Biochemical and Biophysical Research Communications 372 (2008) 236–242

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Morusin induces apoptosis and suppresses NF-κB activity in human colorectal cancer HT-29 cells

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ARTICLE INFO

Article history:
Received 2 May 2008
Available online 14 May 2008

Keywords:
Morusin
Apoptosis
Caspases
NF-κB
Colorectal cancer

ABSTRACT

Morusin is a pure compound isolated from root bark of *Morus australis* (Moraceae). In this study, we demonstrated that morusin significantly inhibited the growth and clonogenicity of human colorectal cancer HT-29 cells. Apoptosis induced by morusin was characterized by accumulation of cells at the sub-G_1 phase, fragmentation of DNA, and condensation of chromatin. Morusin also inhibited the phosphorylation of IKK-α, IKK-β and IκB-α, increased expression of IκB-α, and suppressed nuclear translocation of NF-κB and its DNA binding activity. Dephosphorylation of NF-κB upstream regulators PI3K, Akt and PDK1 was also displayed. In addition, activation of caspase-8, change of mitochondrial membrane potential, release of cytochrome *c* and Smac/DIABLO, and activation of caspase-9 and -3 were observed at the early time point. Downregulation in the expression of Ku70 and XIAP was exhibited afterward. Caspase-8 or wide-ranging caspase inhibitor suppressed morusin-induced apoptosis. Therefore, the antitumor mechanism of morusin in HT-29 cells may be via activation of caspases and inhibition of NF-κB.

Root bark of *Morus australis* (*M. australis*, Moraceae; mulberry tree) has been used in traditional Chinese medicine as antiphlogistic, diuretic, expectorant, antitussive, antiheadache, antipyretic, and antidiabetic [1,2]. Morusin, a prenylated flavonoid isolated from *M. australis* [3], has been demonstrated to inhibit arachidonic acid-, PAF-, and collagen-induced platelet aggregation [4], suppress superoxide anion formation [5], and reduce bacterial activity [6]. Notably, no study has addressed on the antitumor action of morusin. Colorectal cancer is one of the leading causes of death and is a major public health problem in western countries as well as in Taiwan [7]. Development of complementary strategies to reduce the burden of this disease is important.

Apoptosis is a regulated process involving activation of various molecules and initiation of cell death. Induction of apoptosis in tumor cells is a potentially promising approach for cancer therapy [8]. The signaling pathway of apoptosis can proceed via the activation of caspases and recruitment of proteins in Bcl-2 family [9]. Nuclear factor-κB (NF-κB) is also related to regulate gene expression in apoptosis. In addition, activation of NF-κB is associated with tumor promotion and progression of colorectal cancers [10]. The data of this study suggest that the antitumor mechanism of morusin may be via induction of apoptosis as well as inhibition of NF-κB.

Materials and methods

Reagents. M. australis. (Moraceae) plants were collected at Kaohsiyung Hsien, Taiwan. Morusin (99.5%) was purified from the dried roots of *M. australis*, and the structure of morusin has been identified [3]. Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Mouse monoclonal anti-caspase-3 and rabbit polyclonal anti-second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (Smac/DIABLO) antibodies were purchased from IMGENEX (San Diego, CA). Mouse monoclonal anti-caspase-9 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal anti-cytochrome *c* and rabbit polyclonal anti-Bid and anti-tBid antibodies were purchased from BD Pharmingen (San Diego, CA). Mouse polyclonal anti-phosphatidylinositol-3-kinase (PI3K), mouse monoclonal anti-X-linked apoptosis-inhibiting protein (XIAP), anti-phosphoinocitide-dependent protein kinase 1 (PDK1), and anti-receptors for activated C-kinase (RACK1) antibodies were purchased from BD Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-phospho-Akt, anti-poly(ADP-ribose) polymerase (PARP), anti-phospho-PDK1, anti-Akt, anti-IκB kinase complex (IKK)-α, anti-IKK-β, and anti-phospho-IKK-α/IKK-β antibodies

