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Vasoactive intestinal peptide induces cyclooxygenase-2 expression through nuclear factor- κ B in human prostate cell lines. Differential time-dependent responses in cancer progression

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Abstract

The effect of vasoactive intestinal peptide (VIP) on cyclooxygenase-2 (COX-2) expression was analyzed in human prostate non-neoplastic (RWPE-1) as well as cancer androgen-dependent (LNCaP) and independent (PC3) cells. The three cell lines expressed VIP mRNA and VIP peptide, as measured by RT-PCR and immunochemistry, which supports an autocrine/paracrine action of VIP in the prostate gland. VIP levels were progressively higher from non-neoplastic to androgen-dependent and independent cells. Real-time RT-PCR and Western-blotting showed that VIP stimulated both COX-2 mRNA and protein expression in a faster manner as prostate cancer stage progressed (i.e. RWPE1 < LNCaP < PC3 cells). Furthermore, VIP induced higher levels of COX-2 protein expression in cancer cells as compared with non-neoplastic cells. The anti-inflammatory agent curcumin blocked VIP-induced COX-2 expression in all cell lines studied supporting the involvement of nuclear factor- κ B (NF κ B) in such a response. In fact, VIP increased the translocation of the NF κ B p50 subunit to the nucleus and the binding of the active form to its target gene promoter, as measured by Western-blotting and ELISA, respectively. VIP provoked faster responses according to the most aggressive status in cancer progression (androgen-independent situation). These results together with the existence of two NF κ B sites in the COX-2 gene promoter together suggest that COX-2 may be a target for VIP in prostate cancer progression. On the other hand, VIP could be a pro-inflammatory cytokine acting through the NF κ B/COX-2 system.

Keywords: VIP; NF κ B; COX-2; Prostate cancer progression; Inflammation

1. Introduction

Prostate cancer is a major public health concern worldwide since it represents one in ten cases of cancer in men and the highest incidence of any cancer (Jemal et al., 2004). Current treatment modalities include radical prostatectomy, hormonal therapy, cryotherapy and chemotherapy. However, new treatments are needed for either patients that initially exhibit metastatic disease or that do not respond to standard therapy. Most prostate tumours are initially androgen-dependent but become androgen-independent and refractory to hormone withdrawal therapy (Foley et al., 2004). The molecular mechanisms underlying the progression, relapse and tumour outgrowth that lead to incurable disease are poorly understood (Di Lorenzo et al., 2005). The undesired transformation is associated with the appearance of new cell phenotypes that result in angiogenesis, neuroendocrine differentiation and inhibition of apoptosis (Sternberg, 2002; Deusth et al., 2004). This multistep process involves changes in the function of growth factors and other active peptides that regulate cell growth, differentiation and survival, thus making very important the accurate knowledge of the corresponding signal transduction pathways from the cell surface to the nucleus (Vilches et al., 2004; Di Lorenzo et al., 2005).

Cyclooxygenase (COX) catalyzes the formation of prostaglandins from arachidonic acid. COX exists as two distinct isoforms: COX-1 is constitutively expressed in nearly all tissues and is involved in maintaining cellular homeostasis, whereas COX-2 is expressed by an inducible or immediate early response gene by cells involved in acute and chronic inflammatory states (Vane et al., 1998; Smith et al., 2000). There are strong evidences implicating COX-2 in the development of various human cancers which show overexpression of the enzyme as well as increased synthesis of prostaglandins (Zha et al., 2004). COX-2 gene can be induced by a variety of growth

