concentration-dependent manner (2.1, 3.0, and 6.3-fold increase in mono and
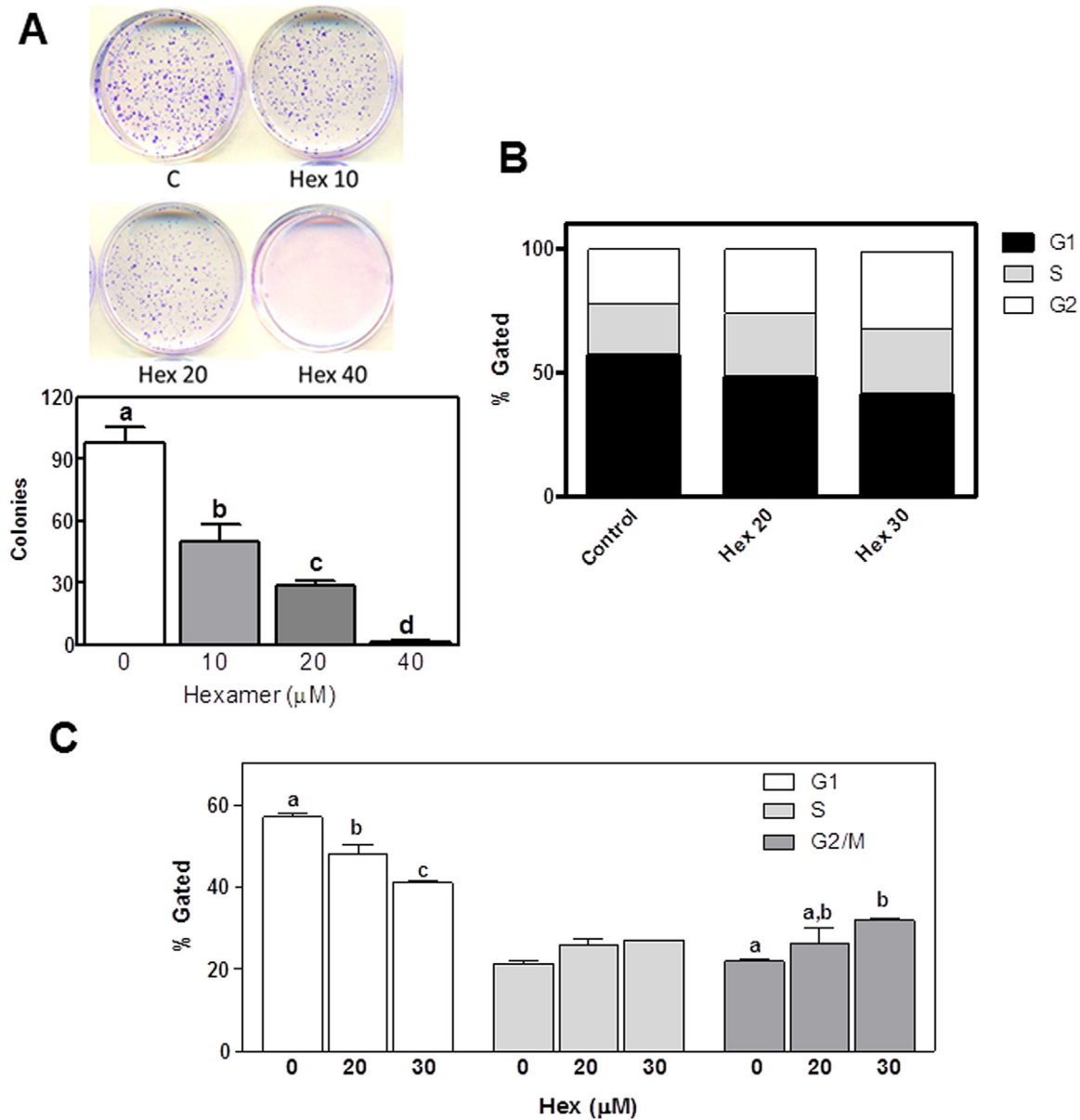
Figure 3. Hex reduced Caco-2 cell colony formation and arrested the cell cycle. (A) The effects of Hex (10–40 μM) on Caco-2 cell colony formation was measured as described in Methods. (B) The effects of Hex (20,30 μM) on Caco-2 cell cycle progression were measured by flow cytometry as described in Methods. (C) The percentage of cells in G_0/G_1, S, and G_2/M was determined from DNA content histograms. Values are shown as means ± SEM of at least 3 independent experiments. Values having different superscripts are significantly different ($P < 0.05$, one way ANOVA test).

oligonucleosomes at 10, 20, and 40 μM Hex, respectively), measured as mono and oligonucleosomes (Figure 4B). Phosphatidylserine externalization, an early-mid apoptotic event, was assessed by Annexin V staining. Incubation of Caco-2 cells with 20–40 μM Hex for 48 h caused an increase in Annexin V positive cells (Figure 4C). Treatment with Hex 20 and 30 μM for 48 h increased the proportion of apoptotic cells 5.6- and 12.1-fold, respectively, compared with controls. We next investigated the potential action of Hex on Caco-2 cell autophagy. The induction of autophagy by Hex is suggested by its capacity to cause a concentration (10–40 μM)-dependent increased protein expression of the autophagy markers Beclin-1 and LC3 (Figure 4D).