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doi:10.1016/j.bbrc.2008.05.023

were purchased from Cell Signaling Technology (Beverly, MA). Z-IETD, Z-VAD, mouse monoclonal anti-Ku70, anti-Bax, anti-Bcl-X_L, rabbit polyclonal anti-DNA fragmentation factor (DFF)-45, anti-DFF-40, anti-IκB-α, anti-phospho-IκB-α, anti-NF-κB p65, goat polyclonal anti-NF-κB p50, anti-phospho-PI3K, anti-mouse conjugated HRP, and bovine anti-goat conjugated HRP secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Goat anti-rabbit conjugated HRP secondary antibody was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell culture. Human colorectal cancer HT-29 cells, human hepatocellular carcinoma Hep 3B cells, human breast adenocarcinoma MCF-7 cells, and human embryonic kidney epithelial HEK293 cells were obtained from ATCC (Rockville, MD). Human peripheral blood mononuclear cells (PBMC) were freshly collected from healthy donors' whole blood at Tainan Blood Bank Center (Tainan, Taiwan). Cells were prepared and maintained in complete Dulbecco's Modified Eagle's Medium (DMEM, GIBCO BRL, Grand Island, NY) in a humidified atmosphere of 5% CO_2 incubator at 37 °C [11].

Cell viability assay. Cytotoxicity was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [11]. After treatment, MTT was added to each well to a final concentration of 0.5 mg/mL. After adding 10% SDS in 0.01 N HCl to each well, the absorbance of each well was determined at 590 nm in a Multiscan photometer (MRX II, Dynatech, McLean, VA).

Colony formation assay. The base layer was 1 mL of 0.6% agar containing DMEM with 10% FCS. The upper layer was made by mixing cells (6×10^4) in 0.1 mL DMEM with 0.9 mL of 0.33% agar in DMEM supplemented with 10% FCS in the presence or absence of morusin. Colonies with diameter larger than 1 mm were counted [11].

Flow cytometry. For cell cycle analysis, cells (1×10^5) were washed with Hepes buffer solution (HBS) and resuspended in 70% ethanol. After centrifugation, the pellets were resuspended in HBS containing 40 μg/mL propidium iodide (PI) and 100 mg/mL RNase A at 37 °C [11]. To measure mitochondrial membrane potential ($\Delta\psi m$), cells were stained with 5 μM rhodamine 123 [11] and then analyzed in a FACScan flow cytometer (Becton–Dickson, Mountain View, CA).

Analysis of nuclear morphology. Cells were fixed with 4% paraformaldehyde in PBS. After permeabilization, cells were stained with 0.5 μg/mL Hoechst 33258 in PBS at 37 °C for 30 min [11]. Changes of nuclear morphology were visualized under fluorescence microscope (Leica DMRBE microscope).

Analysis of DNA fragmentation. Cells (1×10^6) were lysed with lysis buffer [11]. After centrifugation, the supernatants were incubated with a solution containing 4 mg/mL RNase A and 1% SDS at 56 °C for 2 h. After adding proteinase K to a final concentration of 1 mg/mL, the mixtures were incubated at 37 °C overnight. After precipitation, the DNA fragments were separated on a 1% (w/v) agarose gel [11].

Subcellular fractionation and Western blot analysis. Cells (1×10^6) were lysed with lysis AKTP buffer [11]. After centrifuging at 15,000*g* for 10 min, the supernatants were used as the whole cell lysates for immunoblotting [12]. For preparation of cytosolic and mitochondrial fractions, cells were centrifuged at 800*g* and resuspended in TSE buffer [11]. The cell suspensions were then transferred to a Dounce homogenizer (Glas-Col, Terre Haute, IN) and broken with 10 strokes of the Teflon pestle. The cell homogenates were centrifuged at 750*g* at 4 °C for 30 min. The supernatants were centrifuged at 12,000*g* at 4 °C for 30 min. The lysed solutions were centrifuged again at 100,000*g* for 1 h and their supernatants were used as cytosolic fractions. The pellets were then lysed in AKTP lysis buffer and used as mitochondrial fractions. For nuclear fraction preparation, cells were lysed in buffer A [11]. After centrifugation at 11,000*g* at 4 °C for 10 s, the pellets were resuspended in buffer B [11] and used as nuclear fractions.

Electrophoretic mobility shift assay (EMSA). The EMSA of nuclear extracts was performed as described previously [12]. The double-stranded oligonucleotide probe with the consensus binding sequence for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega, Madison, WI) was 3′ end-labeled with digoxigenin-ddUTP using a DIG gel shift kit (Roche Molecular Biochemicals, Mannheim, Germany). A competition assay was carried out by adding a 100-fold excess of the unlabeled oligonucleotides or unlabeled mutant NF-κB oligonucleotides (5′-AGTTGAGGCGACTTTCCCAGGC-3′; Santa Cruz Biotechnology) to the nuclear extracts. The gels were transferred to Hybond-N plus membrane (Amersham Biosciences, UK Limited, Buckinghamshire, UK), dried and subjected to autoradiography.