factors, tumour promoters, oncogenes, and carcinogens. The promoter region of the COX-2 gene contains various transcriptional regulatory elements including those for nuclear factor- κ B (NF κ B), activator protein-1 (AP-1) and cAMP response element binding protein (CREB) (Charalambous et al., 2003; Chun and Surh, 2004). The role of COX-2 in prostate cancer remains unclear, since controversy exists regarding to the detection of COX-2 protein expression and activity in human tissues and established cell lines with reports on increased or even absent expression of COX-2 in prostate cancer (Hussain et al., 2003; Wagner et al., 2005). Moreover, some studies describe that proinflammatory atrophic lesions in prostate, which are thought to be precursors of prostate cancer, express COX-2 (Zha et al., 2001; 2004). Thus, the association of COX-2 to prostate carcinogenesis or cancer progression has led to consider that it may be a rational drug target for prostate cancer prevention. However, laboratory and clinical data make advisable to examine more carefully both the regulation and the function of COX-2 in prostate cancer (McCarthy, 2004; Shigemura et al., 2005).

Vasoactive intestinal peptide (VIP) is a pleiotropic neuropeptide that binds to two classes on membrane receptors (VPAC₁ and VPAC₂ receptors) which are mainly coupled to adenylyl cyclase activation through G α_s protein in many cells and tissues, including normal and cancer prostate gland (García-Fernández et al., 2003; Collado et al., 2005a) as well as prostate cancer cell lines (Gutiérrez-Cañas et al., 2003; Collado et al., 2004). VIP increases the expression of the major angiogenic factor, vascular endothelial growth factor (VEGF) (Collado et al., 2005b; 2006) and induces neuroendocrine differentiation (Collado et al., 2004; Gutiérrez-Cañas et al., 2005) in androgen-dependent prostate cancer LNCaP cells by a mechanism involving cAMP/protein kinase A (PKA), phosphatidylinositol 3'-kinase (PI3-K) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) signalling systems. Furthermore, VIP

protects from apoptosis in androgen-independent prostate cancer PC3 cells (Gutiérrez-Cañas et al., 2005). Angiogenesis, neuroendocrine differentiation and survival of cells are three steps seen in prostate cancer progression to the androgen-independent stage. Thus, both VIP and COX-2 appear to be involved in this evolution towards the most aggressive and worst-prognostic form of the disease. We have previously shown the role of AP-1 in VIP-induced VEGF expression in LNCaP cells (Collado et al., 2005a). VIP and COX-2 relationship and the presence of AP-1 and NFκB regulatory elements in the COX-2 gene promoter led us to study the regulatory role of VIP on COX-2 expression and the involvement of NFκB in such effect in prostate cancer. Tumour progression was monitored by using non-neoplastic as well as androgen-dependent and independent cancer cells.

2. Materials and methods

2.1. Materials

Synthetic VIP was purchased from NeoMPS. Affinity-purified rabbit polyclonal anti COX-2 antibody was obtained from Cayman and it did not react with COX-1. Purified goat polyclonal anti NFκB p50-subunit antibody was purchased from Santa Cruz. The specificity was evaluated by preadsorption with a specific blocking peptide. The secondary antibodies against rabbit and goat serum were from Oncogen and Santa Cruz, respectively. Curcumin (diferuloylmethane) and other chemicals were from Sigma.

2.2. Cell culture

Three human prostate cell lines, which exhibit different features of prostate cancer progression from early stages to androgen independence stages, were used. Non-

neoplastic, immortalized adult human prostatic epithelial cells (RWPE-1) are androgen-responsive and show many normal cell characteristics (Bello et al., 1997). The cancer cell line LNCaP is androgen-responsive and may be induced to acquire characteristics of neuroendocrine cells associated with many prostate adenocarcinomas (Collado et al., 2004). The androgen-unresponsive cell line PC3 (Gutiérrez-Cañas et al., 2003) may be related to recurrent prostate cancers that have achieved androgen independence and may receive only palliative therapy. The three cell lines were obtained from the American Type Culture Collection. RWPE-1 cells were maintained in complete keratinocyte serum-free medium (K-SFM) containing 50 µg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor. LNCaP and PC3 cells were grown and maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS). All culture media were also supplemented with 1% penicillin/streptomycin/amphoterycin B (Life Technologies). The culture was performed in a humidified 5% CO₂ environment at 37°C. When appropriate, cell morphology was analyzed by inverted and phase contrast microscopy with a Nikon Optiphot 2. After the cells reached 70-80% confluence, they were washed with PBS, detached with 0.25% trypsin/0.2% EDTA, and seeded at 30,000-40,000 cells/cm². The culture medium was changed every 3 days.