Hex Induces Apoptotic Cell Death Via the Intrinsic/Mitochondrial Pathway

Results described above indicate that apoptosis is the major pathway involved in Hex-mediated decrease in Caco 2 cell growth. Thus, we next investigated the pathway involved in Hex-induced apoptotic cell death. Caspase 9 is an initiator caspase
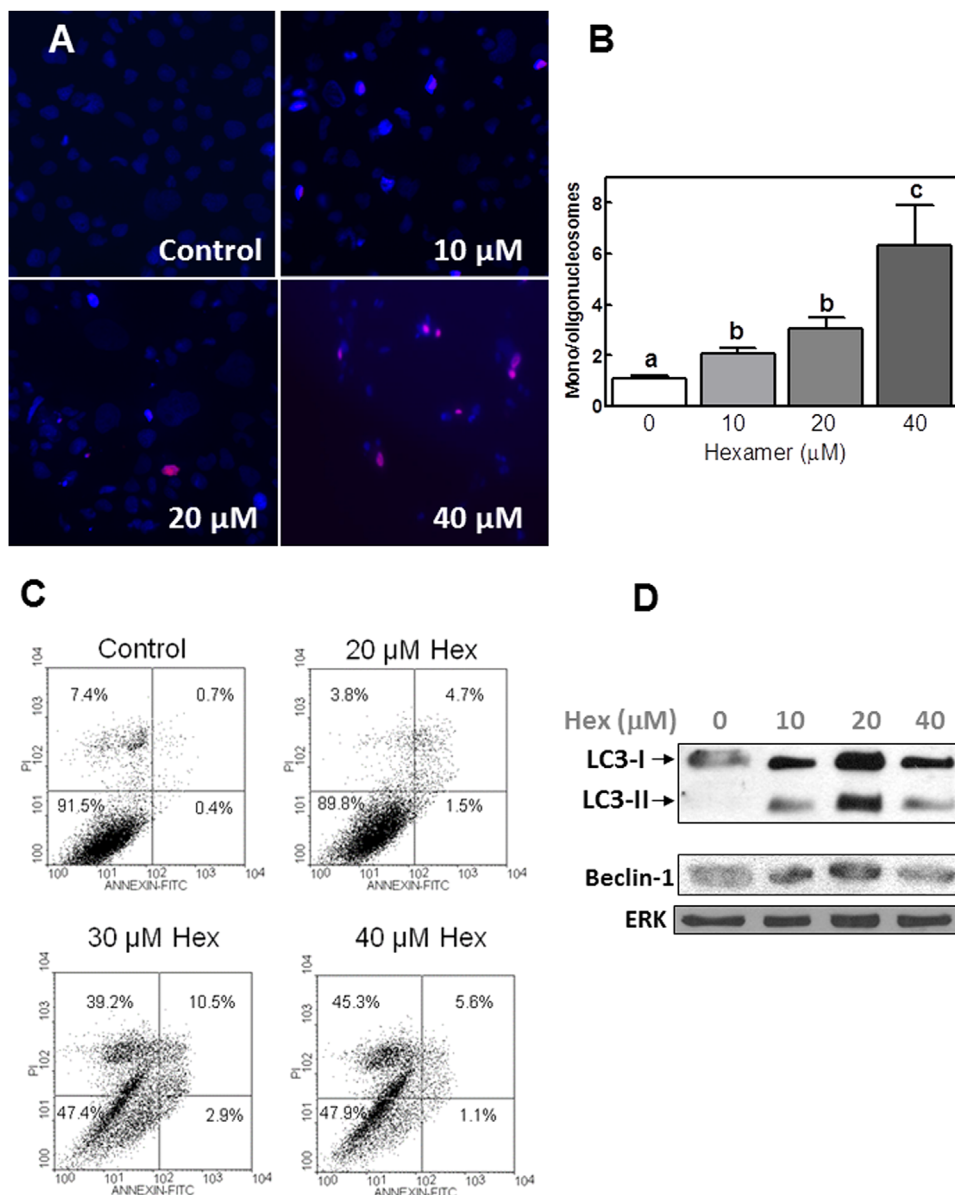
Figure 4. Hex induced CRC cell apoptosis and increased the expression of proteins involved in autophagy. Undifferentiated Caco-2 cells were incubated in the presence of 10–40 μM Hex for 24 (A,B) or 48 h (D). Apoptosis was evaluated as: (A) Hoechst/PI staining and subsequent fluorescence microscopy; (B) mono and oligonucleosomes (DNA fragmentation); (C) phosphatidyl serine externalization (Annexin V staining and subsequent FACS); (D) Beclin-1 and LC3 protein levels were measured by Western blot. For mono and oligonucleosome content data are shown as means ± SEM. At least three independent experiments were done per each assay. Values having different superscripts are significantly different ($P < 0.05$, one way ANOVA test).

