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## Opposite regulation of XIAP and Smac/DIABLO in the rat endometrium in response to $17\beta$ -estradiol at estrus

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### Abstract

During rat estrous cycle, the endometrium proliferates in response to sex steroids and specific endometrial epithelial cells undergo apoptosis in absence of embryonic factors. The central executioner of apoptosis is a family of aspartic acid-specific cysteine proteases known as caspases. Smac/DIABLO is released from the mitochondria during apoptosis and its stimulation promotes caspases activation by neutralizing members of the inhibitor of apoptosis proteins (IAPs) family, such as X-linked inhibitor of apoptosis protein (XIAP). The aim of this study was to investigate the involvement of Smac/DIABLO and XIAP in the control of caspases activation in endometrium of cycling rats. Polyoestrus female rats were sacrificed at each stage of estrous cycle (diestrus, proestrus, estrus, and metestrus). Endometrial protein extracts were collected to perform Western Blot analysis. Alternatively, uterine horns were sectioned for immunohistochemistry (IHC). We and others showed previously the presence of apoptosis at estrus in rat uterine epithelium. In the present study, cleaved caspase-3, -6, and -7 fragments were detected at estrus. IHC confirmed that caspase-3 was present only in luminal and glandular epithelium at estrus. XIAP was highly expressed at estrus in both epithelial and stromal cells. In contrast, expression of Smac/DIABLO was elevated at diestrus, proestrus and metestrus but was minimal at estrus. Treatment of ovariectomized rats with  $17\beta$ -estradiol induced XIAP expression and inhibited Smac/DIABLO protein expression in the endometrium. Cleaved caspase-3, -6, and -7 fragments increased in endometrial protein extracts following  $17\beta$ -estradiol treatment. Expression of NF- $\kappa$ B and I $\kappa$ B proteins, and I $\kappa$ B phosphorylation status were detected in the endometrium but were not influenced by the estrous cycle. These findings suggest that Smac/DIABLO and XIAP are regulated differently and may play important roles in the regulation of endometrial cell fate. Moreover, this study confirms a key role for executioner caspases in the control of apoptotic processes at estrus in the rat uterus.

### Introduction

In the absence of embryonic factors during pre-implantation period in uterine luminal epithelium, glands and stroma cell death is found in a cycling fashion through the estrous cycle by a mechanism known as apoptosis. Estro-

gens and progesterone are directly responsible for the histological and morphological changes in the uterus during estrous cycle. Studies have shown that apoptosis was induced in luminal epithelium at estrus in mouse [1] and rat [2,3]. Other studies have shown that estrogen induces

uterine epithelial cell proliferation and estrogen withdrawal results in cell death [4–6]. However, little is known about the cellular and molecular mechanisms involved in the regulation of apoptosis in the uterus. Our group has recently demonstrated that Akt, a serine/threonine protein kinase also known as PKB, is an important kinase involved in the control of endometrial cell proliferation and its expression and phosphorylation/activation are regulated during the estrous cycle, particularly through the action of 17 $\beta$ -estradiol [7]. We have further showed a decrease in Akt activity at estrus which was accompanied by an increase in apoptosis in luminal epithelial cells [7]. Whether downstream Akt targets might be involved in the regulation of proliferation/cell death in the endometrium is unknown.

X-linked inhibitor of apoptosis protein (XIAP) is a well known inhibitor of caspase-3, -7 and -9 [8,9]. XIAP belongs to a family of IAP genes which represent critical regulatory factors of apoptosis signaling and regulation. The IAP family also includes c-IAP1 (HIAP2), c-IAP2 (HIAP1), NAIP and Survivin [10]. Recently, we demonstrated in rat granulosa cells and human ovarian surface epithelial cancer cells that XIAP overexpression induces Akt phosphorylation/activation [11,12]. Akt is activated by phosphorylation at threonine 308 and serine 473 in response to growth factors or cytokines [13–15] through phosphatidylinositol 3-kinase (PI 3-K). Once phosphorylated Akt has been shown to 1) phosphorylates and blocks the action of several pro-apoptotic proteins such as Bad [14], and 2) block cytochrome C release from the mitochondria through the regulation of Bcl-2 [16]. The kinase Akt has been shown to induces phosphorylation of procaspase-9, suggesting that caspase processing and apoptosis can be directly regulated by protein phosphorylation [17]. This is supported by the observation that an activated form of Akt is able to block apoptosis [18].

At the time of apoptosis induction Smac/DIABLO is released from mitochondria into the cytosol, where it binds to IAPs and enables caspase activation [19]. It has been shown that Smac/DIABLO promotes not only the proteolytic activation of procaspase-3 but also the enzymatic activity of mature caspase-3, both of which depend upon its ability to interact physically with IAPs [19]. Smac/DIABLO is synthesized with an N-terminal mitochondrial targeting sequence that is proteolytically removed during maturation to the mature polypeptide. X-ray crystallography has shown that the first four amino acids (AVPI) of mature Smac/DIABLO bind to a portion of the third BIR (BIR3) domain of XIAP [20]. This N-terminal sequence is essential for binding IAPs and blocking their antiapoptotic effects [20].

Caspases are well known executioners of apoptosis. Once activated from their proactive forms, caspases target important proteins involved in cell proliferation and survival (for a review see [21]). Caspase-3 is one of the key executioner of apoptosis and induces downstream caspase-6 which in turn target cleavage of important structural proteins such as laminin and keratins [22,23]. Among other caspase targets, poly(ADP-ribose) polymerase (PARP, 113–116 kDa), a nuclear enzyme which is activated during DNA damage, is known to be cleaved by caspases-3 and -7 [24]. We have recently demonstrated that Akt is also a target for caspase-3 cleavage, indicating that Akt survival pathway inhibition is an important mechanism for apoptosis activation [12].

Although the regulation and importance of XIAP and Smac/DIABLO has been described in other systems and cell types, their expression has not been reported for the uterus. In the present study, we have investigated the regulation of XIAP, Smac/DIABLO, caspase-3, -6 and -7 during the four stages of estrous cycle (proestrus, estrus, metestrus and diestrus) and further analyses were carried out to determine the possible regulation of XIAP and Smac/DIABLO by 17 $\beta$ -estradiol in ovariectomized rats.

