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Kaempferol Induces Apoptosis in Two Different Cell Lines Via Akt Inactivation, Bax and SIRT3 Activation, and Mitochondrial Dysfunction

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ABSTRACT

Kaempferol (3,4′,5,7-tetrahydroxyflavone) is a flavonoid with anti- and pro-oxidant activity present in various natural sources. Kaempferol has been shown to posses anticancer properties through the induction of the apoptotic program. Here we report that treatment of the chronic myelogenous leukemia cell line K562 and promyelocitic human leukemia U937 with 50 μM kaempferol resulted in an increase of the antioxidant enzymes Mn and Cu/Zn superoxide dismutase (SOD). Kaempferol treatment induced apoptosis by decreasing the expression of Bcl-2 and increasing the expressions of Bax. There were also induction of mitochondrial release of cytochrome *c* into cytosol and significant activation of caspase-3, and -9 with PARP cleavage. Kaempferol treatment increased the expression and the mitochondria localization of the NAD-dependent deacetylase SIRT3. K562 cells stably overexpressing SIRT3 were more sensitive to kaempferol, whereas SIRT3 silencing did not increase the resistance of K562 cells to kaempferol. Inhibition of PI3K and de-phosphorylation of Akt at Ser473 and Thr308 was also observed after treating both K562 and U937 cells with kaempferol. In conclusion our study shows that the oxidative stress induced by kaempferol in K562 and U937 cell lines causes the inactivation of Akt and the activation of the mitochondrial phase of the apoptotic program with an increase of Bax and SIRT3, decrease of Bcl-2, release of cytochrome *c*, caspase-3 activation, and cell death. J. Cell. Biochem. 106: 643–650, 2009.

KEY WORDS: KAEMPFEROL; Bax; Bcl-2; SIRTUINS; APOPTOSIS

The role of free radicals and active oxygen in the pathogenesis of numerous human diseases including cancer, aging, and atherosclerosis is becoming increasingly recognized [Halliwell et al., 1992]. Much attention has been focused on the use of antioxidants, especially natural antioxidants, to inhibit lipid peroxidation or to protect from damage by free radicals. Previous studies have shown that plant phenolic compounds are effective antioxidants. Polyphenol constituents were reported to inhibit lipid peroxidation [Ratty and Das, 1988; Xie and Sastry 1993], to scavenge free radicals [Hanasaki et al., 1994], and to inactivate lipoxygenase [Xie et al., 1993]. However, plant phenolics have sometimes been found to show pro-oxidant properties [Laughton et al., 1989; Gow et al., 1997]. Several flavonoids have been shown to auto-oxidize and generate reactive oxygen species (ROS), such as hydrogen peroxide. They are also capable of reducing Fe^{3+} to Fe^{2+}, resulting in the formation of hydroxyl radicals by reacting Fe^{2+} with H_2O_2.

The flavonoid kaempferol has been shown to generate ROS and to induce apoptosis when used at a 50 μM concentration [Bestwick et al., 2007]. In fact, treatment of glioma or non-small cell lung carcinoma cells with kaempferol activated apoptosis and induced an oxidative stress [Sharma et al., 2007]. In these cells, kaempferol-induced apoptosis proceeded through SOD activation, loss of mitochondrial membrane potential, caspase-3 cleavage, and loss of cell viability [Leung et al., 2007].

Apoptosis, or programmed cell death, is a highly regulated process that involves activation of a series of molecular events

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leading to cell death. Several pathways have been described regulating apoptosis during development, tumorigenesis, and chemical treatments [Reed, 2001]. The expression of several genes has been demonstrated to be critical for the regulation of apoptosis such as caspase cascades and Bcl-2 family proteins. Activation of the caspase cascade is involved in chemical- and agent-induced apoptosis. Activated caspase-9 cleaves and activates the executioner caspase-3 from the inactive pro-caspase-3 [Joza et al., 2002].

Members of the Bcl-2 family proteins can be divided into two sub-families, one being anti-apoptotic and including proteins like Bcl-2, Mcl-1, Bcl-xL, and the other being pro-apoptotic and including proteins like Bax, Bcl-xs, and Bad. Induction of pro-apoptotic Bcl-2 family proteins and inhibition of anti-apoptotic family proteins have been detected in apoptosis induced by chemicals [Lee et al., 2001].

Sirtuins are a family of proteins conserved in all domain of life and involved in many cellular processes from caloric restriction to ageing [Yamamoto et al., 2007]. So far seven sirtuins proteins [SIRT 1–7] have been identified in mammalian cells and they have been shown to posses NAD^+-dependent deacetylase and mono-ADP-rybosyl transferase activity [Saunders and Verdin, 2007]. SIRT3, a member of this family, has a mitochondrial localization [Allison and Milner, 2007]. In particular, SIRT3 has been shown to possess a pro-poptotic activity in several cell lines [Allison and Milner, 2007]. However, little is known about the involvement of this protein in the cell death induced by oxidative stress.