involved in the mitochondrial (intrinsic) apoptotic pathway, while caspase 3 is an effector caspase, Hex caused a time (24–72 h)- and concentration (10–40 μM)-dependent increase in caspase 3 and caspase 9 activities (Figure 5A). To confirm that Hex activated the intrinsic apoptotic pathway, the translocation of Bad to the mitochondria, and the associated cytochrome c release from the mitochondria into the cytosol were measured in Caco-2 cells treated with 20 μM Hex for 24 h. Hex caused a significant decrease in cytosolic Bad, an increase in mitochondrial Bad, an increase in cytosolic cytochrome c, and a decrease in mitochondrial cytochrome c (Figure 5B). The above results indicate that Hex promotes apoptotic cell death via the mitochondrial (intrinsic) pathway.

Hex Inhibits the Akt Pathway

Given the role of Akt in the prevention of apoptosis and of autophagy [30,31], we next investigated the effects of Hex on the activation of this kinase and of Akt downstream targets involved in the regulation of cell

survival. Within 24 h, Hex caused a concentration (10–40 μM)-dependent decrease in Akt phosphorylation at Ser 473, and at a lower extent at Thr 308 (Figure 6A). Upstream, Hex inhibited PI3K phosphorylation (Tyr458) in a concentration-dependent manner. Downstream, Akt inhibited the intrinsic mitochondrial pathway by phosphorylating Bad at Ser136, which prevents Bad translocation to

the mitochondria. Hex caused a concentration -dependent decrease in Bad phosphorylation (Ser 136) (Figure 6B). After 24 h incubation, 20–40 μM Hex also decreased the phosphorylation levels of other Akt target, GSK-3β (Ser 21/9).

To further investigate the relevance Akt inhibition in the anti CRC actions of Hex, we studied the capacity of the allosteric Akt inhibitor MK-2206 to
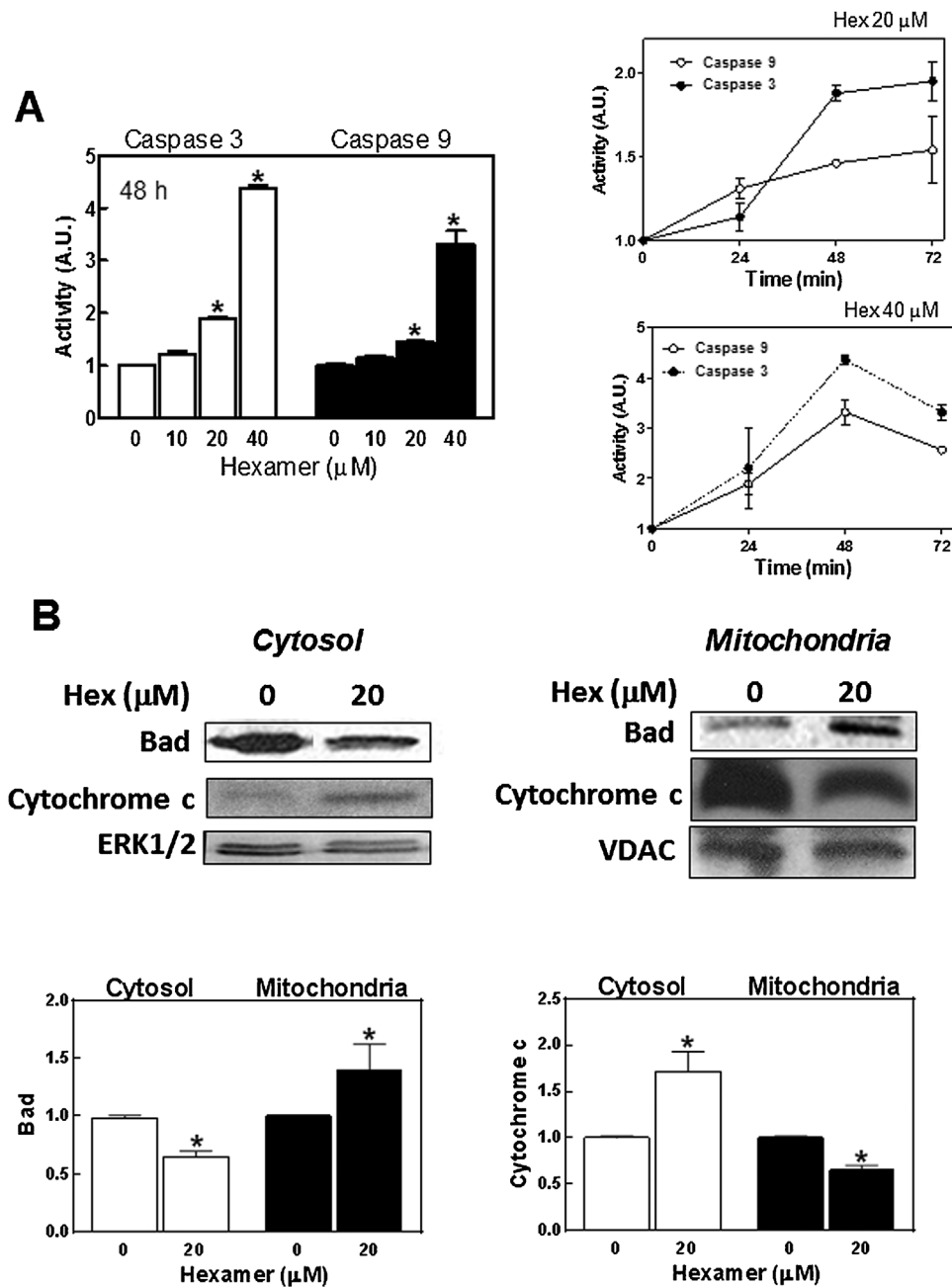