## Materials and Methods

### Reagents

XIAP, Smac/DIABLO, NF- $\kappa$ B, I $\kappa$ B, Phospho-I $\kappa$ B and cleaved-caspases antibodies were obtained from New England Biolabs (Mississauga, ON). Vectastain ABC Kit for rabbit IgG was purchased from Vector Laboratories Inc. (Burlingame, CA). Protease Inhibitor Cocktail Tablets, POD and DAB substrate were purchased from Roche (Laval, QC). 17 $\beta$ -estradiol (E<sub>2</sub>) was purchased from Laboratoire Mat (Québec, QC).

### Animals

Mature Sprague-Dawley female rats (200–225 g) were obtained from Charles River Laboratories Canada. Animals were maintained on standard chow and water, which were available *ad libitum*, in animal facilities illuminated on a normal 12 hour cycle. All procedures were performed in accordance with guidelines of the Canadian Council on Animal Care for the handling and training of laboratory animals and the Good Health and Animal Care Committee of the Université du Québec à Trois-Rivières. Stages of the estrous cycle were confirmed by vaginal smears. Rats with three regular cycles of 4 days were used in these experiments and killed at various stages of the estrous cycle (diestrus, proestrus, estrus and metestrus). Uteri were collected and fixed for immunohistochemical staining (IHC) or endometrial protein extracts collected by scraping the endometrium for Western analysis. To determine the effect of estrogen, rats were ovariectomized for at least 10 days and then injected with (17 $\beta$ -estradiol)

$E_2$ . Animals were treated for a total of 3 days and killed after hormone treatment (72 hours) according to previous preliminary time-course studies done in our laboratory (Leblanc et al., unpublished information) and information found in the literature [25].  $E_2$  was dissolved with sesame oil, and administered by subcutaneous injection. Sesame oil was injected into control animals. The dose administered was 40  $\mu\text{g}/\text{kg}/\text{day}$  ( $E_2$ ).

### **Immunohistochemistry**

The uterus was fixed in 4% paraformaldehyde solution and embedded in paraffin. Tissue sections 7  $\mu\text{m}$  thick were mounted on polylysine-coated slides, deparaffinized, rehydrated, and then heated with 10 mM citrate buffer (pH 6). After two wash with PBS, slides were then incubated with 0.3 % hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity. After washing with PBS, tissues were incubated with blocking serum (Vectastain ABC Kit) at room temperature for 1 h. Then, a primary antibody (XIAP 1:200; Smac/DIABLO 1:100; and cleaved caspase-3 1:100) was added to the slides and incubated at 4°C overnight. After washing 5 min in PBS, tissue sections were incubated for 30 min with 3  $\mu\text{g}/\text{ml}$  biotinylated antibody (anti-rabbit or anti-mouse). Subsequently, slides were washed with PBS and incubated with avidin-biotin complex reagent containing horseradish peroxidase for 30 min. Again washed with PBS for 5 min and colour development was achieved using DAB substrate. The tissue sections were counterstained with haematoxylin. Negative controls were performed using the same protocol without the presence of primary antibody.

### **Protein extraction and Western analysis**

Endometrium from each uterus was scraped using a glass microscope slide and homogenized using a pipette in the lysis buffer (PBS 1X pH 7.4; 1% Nonidet P-40; 0.5% Sodium deoxycholate; 0.1% SDS; Protease Inhibitor Cocktail Tablets (Roche)). Homogenates were centrifuged (12,000  $\times$  g for 20 min at 4°C) to remove insoluble material. The supernatant was recovered and stored at -20°C pending analysis. Protein content was determined with the Bio-Rad DC Protein Assay. Concentrations of reagents found in the lysis buffer were chosen to avoid any interference with the protein assay. Protein extracts (50  $\mu\text{g}$ ) were heated at 94°C for 3 min, resolved by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes using a semidry transfer (Bio-Rad, Mississauga, ON). The membranes were then blocked 2 h at room temperature with PBS containing 5 % milk powder, then incubated with XIAP 1:500; Smac/DIABLO 1:500; Caspase-3 1:1000; Caspase-6 1:1000; Caspase-7 1:1000; NF- $\kappa$ B 1:1000; I $\kappa$ B 1:1000; Phospho-I $\kappa$ B 1:500 and subsequently with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:3000; room temperature for 45

min). All membranes were reprobated with an antibody specific to  $\beta$ -actin which was used as an internal standard. Densitometrical analyses were performed on both films (protein of interest and  $\beta$ -actin) using the GelDoc 2000 and the Quantity One software (Bio-Rad, Mississauga, ON). Results are expressed as a ratio protein of interest/ $\beta$ -actin to correct for loading for each endometrial sample.

