Molecular Cancer

Research

Regulation of COX-2 protein expression by Akt in endometrial cancer cells is mediated through NF-κB/IκB pathway

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Abstract

Background: Cyclooxygenase-2 (COX-2) has been shown to be highly expressed in a broad series of primary endometrial tumors and its expression may be closely associated with parameters of tumor aggressiveness. In human endometrial cancer, tumor suppressor phosphatase tensin homologue (PTEN) is frequently mutated. In the presence of a mutated PTEN protein, Akt phosphorylation levels increase leading to the activation of this survival pathway. The nuclear transcription factor κB (NF-κB) is a well establish regulator of genes encoding cytokines, cytokine receptors, and cell adhesion molecules that drive immune and inflammatory responses. More recently, NF-κB activation has been connected with multiple aspects of oncogenesis, including the control of apoptosis, cell cycle, differentiation, and cell migration. It is known that Akt may act through NF-κB pathway and that COX-2 gene has been shown to be regulated at the promoter level by NF-κB. Recently, we showed that Akt regulates COX-2 gene and protein expressions in phospho-Akt expressing endometrial cancer cells. The present study was undertaken to determine the involvement of NF-κB pathway and IκB (an inhibitor of NF-κB) in the regulation of COX-2 expression and to determine more precisely the downstream targets of Akt involved in this process.

Results: Three different human endometrial cancer cell lines known to have wild type PTEN (HEC 1-A) or a mutated inactive PTEN protein (RL 95-2 and Ishikawa) were used for these studies. Expression IκB and Phospho-IκB were evaluated by Western analysis. The presence of IκB phosphorylation was found in all cell lines studied. There was no difference between cell lines in term of NF-κB abundance. Inhibition of PI 3-K with Wortmannin and LY294002 blocked IκB phosphorylation, reduced NF-κB nuclear activity, reduced COX-2 expression and induced apoptosis. Transfection studies with a dominant negative Akt vector blocked IκB phosphorylation and reduced COX-2 expression. On the opposite, constitutively active Akt transfections resulted in the induction of IκB phosphorylation and up-regulation of COX-2.

Conclusion: These results demonstrate that Akt signals through NF-κB/IκB pathway to induce COX-2 expression in mutated PTEN endometrial cancer cells.

Background

The phosphoinositide 3-kinase (PI 3-kinase) pathway has been implicated in the activation of the proinflammatory transcription factor nuclear factor κB (NF-κB) [1-3]. It has been demonstrated that both the regulatory and the catalytic subunit of phosphatidylinositol 3-kinase (PI 3-K) play a role in NF-κB activation by the tyrosine phosphorylation-dependent pathway [3]. The NF-κB transcription factor is a pleiotropic activator that participates in the induction of a wide variety of cellular genes [4]. In addition to its role in inflammation and immune response, NF-κB has also been implicated in the suppression of apoptosis [5], cellular survival, transformation, and oncogenesis [6]. Predominantly a heterodimeric complex of two polypeptides (p65/RelA and p50), NF-κB lies dormant in the cytoplasm through the binding IκB inhibitory proteins. When phosphorylated on serine 32 and serine 36, IκBα is targeted and degraded by ubiquitin/26 S proteasome pathway liberating the NF-κB heterodimer so that it may translocate to the nucleus and bind DNA. NF-κB binds to *cis*-acting κB in the promoters and enhancers of key cellular genes. Active, DNA-binding forms of NF-κB are dimeric complexes, composed of various combinations of members of the Rel/NF-κB family of polypeptides (p50, p52, c-Rel, v-Rel, RelA (p65), and RelB). Recently, a large-molecular weight complex was identified that is responsible for phosphorylating IκBα and IκBβ. Two key catalytic sub-units of the IκB kinase (IKK) complex were identified as IKKα and IKKβ [7]. Constitutive NF-κB activation appears to have an important role in tumorigenesis. For example, persistent nuclear NF-κB localization and NF-κB-dependent transcription is detected in breast [8], ovarian [9], colon [10], thyroid [11] and prostate [12] tumors. In breast and prostate tumor cells, constitutive NF-κB activity is associated with reduced levels of IκBα that appears related to increased degradation of IκB proteins in these cells [13].

Previous reports indicate that the transcription factor NF-κB can function upstream of cyclooxygenase-2 (COX-2) to control transcription of this gene through the IKK pathway activation [14]. Cyclooxygenase (COX) is the rate-limiting enzyme involved in the biosynthesis of prostaglandins (PG) and exists in two isoforms: COX-1 (constitutively expressed) and COX-2 (the regulated isoform). Cyclooxygenase-2 (COX-2) up-regulation has been found in several type of cancers such as colon carcinomas [15], cervix [16], head and neck [17], bladder [18], pancreas [19], stomach [20], prostate [21] and breast [22]. It is believed that COX-2 and PGs, particularly PGE_2, may be key elements in the evolution of tumor transformation and malignancy. Epidemiological studies showed that nonsteroidal anti-inflammatory drugs (NSAIDs) can be used for cancer prevention [23]. It has been shown that COX-2 expression in colorectal carcinoma cells provides a growth and survival advantage and increases tumor cell invasiveness (see [22] for a review). Additionally, more evidences suggest that COX-2 is highly express in a broad series of primary endometrial tumors and its expression may closely be associated with parameters of tumor aggressiveness [24].