Figure 5. Hex induced apoptotic cell death via the intrinsic/mitochondrial pathway. Undifferentiated Caco-2 cells were incubated in the presence of 10–40 μM Hex for 24–72 h. The occurrence of apoptosis via the intrinsic pathway was evaluated by measuring: (A) caspase 3 and 9 activation; (B) Bad and cytochrome c change of cell compartments by Western blot. ERK1/2 were used as cytosol protein loading control and VDAC for mitochondrial loading control. Values are shown as means ± SEM of at least three independent experiments. Values having different superscripts are significantly different (P < 0.05, one way ANOVA test).

promote Caco-2 cell death. MKK-2206 caused a concentration (1–50 µM)-dependent decrease of Caco-2 cell viability within 24h of incubation (Figure 7A). Hex (20 µM) and MK 2206 showed additive effects reducing cell viability. MKK-2206 induced apoptotic cell death as evaluated by mono/oligonucleosome content (DNA fragmentation) (Figure 7B). Western blots confirmed that

MKK-2206 inhibited Akt phosphorylation at Thr 308 and Ser 473 and the downstream phosphorylation of the Akt targets Bad and GSK-3β (Figure 7C).

Effect of Hex on the Activation of Transcription Factors NF-κB and AP-1

We next evaluated the effects of Hex on the activation of transcription factors that regulate the expression of
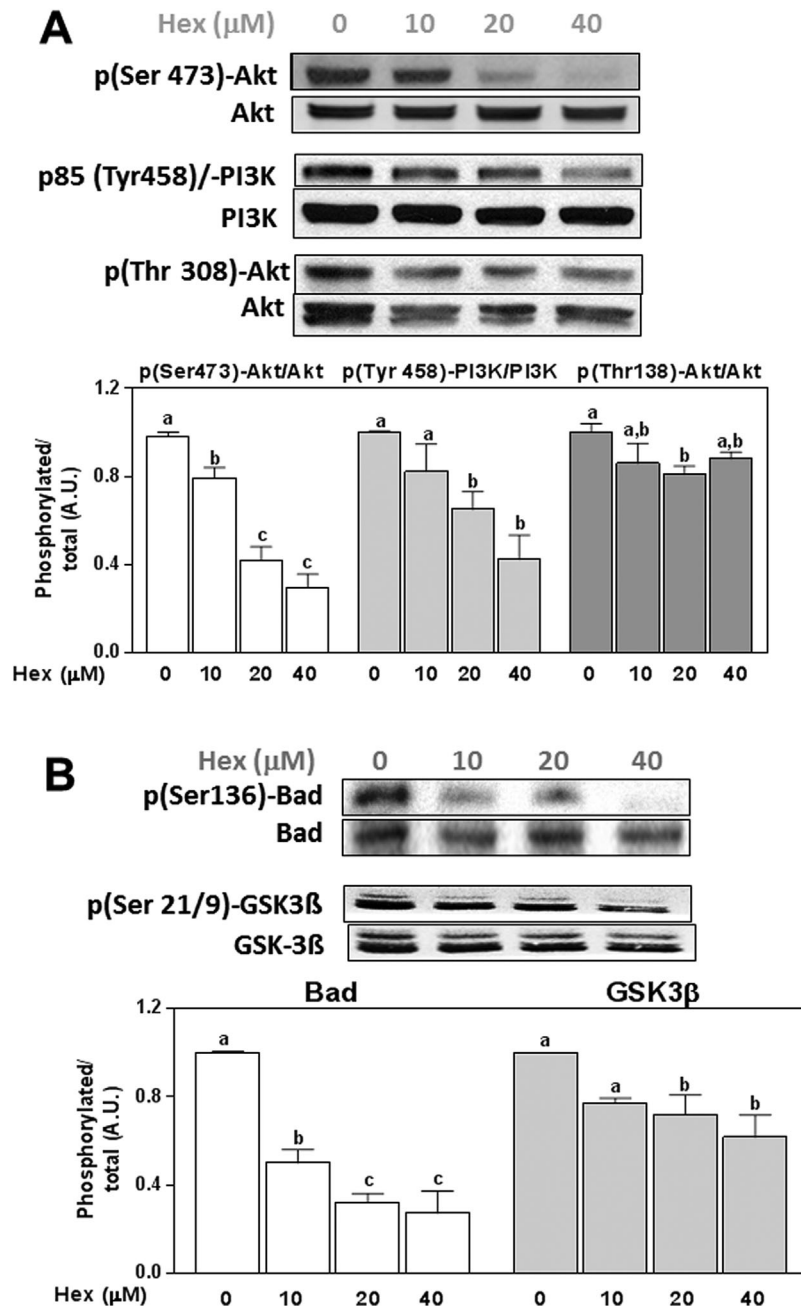

