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Direct Effect of a Gonadotropin-Releasing Hormone Agonist on the Growth of Canine Mammary Tumour Cells

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Abstract Gonadotropin-releasing hormone (GnRH) agonist exert ''in vivo'' an inhibitory action on the growth of hormone-dependent canine mammary tumours (Lombardi et al. [1999] J. Vet. Pharmacol Ther. 22(1):56–61). The present experiments have been performed ''in vitro'' in order to investigate the mechanisms involved in this direct antiproliferative action of GnRH agonists. In particular, the aim was to study whether these compounds might exert their antiproliferative effect by interfering with the stimulatory action of epidermal growth factor (EGF). To this purpose, the effects of GnRH agonist, Goserelin (GnRH-A), on the mitogenic action of EGF, on EGF-activated intracellular signaling mechanisms (intracellular calcium and nitric oxide production) as well as on ATP induced cell proliferation and signalling, and on the binding of EGF receptors have been evaluated in primary culture of canine mammary tumour cells. The results of these ''in vitro'' studies show that GnRH-A counteracts the mitogenic action of EGF and ATP, decreases the EGF/ATP-induced calcium signalling and reduces EGF binding, probably by means of NO-induced $[Ca^{2+}]_i$ downregulation. These data suggest that GnRH agonists may inhibit the proliferation of the tumour cells by interfering with the stimulatory action of EGF. J. Cell. Biochem. 85: 470–481, 2002.

Key words: canine mammary tumour; epidermal growth factor; gonadotropin-releasing hormone

The role of female hormones, especially oestrogen in the development of mammary tumours in mammals, including the bitch is well established [Schneider, 1970; Owen, 1979; Lespagnard et al., 1987]. Ovarian hormones act synergistically with pituitary hormones, especially growth hormone and prolactin to promote the development of mammary tumours [Schneider et al., 1969; Schneider, 1970].

Besides receptors for sex steroids, mammary tumours, as well as established cell lines, express receptors for a wide variety of polypeptide growth factors, which may stimulate or repress cellular proliferation. Mammary tumours can produce growth factors that potentially could modulate their own proliferation in an autocrine fashion (i.e., TGF-α & TGF-β) or with a paracrine mechanism (i.e., EGF, FGF, PDGF, IGF).

The expression of EGF-R and/or their ligands, as well as for other growth factors, is related in mammary tissues to the action of oestrogen and progesterone and to the presence of functional receptors for oestrogen (ER) and progesterone (PGR) [Stewart et al., 1990; Koenders, 1992]. Consequently, several therapeutic approaches to this malignancy are aimed at achieving a blockade of ovarian hormones secretion and/or action [Robustelli della Cuna, 1992; Bajetta et al., 1994]. Gonadotropin-releasing hormone-agonists (GnRH-A) have been shown to be effective to suppress ovarian hormones in bitch, through the downregulation of the pituitary-ovarian axis [McRae et al., 1985; Vickery et al., 1989; Kawakami et al., 1991].

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Despite the fact that surgical castration showed no therapeutic effect on canine mammary tumours [Yamagami et al., 1996; Morris et al., 1998], our previous results showed that Goserelin (GnRH-A), which acts according with a chemical castration, is efficacy on such disease producing tumour shrinkage and reducing the incidence of metastases in bitches with hormone-dependent mammary cancer [Lombardi et al., 1999]. This suggests that GnRH-A efficacy is not due solely to the suppression of gonadal activity but also to the block of hypothalamus-pituitary axis and to a direct action on tumour cells, such hypothesis is supported by the detection of GnRH receptors in canine mammary tumours [Sartin et al., 1995] as well as from the results of Vincze et al. [1991], who have reported that the GnRH agonists significantly inhibit the growth of xenografts of the oestrogen-receptor-negative of human MDA-MB 231 mammary tumour, and the effect of GnRH-A on murine MXT mammary adenocarcinoma which reduce significantly the concentration of EGF binding sites [Szende et al., 1990]. Moreover, recent findings showed that GnRH agonists exert both in vivo and in vitro a direct inhibitory action on the proliferation of human prostate tumour cells by interfering with the stimulatory action of EGF, reducing EGF-R and c-fos expression [Montagnani et al., 1997].

On the other hand, several authors showed that canine mammary tumours differ from the same disease in human [Owen, 1979]. In fact, in human breast cancer, an inverse correlation was observed between ER and EGF-R positivity [Grimaux et al., 1989; Formento et al., 1990; Koenders et al., 1991; Nicholson et al., 1994]. Despite the fact that normal and neoplastic canine mammary tissues express ER, PR and EGF-R [Donnay, 1993; Donnay et al., 1995], a significant direct correlation was observed between the concentrations of ER and EGF-R in canine malignant tumours [Donnay et al., 1996]. These findings suggest that the growing mechanisms of human hormone-dependent breast cancer are different from those of the canine tumours.

Tumour growth factor (TGF-α) and epidermal growth factor (EGF) bind to the same membrane receptor (EGF-R), which is a Class I receptor tyrosine kinase [Baselga et al., 1996] which induce direct PIP_2 hydrolysis, by a direct phosphorylation of PLCs, producing Inositol trisphosphate ($InsP_3$) and variations in $[Ca^{2+}]_i$ sustained by both release from intracellular stores and influx across the plasmalemma [Berridge, 1993; Fantl et al., 1993]. The divalent cation calcium (Ca^{2+}) is used by cells as a second messanger to control many cellular processes, including muscle contraction, secretion, metabolism and neuronal excitability [Berridge, 1993]. Moreover, cytosolic Ca^{2+} plays an important role in the regulation of several cell types [Meldolesi et al., 1991]. Importantly, it participates in the regulation of the cell cycle in proliferating cells and of tumour cells in particular [Dixon et al., 1997]. Moreover, it has long been thought that Ca^{2+} and NO (nitric oxide) work together in the control of cell homeostasis and NO could have appeared as a step in the signalling cascade initiated by the cation. However, the interaction between the two messengers does not exist as a dependence but as a true, bi-directional cross-talk. In fact currently, almost all aspects of Ca^{2+} homeostasis have been reported to involve modulation by NO [Clementi and Meldolesi, 1997]. Therefore, the present experiments have been performed in order to verify if GnRH-A may possess a direct effect on the growth of canine mammary tumour cells and whether a GnRH regulatory system may be present in these cells by interfering with the stimulatory action of epidermal growth factor (EGF). For this purpose, the in vitro effect of Goserelin, on EGF-activated intracellular signalling mechanism (intracellular calcium $[Ca^{2+}]_i$ variations and nitric oxide production), which is a primary step in the mechanism of action of EGF, on EGF-induced cell proliferation and on EGF receptors binding affinity has been evaluated.