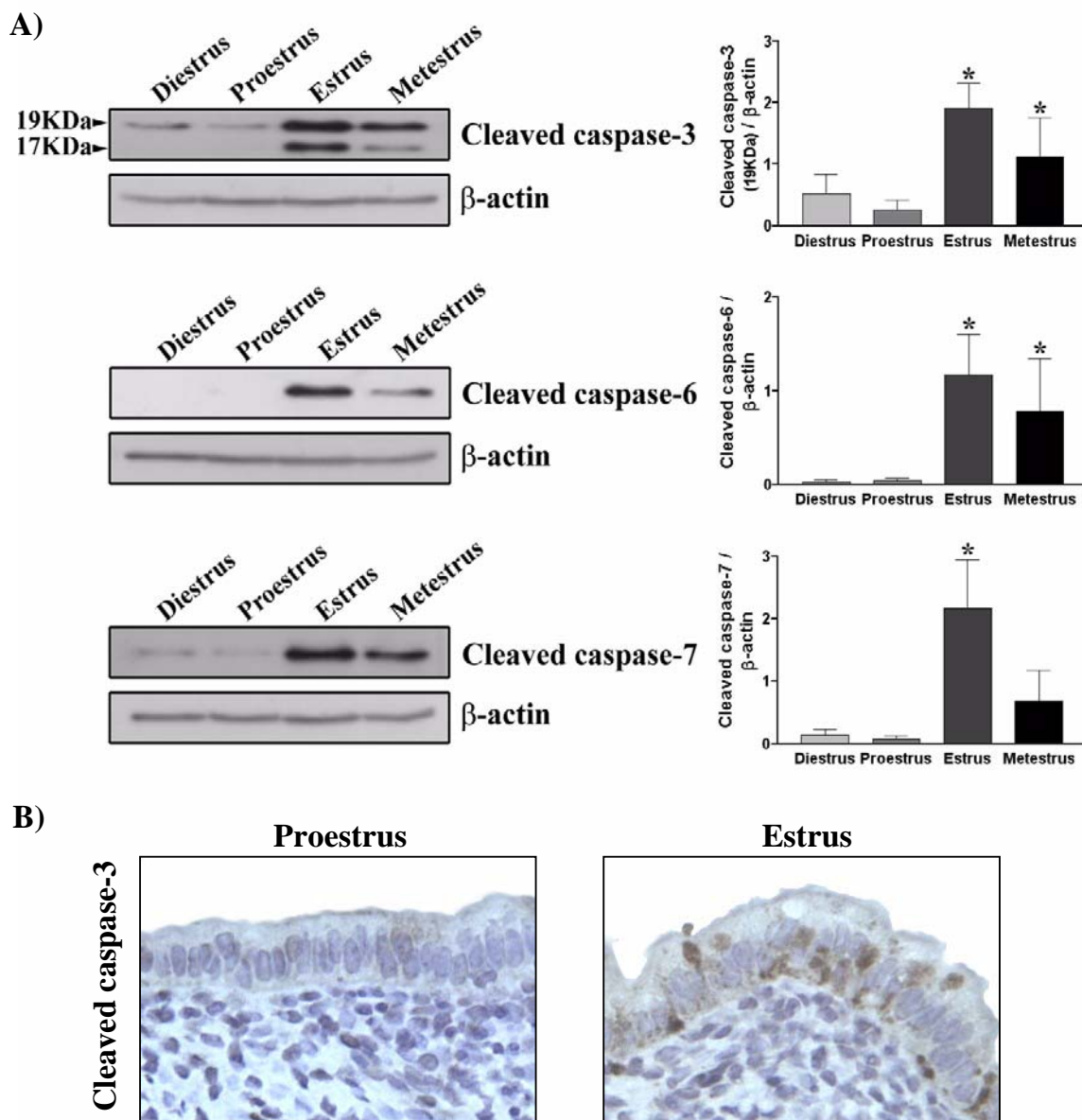
### **Statistical analysis**

Western analyses of cycling animals were repeated four to six times (one rat per extract/day repeated 4 to 6 different times). Endometrial extracts from each rats was assessed individually. Western blot analyses were performed with samples from ovariectomized rats treated with  $E_2$  (4 rats/group). Endometrial extracts from each rats was assessed individually for both studies. Results subjected to statistical analyses were expressed as means  $\pm$  SEM. Data were subjected to one-way ANOVA (PRISM software version 4.0; GraphPad, San Diego, CA). Differences between experimental groups were determined by the Tukey's test. Statistical significance was accepted when  $P < 0.05$ .

## **Results**

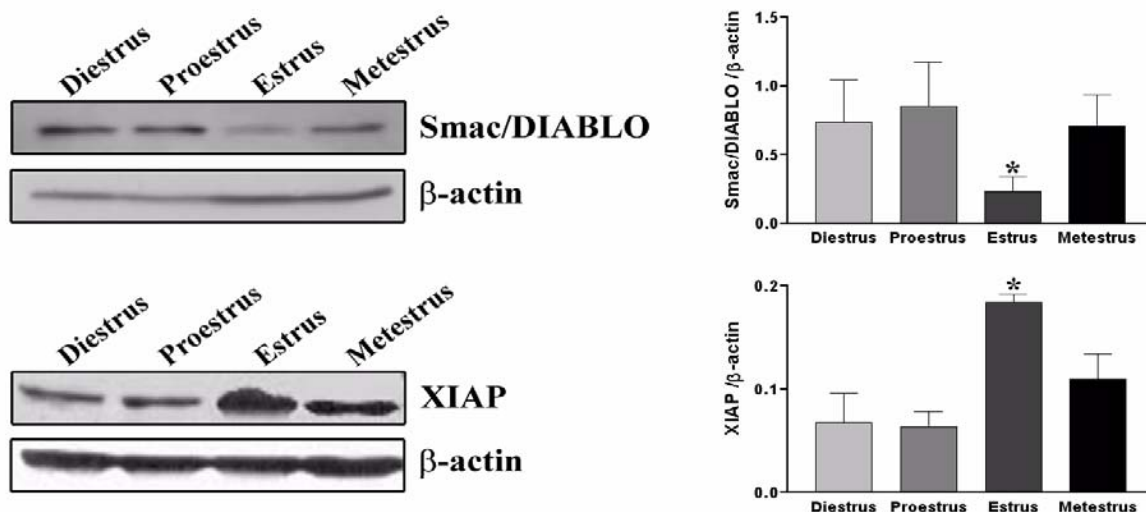
### **Caspases activation during the estrous cycle**

We have shown previously that apoptosis was maximal at estrus and weakly detectable at metestrus, diestrus and proestrus [7]. Apoptosis was mainly located in luminal epithelial cells at estrus. On the other hand, cell proliferation was maximal at proestrus as determined by expression of the cell proliferation marker CDC47/MCM7 [7]. Since executioner caspases such as caspase-3, -6 and -7 are key proteases involved in the execution of apoptosis, we have investigated, using antibodies specific for activated forms (cleaved fragments), their expression in the endometrium throughout estrous cycle. Caspase-3 fragments were detected in greatest amounts at estrus (Fig. 1). Caspase-3 19 KDa fragment was weakly detectable at diestrus and proestrus, maximal at estrus and further reduced at metestrus. Likewise, caspase-3 17 KDa fragment was detected only at estrus and metestrus but was significantly reduced at metestrus compared to estrus. Large cleaved caspase-6 subunit (18 KDa) was absent at diestrus and proestrus, maximal at estrus and reduced at metestrus. Similarly, large cleaved caspase-7 subunit (19 KDa) was weakly detected at diestrus and proestrus, was highly increased at estrus and reduced at metestrus. IHC studies using the antibody specific for cleaved caspase-3 (cleaved caspase-6 and -7 antibodies are not functional for IHC) revealed that cleaved caspase-3 fragment signal was located in apoptotic luminal epithelial cells at estrus and that the signal was absent in proliferative endometrium at proestrus.