Results

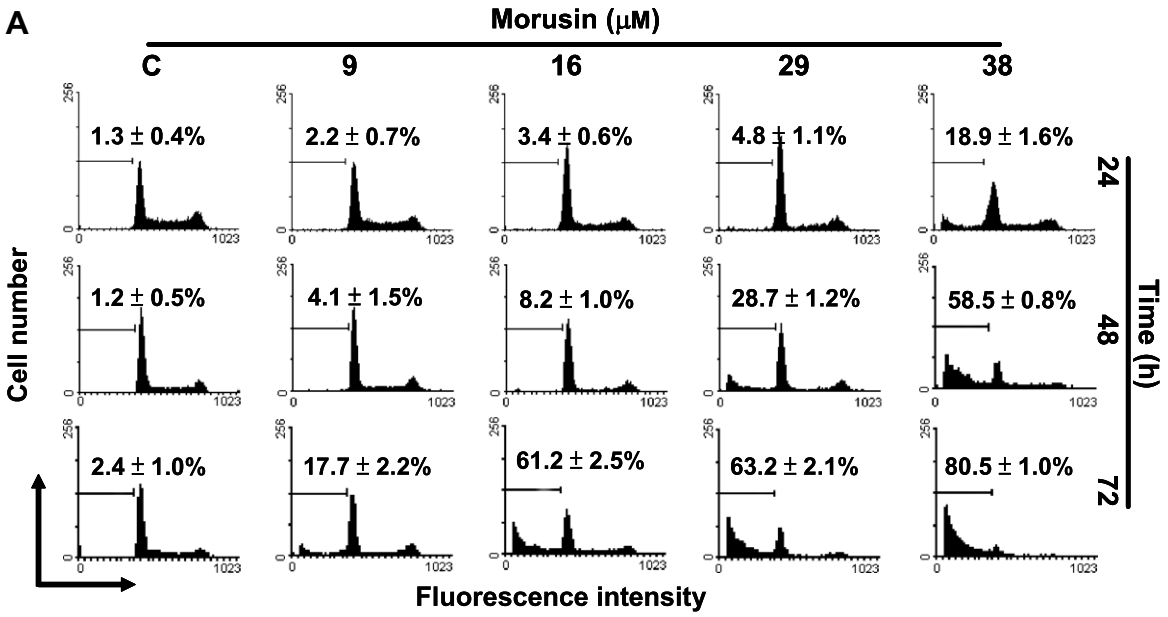
Morusin inhibits growth and induces apoptosis of HT-29 cells

The 50% inhibitory concentration (IC_{50}) for HT-29, Hep 3B and MCF-7 cells on growth was 6.1 ± 0.3, 8.5 ± 1.1 and 12.7 ± 2.1 μM, respectively, at 6 days of post-treatment. The IC_{50} for these cells to form colony at 14 days of post-treatment was 2.8 ± 0.1, 9.2 ± 1.2 and 10.3 ± 1.6 μM, respectively. In spite of this, the IC_{50} of morusin on the growth of HEK293 cells and PBMC by cell viability assay was 23.9 ± 1.1 and 29.8 ± 0.1 μM, respectively. To evaluate the induction of apoptosis, increase in the percentage of sub-G_1 DNA content was determined by flow cytometry. In Fig. 1A, morusin increased the population of sub-G_1 HT-29 cells in a time- and dose-related manner. Enhancement of DNA fragments was also exhibited in a time- and dose-related manner in response to morusin (Supplementary Fig. 1). To further confirm the induction of apoptosis by morusin, the nuclei of HT-29 cells were stained with Hoechst 33258 fluorescence. As shown in Supplementary Fig. 2, the number of cells with condensed chromatin was increased with morusin in a time-related manner.