In this study we investigated the mechanism of kaempferol-induced apoptosis in the K562 and U937 cell lines. We demonstrate that the oxidative stress induced by kaempferol increases Bax and SIRT3 and decreases Bcl-2 levels. This change in expression is followed by the release of cytochrome *c*, caspase-3 activation, inhibition of PI3K and Akt and cell death.

MATERIALS AND METHODS

CELL CULTURES AND TREATMENTS

Human chronic myelogenous leukemia cell line K562 and promyelocitic human leukemia U937 were purchased from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were routinely collected by centrifugation at 700*g* and resuspended in fresh medium at a concentration of 2×10^5/ml. Cell viability was assessed by Trypan Blue exclusion.

In all experiments, kaempferol (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) was dissolved in dimethyl sulfoxide [Me_2SO] and added for 24, 48,and 72 h to a final concentration of 15, 25, and 50 μM as indicated in the figures. Me_2SO had no effect on any of the parameters measured.

MEASUREMENT OF CELL VIABILITY

Cell viability was determined by Trypan blue exclusion and Flow Cytometry as described below.

FLOW CYTOMETRY

Cells [2×10^5] were plated in 6-well plates. The following day the cells were treated with 50 μM Kaempferol. After the treatment the cells were harvested by centrifugation [10 min at 590*g* at 4°C], washed with 5 ml PBS, and resuspended in 500 μl PBS to which 5 ml of cold 70% EtOH was slowly added while stirring. Following overnight incubation at 4°C, cells were centrifuged at 500*g* for 5 min at 4°C and washed once with PBS. The cells were then resuspended in 300 μl of a 20 μg/ml Propidium iodide/250 μg/ml RNase A solution and kept at 37°C for 30 min. DNA content was analyzed on a COULTER EPICS XL Flow Cytometer (Beckman Coulter, Fullerton, CA).

DNA FRAGMENTATION ASSAY

Cells were washed twice with phosphate-buffered saline [PBS] and lysed by addition of a hypotonic solution [1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl pH 7.5]. The supernatant was collected and prepared as previously reported [Herrmann et al., 1994].

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Cells were lysed in 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate. Lysates were centrifugated at 14,000 rpm for 10 min. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA). Equivalent amounts of protein were loaded and electrophoresed on SDS–polyacrylamide gels. Subsequently, proteins were transferred to nitrocellulose membranes (Immobilon, Millipore Corp., Bedford, MA). After blocking with Tris-buffered saline-BSA (25 mM Tris pH 7.4, 200 mM NaCl, and 5% BSA), the membrane was incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer. Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and the reaction was detected with an enhanced chemiluminescence system (Amersham Life Science, Buckinghamshire, UK). Densitometric measurements of the band in Western blot analysis were performed using digitalized scientific software program UN-SCAN-IT.

CYTOSOLIC PROTEIN EXTRACTION AND CYTOCHROME *c* IMMUNOBLOT

Isolation of mitochondria and cytosolic fraction and cytochrome *c* immunoblot were carried out using a modified protocol from Kluck et al. [1997]. Cells were treated with kaempferol and then lysed in lysis buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM PMSF, and 100 μM $NaVO_4$). Samples were then incubated on ice for 20 min and centrifuged at 14,000 rpm for 15 min. The supernatants were collected and protein concentration was determined by the Bradford assay. Equivalent amounts of protein were loaded and electrophoresed on SDS–polyacrylamide gels. The membrane was incubated with the following primary antibodies: rabbit anti-cytochrome *c* (H-104, Santa Cruz, CA), rabbit anti-SIRT3 (Cell Signaling, Boston, MA), rabbit anti-prohibitin (Novus Biologicals Littleton, CO), rabbit anti-β-Actin (Novus Biologicals), followed by incubation with a horseradish peroxidase conjugated anti-rabbit secondary antibody. The reaction was detected with an enhanced chemiluminescence system.

RNA ISOLATION AND NORTHERN BLOT

Total RNAs from control or treated cells were isolated using Tri Reagent (Sigma–Aldrich Chemie GmbH). Aliquots of RNA were electrophoresed on 1% agarose formaldehyde gels and subsequently blotted onto nylon membranes (Hybond N, Amersham, Braunschweig, Germany). The membrane was then UV cross-linked, and hybridized to ^{32}P-labeled probe.

TRANSFECTION AND TRANSDUCTION

K562 cells were stably transfected with an expression vector encoding for human SIRT3. Total RNA was isolated from the cells. A 5′-primer (5′ TCTAGAATGGCGTTCTGGGG 3′) and a 3′-primer (5′ GGATCCC-TATTTGTCTGGTC 3′) were designed and utilized for reverse transcription and polymerase chain reaction amplification of the cDNA for SIRT3 from the isolated total RNA. The fragment was then cloned into a pBk-CMV expression vector (Stratagene, La Jolla, CA). To generate stable clones, K562 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) and then selected with G418 (Sigma–Aldrich Chemie GmbH). Mission™ TRC shRNA lentiviral transduction particles expressing short hairpin RNA (shRNA) targeting SIRT3 and lentiviral negative control particles were purchased from Sigma–Aldrich Chemie GmbH. Stably transduced clones expressing SIRT3 shRNA were generated according to the manufacturer's instructions. Briefly, cells were seeded on a 24-well plate. The following day cells were infected. After 24 h medium was changed with fresh RPMI. Selection of stable clones was started 24 h later with the addition of 3 μg/ml of puromycin. The expression of SIRT3 was confirmed by Western blot assay.