**Figure 1**

Cleaved caspase-3, -6, and -7 expression in rat endometrium during estrous cycle. Polyoestrus rats were sacrificed at each stage of the estrous cycle (diestrus, proestrus, estrus and metestrus) and total endometrial proteins were collected. A) Western blots of cleaved caspase-3, -6 and -7 (one blot presented out of 4). Graphics shows Western blots densitometric analysis. Data represent the mean  $\pm$  SEM of four independent experiments (four different rats). B) Immunohistochemistry of cleaved caspase-3. IHC shown are from one representative experiment (a total of four different uterine sections from four different rats per day of the estrous cycle have been tested). \*Significantly different from diestrus and proestrus ( $p < 0.05$ ).



**Figure 2**

Smac/DIABLO and XIAP expression in rat endometrium during estrous cycle. Total endometrial proteins were collected from polyoestrous cycling rats at each stage of the estrous cycle (diestrus, proestrus, estrus and metestrus). A) Western blots analyses of Smac/DIABLO and XIAP (one blot presented out of 6). Graphics shows Western blots densitometric analysis. Data represent the mean  $\pm$  SEM of four independent experiments (four different rats). \*Significantly different from all other days of estrus cycle ( $p < 0.05$ ).

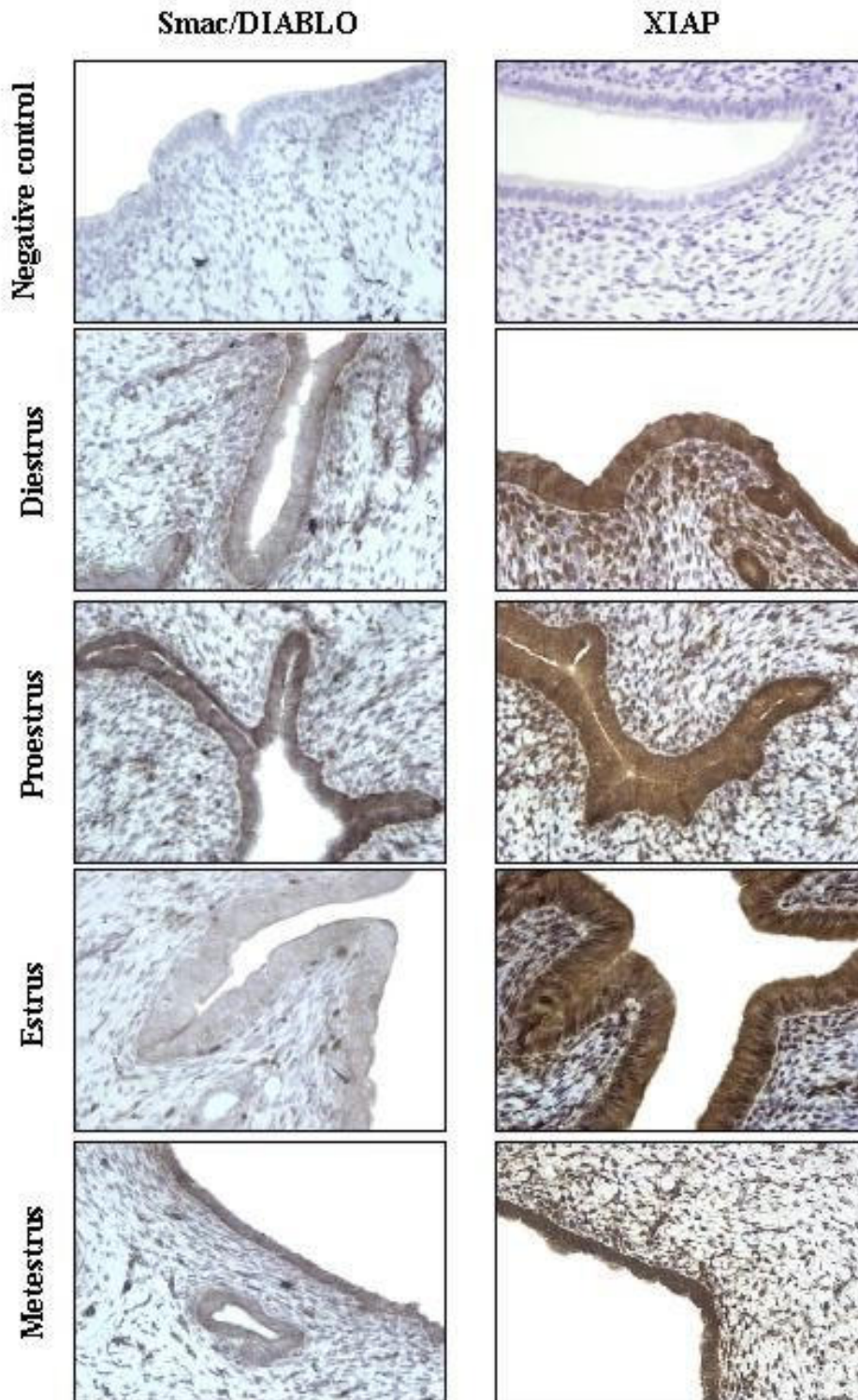
#### **XIAP and Smac/DIABLO expression during the estrous cycle**