MATERIALS AND METHODS

Chemicals

D-ser(But[t])[6]-Arzgly[10]-GnRH (Goserelin acetate) was kindly provided from Astra-Zeneca (Milan, Italy), murine-epidermal growth factor, Dulbecco-MEM, Ham's Nurient Mixture F-12, Sulfanilamide, N-(1-napthyl)ethylendiamine dihydrochloride, Type 1 Collagenase, Trypan blue/PBS, antibodies against α-smooth muscle actin, Nitrate reductase, NADPH, FAD, vimentin, desmin, and keratin were purchased from SIGMA (Milan, Italy), [2,4,6,7-^{3}H]-Estradiol (specific activity 98 Ci/mmole) and [^{3}H]-ORG 2058 [16-alpha-ethyl-21-hydroxy-19-nor-pregn-4-ene-3,20-dione)-6,7-^{3}H] (specific activity 44 Ci/

mmole) were purchased from Amersham Italia, Milan, [^{125}I]-murine epidermal growth factor (specific activity 110 Ci/mmole) was purchased from Nen-Dupont (Italy).

Cell Culture

A tumour mass 3 cm × 5 cm was obtained from the mammary gland of a 8-year-old female Irish setter dog. Histologically, the tumour was diagnosed as a lobular/invasive carcinoma according to Moulton [1990], and bone and lung metastases were demonstrated by radiography.

The tissue was minced and digested for 8 h at 37°C in humidified atmosphere of 5% carbon dioxide in air with 4 ng ml^{-1} collagenase (125 U mg^{-1}) in Dulbecco's Modified Eagle Medium (D-MEM) and Ham's Nurient Mixture F-12, containing 10% Foetal Calf Serum (FCS), 100 IU ml^{-1} Pencillin G and 100 μg ml^{-1} Streptomycin. The digested tissue was filtered through nylon mesh cloth (80 μm), centrifuged at 184*g* for 10 min and cultured as described by Tateyama et al. [1990]. Briefly, the isolated cells were cultured in D-MEM/ F-12 containing 10% foetal calf serum (FCS), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 10 μg ml^{-1} insulin and 1 μg ml^{-1} hydrocortisone at 37°C in humidified atmosphere of 5% carbon dioxide in air and observed daily with an inverted microscope. After 48 h of incubation, the supernatant, containing the tumour cells, which did not initially attach to the plate, was transferred to new flasks in the same medium without insulin and hydrocortisone.

A coverslip with attached cells was washed twice in PBS, fixed in cold acetone (−20°C) and stored at −20°C for 30 min. The cells then reacted with antibodies against α-smooth muscle actin, vimentin, desmin, and keratin (Sigma) overnight at 4°C. The attached antibodies were visualised by an avidin-biotin labelling system (Sigma).

Receptors Assay

Oestrogen and progesterone binding assay. Tumour cytosols were used for the oestrogen (ER), progesterone receptors (PgR) and protein assay. The ER and PgR concentration was measured, using the classical biochemical method (7-point saturation curve with increasing concentration of [2,4,6,7-^{3}H]-Estradiol (specific activity 98 Ci/mmole) for ER assay and [^{3}H]-ORG 2058 (specific activity 44 Ci/mmole) for PgR assay (Amersham Italia, Milan). A solution of dextran-coated charcoal was used for the separation of free and bound ligands [EORTC, 1980]. The aspecific binding was assessed by adding a concentration of 1 μM diethylstilbestrol (DES) or 3 μM of ORG 2058. The number of receptors and the dissociation constant (Kd) were computed, using the Scatchard method [Scatchard, 1949]. Sensitivity of the assay was 3 fentomoles of receptors per milligram of cytosol protein. The cytosolic protein content was estimated by the Lowry method [Lowry et al., 1951]. Cytosols containing less than 2 mg of protein per millilitre were not considered further. The number of sites was expressed in fentomoles per milligram of cytosolic proteins. Each test was repeated twice and the mean was calculated.

GnRH-binding assay. Iodination of tracer ([^{125}I]-Goserelin) and receptor assay were performed according to Chamson-Reig et al. [1997].

D-ser(But[t])[6]-Arzgly[10]-GnRH (Goserelin acetate)(gift from Astra–Zeneca) was used as tracer and unlabelled hormone in the binding assay. GnRH-A was iodinated using a chloramines-T method. Briefly, 2 μg of GnRH-A were iodinated in presence of 2 mCi [125]Iodine and 0.2 μg chloramines-T. Reaction proceeded for 2 min. The procedure was repeated once, and reaction was stopped by transferring to a carboxy-methyl cellulose column and eluting unbound iodine with 2 mM ammonium acetate, and the labelled analogues with 60 mM ammonium acetate. The iodinated analog was stored at 4°C in the last buffer and used within 3 week of preparation. The specific activity of each preparation was assessed by self-displacement in the receptor assay using crude membrane fraction. Specific activities ranged from 400 to 800 μCi/μg. For saturation analysis, membrane protein samples (50 μg) were incubated with 5–8 × 10^{4} cpm [^{125}I]-GnRH-A. Ligand concentration was near saturation, representing about 85% receptor occupancy. Nonspecific binding was determined by addition 1 × 10^{-6} M unlabelled GnRH-A and represented 5–6% of total iodinated tracer. For Scatchard analysis, membranes were incubated with increasing concentration of the labelled analogue (5,000–120,000 cpm). In all cases, tubes were incubated for 120 min on ice and the reaction was terminated by adding 3 mL ice-cold washing buffer. Bound ligands were separated from the free ligands by vacuum filtration on Watman GF/C filters. The filters were washed twice with 3 ml

ice-cold incubation buffer and counted in a Packard Gamma Counter Multi Prias.

EGF-binding assay. EGF receptors were determined using a ligand-binging method according to a modified published method [Lubrano et al., 1993]. Briefly, membrane protein samples (50 μg) were incubated in 20 mM HEPES pH 7.4 containing 1 mM $MgCl_2$ and 1% BSA for 60′ at 25°C with increasing concentrations of [125I]-EGF (0.01–10 nM) in order to obtain saturation curve or with a single concentration of [125I]-EGF (1 nM) in presence or in absence of increasing concentrations of unlabelled EGF or GnRH-A in order to obtain competition curve. Non-specific binding was determined in the presence of unlabelled EGF (10 μM). The reaction was stopped by adding 3 mL ice-cold washing buffer. Bound ligand was separated from free by vacuum filtration on Watman GF/C filters. The filters were washed twice with 3 ml ice-cold incubation buffer, and counted in a Packard Gamma Counter Multi Prias. The number of EGF-receptors (B max) in the sample, the binding affinity (expressed as the dissociation constant [Kd]) and the displacement curve was analysed on a personal computer using the LIGAND software according to the two-site model [Munson and Rodbar, 1980].