Akt is a serine/threonine protein kinase also known as protein kinase B or Rac [25-27]. Akt is an inactive cytosolic protein recruited to the plasma membrane, and activated by phosphorylation at threonine 308 and serine 473 in response to growth factors or cytokines [28-30] via the product of PI 3-K, phosphatidylinositol 3,4,5-triphosphate (PIP_3). Upon phosphorylation, Akt has been shown to phosphorylate and to block the action of several pro-apoptotic proteins such as Bad [29]. Akt also blocks cytochrome C release from the mitochondria through the regulation of Bcl-2 [31] and regulates expression of cIAP-1 [32]. Inhibition of Akt has been shown to induce apoptosis [33-35] and was shown to be a downstream target of NF-κB [36]. Recently, it has been demonstrated that Akt in involved in IKK phosphorylation resulting in NF-κB activation [37]. In a number of different cancers, the tumor suppressor phosphatase tensin homologue (PTEN, a lipid phosphatase) is frequently mutated. PTEN mutations have been found in several types of endometrial cancer [38-41]. PTEN dephosphorylates PIP_3 into inactive PIP_2, which blocks Akt activation. Moreover, we have previously shown that Akt regulates COX-2 gene and protein expressions.

We have demonstrated recently that Akt directly regulates COX-2 gene and protein expression in endometrial cancer cells [42]. The present study was undertaken to determine the involvement of NF-κB pathway and IκB in the regulation of COX-2 expression and to determine more precisely the downstream targets of Akt involved in this process. We hypothesized that PTEN mutation increase Akt activity which may, in turn, be involved in the activation of NF-κB. Our results demonstrate that activity of NF-κB is up-regulated in human endometrial cancer cells expressing phospho-Akt and is responsible for the increase of COX-2 gene expression.

Results

Inhibition of PI 3-K induces apoptosis

We have showed previously that Akt inhibition in mutated PTEN endometrial cancer cells results in inhibition of Akt phosphorylation, downregulation of COX-2 gene and protein expression and stimulation of apoptosis [42]. The present results confirm that PI 3-K inhibition with Wortmannin and LY294002 induce apoptosis (Fig. 1). Furthermore, these results demonstrate that apoptosis is induced in a time-dependent manner in mutated PTEN cells (RL 95-2 and Ishikawa) as demonstrated by Hoechst