Expression of XIAP and Smac/DIABLO proteins has not been reported for the uterus. Because we have demonstrated previously a possible link between XIAP and survival factor Akt [11,12] and further that Akt expression and activity/phosphorylation are regulated by  $17\beta$ -estradiol in rat endometrium [7], we have asked the question whether XIAP and its inhibitor Smac/DIABLO might have important roles in the control of endometrial cell fate. Western blot revealed that XIAP protein was present at all stages of the estrous cycle, but maximal at estrus (Fig. 2; 2.9-fold increase compared to proestrus;  $p < 0.05$ ). IHC revealed that XIAP expression was high in stromal cells and in both luminal and glandular epithelial cells at estrus (Fig. 3). Expression of Smac/DIABLO during estrous cycle was similar at metestrus, diestrus and proestrus but was significantly reduced at estrus (Fig. 2; 3.6-fold decrease compared to proestrus;  $p < 0.05$ ). IHC confirmed downregulation of Smac/DIABLO at estrus and

showed that it was localized mainly in epithelial cells but was also present in stromal cells (Fig. 3).

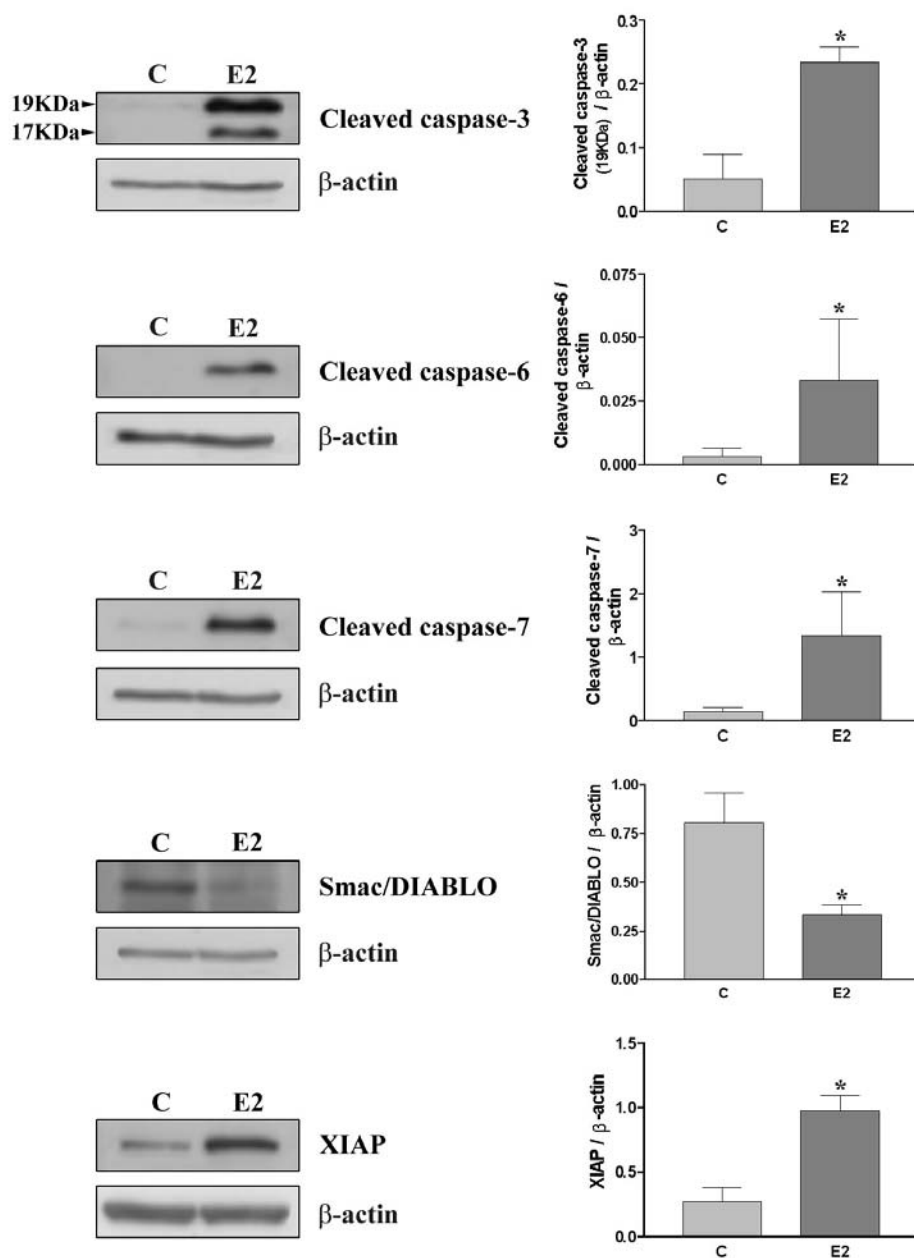
#### **Effect of $17\beta$ -estradiol on XIAP and Smac/DIABLO expression and caspases activation**

To further determine importance of  $17\beta$ -estradiol in the regulation of XIAP and Smac/DIABLO, ovariectomized rats were treated with  $17\beta$ -estradiol and uteri were recovered for Western analysis (Fig. 4) and IHC (Fig. 5) analyses. Our previous results indicated that  $17\beta$ -estradiol increased significantly endometrial cell proliferation (as determined by the expression of CDC47/MCM7) and increased Akt protein expression and its phosphorylation/activity [7]. In the present study, results shows that  $17\beta$ -estradiol induced significantly XIAP protein expression (4.0-fold compared to control;  $p < 0.05$ ) whereas it significantly inhibited Smac/DIABLO protein expression (2.7-fold compared to control;  $p < 0.05$ ) (Fig. 4). IHC analysis in ovariectomized rats confirmed the increase of XIAP expression and inhibition of Smac/DIABLO in the endometrium (Fig. 5). As observed during estrous cycle