2.3. Isolation of RNA and RT-PCR

RWPE-1, LNCaP and PC3 cells ($1.5-3 \times 10^6$ cells in 100 mm cell culture dishes) were stimulated with 0.1 µM VIP in the presence of K-SFM or 10% FBS for different periods of time. Total cell RNA was isolated with Ultraspec RNA reagent (Biotecx) according to the instructions of the manufacturer. Two micrograms of total RNA were reverse-transcribed using 6 µg of hexamer random primer and 200 U M-MLV RT (Life Technologies) in the buffer supplied with the enzyme supplemented with 1,6 µg/ml

oligo dT, 10nM dithiothreitol (DTT), 40 U RNasin (Promega), and 0.5 mM of deoxyribonucleotides (dNTPs). Four microliters of RT reaction were then PCR-amplified with specific primers for COX-2: sense 5'-G ATA CTC AGG CAG AGA TGA TCT ACC C -3', and antisense 5'-CCT TTG GGT CTG GTG CCT GGT CT-3'. For VIP, the primers were: sense 5'-TAA AAG AAG ACA TTG ACA TGT TG-3', and antisense 5'-GAA GTT GTT TTC TTG AAT TAC TT-3'. PCR-conditions were: denaturation at 94°C for 5 min, followed by 40 cycles of 95°C 1 min, 57°C 1 min, 72°C 1 min, and then a final cycle of 10 min at 72°C. The signals were normalized with the β -actin gene expression level; the primers for β -actin were: sense 5'-AGA AGG ATT CCT ATG TGG GCG-3' and antisense 5'-CAT GTC GTC CCA GTT GGT GAC-3'. The PCR products were separated by electrophoresis and visualized in 2% agarose gels. The bands were cut from the gel, eluted, and automatically sequenced with an ABI 377 sequencer (Applied Biosystems).

2.4. Single-step real time quantitative RT-PCR

Real-time quantitative RT-PCR analysis was performed using SYBR Green PCR master mix, in one-step RT-PCR protocol according to manufacturer's protocol (Applied Biosystems). Two nanograms RNA samples were used. The thermal cycling parameters were 30 min at 48°C for RT and 10 min at 95°C for activation of AmpliTaq Gold DNA Polymerase, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers for COX-2 were: sense 5'-TGA CGG GGT CAC CCA CAC TGT GCC CGT CTA-3', and antisense 5'-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG-3' (Shirakawa et al., 2004). The relative quantification was normalized to the β -actin gene expression level. PCR reactions were performed using ABIPrism 7000 SDS (Applied Biosystems). The mean Ct (threshold cycle; cycle at which the increase in signal

associated with an exponential growth of PCR product is first detected) value of stimulated sample was compared to that of unstimulated control sample using the Ct value of β -actin as an internal control. Δ Ct was the difference in Ct values derived from COX-2 gene (in each sample assayed) and β -actin gene, while $\Delta\Delta$ Ct represented the difference between paired samples. The n-fold differential ratio was expressed as $2^{-\Delta\Delta$ Ct (Chang et al., 2002).