TUNEL

Hoechst

A) HEC 1-A
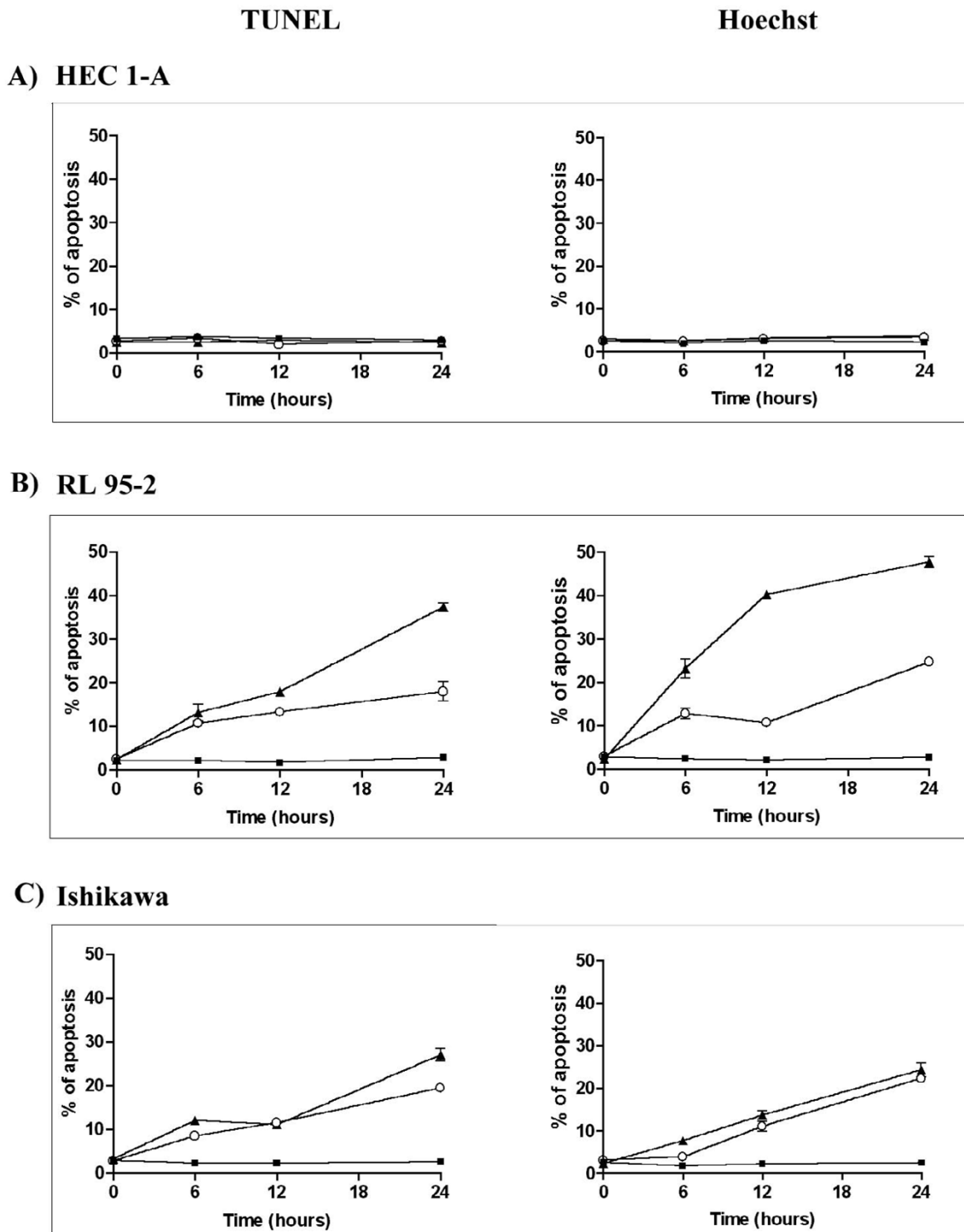
B) RL 95-2

C) Ishikawa

Figure 1
Effect of PI 3-K inhibitors on apoptosis in HEC 1-A, RL 95-2 and Ishikawa cells. Control (■), LY294002 (○) and Wortmannin (▲). 2×10^6 cells were plated for 0, 6, 12, 24 h and cultured in the presence of medium and LY294402 or Wortmannin. Cells were trypsinized, pooled with floating cells and collected for Hoechst nuclear staining (right panel) or TUNEL analysis (left panel) to count apoptotic cells. Data represent the mean ± SEM of 4 independent experiments.

and TUNEL analyses (Fig. 1). However, PI 3-K inhibitors had no effect in non mutated PTEN HEC 1-A cells suggesting that PI 3-K activity is important in the control and inhibition of apoptosis.

Inhibition of the PI 3-kinase/Akt signaling pathway reduces phosphorylation of IκB and activates NF-κB translocation into the nucleus

As we showed previously, mutated PTEN endometrial cancer cell lines (RL 95-2 and Ishikawa) expressed high levels of Akt phosphorylation which was concomitant with the presence of high levels of COX-2 mRNA and protein [42]. In the latter study, there was no Akt phosphorylation found and nearly undetectable COX-2 protein in the wild-type cell line (HEC 1-A). PI 3-K inhibition in RL 95-2 and Ishikawa cells directly blocked Akt phosphorylation and caused a reduction of COX-2 mRNA and protein [42]. We wanted to further investigate the involvement NF-κB/IκB pathway in the regulation of COX-2 by Akt. As hypothesized, the results demonstrate that PI 3-K inhibition results in the reduction IκB phosphorylation in mutated PTEN RL 95-2 and Ishikawa cells (Fig. 2). There was no effect of PI 3-K inhibitors in IκB phosphorylation in HEC 1-A wild-type cells. To further confirm that inhibition of IκB phosphorylation leads to the activation and translocation of NF-κB to the nucleus, a NF-κB Chemiluminescent Assay was used to measure NF-κB activity in the nucleus (Fig. 3). The activity of NF-κB was high in mutated-PTEN human endometrial cancer cells compared to wild-type PTEN HEC 1-A cancer cell line. PI 3-K/Akt inhibition with Wortmannin significantly decreased NF-κB activity in both RL 95-2 and Ishikawa and inhibition had no effect in NEC 1-A cells.

Constitutively active Akt transfections resulted in the induction of IκB phosphorylation and up-regulation of COX-2 expression

To prove further the relationship between Akt, IκB and COX-2 expression, a "gain-of-function" experiment was conducted using a constitutively active (CA) Akt expression vector, which was transfected in the RL 95-2 cell line (Fig. 4). CA-Akt transfection induced Akt and IκB phosphorylation and activity, and decreased total IκB protein. As shown previously [42], CA-Akt transfection induced COX-2 expression.

Dominant negative Akt vector blocked IκB phosphorylation, which leads to the activation of apoptosis

Finally, to confirm that Akt regulates COX-2 gene expression through the NF-κB/IκB pathway, a dominant negative (DN) Akt expression vector was used and transfected in the mutated-PTEN RL95-2 cell line expressing phospho-Akt (Fig. 5). As demonstrated previously, transfection of RL95-2 cells with the DN-Akt decreased Akt phosphor-

ylation and caused a reduction of COX-2 expression [42]. The current study further confirms these observations and demonstrates that transfection with DN-Akt increased total IκB protein and decreased IκB phosphorylation (Fig. 5). In addition, DN-Akt transfection caused an induction of apoptosis in RL 95-2 as observed with the PI 3-K inhibition experiments.

Discussion

The ability of NF-κB to promote cell proliferation, to suppress apoptosis, to promote cell migration, and to suppress differentiation apparently have been co-opted by cellular and viral oncoproteins to promote oncogenesis. It is known that Akt may act through NF-κB pathway [43] and that COX-2 gene has been shown to be regulated at the promoter level by NF-κB [44]. The activity of NF-κB is tightly controlled by inhibitory IκB proteins that bind to NF-κB complexes and thus sequester NF-κB in the cytoplasm. Stimuli such as cytokines promote the serine phosphorylation of IκB and its polyubiquitination and proteosome-mediated degradation and thereby induce NF-κB translocation to the nucleus. Since Akt phosphorylation has been shown to activate NF-κB in other systems, a similar sequence of events might be involved in phospho-Akt expressing RL 95-2 and Ishikawa human endometrial cancer cell lines used in the present study. PTEN is a crucial phosphatase involved in the regulation of Akt phosphorylation: the presence of an active PTEN protein blocks Akt phosphorylation by the dephosphorylation of PI 3-K product, PIP3 [45]. In the presence of a mutated-PTEN protein, activation of Akt generally occurs constitutively. We have demonstrated previously that Akt is constitutively phosphorylated/activated in two mutated-PTEN human endometrial cancer cell lines that have been used in the present study (RL 95-2 and Ishikawa) [32,42]. Whereas, phosphorylation of Akt was absent in one wild-type PTEN cell line (HEC 1-A).