Figure 6. Hex inhibited the Akt signaling pathway. Undifferentiated Caco 2 cells were incubated in the presence of 10–40 µM Hex for 24–72 h. (A) Phosphorylated Akt at Ser473 or Thr308, Akt, p85(Tyr458)-PI3K, and PI3K; and (B) Bad (Ser 136), and GSK-3β (Ser 21/9) phosphorylation were measured by Western blot. The ratios between phosphorylated/total protein content were calculated and results shown as means ± SEM of at least three independent experiments. Values having different superscripts are significantly different ($P < 0.05$, one way ANOVA test).

genes involved in cell proliferation and survival which are found to be over activated in CRC. NF-κB and AP-1 activation was evaluated measuring the transcription factor-DNA binding in nuclear fractions by EMSA. The incubation of cells with 10–40 μM Hex for 24 h caused up to a 2-fold increase in AP-1-DNA binding, while did not affect NF-κB-DNA binding (Figure 8A). Consistently with a lack of effect on NF-κB activation, the expression of the antiapoptotic and NF-κB-regulated proteins Bcl-xL and Bcl-2 remained unchanged after treatment with 10–40 μM Hex (Figure 8B).

DISCUSSION

This paper presents evidence that a hexameric PCA isolated from cocoa exerts anti CRC actions in vitro. Hex suppressed human CRC cell growth by inducing cell death by apoptosis via the mitochondrial pathway, and causing cell cycle arrest in G_2/M phase. In addition, its strong cytokinetic effect was in part mediated by the inhibition of Akt activation and the downstream phosphorylation of select targets, particularly Bad.