Effect of morusin on mitochondria-related protein expression in HT-29 cells

As shown in Fig. 1B, expression of inactive pro-apoptotic Bid was decreased in the cytosol, whereas its active form of truncated Bid (tBid) was increased in the mitochondria. Translocation of another pro-apoptotic protein, Bax, from the cytosol to the mitochondria (~5-fold) was also revealed at 12–72 h (Fig. 1B). Although total expression of anti-apoptotic Bcl-X_L was not affected, a significant decrease in mitochondrial Bcl-X_L was displayed at 48 h and reached the lowest level (60% decrease) at 72 h (Fig. 1B). Downregulation of Ku70 has been reported to enhance Bax-mediated apoptosis [13]. In Supplementary Fig. 3, significant decrease in the level of total and cytosolic Ku70 was observed at 48 h. The expression of cytosolic Ku70 was almost undetectable at 72 h. On the other hand, nuclear expression of Ku70 remained the same (Supplementary Fig. 3).

To ascertain the role of mitochondria in morusin-treated HT-29 cells, $\Delta\psi_m$ was evaluated by flow cytometry. In Fig. 2A, time- and dose-related increase in the percentage of cells with damaged mitochondria was observed. Change of $\Delta\psi_m$ is associated with increase in the permeability of outer mitochondrial membrane which allows efflux of apoptogenic proteins to the cytosol [14]. To address this, the release of cytochrome *c* and Smac/DIABLO from the mitochondria to the cytosol was determined by immunoblotting. As shown in Fig. 2B, increase in cytochrome *c* and Smac/DIABLO in the cytosol was first observed at 12 h (1.8- and 2.4-fold, respectively) and reached the highest level at 72 h (9.9- and 5.4-fold, respectively). As expected, the expression of mitochondrial cytochrome *c* and Smac/DIABLO was decreased with time (80



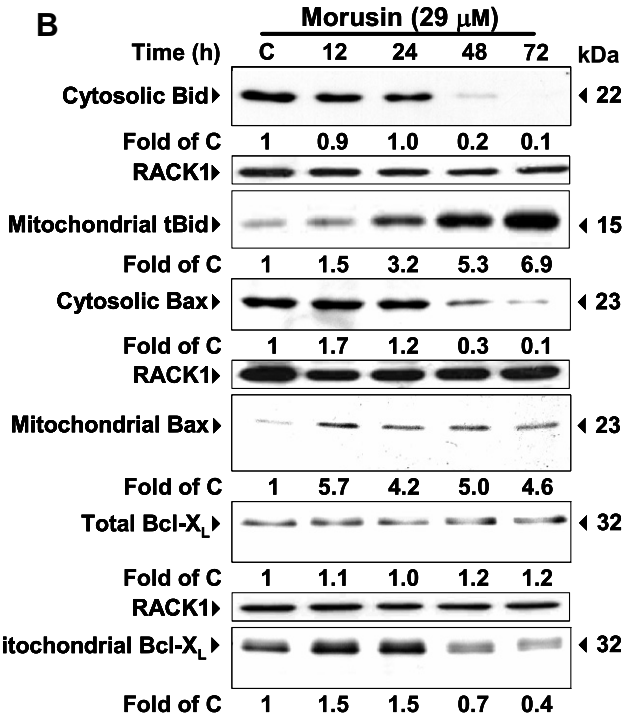


Fig. 1. Effects of morusin on HT-29 cells. (A) Morusin-induced apoptosis in HT-29 cells. Cells (1×10^5) on 6-well dish were stained with PI and analyzed by flow cytometry for DNA content. The percentages in the figure indicate the proportion of apoptotic cells at the sub-G_1 phase. (B) Alternation in expression of Bcl-2 family in morusin-induced HT-29 cells. Total cell lysates, cytosolic or mitochondrial fractions of HT-29 cells (1×10^6) were subjected to Western blotting. The intensity of individual protein signal was normalized to that of RACK1, with control levels arbitrarily set to 1. Morusin was dissolved in dimethyl sulfoxide (DMSO) and then diluted with DMEM to yield the final concentrations. The control cells (C) were treated with DMEM containing 0.1% DMSO. Results are representative of three independent experiments.

and 70% decreases at 72 h, respectively) (Fig. 2B). Cytosolic Smac/DIABLO has been reported to bind XIAP and neutralize its anti-apoptotic activity [15]. In this study, decrease in total XIAP was first detected at 24 h (20% decrease) and reached the lowest (90% decrease) at 72 h (Fig. 2B).