RESULTS

KAEMPFEROL-INDUCED DNA FRAGMENTATION AND SUPEROXIDE DISMUTASE (SOD) EXPRESSION

To evaluate the apoptotic effect of kaempferol, the K562 and U937 (data not shown) cell lines were treated with this compound at concentrations of 15, 25, and 50 μM for 24, 48, and 72 h. Genomic DNA was isolated from cells, harvested at the indicated times, and subjected to electrophoretic separation on agarose gel as shown in Figure 1A. The polynucleosomal laddering pattern showed that DNA fragmentation was time- and concentration-dependent. In fact, the characteristic apoptotic ladder was detected when the cells were treated with 15 and 25 μM, and was clearly induced with 50 μM kampferol (Fig. 1A). The DNA fragmentation started at 24 h treatment and lasted up to 72 h. At this time point, 83% of the cells were apoptotic (Fig. 1B).

Based on these results, 50 μM kaempferol was selected as a representative concentration for the rest of the experiments. Previous reports have shown that kaempferol posses a pro-oxidant activity, in different cell lines, when used at concentration above 25 μM [Bestwick et al., 2007]. In order to study whether treatment of K562 with 50 μM kaempferol changed the mRNA expression of Cu/Zn SOD and Mn SOD, we performed Northern blotting analysis. Figure 1C shows that 50 μM kaempferol induced a gradual increase in Cu/Zn SOD and Mn SOD mRNA expression level up to 72 h.

EFFECTS OF KAEMPFEROL ON Bax AND Bcl-2 EXPRESSION

Bcl-2 family members are involved in the maintenance of cell viability. Bcl-2 and Bcl-xL have been shown to inhibit apoptosis, whereas Bid, Bad, Bcl-xs, and Bax have been reported to enhance apoptosis [Lee et al., 2001]. Alterations in the expression level of members of the Bcl-2 family proteins may regulate the commitment of K562 and U937 cells to apoptosis. Therefore, we performed Western blotting to examine the levels of expression of the pro-apoptotic protein Bax and the anti-apoptotic proteins Bcl-2 and Bcl-xL in K562 cells. When these cells were treated with kaempferol an increase in Bax and a correspondent decrease in Bcl-2 expression was observed (Fig. 2A). Such a change in expression started at 24 h treatment when only 30% of the cells were dead (Fig. 1B). Similarly, when U937 cells were treated with kaempferol an increase in Bax

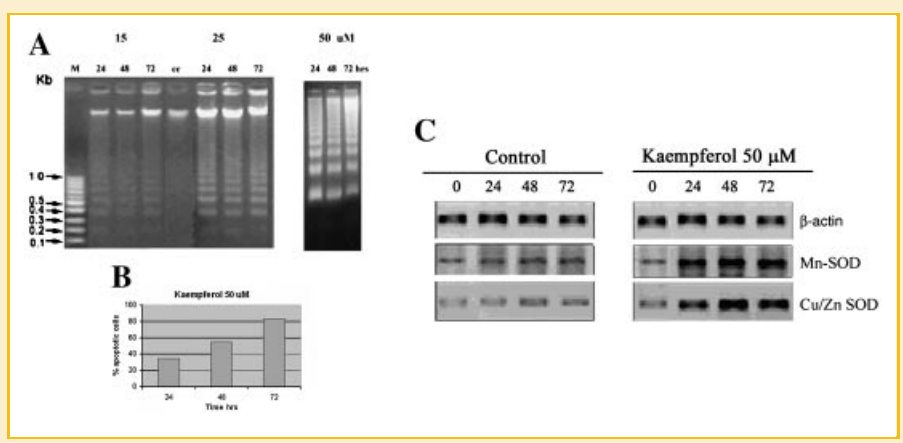

Fig. 1. Kaempferol exposure induces DNA fragmentation, cell death and SOD expression in K562 cells. A: K562 cells were either left untreated or treated with 15, 25, or 50 μM kaempferol as indicated. DNA fragmentation was measured after 24, 48, and 72 h treatment. CC, control cells. Agarose gels are representative of at least three separate experiments. B: Cell death measured by Flow Cytometry analysis in K562 cells treated with 50 μM kaempferol for 24, 48, and 72 h. Results are the mean ± SD of three separate experiments. C: K562 cells were either left untreated or treated with 50 μM kaempferol for the indicated times. RNA was extracted, electrophoresed and hybridized with a labeled probe for Mn SOD and for Cu/Zn SOD as described under Materials and Methods Section. β-Actin was used as loading control. Blots are representative of at least three separate experiments.