Cell Proliferation Experiments

The effect of GnRH agonist on the EGF-stimulated growth of canine mammary tumour cells was studied by plating 5×10^4 cells/ml of medium supplemented with 1% of FCS with different concentration of GnRH and EGF used alone or in association. The final ethanol concentration never exceeded 0.1%. Cell culture were set up in triplicate for each drug concentration and control dishes were run in parallel. After 24–48 h of treatment, cell were collected by trypsinisation and counted in a hemocytometer. Cell viability was assessed by trypan blue dye exclusion.

$[Ca^{2+}]_i$ measurement. Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured by using the ratiometric fluorescent indicator dye Fura2-AM, the membrane-permeant form of Fura2-AM. Cell monolayers, gently detached and washed, were resuspended in Krebs Ringer (KRH) medium with Ca^{2+}-sensitive dye Fura2-AM for 30 min at 25°C and then maintained at 37°C until use. Cells were subsequently centrifuged and resuspended in KRH supplemented with 250 μM sulfinpyrazone to prevent dye leakage. The samples were then transferred to a thermostat-equipped cuvette (37°C), maintained under continuous stirring and analysed in a SPEX spectrophotofluorimeter. The experiments were initiated by incubation of cell aliquots (4×10^6 cells) with GnRH agonist (Goserelin) for 5 min. In the experiments in which Ca^{2+} release and Ca^{2+} influx were analysed separately, cell samples were resuspended in a KRH medium from which $CaCl_2$ had been omitted, and 10 μM EGTA had been added. After stimulation with EGF or ATP, the recording was continued until the end of the first $[Ca^{2+}]_i$ peak (intracellular Ca^{2+} release). Ca^{2+} (2 mM) was then introduced into the medium, and the ensuing second peak (Ca^{2+} influx) was recorded. At the end of the experiment, calibration was performed determining minimal fluorescence induced by 0.1% Triton X-100 in presence of 25 mM EGTA (F_{min}) and maximal fluorescence induced by 2 mM $CaCl_2$ (F_{max}). $[Ca^{2+}]_i$ was calculate according to the method of Grynkiewicz et al. [1985]. Graphs show the mean ± standard deviation values of 6–10 experiments.

Nitrite and Nitrate Assays

The production of nitrite (NO_2^-) and nitrate (NO_3^-), stable metabolites of nitric oxide (NO), was determined in the supernatant of cell cultures incubated in elsewhere cited experimental conditions by the Griess reagent [Chandler et al., 1995], which contained 1 part 0.75% sulfanilamide in 0.5 N HCl to 1 part 0.75% N-(1-napthyl)ethylendiamine dihydrochloride in water. Nitrate was reduced to nitrite by nitrate reductase 0.4 U/mL, in the presence of 10 mM NADPH and 2.5 mM FAD and then assayed as nitrite. The plates were incubated with the Griess Reagent at 25°C under reduced light for 10 min. Absorbance was read at 550 nm using a Perkin Elmer UV spectrophotometer. The concentration of NO was calculated on a calibration curve (range: 0.125–8 μg/ml), prepared using dilutions of sodium nitrate in the plating medium. Protein was determined by the method of Lowry et al. [1951], and the data were expressed as nanomole of nitrite per milligram of protein per 24 h. The sensitivity of the assay was 0.125 μg/ml.

Statistical Analysis

Statistical analysis was performed by means of Student's *t* test. Data are expressed as

mean ± standard deviation of three experiments. An error probability with $P < 0.05$ was selected as significant. All experiments were performed in duplicate and the mean was used for analysis. IC_{50} was calculed according to Cheng and Prusoff [1973].

RESULTS

The cultured cells showed that morphological characteristics were in almost complete conformity with those of epithelial cells, as confirmed by immunohistochemical examination with antivimentin and antidesmin antiserum.

Tumour cells were positive for oestrogen (ER) and progesterone receptors (PgR) and GnRH receptors (ER: 33.4 ± 3.2 fmoles/mg of proteins; PgR: 18 ± 6 fmoles/mg of proteins GnRH: 22.3 ± 3.1 fmoles/mg of proteins), and the doubling time was 24 h.

[^{125}I]-mEGF binding in canine mammary tumour cells was performed using concentrations of [^{125}I]-mEGF ranging from 0.1 to 10 nM. The data for the specific binding are given in Figure 1. Saturation analysis of [^{125}I]-mEGF binding revealed two receptors. The first, at high affinity binding, with an affinity (Kd) of 0.043 ± 0.009 nM and a B_{max} value of 7.9 ± 1.3 fmoles/mg of protein (n = 5). The second, at low affinity binding, with an affinity (Kd) of 5.1 ± 1.7 nM and a B_{max} value of 84.01 ± 15.1 fmoles/mg of protein (n = 5).

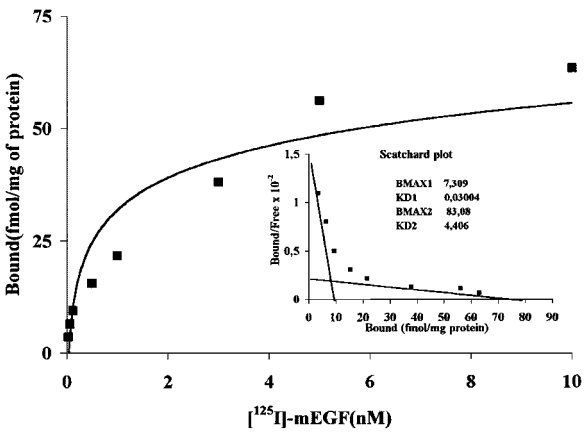

Fig. 1. Saturation analysis of [^{125}I]-mEGF binding in canine tumour mammary cell membranes. Data represent a typical experiment performed in triplicate. Essentially similar data were obtained in at least two other experiments on different preparation (see text for meaned values). Scatchard plot analysis: the equilibrium binding parameters Kd_1 and B_{max1} were 0.03 nM and 7.3 fmol/mg protein and Kd_2 and B_{max2} were 4.4 nM and 83.08 fmol/mg protein.