Morusin induces caspase activity

Apoptosis can also be regulated by members of caspases. In Fig. 2C, morusin significantly increased the cleavage form of active caspase-8 at 12 h of treatment, and this activation sustained to 72 h. Increase in the active form of cleaved caspase-9 (Fig. 2C) and caspase-3 (Fig. 2C) was also observed at 12–72 h. As expected, increase in cleaved PARP (2.6-fold), a substrate of caspase 3, was observed in the nuclei at 48 h of treatment (Supplementary Fig. 4). In Supplementary Fig. 4, expression of DFF-45 was not reduced greatly, while the protein level of DFF-35 was decreased at 48 h and reached the lowest (80% decrease) at 72 h. In addition, increase in nuclear DFF-40 was observed (Supplementary Fig. 4). Of note, admin-

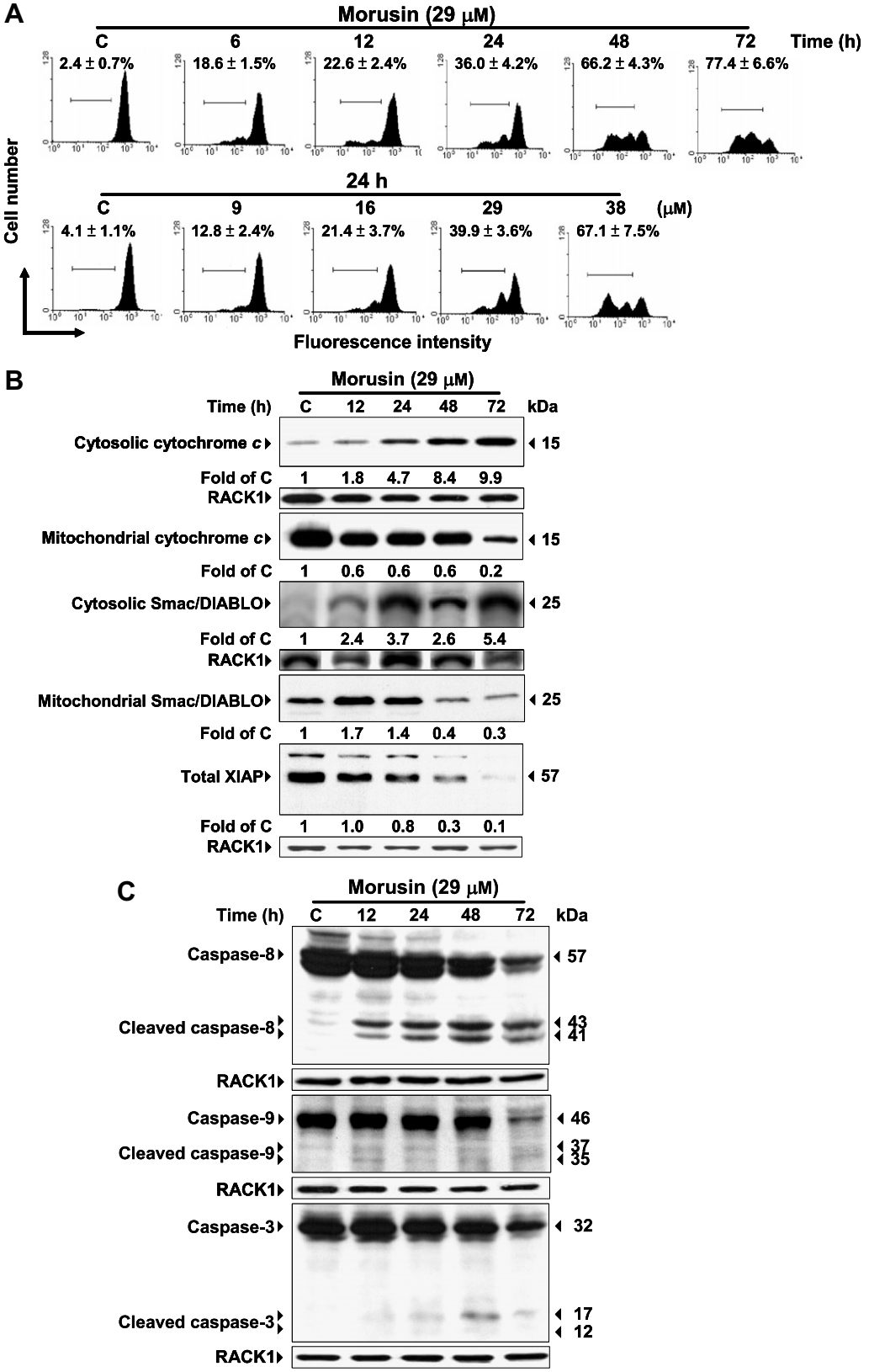


Fig. 2. Effect of morusin on mitochondria, caspases and their target proteins in HT-29 cells. (A) The effect of morusin on $\Delta\psi_m$ was evaluated by flow cytometry after staining the cells with rhodamine 123. The percentages in the figure indicate the proportion of apoptotic cells with the changes of $\Delta\psi_m$. (B) Subcellular distribution of cytochrome *c* and Smac/DIABLO, and level of XIAP in morusin-treated cells. (C) Expressions of caspase-8, -9 and -3. Whole cell lysates were subjected to Western blotting. Results are representative of three independent experiments.