Indeed, our results demonstrate that the presence of IκB phosphorylation was found in all cell lines studied. There was no difference between cell lines in term of NF-κB abundance indicating that NF-κB expression is not involved in the regulation of COX-2 gene expression. However, NF-κB was shown to be activated and present in the nucleus of the two mutated-PTEN endometrial cancer cells (RL 95-2 and Ishikawa) expressing phospho-Akt. Thus, the presence of a wild-type PTEN protein results in the reduction of Akt activity/phosphorylation leading to the inhibition of IκB phosphorylation and the sequestration of NF-κB. On the opposite, the presence of a mutated PTEN protein enables Akt phosphorylation, which in turn may phosphorylate IκB allowing NF-κB to be translocated to the nucleus to induce transcription of genes involved in cell survival. These results demonstrate that the ability of PTEN to negatively regulate the PI 3-K/Akt/NF-κB
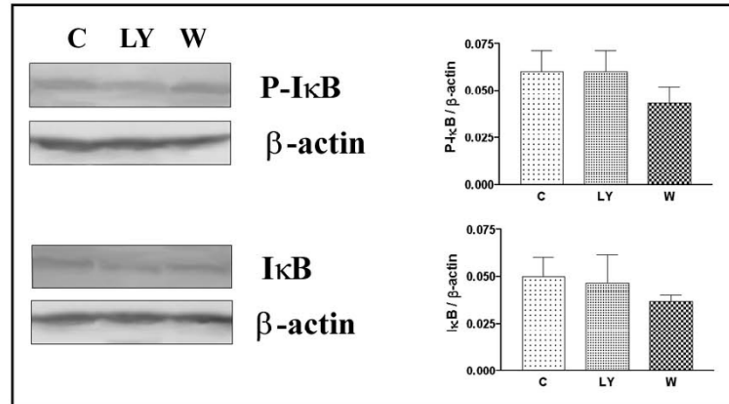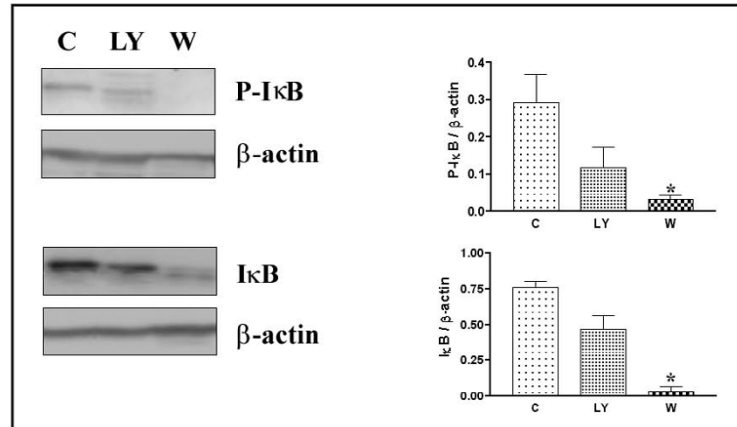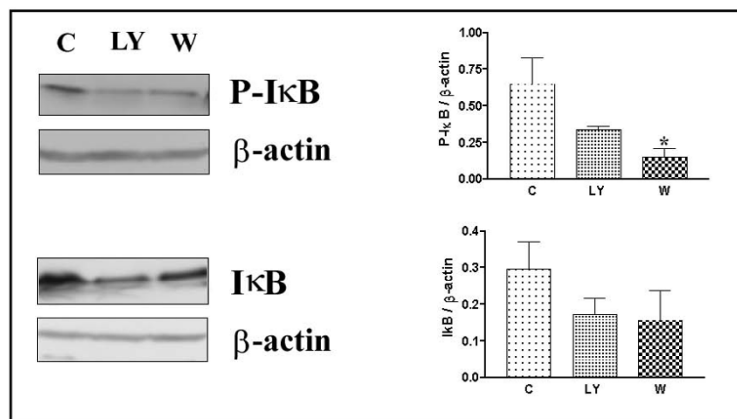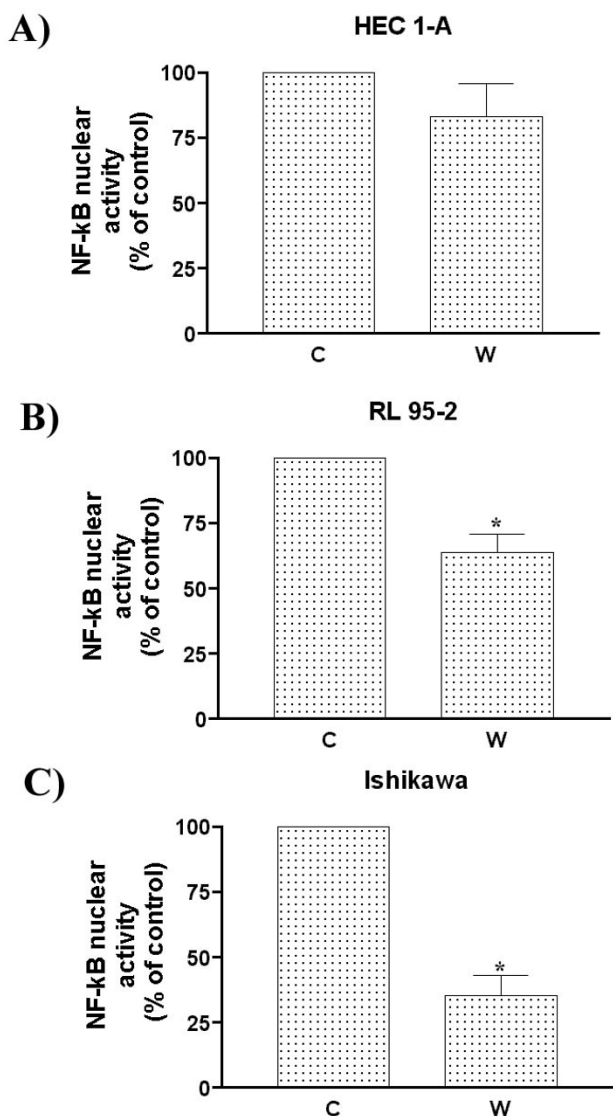
A) HEC-1-A