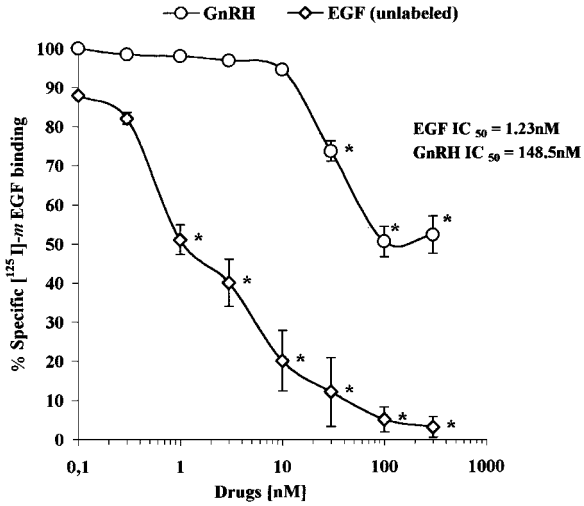

Fig. 2. GnRH or mEGF effect on specific [^{125}I]-mEGF binding to canine tumour mammary cells membranes. Specific [^{125}I]-mEGF binding was determined, as described in Materials and Methods, and specific binding in the absence unlabelled mEGF was rapresented as 100%. The final concentration of [^{125}I]-mEGF in the binding medium was 1 nM. Data are the mean ± SD of three experiments conducted in duplicate. *$P < 0.05$ compared with [^{125}I]-mEGF binding alone.

In Figure 2 are reported the results of binding competition in which a single concentration 1 nM of [^{125}I]-mEGF and increasing concentration of GnRH-A (range 0.1–300 nM) or unlabelled EGF (range 0.1–300 nM) were used. As predictable, unlabelled EGF competes in a specific manner with [^{125}I]-mEGF for EGF-R with an IC_{50} of 1.23 ± 0.22 nM. GnRH-A instead competes with [^{125}I]-mEGF only at high concentration. In fact, the IC_{50} for GnRH-A corresponded to 138 ± 2.5 nM.

In Figure 3, instead, are reported the results of binding competition in which a single concentration 1 nM of [^{125}I]-mEGF, an equimolecular dose of unlabelled EGF and increasing concentration of GnRH-A (0.1–300 nM) were used. As shown, GnRH-A was able to significantly compete with [^{125}I]-mEGF for EGF-R starting with 3 nM concentration in a dose-dependent manner, until a percentage of inhibition of 85% and with an IC_{50} of 28.5 ± 4.7 nM.

Results obtained with different concentration, using GnRH-A, ATP, and EGF alone on cell proliferation, in canine mammary tumour cells are reported in Figure 4A. After 24 h of incubation with different concentrations of EGF, ATP or GnRH-A (1, 5, 10, 30 and 100 μM), the 50% increase in cell growth was given by 10 μM (+ 58.5 ± 3.1%) for EGF and 50 μM (+ 45.2 ±

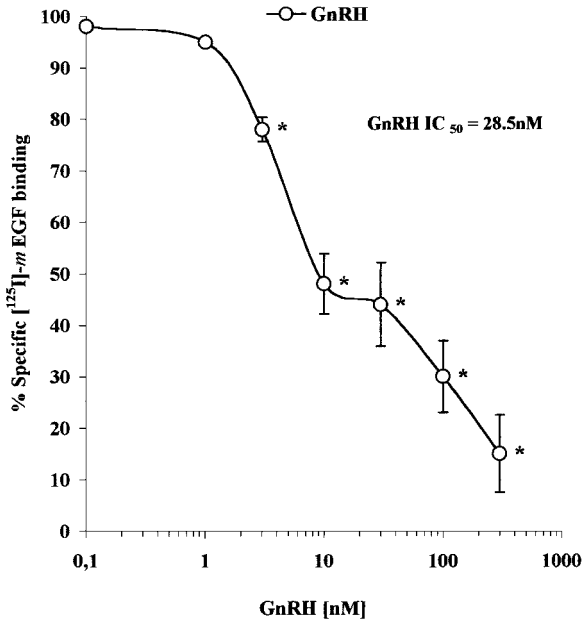

Fig. 3. GnRH effect on specific [^{125}I]-mEGF binding to canine tumour mammary cell membranes was determined as described in Materials and Methods, and specific binding in the presence of 1 nM unlabelled mEGF was represented as 100%. The final concentration of [^{125}I]-mEGF in the binding medium was 1 nM. Data represent the mean ± SD of three experiments conducted in duplicate. *$P < 0.05$ compared with [^{125}I]-mEGF + mEGF 1 nM binding obtained.

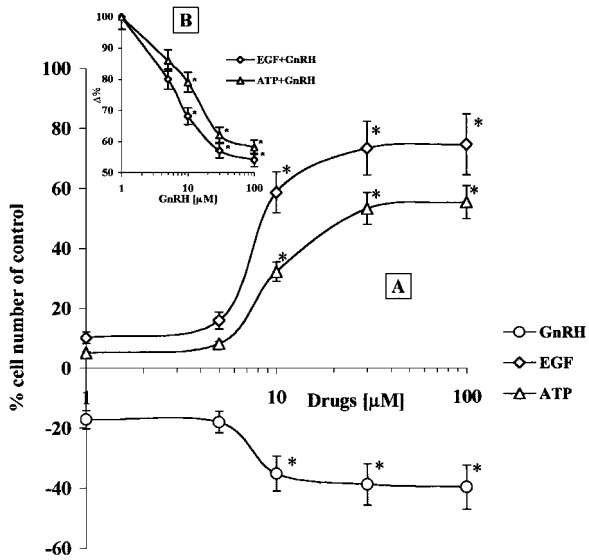

Fig. 4. Effect of GnRH, EGF, or ATP on cellular proliferation. **A**: 10^6 canine mammary tumour cells were treated for 24 h at 37°C in humidified atmosphere of 5% carbon dioxide with increasing concentrations of GnRH or EGF or ATP. Cell number is expressed as percentage of control (mean ± SD). *$P < 0.05$ drug treatment versus control. **B**: GnRH effect on EGF or ATP-induced proliferative activity. Data obtained by addition of EGF or ATP at single concentration of 1 μM on cells treated with increasing concentrations of GnRH for 24 h. Proliferative activity in absence of GnRH was expressed as 100%. *$P < 0.05$ GnRH treatment versus EGF or ATP treated cells in absence of GnRH.

2.3%) for ATP, while GnRH-A was able to significantly reduce tumour cell proliferation only at the concentration of 10^{-6} M and above.

Similarly, in order to evaluate the inhibiting effect of GnRH-A on EGF or ATP-induced cell proliferation, a dose effect experiment was performed in which EGF and ATP were used at the single dose of 10 μM in presence of increasing concentration of GnRH-A (1, 5, 10 and 30 μM) (Fig. 4B). Addition of GnRH-A significantly decreased EGF and ATP-induced tumour cell proliferation since the equimolar dose of 5 μM and such effect was dose dependent. When GnRH-A and EGF were used in association, the concentration of EGF that induced close to 50% increase in cell proliferation shifted from 10 μM over 1 mM when used in presence of GnRH and 1.4 mM in presence of ATP (data not shown).