**Figure 3**

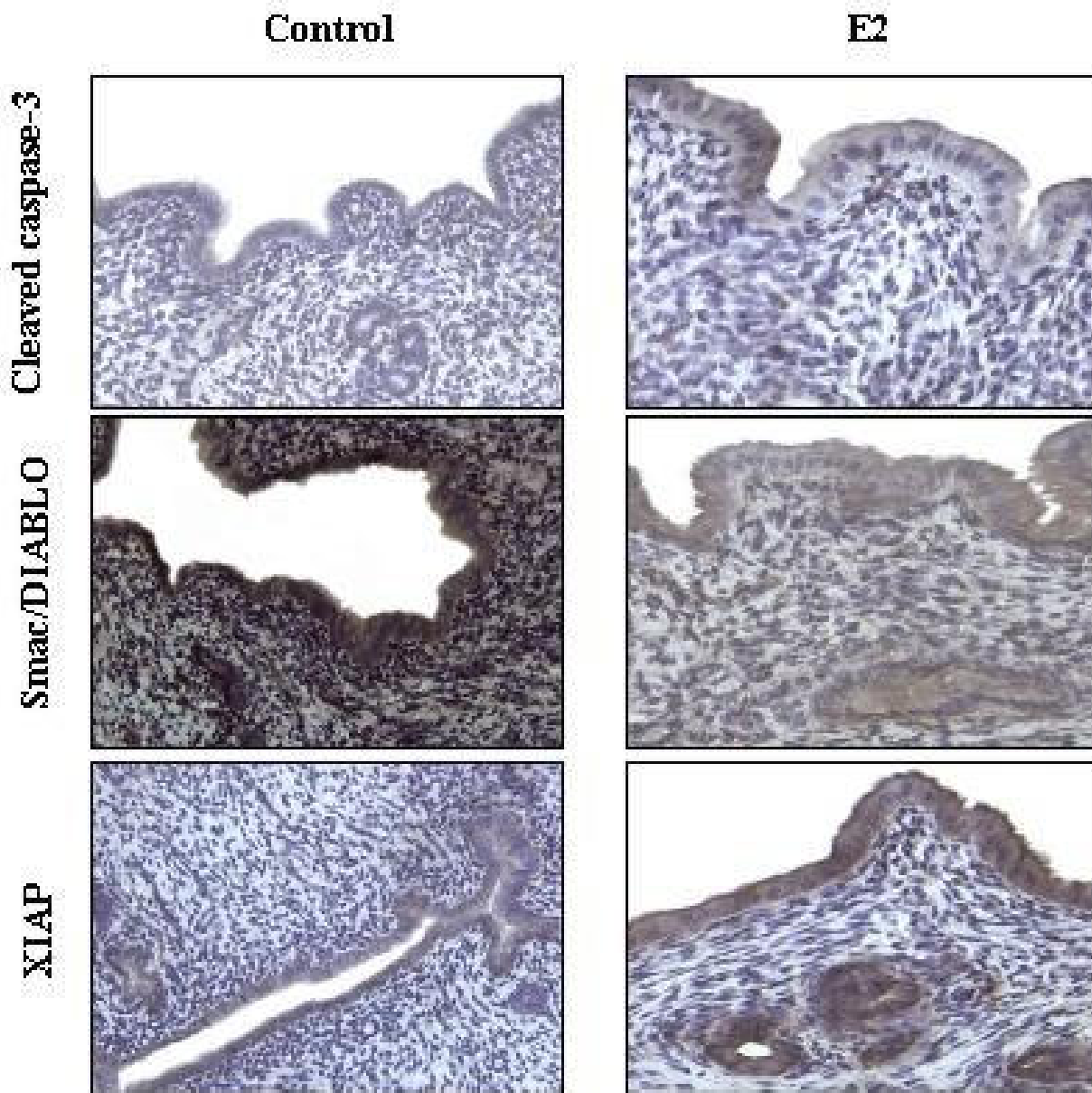
Immunohistochemistry of Smac/DIABLO and XIAP in rat endometrium during estrous cycle. IHC shown are from one representative experiment carried out on four different uterine sections (a total of four different uterine sections from four different rats per day of the estrous cycle have been tested). Negative control: primary antibody absent.



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**Figure 4**

Expression of cleaved caspase-3, -6, -7, Smac/DIABLO and XIAP in response to 17 $\beta$ -estradiol (E<sub>2</sub>) in ovariectomized rats. Ovariectomized rats received daily subcutaneous injections of E<sub>2</sub> (40  $\mu$ g/Kg/day) or vehicle for control group for 3 days. Graphics shows Western blots (one blot presented out of 4) densitometric analysis. Data represent the mean  $\pm$  SEM of four independent experiments (4 rats per group). \*Significantly different from control (p < 0.05).



**Figure 5**  
 Immunohistochemistry of Smac/DIABLO and XIAP in rat endometrium of treated ovariectomized rats. Ovariectomized rats received daily subcutaneous injections of E<sub>2</sub> (40 µg/Kg/day) or vehicle for control group for 3 days. IHC shown are from one representative experiment per group (a total of four different uterine sections from four different treated ovariectomized rats have been tested).

(Fig. 3), XIAP and Smac/DIABLO proteins were found mainly in epithelial cells but were also present in stroma (Fig. 5). The effect of 17β-estradiol on the regulation of

XIAP and Smac/DIABLO support the mitogenic role of 17β-estradiol and its involvement in cell survival.



### **NF- $\kappa$ B pathway regulation through the estrous cycle**

The NF- $\kappa$ B pathway has been shown to be activated by Akt in several cell types (for a review [26]). Furthermore, it has been shown that XIAP expression is up-regulated once NF- $\kappa$ B is activated and translocated to the nucleus [27,28]. The activation of NF- $\kappa$ B proceeds through the phosphorylation of its inhibitor, I $\kappa$ B $\alpha$ , that targets it for polyubiquitination and subsequent enzymatic degradation by the 26S ubiquitin proteasome [29]. Since we have showed an increase of Akt phosphorylation in response to E<sub>2</sub> [7], we sought to determine if NF- $\kappa$ B/I $\kappa$ B pathway might be involved in the regulation of XIAP expression in response to 17 $\beta$ -estradiol. The present study shows that NF- $\kappa$ B (both p50 and p65 subunits) and I $\kappa$ B proteins were both expressed in the endometrium but were not influenced by the estrous cycle (Fig. 6). Since I $\kappa$ B phosphorylation status is a sign of NF- $\kappa$ B activation, we also carried out Western analyses using a phospho specific I $\kappa$ B antibody (Fig. 6C). The results showed that phosphorylation of I $\kappa$ B was detected at all days of estrous cycle but was not influenced by the estrous cycle. These results demonstrate that NF- $\kappa$ B/I $\kappa$ B pathway is not involved in the regulation of XIAP expression in the cycling rat uterus.