B) RL-95-2

C) Ishikawa

Figure 2
Effect of PI 3-K inhibitors on IκB expression and phosphorylation in HEC-1-A, RL-95-2 and Ishikawa cells. Western analysis was performed on cell protein lysates from pooled attached and floating cells. β-actin was used as control to correct for loading. Densitometric analyses were performed using BIO RAD gel doc system and are presented as a ratio (value/β-actin). 2×10^6 cells were plated for 24 h and cultured in medium in the presence or absence of LY294402 or Wortmannin. Data represent the mean ± SEM of 4 independent experiments. * $p < 0.05$ compared to control.

A) HEC 1-A

B) RL 95-2

C) Ishikawa

Figure 3
NF-κB activity in response to Wortmannin. HEC 1-A,
RL 95-2 and Ishikawa cells were treated with Wortmannin
for 24 hours and cells were recovered and lysed. Nuclear cell
lysates were recovered and NF-κB activity was measured
using the Chemiluminescent NF-κB Assay. Data represent
the mean ± SEM of 4 independent experiments. * p < 0.05
compared to control.

pathway may be important to its role of tumor suppressor
protein.

In the present study, we have investigated the role of PI 3-
kinase in NF-κB activation. Various PI 3-kinase inhibitors
such as Wortmannin, LY294002 and dominant-negative

Akt expression vector were used to fully prove the involve-
ment of PI 3-K/Akt pathway in the regulation of NF-κB
activity. Inhibition of PI 3-K with Wortmannin and
LY294002 blocked Akt and IκB phosphorylation and
reduced COX-2 expression in RL 95-2 and Ishikawa cells.
However, PI 3-K inhibitors had no effect in HEC 1-A cells
(a cell line with non detected Akt phosphorylation and no
detectable level of COX-2) confirming that COX-2 is a tar-
geted gene downstream of Akt. Transfection studies with a
dominant negative Akt vector in mutated-PTEN RL 95-2
cells blocked IκB phosphorylation, increased IκB expres-
sion and leaded to the activation of apoptosis. These PI 3-
K/Akt inhibition studies demonstrate that PI 3-K and Akt
are required for NF-κB activation.

To further confirm the involvement of Akt and NF-κB /IκB
pathway in the control of COX-2 expression, transfections
with a constitutively active (CA) Akt expression vector
were carried out using RL 95-2 cells. As hypothesized, CA-
Akt transfection induced COX-2 expression and con-
firmed the results obtained with DN-Akt inhibition and PI
3-K inhibition studies. Moreover, these transfections
resulted in the induction of IκB phosphorylation. The
subsequent degradation of IκB allows the release and
translocation of NF-κB to the nucleus. Recent evidences
also suggest that CCAAT/enhancer-binding protein beta
(C/EBPβ), a transcription factor, is involved downstream
Akt activation pathway [46-48]. Studies have demon-
strated that C/EBPβ is an essential transcription factor for
COX-2 gene regulation [49,50] indicating that activation
of C/EBPβ by Akt may be in part responsible for COX-2
gene expression. Another study showed that inactivation
of GSK-3β through activation of Akt plays an important
role in the UVB induction of COX-2 transcription [51].
Thereby, C/EBPβ and GSK-3β may be other different tar-
gets following Akt phosphorylation to activate cell sur-
vival through COX-2 gene expression and PGE$_2$ secretion.