The consumption of PCA has been found in epidemiological studies to be associated with a lower CRC risk. Significantly, the risk of CRC and gastric cancers decreased with increasing PCA polymerization degree [32]. Previous studies have demonstrated that in vitro, extracts enriched in PCA can induce apoptosis and inhibit cell proliferation in CRC cell lines [14,33,34]. However, it has remained unclear which constituents in PCA-rich extracts are active, and the mechanism involved in their beneficial actions. Thus, we undertook this study in order to clarify which constituents in PCA-rich extracts could have the most potent cytokinetic activity. Hex strongly reduced the viability of six human CRC cell lines (Caco-2, HCT-116, HT-15, HT-29, LoVo, and SW-480). On the other hand, the monomer ((−)-EC)
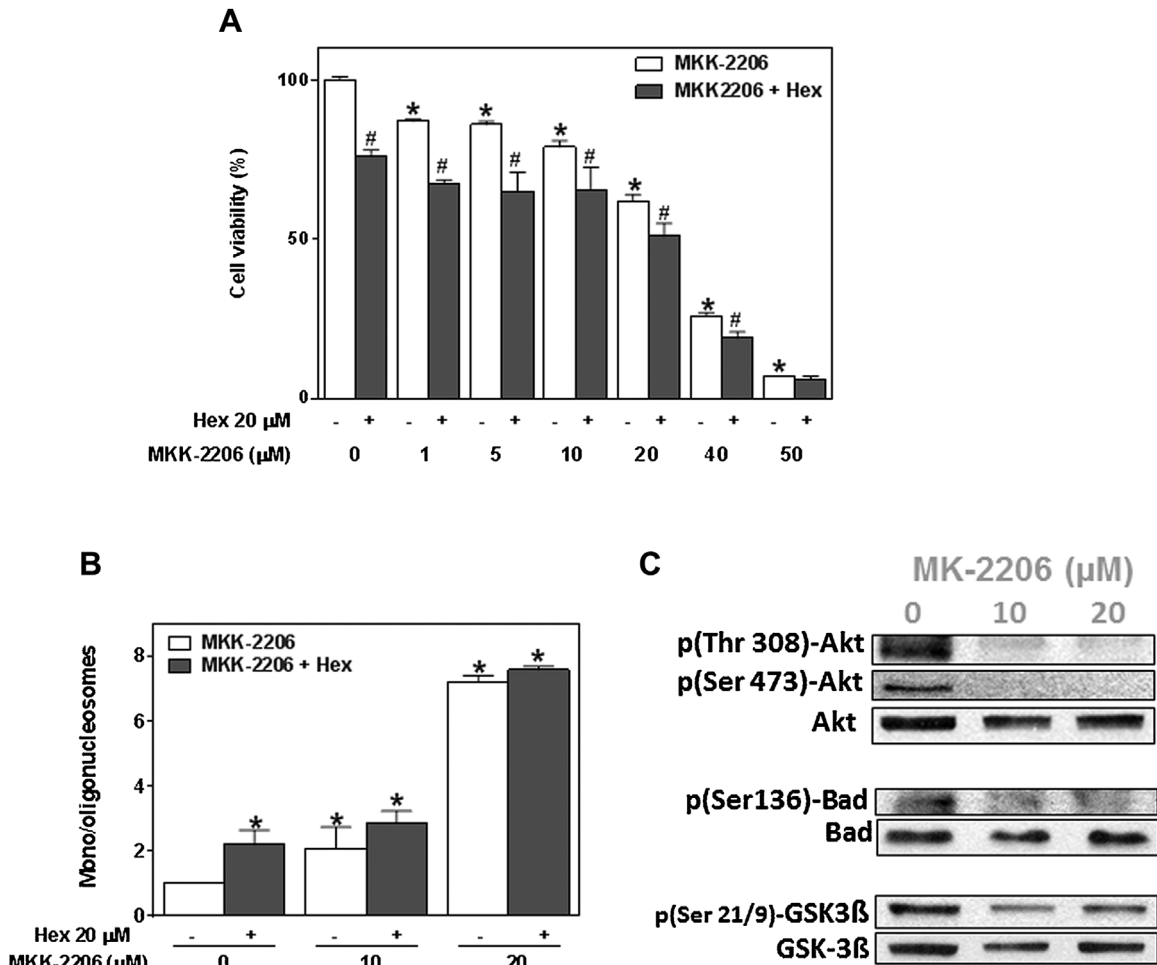