### **Discussion**

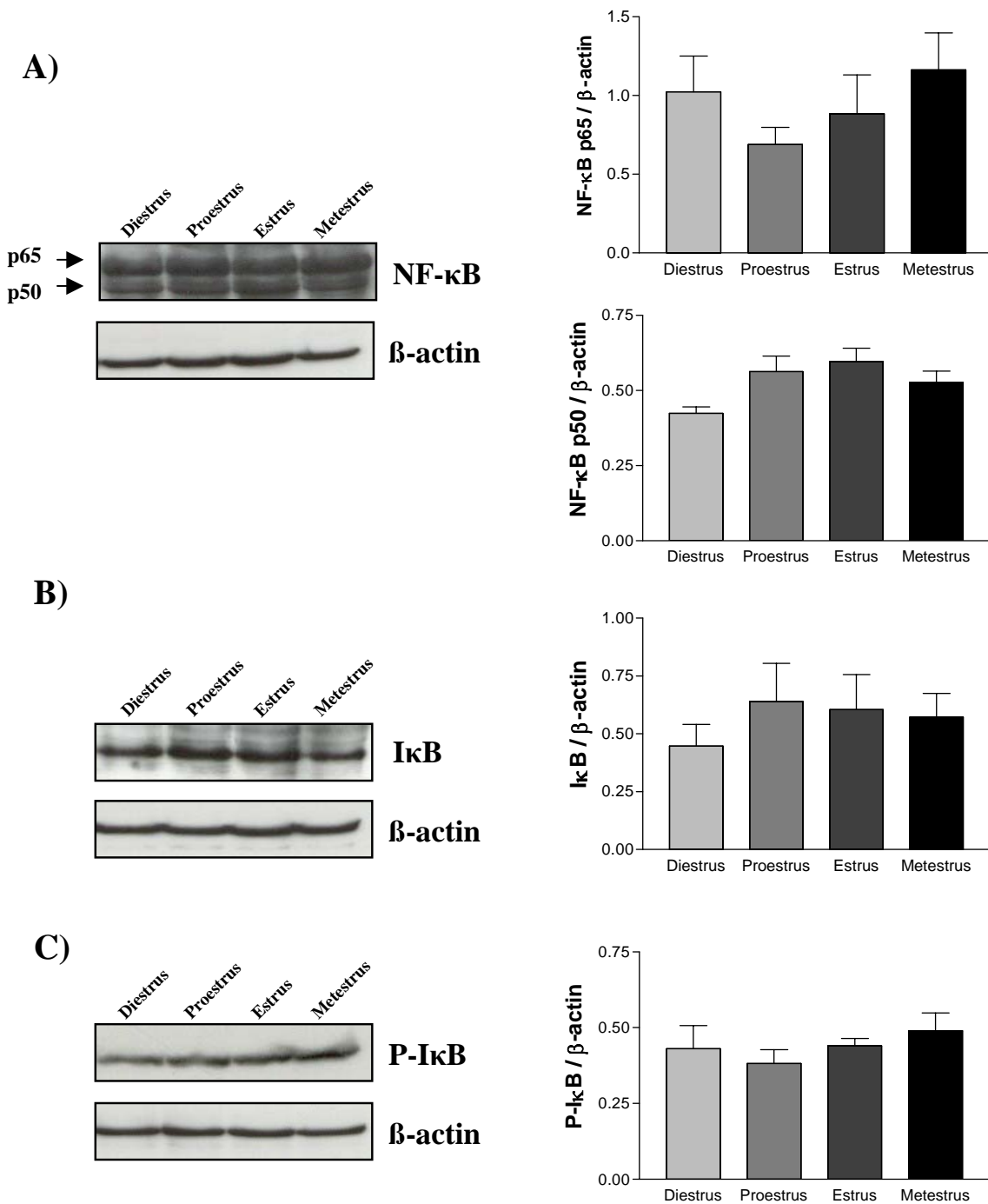
We and other have demonstrated the presence of apoptosis in the rat [2,3,7] and mouse [1] endometrium during the estrous cycle. Although our recent study showed that regulation of Akt survival pathway is a key factor involved in the regulation of endometrial cell fate during estrous cycle [7], molecular and cellular mechanisms involved in the regulation of apoptosis in the uterus are poorly documented in the literature. Since menses are absent in rodent specie, there must be precise intra-cellular mechanisms involved in the regulation of cell fate in the endometrium after estrogen withdrawal in the absence of embryonic factors. We have previously demonstrated in human ovarian cancer cells [12] and rat granulosa cells [11] the importance of XIAP and Akt expression/activity. In the present study, regulation of XIAP and Smac/DIABLO have been investigated in rat uterus to determine their importance and their possible influence on endometrial cell survival/death in relation to the stage of estrous cycle.

In the present study, expression of XIAP was maximum at estrus and increased in response to E<sub>2</sub> in ovariectomized rats. XIAP was strongly expressed in luminal and glandular epithelia and stroma during estrus, perhaps to protect these cells from apoptosis. Although stimulation of XIAP by E<sub>2</sub> is consistent with mitogenic activity and apoptosis inhibitory activity, it is not clear why XIAP expression does not increase during proestrus. A recent study showed that XIAP may be directly regulated by the presence of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in rat granulosa cells

[27]. Since it has been shown that TNF- $\alpha$  mRNA expression was high at estrus and was regulated by estrogen in the mouse uterus [30], a similar process may be involved in the endometrium. Thus, action of E<sub>2</sub> on XIAP expression might be indirect and might involve specific factors regulated upstream and/or downstream by this hormone. Whether involvement of TNF- $\alpha$  or other growth factors/cytokines might be involved in this process remains to be elucidated.