COX-2 has been shown to contribute to tumorigenesis
and the malignant phenotype of tumor cells by different
mechanisms, including: (1) inhibition of apoptosis; (2)
increased angiogenesis; (3) increased invasiveness; (4)
modulation of inflammation/immuno-suppression; and
(5) conversion of procarcinogens to carcinogens (see [52]
for a review). An evident correlation between COX-2
expression and inhibition of apoptosis has been estab-
lished, associated with increased PGE$_2$ levels resulting in
modulation of pro- and anti-apoptotic factors such as Bcl-
2 [53]. We have showed previously that COX-2 inhibition
with NS-398 in RL 95-2 and Ishikawa cells results in the
inhibition of Akt phosphorylation and induction of apop-
tosis suggesting that the Akt/NF-κB/COX-2 pathway is an
important point of control of cell survival.
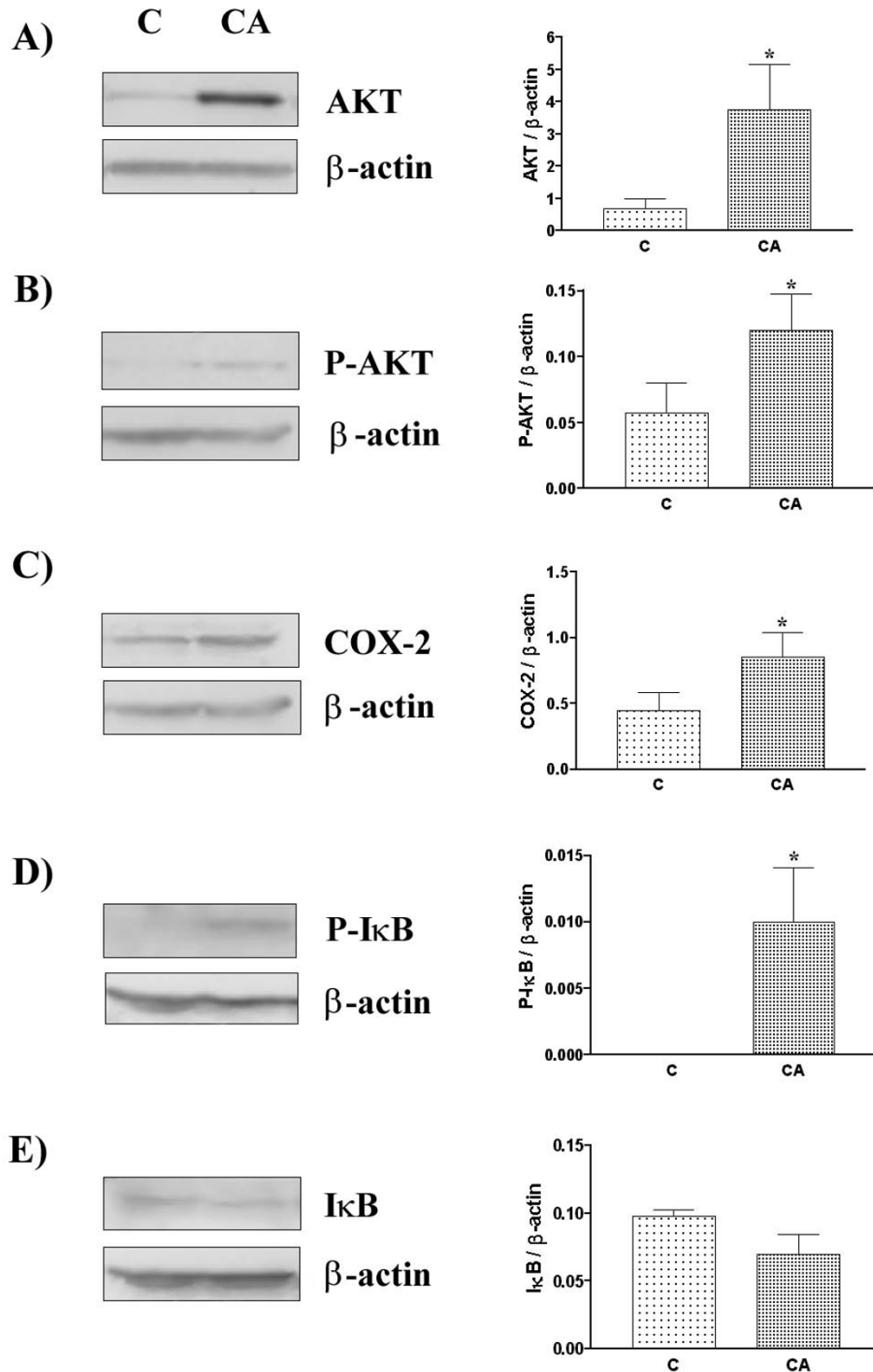
Figure 4
Constitutively active Akt action on IκB activity and COX-2 protein expression. RL 95-2 cells were transfected with constitutively active (CA) Akt expression vector or control vector and (A) Akt protein, (B) phospho-Akt, (C) COX-2, (D) Phospho-IκB et (E) IκB protein levels were measured by Western analysis. β-actin was used as control to correct for loading. Densitometric analyses were performed using BIO-RAD gel doc system and are presented as a ratio (value/β-actin). Data represent the mean ± SEM of 4 independent experiments. * $p < 0.05$ compared to control.
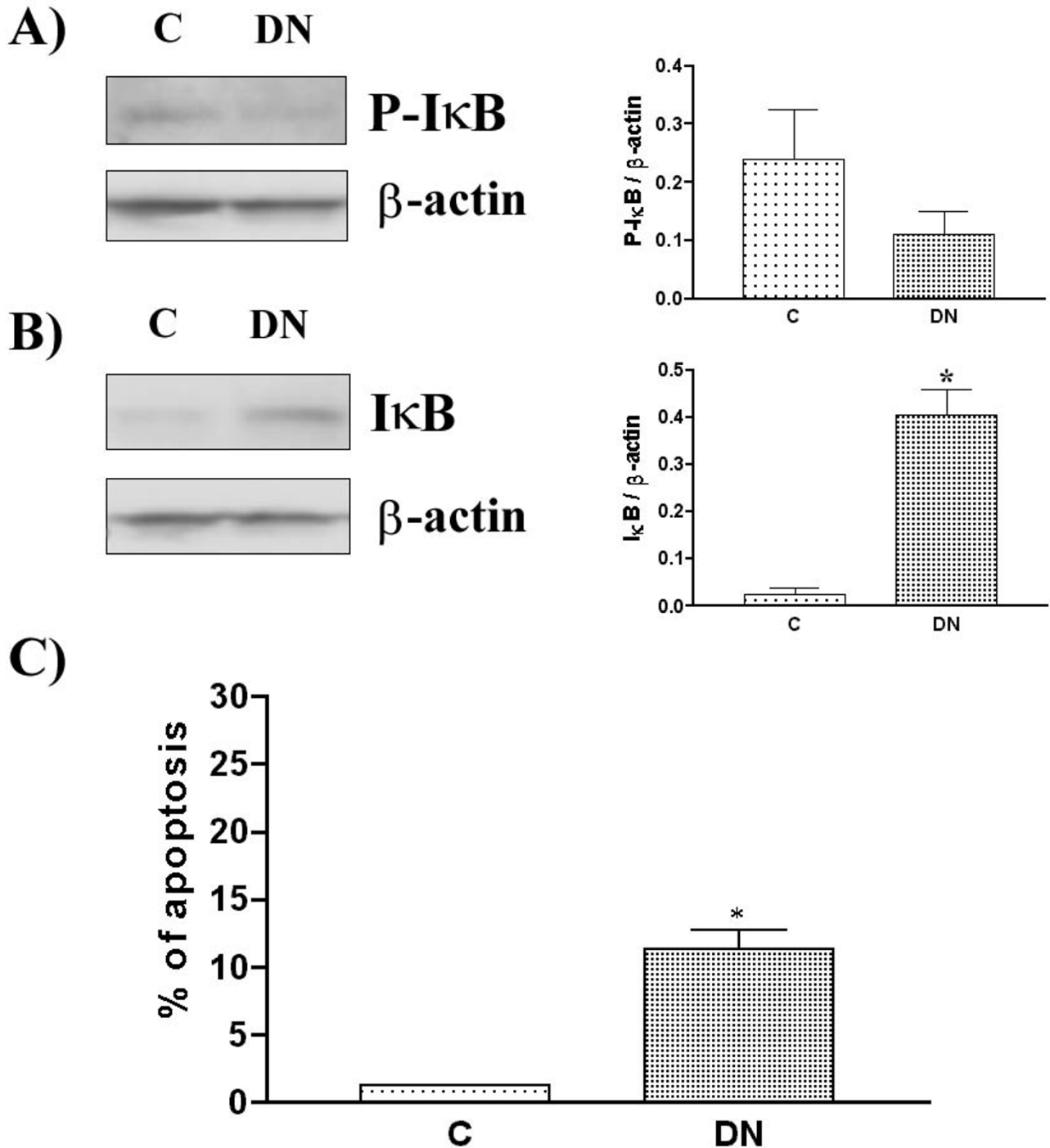
Figure 5
Dominant negative Akt action on IκB activity. RL 95-2 expressing phospho-Akt cells were transfected with Akt dominant negative (DN) vector or control vector and (A) phospho-IκB and (B) IκB protein levels were measured by Western analysis. β-actin was used as control to correct for loading. Densitometric analyses were performed using BIO-RAD gel doc system and are presented as a ratio (value/β-actin). (C) Cells were trypsinized, pooled with floating cells and collected for Hoechst nuclear staining to count apoptotic cells. Data represent the mean ± SEM of 4 independent experiments. * $p < 0.05$ compared to control