Figure 7. Effects of the Akt inhibitor MKK-2206 on CRC cell viability and apoptosis. Undifferentiated Caco 2 cells were incubated in the absence or presence of 1–50 μM MKK-2206 with or without 20 μM Hex for 24 h. (A) Cell viability was measured as described in Methods. (B) Apoptosis was evaluated as mono and oligonucleosomes (DNA fragmentation). Values are shown as means ± SEM of at least three independent experiments. (C) Phosphorylated Akt at Ser473 or Thr308, Akt, Bad (Ser 136), and GSK-3β (Ser 21/9) phosphorylation were measured by Western blot.
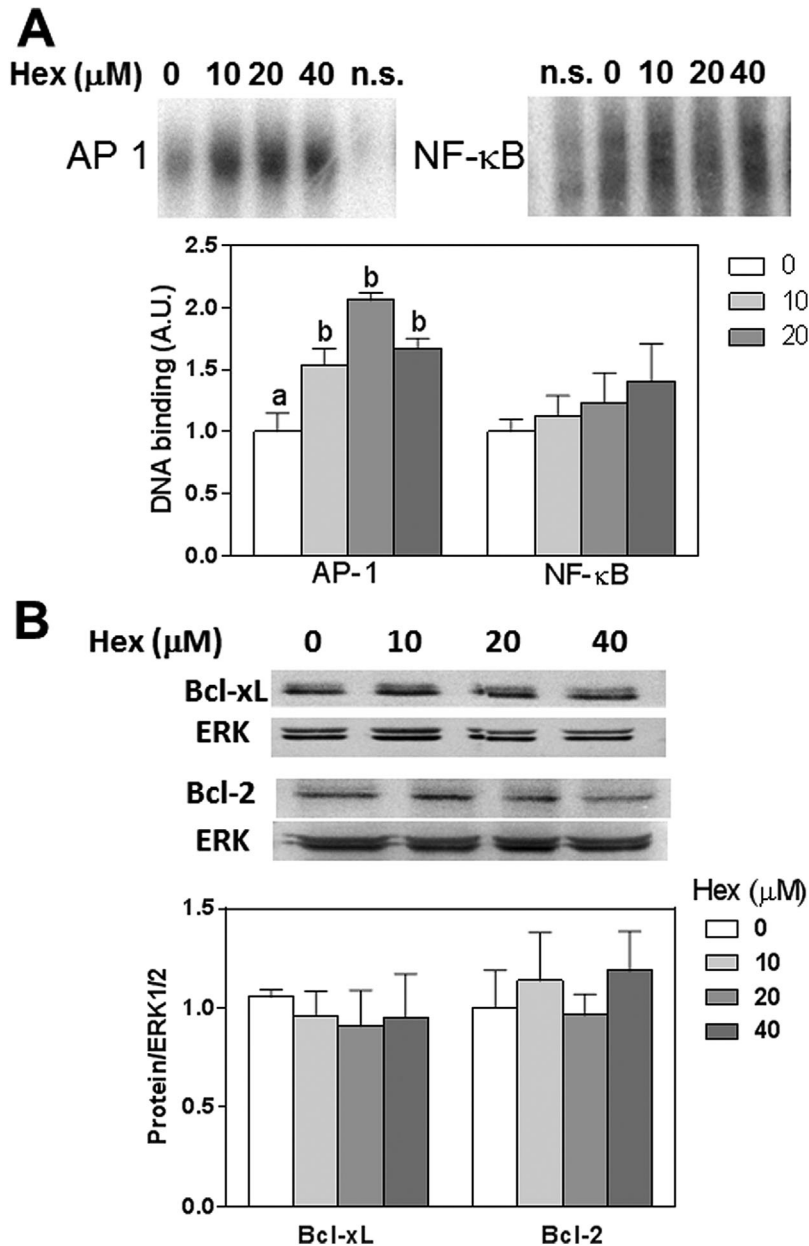
Figure 8. Effects of Hex on AP-1 and NF-κB-DNA binding and expression of NF-κB-regulated antiapoptotic proteins. Undifferentiated Caco-2 cells were incubated in the presence of 10–40 μM Hex for 24 h. (A) AP-1- and NF-κB-DNA binding was measured by EMSA. n.s: control of specificity was done by pre incubating an untreated nuclear fraction sample with a 100-fold molar unlabeled oligonucleotide. (B) Protein levels of Bcl-xL and Bcl-2 were measure by Western blot using ERK1/2 levels as loading controls. For (B) the ratios between Bcl-xL and Bcl-2/ERK1/2 were calculated. Results are shown as means ± SEM of at least three independent experiments. Values having different superscripts are significantly different ($P < 0.05$, one way ANOVA test).

or the trimeric PCA had minor effects, if any, on CRC cell viability. These results are consistent with other studies suggesting a direct relationship between a higher PCA degree of polymerization and a decrease in CRC cell viability [35,36]. The Hex used in this study is purified from cocoa extract and consists primarily of EC and (+)-catechin subunits. Hence, the anti CRC actions of Hex can be specifically attributed to this type of PCA which do not contain galloylated subunits. It is noteworthy that Hex had no effect on the viability of Caco-2 cells differentiated into epithelial intestinal cells. This suggests a differential effect of Hex on proliferating CRC cells, with respect to the differentiated epithelium, which would be central to a CRC preventive effect.

The concentrations of Hex observed to decrease CRC cell viability could be attainable in the colon. This is supported by our previous studies done in rats

and pigs fed diets containing 0.25 and 1% (w/w) GSE, respectively [11,37]. Furthermore, in rats fed with GSE-supplemented diets, the content of PCA levels tends to increase in feces as a function of days in the diet [11]. This suggests a potential accumulation of PCA in the gastrointestinal tract on a basis of regular dietary consumption. Other factors may affect the availability of PCA to cells in the intestinal epithelium, including PCA distribution in the colonic content which may not be uniform, being the hydrophilic PCA potentially concentrated in fecal water.

Hex reduced Caco-2 cell growth through the induction of apoptosis and the inhibition of the progression of the cell cycle at G_2/M phase. Although the capacity of Hex to block the cell cycle progression can be an important anticancer mechanism, its apoptotic effect emerges as Hex's main cytokinetic mechanism. The induction of apoptosis by Hex occurs via the mitochondrial pathway as evidenced by, the translocation of Bad to the mitochondria, the release of cytochrome c to the cytosol, and the activation of caspase 9 and 3. Accordingly, PCA extracted from apples were shown to induce apoptosis in melanoma and mammary tumor cells through the mitochondrial pathway [34].