istration of caspase-8 specific inhibitor (Z-IETD) or wide-ranging caspase inhibitor (Z-VAD) significantly suppressed morusin-induced increase in sub-G_1 DNA content (Supplementary Fig. 5).

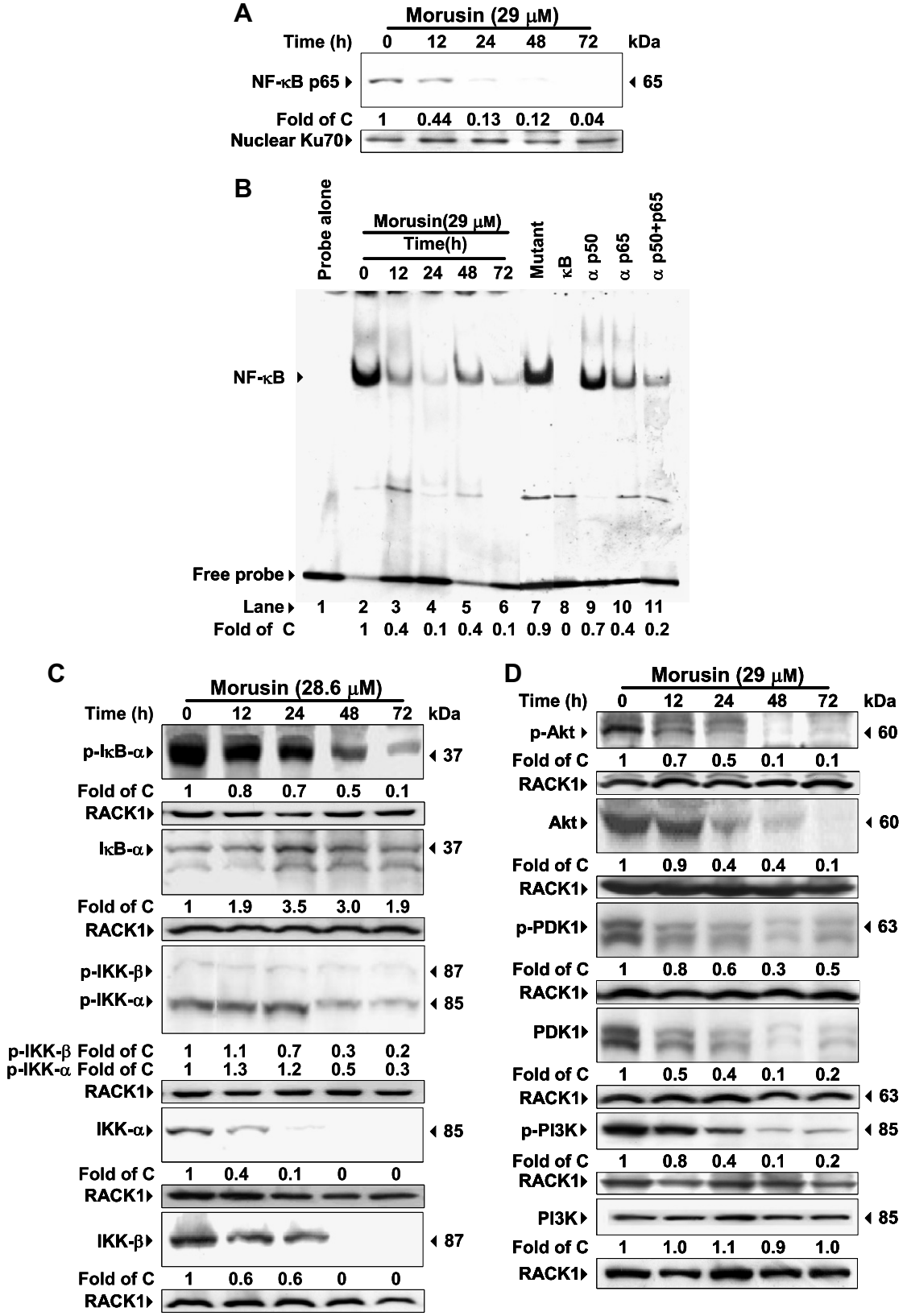


Fig. 3. Inhibition of NF-κB activity and its related proteins in morusin-treated HT-29 cells. (A) Nuclear fractions were subjected to Western blotting to determine the expression of NF-κB. (B) Determination of NF-κB DNA binding activity by EMSA. Except for the free probe control (lane 1), nuclear proteins (10 μg) were incubated with a Dig end-labeled NF-κB oligonucleotide. The NF-κB band was confirmed by competition of the nuclear protein obtained from the HT-29 cells treated with vehicle with a 100-fold excess of unlabeled mutant κB (lane 7) or unlabeled κB (lane 8). The nuclear protein was incubated in an EMSA-binding reaction with an anti-p50 (5 μl, lane 9), anti-p65 (5 μl, lane 10) or anti-p50 (5 μl) plus anti-p65 (5 μl, lane 11) antibodies. The NF-κB-DNA binding activity (lanes 2–6) was quantified by densitometry, with control levels arbitrarily set to 1. (C) Whole cell lysates were subjected to Western blotting to determine the phosphorylation of IκB-α, IKK-α and IKK-β. (D) Whole cell lysates were subjected to Western blotting to determine the phosphorylation of Akt, PDK1 and PI3K. Results are representative of three independent experiments.

Morusin decreases NF-κB activity in HT-29 cells

Morusin dramatically decreased nuclear NF-κB p65 subunit at 12 h (more than 50% decrease) of treatment and reached the lowest level at 24–72 h (more then 90% decrease) (Fig. 3A). Consistently, significant decrease in DNA-binding activity of nuclear NF-κB was detected by EMSA at 12–72 h, ranging from 60 to 90% decrease (Fig. 3B, lanes 3–6). The NF-κB bands were confirmed by observing the elimination of a NF-κB band in the presence of unlabeled κB (Fig. 3B, lane 8). Unlabeled mutant κB had no effect (Fig. 3B, lane 7). The involvement of NF-κB subunits was also confirmed by showing the suppression of NF-κB bands in the presence of anti-p50 and/or anti-p65 antibodies (Fig. 3B, lanes 9–11). To characterize the signaling pathway in the inhibition of NF-κB