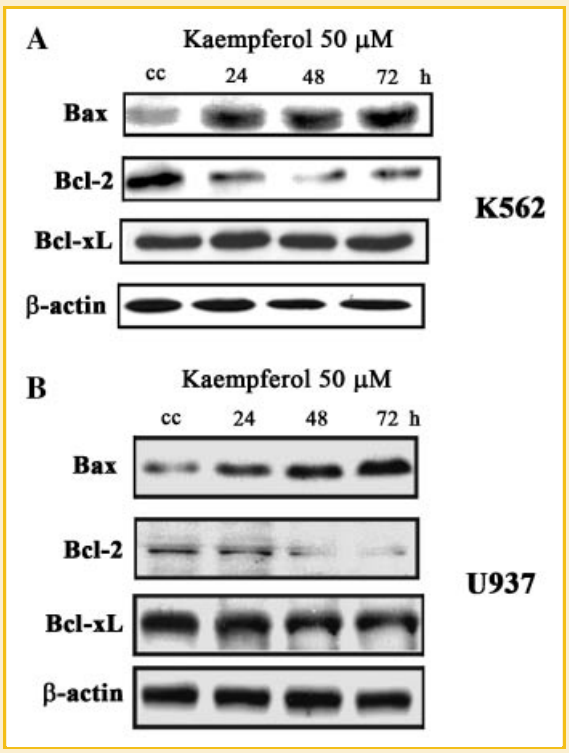

Fig. 2. Kaempferol exposure induces an increase in Bax and a decrease in Bcl-2. A: K562 cells were either left untreated or treated with 50 μM kaempferol for 24, 48, and 72 h. Whole cell lysates were obtained. Equivalent amounts of protein were loaded, electrophoresed on SDS–polyacrylamide gel and transferred to nitrocellulose membranes. The presence of Bax, Bcl-2, and Bcl-xL was observed using the appropriate primary antibody followed by incubation with a secondary antibody and enhanced chemiluminescence as described under Materials and Methods Section. β-Actin was used as loading control. Blots are representative of at least three separate experiments. B: U937 cells were either left untreated or treated with 50 μM kaempferol for 24, 48, and 72 h. Whole cell lysates were obtained. Equivalent amounts of protein were loaded, electrophoresed on SDS–polyacrylamide gel and transferred to nitrocellulose membranes. The presence of Bax, Bcl-2 and Bcl-xL was observed using the appropriate primary antibody followed by incubation with a secondary antibody and enhanced chemiluminescence as described under Materials and Methods Section. β-Actin was used as loading control. Blots are representative of at least three separate experiments.

and a decrease in Bcl-2 protein levels was observed (Fig. 2B). By contrast, we did not notice any change in the Bcl-xL protein along the treatment in the two cell lines examined (Fig. 2A,B). In both cases, β-Actin was considered as a loading control.

CYTOCHROME *c* RELEASE IN KAEMPFEROL-TREATED K562 CELLS

The release of cytochrome *c* from the mitochondria plays a major role during the apoptotic process. We examined whether the increase in Bax and the decrease in Bcl-2 was accompanied by a mitochondrial release of cytochrome *c* into the cytosol. Figure 3A shows that kaempferol treatment of K562 cells caused a time-dependent increase of cytochrome *c* in the cytosolic fraction with a correspondent decrease in the mitochondrial fraction. The release of cytochrome *c* was observed after 24 h of treatment and continued up to 72 h (Fig. 3A). Similar results were obtained with the U937 cell line (not shown).

CASPASES ACTIVATION IN KAEMPFEROL-TREATED K562 CELLS

Activation of caspases is a crucial point when the cells become committed to apoptosis. To understand the caspase cascade during kaempferol treatment, we investigated the activation of the initiator caspase-9 and the executioner caspase-3. Cleavage of caspase-3 and -9 after treatment of K562 cells with kaempferol (50 μM) was studied using specific antibodies. Figure 3B shows that caspase-9 and -3 were activated after 24 h treatment. Such activation was clear after 48 h and reached its peak at 72 h. Additionally, the caspases:procaspases ratio exhibited a time-dependent increase in kaempferol-treated cells (Fig. 3B).

In order to confirm the activation and execution of apoptosis by caspase-3, we studied the cleavage of PARP, a well-known caspase-3 substrate. As shown in Figure 3B, the cleavage of PARP followed the activation of caspase-3 and -9. Similar results to those reported in Figure 3A,B were obtained with the U937 cell line (not shown).