2.5- Protein isolation and Western blotting

RWPE-1, LNCaP and PC3 cells ($1.5-3 \times 10^6$ cells in 100 mm cell culture dishes) were stimulated with 0.1 μ M VIP for different time periods. Cells were washed twice with ice-cold PBS and then harvested, scraped into ice-cold PBS, and then pelleted by centrifugation at 500 x g for 5 min at 4°C. In order to obtain nuclear extracts, the packed cells were resuspended in 10 mM Tris-HCl (pH 7.5), 1.5 mM MgCl₂, and 10 mM KCl freshly supplemented with 2 mM DTT, 0.4 mM phenyl methylsulfonylfluoride (PMSF), 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 2 μ g/ml pepstatin, and 1 mM Na₃VO₄. Cells were kept on ice for 10 min, and then nuclei were pelleted by centrifugation at 17,000 x g for 10 min at 4°C. The pellet was resuspended into 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, 20% glycerol and 1.5 mM MgCl₂, freshly supplemented with the protease and phosphatase inhibitors listed above, and rotated for 30 min in a cold room. The extract was cleared by centrifugation at 20,000 x g for 30 min at 4°C. For preparation of cytosolic extracts the packed cells were resuspended into 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 5 mM NaF, 1 mM Na₃VO₄, and 1 mM NaN₃. Cells were kept on ice for 15 min, then 0.5% Nonidet NP-40 was added and the cytosolic extract was pelleted by centrifugation at 25,000 x g

for 40 s at 4°C. For preparation of cell lysates, the cells were kept on ice for 30 min in a solution containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl, 0.5% Nonidet NP-40, 2 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 5 µg/ml pepstatin. Thereafter, the cells were pelleted by centrifugation at 4,000 x g for 5 min at 4°C. Proteins from cell lysates or nuclear (20-30 µg) and cytosolic (60 µg) extracts were denatured by heating. Then, they were resolved by 10% SDS-PAGE, and blotted onto a nitrocellulose membrane (BioTrace/NT) overnight in 50 mM Tris-HCl, 380 mM glycine, 0.1% SDS, and 20% methanol. Rabbit anti-COX-2 (working dilution 1:500) or goat anti-NFκB p50 subunit (1:500) antibodies were added followed by incubation for 2 h at room temperature. After treatment for 1 h at room temperature with the corresponding secondary antiserum (1:2,000 for anti-rabbit serum and 1:4,500 for anti-goat serum) the signals were detected with enhanced chemiluminescence reagent (Pierce) using β-actin antibody as loading control.

2.6. VIP determination

VIP levels in the cytosolic cell fraction were determined by an enzyme immunoanalysis (EIA) with a commercially available kit according to the manufacturer's instructions (Phoenix). The antiserum had 100% cross-reactivity with human VIP and VIP(10-28), and <0.02% with the related peptide PACAP-27.

2.7. Measurement of NFκB nuclear binding activity

Binding of NFκB p50 subunit to the NFκB binding sequence 5'-GGGACTTCC-3' was measured with the ELISA-based StreeXpress NFκB p50 kit (Stressgen) using 10 µg of cell nuclear extracts. The assay was performed exactly according to the manufacturer's instructions. The captured active form of the NFκB p50

subunit was incubated with a NF κ B p50 antibody specific for an epitope which is accessible only when p50 subunit has bound to its target DNA. Thereafter, a secondary antibody conjugated to horseradish peroxidase was added. The assay was developed with a chemiluminiscent substrate and the signal was detected using a luminometer (Wallac Victor 1420 multilabel counter).

2.8. Data analysis

Quantification of band densities was performed using the Quantity One Program (Bio-Rad). Data shown in the figures are representative of at least three different experiments. The results are expressed as the mean \pm S.E.M. and were statistically treated following the Bonferroni's test for multiple comparisons after one-way analysis of variance (ANOVA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Expression of VIP mRNA and VIP peptide