activity in morusin-treated HT-29 cells, change of its upstream regulators was determined. As shown in Fig. 3C, decrease in IκB-α phosphorylation (20 and 90% decrease at 12 and 72 h, respectively) and increase in total IκB-α protein expression (1.9- and 3-fold increase at 12 and 48 h, respectively) were observed. Furthermore, morusin significantly decreased the phosphorylation (30% decrease for IKK-β at 24 h and 50% decrease for IKK-α at 48 h) and total protein expression (60 and 40% decrease at 12 h) of both IKK-α and IKK-β. Subsequently, the effect of morusin on the phosphorylation of PI3K and Akt was examined. As shown in Fig. 3D, dephosphorylation of both PI3K and Akt in HT-29 cells was first observed at 12 h and sustained to 72 h of morusin treatment, ranging from 20 and 30% decrease at 12 h to 80 and 90% decrease at 72 h for PI3 K and Akt, respectively. A study also indicates that phosphorylation of Akt can be triggered by PDK1 [16]. In Fig. 3D, we observed dephosphorylation of PDK1 at 12 h (20% decrease) of morusin treatment.

Discussion

The constitutive activation of NF-κB in the colon cells has been reported to increase the production of proinflammatory molecules and result in colitis and colon cancer [10]. Drug resistance has also been reported to associate with NF-κB [17]. In the present study, morusin significantly suppressed the constitutive activity of NF-κB in HT-29 cells (Fig. 3A–D). Dephosphorylation of IKK-α, IKK-β and IκB-α, elevation of IκB-α protein expression (Fig. 3C) and involvement of p50/p65 (Fig. 3A and B) suggest that the inactivation of NF-κB by morusin may be via the classical pathway [18]. In addition to these observations, dephosphorylation of PI3K, Akt, and PDK1 in HT-29 cells was displayed at 12–72 h (Fig. 3C and D). In the PI3K-dependent pathway, phosphorylation of its downstream molecules Akt and then IKK-α is required [18]. A report also indicates that PDK1 resides upstream of Akt to control its phosphorylation status [16]. In conjunction with the reported cascade, it appears that the inhibition of NF-κB activity by morusin may result from the suppression of PDK1, PI3K, and subsequently sequential inhibition of Akt and IKK in HT-29 cells.