EFFECTS OF KAEMPFEROL ON SIRT3 EXPRESSION

Sirtuins are a family of proteins involved in many cellular processes including nutritional deprivation, DNA damage, cell cycle arrest, and cell viability [Yamamoto et al., 2007]. However, the role of members of this family in the apoptotic pathway is largely unknown. Recently, studies by Allison and Milner (2007) have shown that SIRT3 is required for apoptosis in HCTT 116 human epithelial cells. In order to elucidate whether treatment of K562 and U937 cells with 50 μM kaempferol can influence the mRNA and protein expression of SIRT3, we performed Northern and Western blotting analysis. Figure 4A shows that treatment of K562 and U937 cells with kaempferol increased the mRNA expression of SIRT3. Figure 4B shows that kaempferol treatment also increased the protein levels of SIRT3. Furthermore, SIRT3 protein showed a mitochondrial localization in kaempferol-treated K652 cells (Fig. 4B). In fact, we could not detect any SIRT3 in the cytosolic fraction (Fig. 4B). As shown in Figure 4B the content of both the mitochondrial marker prohibitin and the cytosolic marker actin did not vary during the experiment. Similar results were obtained with the U937 cell line (not shown).

EFFECTS OF SIRT3 SILENCING AND OVEREXPRESSION ON KAEMPFEROL-INDUCED CELL DEATH

To further explore the role of SIRT3 in kaempferol-induced cell death, two different clones of K562 were produced. One transduced with lentiviral particles expressing SIRT3 shRNA and one transfected with a mammalian expression vector overexpressing SIRT3. Figure 5A shows the silencing (left panel) and the overexpression (right panel) of SIRT 3. β-Actin was used as loading control. Furthermore, to exclude the possibility that other sirtuins were affected by the transduction or transfection procedure as well, the levels of SIRT1 were measured in the clones and shown to be identical to those of the wild type K562 cells (Fig. 5A). Treatment of SIRT3 overexpressing cells with kaempferol resulted in an increased cell killing as shown by the pictures and Flow Cytometry analysis of Figure 6B. By contrast, SIRT3 silencing did not have any effect on

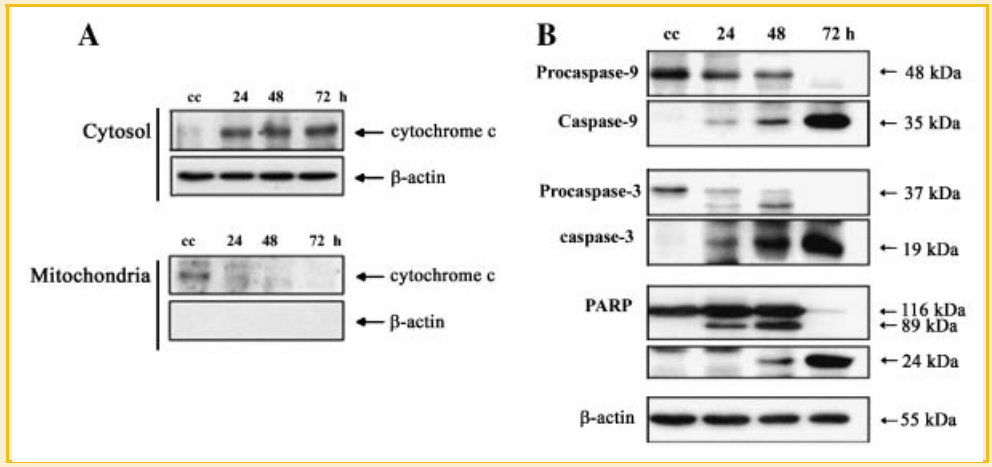

Fig. 3. Cytochrome *c* release and activation of caspase-3 and -9 in kaempferol-treated cells. A: K562 cells were either left untreated or treated with 50 μM kaempferol for 24, 48, and 72 h. The cells were then processed to obtain mitochondrial and cytosolic fractions as described under Materials and Methods Section. The content of cytochrome *c* and β-Actin was determined by SDS–PAGE and Western Blotting. Blots are representative of at least three separate experiments. B: K562 cells were either left untreated or treated with 50 μM kaempferol for 24, 48, and 72 h. Whole cell lysates were obtained. The content and the processing of caspase-3, -9, and PARP was determined by SDS–PAGE and Western Blotting. β-Actin was used as loading control. Blots are representative of at least three separate experiments.

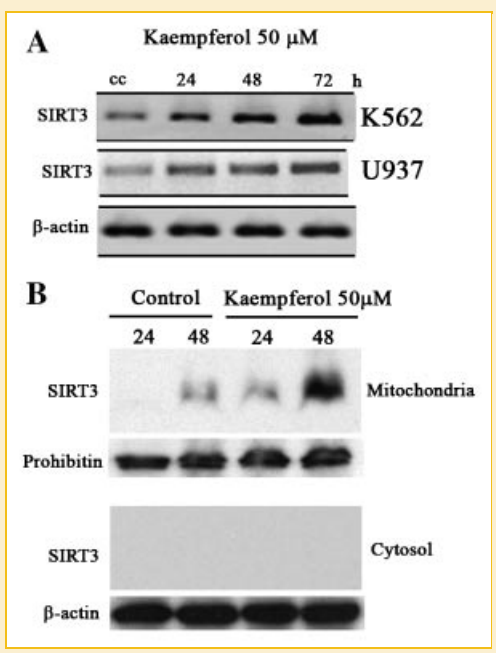

Fig. 4. Kaempferol exposure induces an increase in SIRT3. A: K562 and U937cells were either left untreated or treated with 50 μM kaempferol for the indicated times. RNA was extracted, electrophoresed, and hybridized with a labeled probe for SIRT3 as described under Materials and Methods Section. β-Actin was used as loading control. Blots are representative of at least three separate experiments. B: K562 cells were either left untreated or treated with 50 μM kaempferol for the indicated times. Mitochondrial and cytosolic fractions were obtained and the presence of SIRT3, prohibitin and β-Actin was determined by Western blot assay as described under Materials and Methods Section. Blots are representative of at least three separate experiments.