Conclusion

In summary, the present study demonstrates a crucial role for Akt in the regulation of NF-κB expression through the phosphorylation of IκB in human endometrial cancer cells. The results demonstrate that Akt signals through NF-κB/IκB pathway to induce COX-2 gene and protein expression. There is compelling evidence that NF-κB is deregulated in many forms of cancer and its inhibition is a logical therapy for certain cancers and for adjuvant approaches to cancer therapy. Indeed, this study shows that NF-κB/IκB pathway could be a good target for gene therapy in endometrial cancers. Further studies on other signaling factors/transcription factors such as GSK-3β and C/EBPβ activation/phosphorylation will provide more insight into the complex mechanisms by which Akt regulates COX-2 gene expression in human endometrial cancer cells.

Methods

Reagents

Wortmannin, LY294002, MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) and Hoechst 33258 were obtained from Sigma (St. Louis, MO). DMEM-F12, Mc Coy's 5A medieum, FBS serum and PCR primers were purchased from Invitrogen (Burlington, ON). Anti-human PhosphoPlus Akt (Ser473), Akt, IκBα and Phospho-IκBα antibodies were obtained from Cell Signaling Technology and anti-human COX-2 were obtained from Cedarlane Laboratories (Hornby, ON). Secondary horse radish peroxidase (HRP)-conjugated anti-rabbit antibody was purchased from BioRad (Mississauga, ON). Dominant negative (DN) and constitutively active (CA) Akt vectors were generously provided by Dr Zhenguo Wu, Hong Kong University of Science and Technology.