The expression of VIP was analyzed at both mRNA and peptide levels by means of RT-PCR and immunochemical experiments. Photomicrographs are presented in Fig. 1 (A) to illustrate the morphological appearance of the human prostate cell lines used, i.e. non neoplastic (RWPE-1), and cancer androgen-responsive (LNCaP) or androgen-independent (PC3) cells. As shown in Fig. 1 (B1), RT-PCR using cDNA primers specific for a segment of the human VIP gene gave a product of the expected size in the three cell lines. The expression of β -actin mRNA served as internal control for the amount of RNA template in each RT-PCR aliquot: the single product form observed in all the samples suggested that there was no degradation in the RNA preparation. Semiquantitative analysis (Fig. 1, B2) of the bands showed increasing VIP mRNA

levels when advancing from non-neoplastic to androgen-independent conditions (RWPE-1 < LNCaP < PC3 cells). Thereafter, we evaluated whether the observed mRNA translated into the corresponding peptide (Fig. 1, C). The presence and concentration of VIP in cytosolic extracts were analysed by EIA. All the extracts gave an immunopositive response that increased similarly to that observed at the mRNA level (RWPE-1 < LNCaP < PC3 cells).

3.2. Effect of VIP on COX-2 mRNA expression

To investigate the effect of VIP on COX-2 signalling pathway, non-neoplastic (RWPE-1) and cancer (LNCaP and PC3) cells from human prostate were stimulated with 0.1 μ M VIP for different time periods. After extraction of total RNA, both RT-PCR and single-step real-time quantitative RT-PCR were carried out (Fig. 2). COX-2 mRNA expression was detected by RT-PCR in all conditions tested both before and after VIP addition. Real-time quantitative experiments indicated the time dependence of VIP effect upon COX-2 mRNA expression. Whereas 0.1 μ M VIP nearly doubled basal COX-2 mRNA levels in the three cell lines, important differences existed in the time-course pattern. Significant responses were observed at 4-6 h in RWPE-1, 30 min in LNCaP, and 15-30 min in PC-3 cells. These results suggest that VIP enhancement of COX-2 mRNA expression accelerates with cancer progression from normal to cancer androgen-independent stages.

3.3. Effect of VIP on COX-2 protein expression

Measurement of COX-2 protein levels by means of Western-blot analysis and further densitometry showed that the three cell lines studied displayed a positive response to 0.1 μ M VIP (Fig. 3). The stimulation of COX-2 protein expression was

substantially lower in normal cells than that for cancer cells. It is noteworthy that different time-course patterns were seen again since the response was faster with the status of development and behaviour of prostate cancer, i.e. from non-malignant RWPE-1 to cancer LNCaP and PC3 cancer cells. This feature correlates VIP effect on mRNA levels with translation into changes in protein levels of COX-2.

3.4. Effect of curcumin on VIP-induced COX-2 protein expression

In order to analyze the mechanisms by which VIP induces expression of COX-2 in prostate non-neoplastic and cancer cells, we used curcumin (diferuloylmethane). This yellow pigment of turmeric is known to suppress different stimuli on the activation of transcription factors such as NF κ B or AP-1 that interact with gene promoter regions (Aggarwal et al., 2006). Results in Fig. 4 show that the activation of COX-2 enzyme expression by VIP was completely abolished by curcumin in the three cell lines, as studied by Western blotting in cell lysates. For these experiments, after preincubation in the absence or the presence of 10 μ M curcumin, the cells were treated or not with 0.1 μ M VIP for 18 h (RWPE-1), 2 h (LNCaP) or 1 h (PC3), i.e. when VIP had been seen to be more effective.

3.5. Effect of VIP on NF κ B activation

To investigate whether VIP stimulatory activity on COX-2 production in prostate cell lines may be related to NF κ B-dependent COX-2 gene transcription, we evaluated the effect of VIP upon NF κ B nuclear translocation and binding activity. Measurement of NF κ B p50 subunit levels by Western-blotting after cell incubation in the absence or the presence of 0.1 μ M VIP showed that VIP stimulated p50 translocation from cytosol to nucleus for binding to its target DNA in RWPE-1 (Fig. 5),

LNCaP (Fig. 6) and PC3 (Fig. 7) cells. Interestingly, this VIP effect upon NF κ B p50 subunit translocation to the nucleus resulted in similar observations when studying p50 subunit binding activity as measured by an ELISA procedure that was specific for the captured active form of NF κ B p50 subunit bound to its target DNA (Fig. 8).