kempferol-induced cell death as compared to the wild type K562 cells (Fig. 5B). Figure 5C shows the percentage of cell killing measured by Trypan blue exclusion after 24 h of Kaempferol treatment in wt, SIRT3 silenced and SIRT3 overexpressing K562 cells. SIRT3 overexpression increased the number of cells killed by kaempferol to 52% from the 28% of the wt type. By contrast, SIRT3 silencing did not show any difference in kaempferol-induced cell death as compared to the K562 wt cells (Fig. 5C).

INHIBITION OF PI3K EXPRESSION AND AKT PHOSPHORYLATION BY KAEMPFEROL IN K562 AND U937 CELLS

The PI3K/Akt signaling pathway and its downstream transcription factors have been studied in detail for their role in cell proliferation, survival, cycle control, as well as other cellular functions. Accumulating evidence shows that deregulation of this pathway also plays an essential role in cancer development. We first determined the effect of kaempferol on PI3K protein expression in both K562 and U937 cell lines. Western blot analysis revealed that kaempferol (50 μM) caused inhibition in the expression of regulatory (p85) subunit of PI3K in both cell lines (Fig. 6A). Furthermore, treatment of K562 and U937 cells with kaempferol (50 μM) inhibited phosphorylation of Akt at both Ser473 and Thr308 (Fig. 6B). Kaempferol treatment did not change the total content of Akt in the two cell lines examined (Fig. 6A,B).

DISCUSSION

Kaempferol, a natural compound present in fruits and other vegetables, has been shown to possess both antioxidant and pro-oxidant activity based on the concentration used. Previous studies have shown that 50 μM kaempferol has a pro-oxidant activity inducing apoptosis in both human non-small cell lung carcinoma