Cell culture

Human endometrial cancer cells (HEC 1-A and RL 95-2) were obtained from ATCC. Ishikawa cells were generously provided by Dr Sylvie Mader, Université de Montréal, Canada. Cells were cultured in 75 cm^2 bottles at 37°C in an atmosphere of 5% CO_2. Ishikawa cells were maintained in DMEM-F12 supplemented with 2.438 g/L of $NaHCO_3$, FBS (10%) and gentamycin (50 μg/ml). HEC 1-A cells were grown in Mc Coy's 5A medium supplemented with 2.2 g/L of $NaHCO_3$, FBS (10%) and gentamycin (50 μg/ml). RL 95-2 were cultured in DMEM-F12 supplemented with 1.75 g/L of $NaHCO_3$, HEPES (5 μM), insulin (2.5 μg/ml), FBS (10%) and gentamycin (50 μg/ml). 1 × 10^6 cells were plated in log growth phase into 6 wells plates for 24 hrs in the above culture medium prior to initiation of treatment. Wortmannin dose (50 μg/ml) and 24 hours time were chosen following dose-responses and time-course preliminary studies.

Transfections

Cells were plated at a density of 4 × 10^5 cells/well in six-well plates 24 hours before transfection. RL 95-2 cells were transfected with DN-Akt and CA-Akt vectors. Transient transfection of the cells was carried out with 1 μg of DNA/well using Effectene (Qiagen, Mississauga, ON), according to the protocol suggested by the manufacturer. Empty vector was used as the transfection control. Transfection efficiencies were determined by Western analysis using an anti-Akt antibody.

NF-κB cheluminescent assay

The BIOXYTECH NF-κB Chemiluminescent Assay (Medicorp, Montreal, QC) employs an oligonucleotide containing the DNA binding NF-κB consensus sequence bound to a 96-well plate. NF-κB present in the sample binds specifically to the oligonucleotide coated on the plate. The DNA bound NF-κB is selectively recognized by the primary antibody (p50 and p105 specific). A secondary antibody-alkaline phosphatase conjugate binds to the primary antibody. Then, the Relative Light Units (RLU) is measured using a chemiluminescence detector after addition of the alkaline phosphatase substrate.

Hoechst staining

Following treatment, both floating and attached cells were resuspended in 10% formalin containing Hoechst 33258 for 24 hours at 4°C. Hoechst nuclear staining was viewed and photographed using a Olympus BX60 fluorescence microscope and a Coolsnap-Pro CF digital Camera (Carsen Group, ON). Cells with typical apoptotic nuclear morphology (nuclear shrinkage, condensation and fragmentation) were identified and counted, using randomly selected fields on numbered photographic slides, of which the "counter" was not aware of the treatment, so as to avoid experimental bias. A minimum of 200 cells per treatment group was counted in each experiment and results are presented as a percentage of apoptotic cells/non-apoptotic cells.

Terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL)

Cells (floating and attached) were pooled, placed on a positively charged microscope slide, dried and rinsed with PBS. Slides were incubated with proteinase K (20 μg/ml) for 30 min at room temperature. Slides were washed twice with PBS and endogenous peroxidase was inactivated with 0.3 % hydrogen peroxide in methanol for 30 min. Slides were rinsed with buffer and incubated with 10 mM citrate solution for two minutes on ice. Then, tissue sections were rinsed with PBS and incubated with TdT labelling reaction (In Situ Cell Death Detection, POD, Roche) for 30 min at 37 °C in humidified environment. Slides were washed three times in PBS and tissue sections were blocked with BSA 3% for 20 min at room temperature.

Converter-POD solution was added and incubated 30 min at 37°C in humidified environment. Slides were washed 5 min in PBS and color development was achieved by incubation using DAB substrate. Cells were finally counterstained with hematoxylin. Negative control was performed using the same protocol without TdT enzyme. TUNEL positive cells were counted as described with the Hoechst nuclear staining assay.

Protein extraction and Western analysis
Cells (both floating and attached) were trypsinized, lysed in lysis buffer (PBS 1 × pH 7.4; 1% Nonidet P-40; 0.5% Sodium deoxycholate; 0.1% SDS; Protease Inhibitor Cocktail Tablets (Roche)), frozen and thawed three times, and centrifuged (13000 × g, 20 min at 4°C) to remove insoluble material. Supernatant was recovered and stored at -20°C pending analysis. Protein content was determined with the Bio-Rad DC Protein Assay according to manufacturer instructions. Protein extracts (50 μg) were heated (95°C, 3 min), resolved by 10% SDS-Polyacrylamide gel electrophoresis (PAGE) and electro-transferred to nitrocellulose membranes (15 V, 30 min) using a semi-dry transfer (Bio-Rad, Mississauga, ON). Membranes were then blocked (2 hrs, RT) with PBS containing 5% milk powder + 0.05% Tween 20, then incubated with anti-COX-2 (1:1000), anti-Akt (1:1000), anti-Phospho-PKB/Akt (1:250), anti-IκBα (1:1000), anti-Phospho-IκBα (1:500) (overnight, 4°C), and subsequently with Horse radish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:3000; RT, 45 min). Peroxidase activity was visualized with the ECL kit (Amersham, Arlington Heights, IL), according to the manufacturer's instructions.

Statistical analysis
All experiments were repeated at least three times. Data were subjected to one-way ANOVA or student t test (PRISM software version 4.0; GraphPad, San Diego, CA). Differences between experimental groups were determined by the Tukey's test.

Authors' contributions
MESG drafted the paper. MESG, VG and SP performed the experiments. EA conceived the study, participated in its design and coordination, and wrote final version of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

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