In intracellular calcium experiments, in order to analyse separately calcium influx from extracellular medium and release from intracellular stores, suspensions of fura-2 loaded canine mammary tumour cells were incubated at 37°C in KRH medium, in presence or in absence of 1 mM Ca^{2+}. Figure 5 shows the effect of increasing concentrations of EGF, ATP and GnRH-A used alone on calcium entry (KRH medium containing 1 mM Ca^{2+}) from the extracellular space. As shown, while EGF and ATP significantly increased calcium entry in a dose-dependent manner, GnRH-A did not modify intracellular calcium concentrations. In Figure 6, the results of the treatment of canine mammary tumour cells with a single dose of EGF or ATP (1 μM) and increasing concentrations of GnRH-A in a 1 mM Ca^{2+} KRH medium are reported. GnRH-A was able to significantly decrease EGF and ATP-induced increase of intracellular calcium concentration in a dose-dependent manner and the IC_{50} for GnRH-A was 1.05 ± 0.04 and 1.82 ± 0.3 nM for EGF and ATP, respectively.

Figure 7 shows the effect of increasing concentrations of EGF, ATP, and GnRH-A used alone on calcium release from intracellular stores (KRH medium without 1 mM Ca^{2+}, in presence of 10 μM EGTA). As shown, while EGF and ATP significantly increased calcium entry in a dose-dependent manner, GnRH-A, at all the

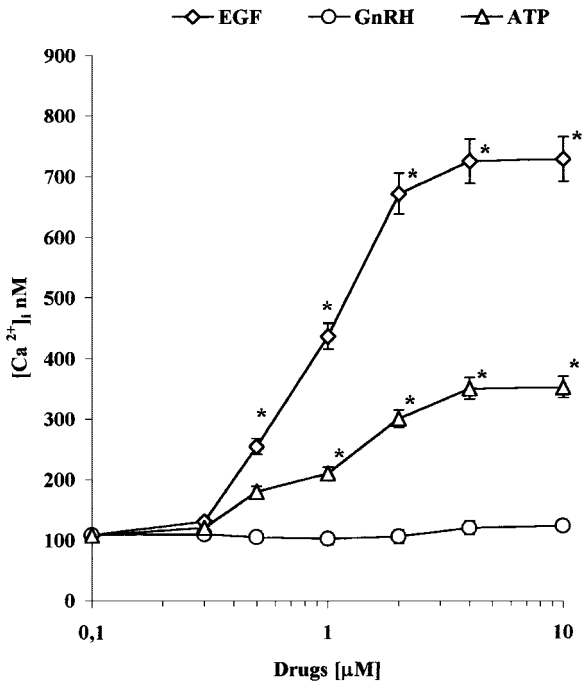

Fig. 5. Effect of increasing concentrations of EGF, ATP, and GnRH-A used alone on calcium entry (KRH medium containing 1 mM Ca^{2+}) from the extracellular space. Data represent the $[Ca^{2+}]_i$ values (mean ± SD) obtained in 6–10 distinct experiments performed in duplicate. *$P < 0.05$ versus intracellular calcium basal levels.

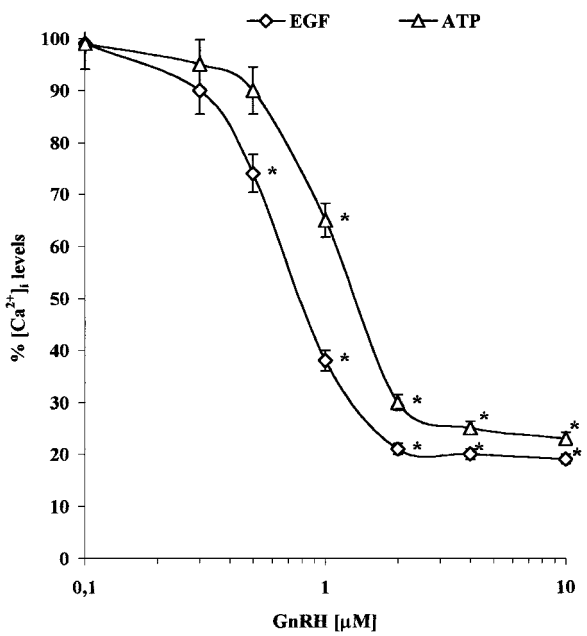

Fig. 6. Effect of increasing concentrations of GnRH-A in canine mammary tumour cells treated with a single dose of EGF or ATP (1 μM) in a 1 mM Ca^{2+} KRH medium (calcium influx). Data represent the Δ% of intracellular calcium values in presence of increasing concentrations of GnRH versus single drug treatment (ATP or EGF) expressed as 100%. *$P < 0.05$ versus $[Ca^{2+}]_i$ levels obtained in absence of GnRH.

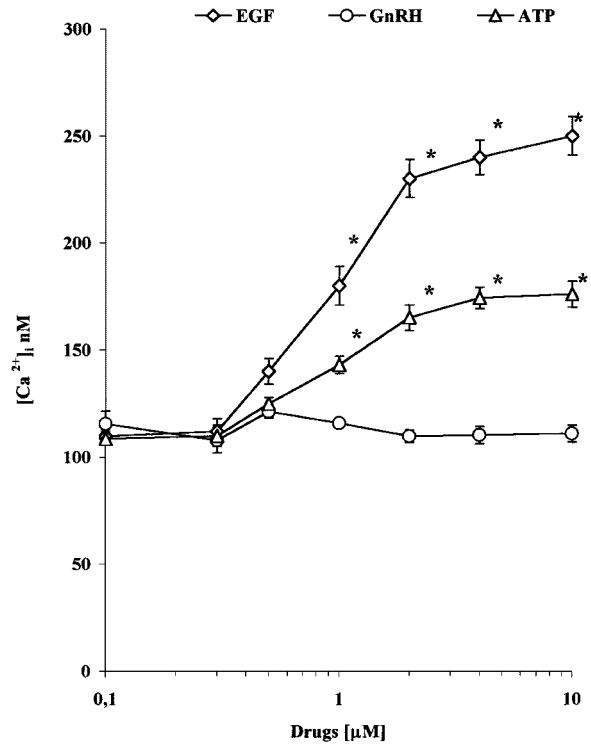

Fig. 7. Effect of increasing concentrations of EGF, ATP, and GnRH-A used alone on calcium release from intracellular stores (KRH medium without 1 mM Ca^{2+}, in presence of 10 μM EGTA). Data represent the $[Ca^{2+}]_i$ values (mean ± SD) obtained in 6–10 distinct experiments performed in duplicate. *$P < 0.05$ versus intracellular calcium basal levels.