Recently, NF-κB is found to promote cell survival by upregulation of XIAP expression [19]. In the present study, significant decrease in total XIAP protein expression by morusin was observed (Fig. 2B), which may further promotes the activation of caspase 9 [20]. NF-κB can also increase the expression of anti-apoptotic Bcl-X_L to promote cell survival [21]. However, we did not observe the change in the expression of Bcl-X_L (Fig. 1B). Although downregulation in XIAP expression implies the involvement of NF-κB in morusin-induced apoptosis [19], the use of Z-IETD or Z-VAD suppressed morusin-induced apoptosis (Supplementary Fig. 5). The weights of NF-κB in morusin-induced apoptosis of HT-29 cells need further investigation.

Caspase-8 is a key initiator caspase which triggers the cleavage of Bid into tBid and results in dysfunction of mitochondria and release of apoptosome to activate caspase-9 and -3 [15]. In the present study, activation of caspase-8, -9 and -3 is detected in response to the treatment of morusin (Fig. 2C). In concert with the activation of caspase-8 at 12 h, increase in mitochondrial tBid was observed (Fig. 1B). Significantly increase in $\Delta\psi_m$ (Fig. 2A), release of cytochrome *c* and Smac/DIABLO (Fig. 2B), and activation of caspase-9 (Fig. 2C) were also displayed at 12 h of treatment. Thus, apoptosis of HT-29 cells by morusin at the early time point (12 h) may be proceeded by activation of caspase-8, increase in mitochondrial tBid, dysfunction of mitochondria, release of cytochrome *c* and Smac/DIABLO, and activation of caspase-9 and -3. PARP is a substrate of caspase-3 [22]. Active caspase-3 can also catalyze DFF-45 and DFF-35 [23,24]. Cleavage of PARP and translocation of DFF-40 (Supplementary Fig. 4) in nuclei indicate the activity of caspase-3 (Fig. 2C) and also suggest the results of DNA fragmentation (Supplementary Fig. 1). In addition, morusin did not significantly alter total or cytosolic Ku70 (Supplementary Fig. 3), or mitochondrial Bcl-X_L (Fig. 1B) before 48 h of treatment, which suggests that Ku70 and Bcl-X_L proteins are not critical in the early morusin-induced mitochondrial dysfunction. Of interest, the apoptosis phenomena (Fig. 1A and Supplementary Figs. 1 and 2) were also elevated greatly at 48 h compared with those at 24 h. Taken together, the apoptotic characteristics appear at 24 h may be mainly due to the activation of caspase-8, -9, -3 and DFF-40 at 12 h, and the decrease in XIAP at 24 h. The involvement of PARP, Ku70 and Bcl-X_L at the later time point (48 h) further assists the process of death signaling cascades. Although Fas expression has been reported to play a critical role for induction of apoptosis in HT-29 cells [25], morusin-induced apoptosis of HT-29 cells may not be death receptor Fas-related since administration of anti-Fas neutralization antibody (ZB-4) did not change morusin-induced increase in the percentage of sub-G_1 DNA content (unpublished data).

In conclusion, morusin might act through the following mechanism to induce apoptosis in human colorectal cancer HT-29 cells: morusin may activate caspase-8 to elevate mitochondrial tBid and Bax to permeabilize the outer mitochondrial membrane, allowing efflux of cytochrome *c* and Smac/DIABLO into cytosol. Cytosolic cytochrome *c* and Smac/DIABLO may subsequently cause the activation of caspase-9 and -3 to increase nuclear DFF40 and cleaved PARP for the results of DNA fragmentation. Morusin may also decrease phosphorylation of PDK1, PI3K and then Akt to downregulate the phosphorylation of IKK-α, IKK-β and IκB-α to inhibit the activity of NF-κB which therefore suppresses the inhibitory effect of XIAP to support the progress of apoptosis.

Acknowledgment

This study was supported by grants from the National Science Council, Taiwan, Republic of China (NSC 95-2313-B-309-001).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.023.

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