In contrast, Smac/DIABLO was decreased at estrus and E<sub>2</sub> treatment in ovariectomized rats reduced its expression. These results directly support the mitogenic action of estrogens on endometrial cells. Since Smac/DIABLO is a pro-apoptotic protein, its down-regulation may be sufficient to activate XIAP to inhibit caspase activity. Smac/DIABLO down-regulation also support our results obtained at estrus showing apoptosis predominantly in luminal epithelial cells [7] and suggest that this pro-apoptotic factor might have a key function in triggering apoptosis at this particular time of rat estrous cycle. There is currently no information available in the literature demonstrating that E<sub>2</sub> modulates Smac/DIABLO expression and the present findings are the first to demonstrate this mechanism. E<sub>2</sub> treatment resulted in the cleavage of caspase-3, -6 and -7 in endometrial cells. This results was contradictory to the fact that E<sub>2</sub> induces endometrial cells proliferation rather than inducing activation of the caspase cascade and apoptosis. Although it has been shown that E<sub>2</sub> triggers apoptosis in a preosteoclastic cell line *in vitro* [31] and in thymocytes of ovariectomized mice *in vivo* [32], it is unlikely that E<sub>2</sub> induced apoptosis in the endometrium. This can be explained by the fact that following injection of ovariectomized rats with E<sub>2</sub>, latent endometrial cells are forced to proliferate and they might be in a situation of stress. Indeed, controlling the balance between survival and death factors is a key mechanism in order to maintain homeostasis in the proliferating endometrium. Increased caspase cleavage is not necessarily a sign of apoptosis activation; caspases activity can be blocked by the presence of high levels of XIAP (or other known or unknown inhibitor of apoptosis proteins such as cIAPs [33]) and the presence of low levels of Smac/DIABLO. This particular mechanism in endometrial cells might be important in order to rapidly induce apoptosis at the appropriate timing (at estrus for instance) through the simple inhibition or degradation of XIAP protein through its ubiquitin protein ligase activity its degradation in proteasomes in response to apoptotic stimuli [34].

Another explanation for the induction of caspase activation in response to E<sub>2</sub> might be due to the presence and activity of E<sub>2</sub> metabolites. For example, 2-Methoxyestradiol (2ME2), a natural metabolite of E<sub>2</sub>, is a potent antitumor and antiangiogenic agent [35] and has been shown to



**Figure 6**  
 NF-κB, IκB and phospho-IκB expression in rat endometrium during estrous cycle. Total endometrial proteins were collected from polyoestrous cycling rats at each stage of the estrous cycle (diestrus, proestrus, estrus and metestrus). A) Western blots analyses of NF-κB, IκB and phospho-IκB (one blot presented out of 6). Graphics shows Western blots densitometric analysis. Data represent the mean ± SEM of six independent experiments (six different rats)

induce apoptosis is several cell type (for a review see [36]). Furthermore, 2ME2 is currently tested in clinical trials for cancer therapies [36]. 2ME2 is a major metabolite of estradiol in human serum and its concentration peaks at mid-cycle, like that of estradiol [37]. Studies have shown that 2ME2 acts through other signaling pathways than  $E_2$  [38,39]. A recent study revealed that 2ME2 treatment resulted in up-regulation of death receptor 5 (DR5) protein expression *in vitro* and *in vivo* and rendered cells more sensitive to the cytotoxic activities of the DR5 ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [40]. In addition, the latter study demonstrated that 2ME2-induced apoptosis required activation of caspase-8, caspase-9, and caspase-3 [40]. Another recent study also showed induction of caspase-3 and apoptosis in response to 2ME2 in gastric carcinoma cells [41]. These observations established in other systems and cell types suggest that 2ME2 could be an element of investigation in order to determine how  $E_2$  might be involved in the induction of caspase cleavage in rat endometrium and the possibility that activation of caspases observed might not be a direct action of  $E_2$ .

Recent studies demonstrated that the transcriptional nuclear factor kappa-B (NF- $\kappa$ B) is a direct downstream target of phosphorylated Akt [42]. NF- $\kappa$ B is sequestered (p65 and p50 subunits) in the cytoplasm by the I $\kappa$ Bs inhibitors which are phosphorylation targets of Akt. Upon phosphorylation I $\kappa$ Bs are released and degraded through ubiquitination and NF- $\kappa$ B enter nucleus for gene expression [43]. In the literature, we found that XIAP promoter is a target for NF- $\kappa$ B [27,28]. Since we have recently demonstrated the activation of Akt in response to 17 $\beta$ -estradiol in rat endometrium and that Akt has been shown to activate NF- $\kappa$ B signaling pathway [42], we wanted to know if Akt/ NF- $\kappa$ B/I $\kappa$ B pathway might be involved in the regulation of XIAP expression. Results of the present study showed that there was no difference in the expression of NF- $\kappa$ B and I $\kappa$ B nor I $\kappa$ B phosphorylation during the four stages of estrous cycle which exclude the involvement of this pathway in the regulation of XIAP or Smac/DIABLO expression. Other pathways downstream of Akt known to be regulated by this kinase are currently investigated in our laboratory such as eNOS [44,45], Bad [46], glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) [47,48], and caspase-9 [49]. A recent report further demonstrated that insulin-like growth factor-1 (IGF-1) induced activation of Akt, NF- $\kappa$ B and induced phosphorylation of Forkhead transcription factor (FKHR), which in turn up-regulated a series of intracellular anti-apoptotic proteins such as XIAP [50]. Because, FKHR is also a well known target for Akt action [51], phosphorylation, activity and regulation of this transcription factor in the uterus needs to be investigated.

In conclusion, the present study showed for the first time the presence and regulation of XIAP and Smac/DIABLO in the rat endometrium and further demonstrated that these two survival factors are regulated in the opposite direction by  $E_2$ . Further analysis will be necessary to determine more specifically, the intra-cellular and molecular signal transducers involved in the process of apoptosis in the rat reproductive tract and to determine the mechanism by which XIAP and Smac/DIABLO are regulated.

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