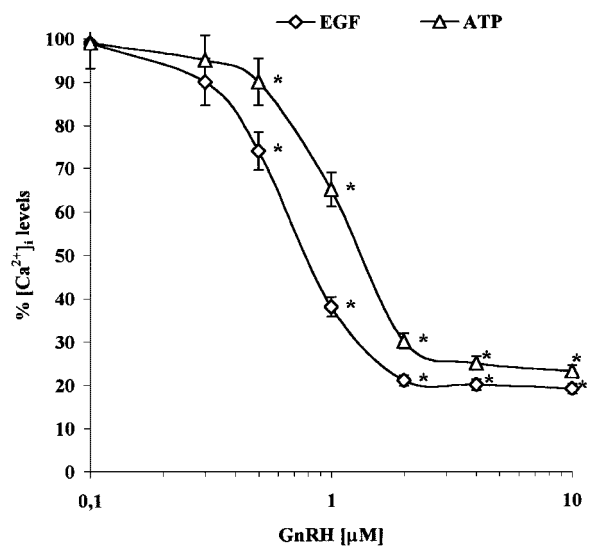

Fig. 8. Effect of increasing concentrations of GnRH-A in canine mammary tumour cells treated with a single dose of EGF or ATP (1 μM) in KRH medium without 1 mM Ca^{2+}, in presence of 10 μM EGTA (calcium release from intracellular stores). Data represent the Δ% of intracellular calcium values in presence of increasing concentrations of GnRH versus single drug treatment (ATP or EGF) expressed as 100%. *$P < 0.05$ versus $[Ca^{2+}]_i$ levels obtained in absence of GnRH.

used concentrations, did not modify intracellular calcium concentrations. In Figure 8, the results of the treatment of canine mammary tumour cells with a single dose of EGF or ATP (1 μM) and increasing concentrations of GnRH-A in KRH medium without Ca^{2+} are reported. In our experimental conditions, GnRH-A significantly decrease EGF and ATP-induced increase of intracellular calcium concentration in a dose-dependent manner and the IC_{50} for GnRH-A was 0.85 ± 0.09 and 1.45 ± 0.07 nM for EGF and ATP, respectively.

Both EGF, ATP, and GnRH-A, increased the nitric oxide concentration in canine mammary tumour cells after 24 h of incubation (Fig. 9A). Epidermal growth factor increased the NO production above $341.9 \pm 24\%$, ATP increased the NO production above $258 \pm 18\%$, while GnRH at the concentration of 100 μM increased the NO production of $58.4 \pm 3.1\%$. The IC_{50} corresponded to 0.9 ± 0.045 and 1.05 ± 0.07 μM for EGF and ATP, respectively and 8.5 ± 1.7 μM for GnRH-A. In Figure 9B are reported the results of increasing concentrations of GnRH-A on NO production in tumour cells treated with a single concentration of EGF or ATP (5 μM). As shown, GnRH-A increased NO production from a concentration of 1 μM with a maximum effect of 138.7 ± 5.3 and $82.2 \pm 3.5\%$, for EGF and ATP, respectively.

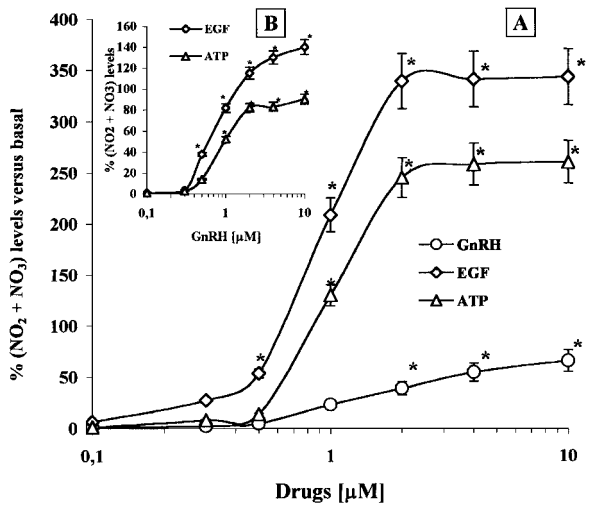

Fig. 9. Activity of GnRH, EGF, or ATP on NO production. **A**: 10^6 canine tumour mammary cells were treated for 24 h at 37°C in humidified atmosphere of 5% carbon dioxide with increasing concentrations of GnRH or EGF or ATP. After 24 h, nitric oxide ($NO_2 + NO_3$) levels were assayed by the Griss reagent. Data represent the Δ% of nitric oxide values obtained in presence of increasing concentrations of GnRH, ATP, or EGF versus basal values. $^*P < 0.05$ versus NO basal levels. **B**: Effect of increasing concentrations of GnRH-A in canine mammary tumour cells treated with a single dose of EGF or ATP (5 μM) on nitric oxide production. Data represent the Δ% of nitric oxide values in presence of increasing concentrations of GnRH versus single drug treatment (ATP or EGF) expressed as 0%. $^*P < 0.05$ versus NO levels obtained in absence of GnRH.

DISCUSSION

GnRH agonists have been shown to be effective for the treatment of hormone dependent mammary carcinoma because of their ability to suppress oestrogen and progesterone secretion, through the downregulation of the pituitary-testicular axis [McRae et al., 1985; Vickery et al., 1989; Kawakami et al., 1991; Lombardi et al., 1999]. In the present study, we evaluated the direct in vitro effect of Goserelin on EGF-activated intracellular signalling mechanism, such as calcium and nitric oxide trasduction pathways; on EGF-induced tumour cells proliferation and on the binding affinity of EGF receptors.

The results of our work clearly indicate that GnRH-A may have a direct anti-proliferative effect on the growth of canine mammary tumour cells, counteracting the stimulatory action of EGF. Moreover, GnRH showed an anti-proliferative activity also in presence of a not specific proliferative stimulus, such as ATP.

ATP has been shown to induce changes in proliferation in several types of nucleated cell types [Corr and Burnstock, 1994; Malam-Souley et al., 1996; Janssens and Boeynaems, 2001], acting via multiple P2 nucleotide receptor subtypes to increase intracellular calcium concentration ($[Ca^{2+}]_i$) [Kitajima et al., 1994; Kalthof et al., 1996; Wagstaff et al., 2000] and stimulates cell proliferation [Wang et al., 1992]. ATP appears to coordinately regulate protein synthesis, DNA synthesis and expression of immediate-early and delayed-early genes [Kitajima et al., 1994; Malam-Souley et al., 1996]. The ATP-induced increase cell proliferation is synergistic with growth factors, such as insulin, insulin-like growth factor-1, epidermal growth factor and platelet-derived growth factor (PDGF) [Wang et al., 1992].

So, ATP is a physiological stimulus that efficiently signals through an elevation of the $[Ca^{2+}]_i$ levels in several transformed epithelial cell lines [Dixon et al., 1997]. Thus, stimulation by ATP was used throughout as an indicator of integrity of cell calcium signalling and for comparison with GnRH-A effect on EGF-induced calcium fluxes.