4. Discussion

The present study reveals that VIP induces COX-2 expression at both mRNA and protein levels in human prostate non-neoplastic (RWPE-1) and cancer (LNCaP and PC3) cells. VIP is one of the best studied neuropeptides present in the prostate gland as shown by observations in tissue pieces as well as in transformed cell lines. Various reports on LNCaP, PC3 and other human prostate cancer cell lines support the involvement of VIP in important processes such as promotion of angiogenesis (Collado et al. 2005b; 2006), neuroendocrine cell differentiation (Collado et al., 2004; Gutiérrez-Cañas et al., 2005) and protection from apoptosis (Gutiérrez-Cañas et al., 2003). These are crucial features that emerge during prostate cancer progression towards the most aggressive, androgen-independent state.

First of all, we analyzed whether VIP was expressed in the three classes of cells. Both VIP mRNA and peptide were detected by RT-PCR and immunochemistry, respectively, in normal RWPE-1 cells as well as in cancer androgen-dependent (LNCaP) and independent (PC3) cells. VIP expression increased from non-neoplastic to advanced cancer status of cells. This is the first demonstration of VIP expression in a human non-neoplastic prostate cell line and it confirms previous observations in prostate cancer LNCaP (Collado et al., 2006) and PC3 (Gutiérrez-Cañas et al., 2003) cell lines as well as in human normal prostate and prostate carcinoma specimens (Collado et al., 2005a) that also express VIP receptors. From our present and previous

observations, VIP appears to act at the prostatic level as an autocrine/paracrine factor in both normal conditions and prostate cancer progression.

The main aim of this study was to identify the putative action of VIP upon COX-2 expression since both agents have been associated with carcinogenesis, cell growth and survival, angiogenesis, invasiveness and other events in a variety of tumour types including prostate cancer (Gutiérrez-Cañas et al., 2003; 2005; Zha et al., 2004; Collado et al., 2004; 2005b). However, a common consensus for COX-2 expression and role in prostate tumorigenesis has not been established to date. In general, COX-2 expression in normal prostate tissue is either nonexistent or weak, whereas it increases in prostate cancer tissues with advancing tumour stage (Hussain et al., 2003; Wagner et al., 2005; Davis et al., 2005). On the other hand, elevated COX-2 levels have been found in benign proliferative hyperplastic lesions, high-grade prostatic intraepithelial neoplasia (HGPIN) and areas of the prostate with postinflammatory hyperproliferation which are considered precursors to prostate cancer (Zha et al., 2001; 2004; Wang et al., 2004). Both LNCaP and PC3 cell lines have been shown to express detectable amounts of COX-2 mRNA and protein (Wagner et al., 2005; Kirschenbaum et al., 2001). Present results show that in the human prostate cell lines, RWPE-1, LNCaP, and PC3, COX-2 mRNA and protein expression were consistently detected under basal conditions and it could be induced transiently by VIP. Interestingly, VIP-induced COX-2 protein expression was higher in cancer cells as compared with non-neoplastic cells. As expected, VIP effect upon COX-2 mRNA expression preceded that on COX-2 protein expression. However, it was noticeable that both developed faster with prostate cancer progression from normal to androgen-dependent and independent cancer stages as represented by the three cell lines used. These observations are likely related to features as the extensive cell proliferation, angiogenesis and apoptosis inhibition that

characterize advanced prostate cancer (McCarthy, 2004) so that cell susceptibility to VIP appears to increase during malignant transformation. However, additional studies on VIP effect upon prostaglandin production are advisable since it would not be prudent to assume that COX-2 expression correlates with COX-2 activity and, furthermore, it would add links between inflammation and cancer at the prostate level (Zha et al., 2001; Hussain et al., 2003).