Hex is a large molecule that is not absorbed by intestinal cells. However, we have previously shown that Hex interacts with the cell membrane [38], particularly with lipid rafts [24] and modulates signaling pathways that can be potentially oncogenic [23]. In this regard, Hex protects differentiated Caco-2 cells from the deleterious effects of the secondary bile product deoxycholic acid [23] and of proinflammatory cytokines [29].

The Akt pathway is overactivated in numerous solid tumors, including CRC [39]. Forty percent of CRC carry mutations that lead to Akt activation [39,40]. Akt plays a central role in the regulation of cell growth and survival by phosphorylating multiple targets including Bad [41] and GSK-3β (reviewed in [42]). Bad phosphorylation by Akt determines its retention in the cytosol. Supporting the involvement of Akt in Hex pro-apoptotic action, Hex mediated Akt inhibition was associated to decreased Bad phosphorylation and consequent increased mitochondrial translocation. Inhibition of GSK-3β phosphorylation by Hex can also underlie its proapoptotic and antiproliferative actions. GSK-3β is a multifunctional serine/threonine protein kinase that regulates important cellular pathways, depending on its substrates for phosphorylation (e.g., β-catenin, cyclin D1, c-myc, c-Jun) [43]. Increased GSK-3β expressions is observed in gastrointestinal-related cancers [44] while GSK-3β inhibition alters proliferation and survival of CRC in vivo [45]. Consistently, both Bad and GSK-3β phosphorylations were inhibited by MK-2206, an allosteric inhibitor of Akt, that has been shown to enhance the anti-cancer action of regularly used chemotherapeutic drugs [46]. In fact, in IGFR-1-dependent CRC cells, MK-2206 induces cell death through caspase dependent and independent mechanisms [47]. We observed that MK-2206 inhibited Akt and downstream targets (Bad, GSK-3β), causing a decrease in cell viability by promoting apoptotic cell death within 24 h, and having an additive effect with Hex. These results further stress the relevance of Akt inhibition in the pro-apoptotic and anti-CRC actions of Hex.

The PI3K/Akt pathway also modulates cell survival by downregulating autophagy [22]. Although the role of autophagy in cancer depends on its stage, it is clear that tumor initiation is prevented by a functional autophagy [30]. In Caco-2 cells, Hex caused an increased expression of proteins involved in autophagy: Beclin-1, and LC3-I and II. Supporting the involvement of the Akt pathway in the modulation of autophagic genes by Hex, constitutive activation of Akt has been found to decrease the expression of both Beclin-1 and LC3-I and II [48]. The antiapoptotic proteins Bcl-2 and Bcl-xL interact with Beclin-1 inhibiting autophagy [30]. However, Hex did not affect Bcl-2 and Bcl-xL expression levels neither the upstream activation of transcription factor NF-κB.

NF-κB and AP-1 are constitutively active in many solid tumors including CRC [49]. While Hex did not affect NF-κB, it caused AP-1 activation in Caco-2 cells. Numerous studies have shown that AP-1 may regulate cell growth, differentiation, apoptosis and tumorigenesis [50]. Thus, therapeutic inhibition of AP-1 activity has attracted considerable interest [51]. Because treatment with Hex led to cell death by apoptosis, still when AP-1 is being activated, this suggests that in Caco-2 cells following Hex treatment, AP-1 does not play a major role in the resistance against apoptosis.

The impact of diet on CRC is a major area of research interest when considering the potential environmental factors that could be modified as strategies in the prevention of CRC development. The association between fruit and vegetable consumption and CRC risk is controversial. A meta-analysis of prospective studies found a weak but statistically significant nonlinear inverse association between them [6]. It is reasonable to consider that the concept of fruit and vegetable consumption as a whole has numerous potential variables. For example, the presence of multiple components and the variation in their relative proportion in different fruits and vegetables, as well as changes in their levels for a particular fruit/vegetable depend on seasonal, cooking, and other variables. This stresses the need to understand the potential CRC preventive action of individual components in fruits and vegetables.

In conclusion, our current findings provide a plausible mechanism to explain epidemiological evidence of an inverse relationship between CRC risk and PCA consumption. PCA can be found in large

amounts throughout the gastrointestinal tract after consumption of PCA-containing extracts [10,11]. Although not absorbable, Hex can exert local effects by interacting with the cell membrane [24]. This work showed that exposure to a large PCA (hexamer) reduces human CRC cell growth by inducing apoptosis and cell cycle arrest, downregulating the oncogenic Akt signaling pathway. Thus, consumption of fruit and vegetables rich in PCA could help reduce the growth of colon cancer cells, thus attenuating CRC development.

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