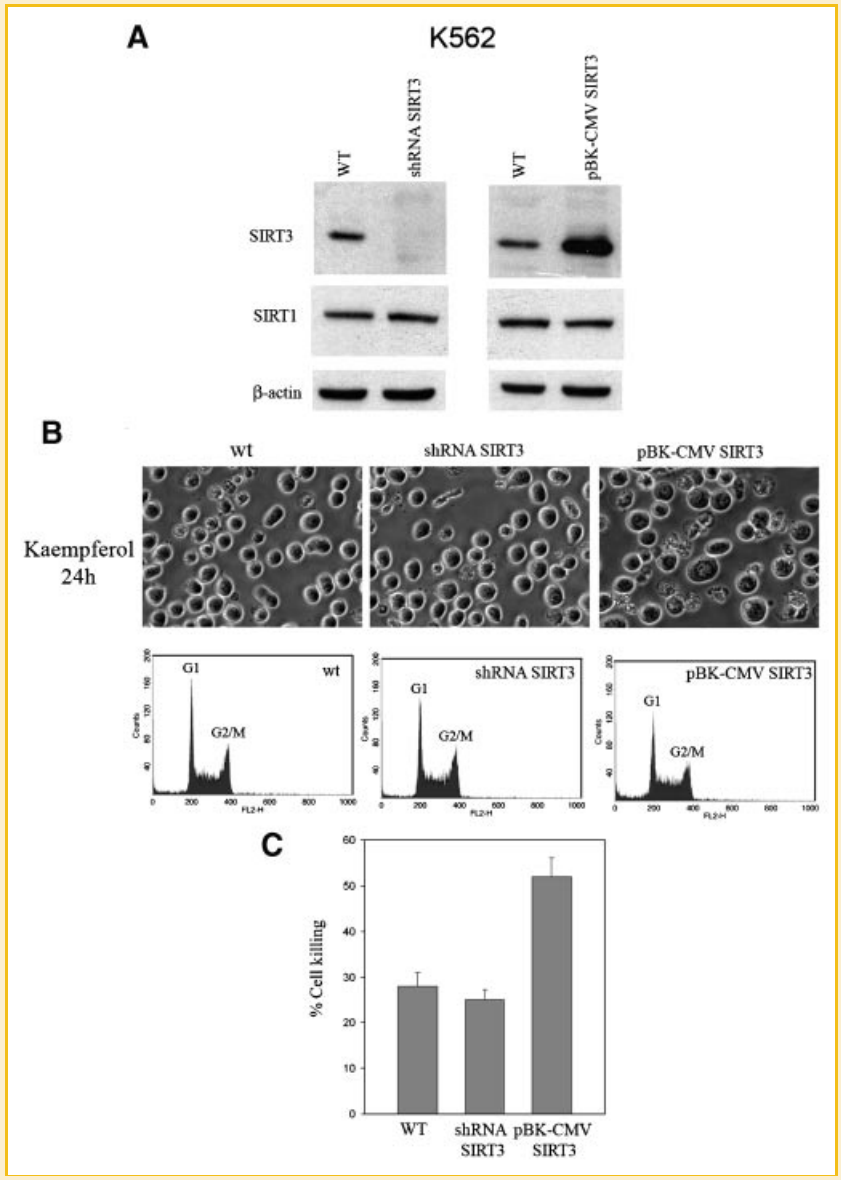

Fig. 5. SIRT3 overexpression increases the sensitivity of K562 cells to kaempferol. A: K562 cells were transduced with lentiviral particles expressing SIRT3 shRNA or with a mammalian expression vector expressing SIRT3. SIRT3 silencing and overexpression was confirmed on selected clones by Western blot analysis of whole cells lysates. β-Actin was used as loading control. Blots are representative of at least three separate experiments. B: Phase contrast images of wt, SIRT3 silenced, and SIRT3 overexpressing K562 cells treated with kaempferol for 24 h. The correspondent cell cycle analysis by Flow Cytometry is shown below each image. C: Cell death measured by Trypan blue exclusion in wt, SIRT3 silenced, and SIRT3 overexpressing K562 cells treated with 50 μM kaempferol for 24 h. Results are the mean ± SD of three separate experiments.

cell line H460 and in glioblastoma cell lines LN229, U87MG, and T98G [Bestwick et al., 2007; Sharma et al., 2007].

In the present study, we show that 50 μM kaempferol can induce apoptosis in human chronic myelogenous leukemia cell line K562 and promyelocitic human leukemia U937.

The pro-oxidant properties of kaempferol have been examined by studying the mRNA expression of antioxidant enzymes, such as SOD. We showed that the mRNA levels of Mn SOD and Cu/Zn SOD were increased after kaempferol treatment (Fig. 1C). It is well known that SOD catalyzes the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. This result may suggest that kaempferol induces apoptosis, indirectly, via the induction of ROS. Therefore, the overexpression of Mn SOD and Cu/Zn SOD mRNA represents a cellular mechanism aimed to maintain cellular redox status in order to prevent kaempferol-induced apoptosis. A similar observation has been recently reported by, Jeong et al. [2008] by showing that treatment of a glioma cell line with 50 μM kaempferol increased ROS production and that kaempferol-induced cell death was prevented by two antioxidants such as *N*-Acetylcysteine and glutathion. The authors concluded that ROS generation is involved in kaempferol-induced cell death. [Jeong et al., 2008].

Members of the Bcl-2 family of proteins are critical regulators of the apoptotic pathway. Bcl-2 is an upstream effector molecule in the

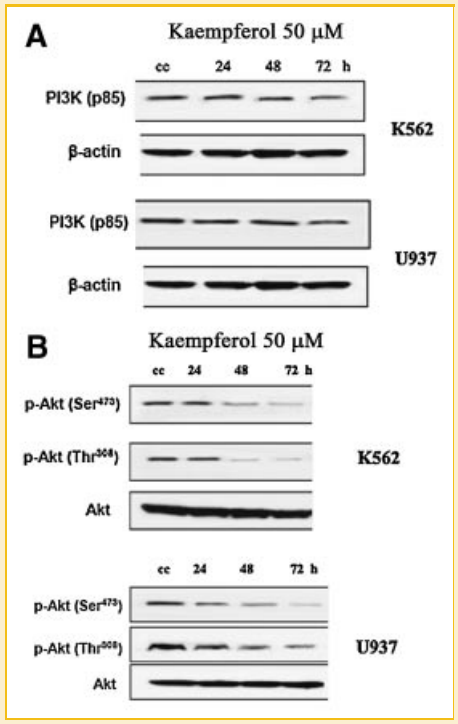

Fig. 6. Inhibitory effects of kampferol on PI3K (p85) expression and Akt phosphorylation. A: Whole cell lysates were obtained from K562 and U937 cells. Equivalent amounts of protein were loaded, electrophoresed on SDS–polyacrylamide gel, and transferred to nitrocellulose membranes. The inhibitory effect of kampferol on PI3K (p85) in both cell lines was observed using the appropriate primary antibody followed by incubation with a secondary antibody and enhanced chemiluminescence as described under Materials and Methods Section. β-Actin was used as loading control. Blots are representative of at least three separate experiments. B: Whole cell lysates were obtained from K562 and U937 cells. Equivalent amounts of protein were loaded, electrophoresed on SDS–polyacrylamide gel and transferred to nitrocellulose membranes. The inhibitory effects of kampferol on phosphorylation of Akt (Ser473 and Thr308) and the levels of total Akt in both cell lines were determined using the appropriate primary antibody followed by incubation with a secondary antibody and enhanced chemiluminescence as described under Materials and Methods Section. β-Actin was used as loading control. Blots are representative of at least three separate experiments.