To identify putative mechanisms through which VIP upregulates COX-2 expression, we studied the effect of curcumin (diferuloylmethane) upon COX-2 promoter activity induced by VIP. We found that this anti-inflammatory agent suppressed VIP-induced COX-2 expression in non-neoplastic and cancer cell lines from human prostate. It has been shown that curcumin inhibits NF κ B activation and down-regulates NF κ B-regulated gene products (i.e. COX-2 protein) involved in cell proliferation and survival, invasion, angiogenesis, metastasis and inflammation (Aggarwal et al., 2003; 2006). In this work, we report that curcumin abrogates VIP-induced COX-2 promoter activity in non-neoplastic and cancer prostate cells, which supports the involvement of NF κ B in such effect. It was reinforced by observations on that incubation of cells with VIP resulted in: a) increased translocation of NF κ B p50 subunit to the nucleus, as measured by Western-blotting, and b) increased binding of the active NF κ B p50 subunit to its target gene promoter, as measured by an ELISA-based procedure. Again, these responses were accelerated with cancer status progression. The NF κ B transcription factor is a heterodimer formed by the p50 and p65 proteins which is implicated in oncogenesis in various tissues, including the prostate gland where its nuclear localization has been associated with an increased risk of cancer recurrence (Fradet et al., 2004). Whereas VIP appears to downregulate NF κ B in the immune system (Delgado and Ganea, 2003) and bone (Juarraz et al., 2005), it is not the case at

the prostatic level as shown in the present study. Our observations agree with previous data on that angiogenesis, invasion and metastasis (i.e. features that are closely related to VIP activity) (Collado et al., 2005b; 2006) are inhibited by blockade of NF κ B activity in human prostate cancer cells (Huang et al., 2001). On the other hand, it is interesting to note that PI3-K is part of the NF κ B signalling pathway (Hughes-Fulford et al., 2006) but it is also involved in VIP signalling (Collado et al., 2004; Gutiérrez-Cañas et al., 2005) in prostate cancer. Also at this level, AP-1 (fos/jun) has been included in the VIP signalling mechanisms (Collado et al., 2005b) together with the well characterized PKA/CREB pathway (Collado et al., 2004; Gutiérrez-Cañas et al., 2005).

Progression of prostate cancer from androgen-dependent to androgen-independent advanced conditions appear to be modulated by many growth signals resulting in increased angiogenesis and cell survival. The current study makes COX-2 a target for these VIP effects by means of the NF κ B pathway since the COX-2 gene promoter exhibits two NF κ B sites (Charalambous et al., 2003). Indeed, COX-2 must be considered a triple target for VIP since the gene promoter also contains sites for CREB and AP-1 (Chun and Surh, 2004). VIP, acting in an autocrine/paracrine manner, would act as a facilitating agent of prostate cancer development through angiogenesis, invasion, and metastasis. Moreover, cells are oversensitive and present fast responses to VIP with prostate cancer progression to the poor prognostic disease status. On the other hand, present results also support a role of VIP as a proinflammatory cytokine which could be involved in prostate cancer progression acting through regulation of COX-2 expression. Finally, the potential therapeutic role of VIP antagonists combined with selective COX-2 inhibitors should be considered for prostate cancer. In this context, we have recently studied the antagonistic action of a growth-hormone-releasing hormone upon VIP receptor function in this malignancy (Collado et al., 2005a). This promising

molecule (JV-1-53) and other related peptide analogs inhibit the proliferation of LNCaP cells in vitro as well as tumour growth in nude mice bearing PC3 human prostate carcinomas (Rekasi et al., 2000; Plonowski et al., 2002). However, contradictory results on usefulness of some available COX-2 inhibitors have been reported at this level (McCarthy, 2004; Shigemura et al., 2005; Wagner et al., 2005). The relative resistance showed by various prostate cancer cell lines to COX-2 inhibitor therapy makes those molecules less effective against prostate cancer than other tumour types if the primary mode of action is by direct inhibition of tumour cell growth. Thus, new compounds with this activity are needed to discern their therapeutic abilities in prostate cancer.