Our data agree with findings obtained in several types of human cancer cells in which an inhibitory activity of GnRH-A on the oestrogen-stimulated growth of tumour cells has been demonstrated [Neri et al., 1990; Marini et al., 1994; Sica et al., 1994, 2001]. In fact, although it was considered originally that epidermal growth factor showed its mitogenic action through separate pathways, there is a growing body of evidence suggesting that the EGF and oestrogen-mediated signalling pathways are intertwined [Improta-Brears et al., 1999]. However, it must be pointed out that in our experimental conditions we did not completely eliminate oestrogen stimuli, since phenol red (present in the medium routinely employed) has been shown to have oestrogenic effect [Sica et al., 2001].

As far as the expression of steroid receptors and epidermal growth factor receptor is concerned, our findings demonstrate that both ER, PgR, GnRH and EGF receptors are expressed in our canine mammary tumour cells. Moreover, Scathchard plot analysis showed the presence of two binding sites for EGF-R with different binding affinity in canine mammary tumour cells. Binding competition experiments clearly demonstrated that GnRH was able to affect EGF binding reducing binding affinity of [^{125}I]-EGF. Such data are in agreement with the results obtained in human and murine tumour cells in which a phosphorylation of EGF-R [Vincze et al., 1991] and a significant reduction of the EGF binding sites was demonstrated in presence of GnRH-A [Szende et al., 1990].

Concerning calcium signalling experiments, our data demonstrated that GnRH-A was able to reduce calcium proliferative stimuli acting both on a specific proliferative stimulus, such as EGF, and on an aspecific proliferative stimulus, such as ATP.

We found that the response to ATP in canine mammary tumour cells has two components, similarly to what has been observed in MCF-7 cells. A first component is fast and of short duration and probably depends on IP_3 stimulation of Ca^{2+} release from internal stores, whereas the second one is more prolongated and is due to extracellular calcium entry. Indeed, as Ca^{2+} is removed by adding 2 mM EGTA, the second component is eliminated and is restored by subsequent addition of Ca^{2+}. Our results showed that GnRH-A was able to reduce both EGF and ATP induced Ca^{2+} rises both from released from internal stores and extracellular calcium entry.

Interestingly, Goserelin, in a dose that was maximal to exert its endocrine action, did not induce a typical calcium response in Canine mammary tumour cells.

The effect of GnRH on calcium mobilization is controversial. Although the classical mechanism of action of GnRH implies stimulation of adenylate cyclase and cAMP production [Leung and Steele, 1992], the regulation of steroidogenesis by GnRH may be exerted through the stimulation of multiple pathways. The activation of PLCs and intracellular calcium increases have been involved in the action of gonadotropins in the ovary in several species, including the mouse, swine, hen and cow [Asem et al., 1987; Davis et al., 1987; Flores et al., 1991; Gudermann et al., 1992]. In other species, such as sheep and rats, GnRH did not induce calcium transients in the ovary [Davis et al., 1986; Wang et al., 1989; Wiltbank et al., 1989], although increases in $InsP_3$ formation after GnRH treatment were observed in these species [Jacobs et al., 1991; Davis et al., 1989]. In our experimental conditions, GnRH induced significant calcium increases in MCF-7 human breast cancer cells (data not shown) in a variety of concentrations but no effect on intracellular calcium was observed in canine mammary tumour cells under stimulation.

The results here reported suggest that NO may have a role in the chain of intracellular events elicited by activation of epidermal growth factor receptors and in the downregulation of calcium signalling by GnRH. While the Ca^{2+} storage machinery is unaffected by the treatment with NO, the gaseous messenger is shown to negatively modulate PIP_2 hydrolysis and the ensuing generation of IP_3 [Clementi et al., 1995]. An important consequence is the reduction of the growth factor-induced release of Ca^{2+} from the intracellular stores.

In fact, GnRH-A, used in association with proliferating stimuli, such as EGF and ATP, significantly increased nitric oxide production and affecting both calcium signalling as well as cell proliferation in canine mammary tumour cells.

The fact that NO-induced negative signal modulations was already observed in several types of neuronal cell lines, strongly suggests that it may have a wider meaning [Clementi et al., 1995].

Moreover, their appearance with all the growth stimuli we have employed, i.e., EGF and ATP, suggest that these effects are generated at the level of the common signal cascade activated after receptor binding rather than at a level of receptors themselves.

In conclusion, our findings seem to suggest that GnRH analogues may be effective in reducing the growth of epidermal growth factor-receptors/estrogen receptors positive canine mammary tumour cells at concentrations which are consistent with current therapeutic doses. Such effect was mediated both by affecting EGF-binding and reducing calcium signalling probably by means of NO-induced downregulation of $[Ca^{2+}]_i$ fluxes.

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REFERENCES

Asem EK, Molnar LL, Hertelendy F. 1987. Luteinizing hormone-induced intracellular calcium mobilization in granulose cells: comparison with forskolin and 8-bromo-adenosine 3′,5′-monophosphate. Endocrinology 120:853–859.

Bajetta E, Celio L, Zilembo N, Bono A, Galluzzo D, Zampino MG, Longhi A, Ferrari L, Buzzoni R. 1994. Ovarian function suppression with the gonadotrophin-releasing hormone (GnRH) analogue goserelin in premenopausal advanced breast cancer. Tumori 80:28–32.

Baselga J, Mendelsohn J, Kim YM, Pandiella A. 1996. Autocrine regulation of membrane transforming growth factor-alpha cleavage. J Biol Chem 271:3279–3284.

Berridge MJ. 1993. Inositol trisphosphate and calcium signalling. Nature 361:315–325.

Brodey RS, Goldschmidt MH, Roszel JR. 1983. Canine mammary gland neoplasms. J Am Anim Hosp Assoc 19:61–90.

Chamson-Reig A, Lux-Lantos VAR, Tesone M, Lebertun C. 1997. GnRH receptors and GnRH endocrine effects on luteoma cells. Endocrine 6:165–171.

Chandler LJ, Kopnisky K, Richards E, Crews FT, Sumners C. 1995. Angiotensin II decreases inducible nitric oxide synthase expression in rat astroglial cultures. Am J Physiol 268:C700–C707.

Cheng Y, Prusoff WH. 1973. Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.

Clementi E, Meldolesi J. 1997. The cross-talk between nitric oxide and Ca2+: a story with a complex past and a promising future. Trends Pharmacol Sci 18:266–269.

Clementi E, Sciorati C, Riccio M, Miloso M, Meldolesi J, Nisticò G. 1995. Nitric oxide action on growth factor-eliced signals. J Biol Chem 22:22277–22282.

Corr L, Burnstock G. 1994. Analysis of P2-purinoceptor subtypes on the smooth muscle and endothelium of rabbit coronary artery. J Cardiovasc Pharmacol 23:709–715.