apoptotic pathway and is identified as a potent suppressor of apoptosis [Oltersdorf et al., 2005]. Bcl-2 has been shown to form a heterodimer with the pro-apoptotic member Bax and might thereby neutralize its pro-apoptotic effects. Therefore, alterations in the levels of Bax and Bcl-2, that is, the ratio of Bax/Bcl-2 is a decisive factor and plays an important role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death. In our study, a decrease in Bcl-2 and increase in Bax protein expression was observed in both cells lines (Fig. 2A,B), hence the ratio of Bax to Bcl-2 was altered in favor of apoptosis. Our results suggest that up-regulation of Bax and down-regulation of Bcl-2 may be another molecular mechanism through which kaempferol induces apoptosis. It has been shown that mitochondria contain and release proteins such as cytochrome *c* that are involved in the apoptotic cascade. Cell-free systems demonstrate that mitochondrial products are rate limiting for the activation of caspases and endonucleases in cell extracts [Martinou et al., 2000]. Functional studies indicate that drug-induced opening or closing of the mitochondrial megachannel (permeability transition pore) can induce or prevent apoptosis [Tsujimoto and Shimizu, 2000]. These experiments indicate that cytochrome *c* is a key factor in apoptosis and that its release further activates caspases, resulting in the appearance of apoptotic features. Our study confirms that cytosolic cytochrome *c* was increased in K562 cells after treatment with kaempferol (Fig. 3A). Following the release of cytochrome *c*, we observed an activation of both caspase-3 and- 9 that were cleaved after 24 h treatment with 50 μM kampferol (Fig. 3B). In fact, cleavage of PARP, a caspase-3 substrate, was evident after 48 h (Fig. 3B).

The phosphatidyl inositol 3-kinase (PI3K)/Akt pathway is activated downstream of a variety of extracellular signals and activation of this signaling pathway impacts a number of cellular processes including cell growth, proliferation and survival. The alteration of components of this pathway, through either activation of oncogenes or inactivation of tumor suppressors, disrupts a signaling equilibrium, and can thus lead to cellular transformation [Hanahan and Weinberg, 2000]. We found that treatment of K562 and U937 cells with kaempferol reduced both the protein expression of PI3K (p85) and the phosphorylation of Akt at Thr308 and Ser473 (Fig. 5A,B). The Akt family has been shown to be the primary downstream mediator of the effects of PI3K and regulates a variety of cellular processes through the phosphorylation of a wide spectrum of downstream substrates. Indeed, deregulation of the PI3K/Akt signaling pathway can lead to an alteration of all the aspects of cell physiology that comprise the hallmarks of cancer.

The sirtuin protein family has been proposed to be involved in cellular stress response pathways including DNA damage, cell cycle arrest, and apoptosis [Saunders and Verdin, 2007; Yamamoto et al., 2007]. A member of this family, SIRT3 has a mitochondrial localization [Allison and Milner, 2007; Yang et al., 2007]. In particular, SIRT3 has been shown to possess a pro-apoptotic function in several cell lines [Allison and Milner, 2007]. We demonstrated an increase in SIRT3 mRNA that was present at 24 h and continued after 48 and 72 h of kaempferol treatment (Fig. 4A). Such mRNA increase was accompanied by an increase of SIRT3 protein that localized in the mitochondria of the treated cells (Fig. 4B). In order to elucidate the role of SIRT3 in the apoptotic pathway activated by kaempferol, we generated both K562 cells stably silenced for SIRT3 and K562 cells stably overexpressing SIRT3. When these clones were treated with kaempferol there was a significant increase in cell killing in the SIRT3 overexpressing clones, whereas SIRT3 silenced clones did not show any difference from the wt K562 cells (Fig. 5). The effects observed in the presence of an overexpression of SIRT3 suggest that an accumulation of this protein is needed in order to have a biological effect. The absence of an effect in the SIRT3 silenced clones will require further investigation but is in agreement with what observed by Allison and Milner (2007) in HCTT 116 cells. In fact, transfection of these cells with a SIRT3 siRNA prevented the killing from Bcl-2 silencing but did not result in an increased protection from other apoptotic stimuli [Allison and Milner, 2007].

Together, these results establish a kaempferol-mediated death-signaling cascade in K562 and U937 cells involving inhibition of PI3K and Akt resulting in a series of cellular events. The downstream pathway involves Bax overexpression, down-regulation of Bcl-2 and release of cytochrome *c*. Furthermore, the mechanism of kaempferol-induced apoptosis involves also the increase in SIRT3. In fact, SIRT3 overexpression increased the sensitivity of K562 cells to kaempferol. Such observation and the mitochondrial localization of SIRT3, may suggest a role for this sirtuin in the apoptotic mitochondrial pathway. Further experiments in other cell lines and with different apoptotic stimuli will be necessary in order to clarify the role of SIRT3 and other sirtuins in the apoptotic process. Finally, these results are of significance because kaempferol, a natural compound, may have clinical applications in the treatment of leukaemia with both direct inhibitory effects and enhancing effects of other chemotherapy with a low toxicity.

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