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FIGURE LEGENDS

Fig. 1. Expression of VIP mRNA and VIP peptide in human prostate cell lines. A: morphological images of non-neoplastic (RWPE-1) and cancer androgen-dependent (LNCaP) or independent (PC3) cells. B1 and B2: RT-PCR analysis of VIP mRNA; the β -actin transcript was analyzed as a control. C: VIP quantification by EIA; values are the mean \pm S.E.M. * P < 0.05 vs. RWPE-1 cells (n = 3).

Fig. 2. Time-dependent VIP-induced expression of COX-2 mRNA. Cells were stimulated with 0.1 μ M VIP for the time periods indicated. Total RNA was submitted to real-time RT-PCR quantification for COX-2 mRNA. The β -actin transcript was analyzed as a control. Data in each bar are the mean \pm S.E.M. * P < 0.05, ** P < 0.025, *** P < 0.01 vs. control (n = 3).

Fig. 3. Time-dependent VIP-induced expression of COX-2 protein. Cells were stimulated with 0.1 μ M VIP for the time periods indicated. After preparation of cell lysates, Western blotting using antibodies to COX-2 and β -actin followed by densitometry of the bands gave the results shown. Data in each bar are the mean \pm S.E.M. * P < 0.05, ** P < 0.025, *** P < 0.01 vs. control (n = 3).

Fig. 4. Effect of curcumin on VIP-induced expression of COX-2 protein. After pretreatment for 1 h in the presence or absence of 10 μ M curcumin, cells were stimulated for 18 h (RWPE-1), 2 h (LNCaP) or 1 h (PC3) with 0.1 μ M VIP. Thereafter, cell lysates were prepared and assayed for COX-2 and β -actin levels by Western blotting and densitometry. Data in each bar are the mean \pm S.E.M. ** P < 0.025, *** P < 0.01 vs. control; ### P < 0.01 vs. VIP (n = 3).

Fig. 5. Time-dependent effect of VIP on NF- κ B p50 subunit translocation in RWPE-1 cells. Cells were incubated with 0.1 μ M VIP for the time periods indicated. Thereafter, cytosolic and nuclear extracts were prepared and assayed for NF- κ B p50 subunit and β -actin levels by Western blotting and densitometry. Data in each bar are the mean \pm S.E.M. * P < 0.05, ** P < 0.025 vs. control (n = 3).

Fig. 6. Time-dependent effect of VIP on NF- κ B p50 subunit translocation in LNCaP cells. Cells were incubated with 0.1 μ M VIP for the time periods indicated. Thereafter, cytosolic and nuclear extracts were prepared and assayed for NF- κ B p50 subunit and β -actin levels by Western blotting and densitometry. Data in each bar are the mean \pm S.E.M. *** P < 0.01 vs. control (n = 3).

Fig. 7. Time-dependent effect of VIP on NF- κ B p50 subunit translocation in PC3 cells. Cells were incubated with 0.1 μ M VIP for the time periods indicated. Thereafter, cytosolic and nuclear extracts were prepared and assayed for NF- κ B p50 subunit and β -actin levels by Western blotting and densitometry. Data in each bar are the mean \pm S.E.M. ** P < 0.025, *** P < 0.01 vs. control (n = 3).

Fig. 8. Time-dependent effect of VIP on NF- κ B p50 subunit binding to DNA. Cells were incubated with 0.1 μ M VIP for the time periods indicated. Thereafter, nuclear extracts were prepared and assayed for NF- κ B p50 subunit binding activity by an ELISA-based procedure (see Methods). Data in each bar are the mean \pm S.E.M. ** P < 0.025, *** P < 0.01 vs. control (n = 3).