Davis JS, West LA, Farese RV. 1986. Gonadotropin-releasing hormone (GnRH) rapidly stimulates the formation of inositol phosphates and dyacylglycerol in rat granulose cells: further evindence for the involvement of Ca^{2+} and proteine kinase C in the action of GnRH. Endocrinology 118:2561–2571.

Davis JS, Weakland LL, Farese RV, West LA. 1987. Luteinizing hormone increases inositol trisphosphate and cytosolic free Ca^{2+} in isolated bovine luteal cells. J Biol Chem 262:8515–8521.

Davis JS, Weakland LL, Coffey RG, West LA. 1989. Acute effects of phorbol esters on receptor-mediated IP3, cAMP, and progesterone levels in rat granulose cells. Am J Physiol 256:E368–E374.

Dixon CJ, Bowler WB, Fleetwood P, Gitny AF, Gallagher JA, Caron JA. 1997. Extracellular nucleotides stimulate proliferation in MCF-7 breast cancer cells via P2-purinoceptors. Br J Cancer 75:34–39.

Donnay I. 1993. Hormono-dependance des tumeurs mammaries de la chienne–Etude epidemiologique et variation des recepteurs a oestrogenes, progesterone et epidermal growth factor dans les tissus mammaries normaux et pathologiques. PhD thesis, University of Liege Belgium.

Donnay I, Rauis J, Develeeschouwer N, Wouters-Ballman P, Leclerq G, Verstegen J. 1995. Changes in concentration and affinities of oestrogen, progesterone and epidermal growth factor receptor during the oestrus cycle in the normal canine mammary gland and uterus. Vet Res Commun 19:101–113.

Donnay I, Develeeschouwer N, Wouters-Ballman P, Leclerq G, Verstegen J. 1996. Relationship between receptors for epidermal growth factor and steroid hormones in normal, dysplastic and neoplastic canine mammary tissues. Res Vet Sci 60:251–254.

EORTC (European Organization for Research and Treatment Cancer). 1980. Breast Cancer Cooperative Group: revision of the standards for the assessment of hormone receptors in human breast cancer. Eur J Cancer 16: 1513–1516.

Evans JM. 1978. Oestrus control in the bitch. Proceeding University Sidney Post Graduate Commitee In Veterinary Science Reference 37:35–52.

Fantl WL, Johnson DE, Williams LT. 1993. Signalling by receptor tyrosine kinases. Annu Rev Biochem 62:453–481.

Flores JA, Veldhuis JD, Leong DA. 1991. Angiotensin II unduces calcium release in a subpopulation of single ovarian (granulose) cells. Mol Cell Endocrinol 81:1–10.

Formento JL, Francoual M, Pormento P, Etienne M, Fischel JL, Namer M, Frenay M, Francois E, Milano G. 1990. Epidermal growth factor receptor assay: validation of a single point method and application to breast cancer. Breast Cancer Res Treat 17:211–219.

Grimaux M, Romain S, Remvikos Y, Martin PM, Magdelenat H. 1989. Prognosis value of epidermal growth factor

receptor in node positive breast cancer. Breast Cancer Res Treat 14:77–90.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca2 + indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Gudermann T, Birnbaumer M, Birnbaumer L. 1992. Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca2 + mobilization. J Biol Chem 267:4479–4488.

Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell P. 1999. Estrogen-indiced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium Proc Natl Acad Sci USA 96: 4686–4691.

Jacobs AL, Homanics GE, Silver WJ. 1991. Activity of phpspholipase C in ovine luteal tissue in response to PGF2a, PGE2 and luteinizing hormone. Prostaglandins 41:495–500.

Janssens R, Boeynaems JM. 2001. Effects of extracellular nucleotides and nucleosides on prostate carcinoma cells. Br J Pharmacol 132(2):536–546.

Kalthof B, Bechem M, Flocke K, Pott L, Schramm M. 1996. Kinetics of ATP-induced Ca^{2+} transients in cultured pig aortic smooth muscle cells depend on ATP concentration and stored Ca^{2+}. J Physiol (Lond) 466:245–262.

Kawakami E, Tonsho H, Tsutsui T, Ogasa A. 1991. Effects of LHRH-analogue treatment of spermatogenic dysfunction in the dog. Int J Androl 14:441–452.

Kitajima S, Ozaki H, Karaki H. 1994. Role of different subtypes of P2 purinoceptor on cytosolic Ca^{2+} levels in rat aortic smooth muscle. Eur J Pharmacol 266:263–267.

Koenders PG. 1992. Epidermal growth factor receptor and its ligand(s): associations with prognonsis of patients with breast cancer. PhD thesis. Ninmegen, The Netherlands.

Koenders PG, Beex LVM, Benraad TJ. 1991. Epidermal growth factor receptor-negative tumours are predominantly confined to the subgroup of estradiol receptor-positive human primary breast cancers. Cancer Res 51: 4544–4548.

Lespagnard L, Kiss R, Danguy A, Legros N, Lenglet G, Devleeschouwer N, Paridaens R. 1987. In vitro studies of canine mammary tumors: influence of 17-beta-estradiol and progesterone on cell kinetics parameters. Oncology 44:292–301.

Leung PCK, Steele GL. 1992. Intracellular signaling in the gonads. Endocr Rev 13:476–498.

Lombardi P, Florio S, Pagnini U, Crispino A, Avallone L. 1999. Ovarian function suppression with a GnRH analogue: D-ser(But[t])[6]-Arzgly[10]-LHRH (Goserelin) in hormone dependent canine mammary cancer. J Eur Vet Pharmacol Ther 22:56–61.

Lowry OH, Rosembrough NJ, Farr AL, Randall RJ. 1951. Protein mesurement with the folin phenol reagent. J Biol Chem 193:265–275.

Lubrano C, Toscano V, Petrangeli E, Spera G, Trotta MC, Rombola N, Frati L, Di Silverio F, Sciarra F. 1993. Relastionship between epidermal growth factor and its receptor (EGF-R) in human benign prostatic hyperplasia. J Steroid Biochem Mol Biol 46:463–468.

Madewell BR, Theilen GH. 1987. Tumours of the mammary gland. In: Theilen GH, Madewell BR, editors. Veterinary cancer medicine. 2nd edition. Philadelphia: Lea and Febiger. p 518–552.

Malam-Souley R, Seye C, Gadeu AP, Loirand G, Pillois X, Campan M, Pacaud P, Desgranges C. 1996. Nucleotide receptor P2u partially mediates ATP-induced cell cycle progression of aortic smooth mucle cells. J Cell Physiol 166:57–65.

Marini L, Iacopino F, Xchinzari G, Robustelli della Cunaet FS, mantovani G, Sica G. 1994. Direct antiproliferative effect of triptorelin on human breast cancer cells. Anticancer Res 14:1881–1885.

McRae GI, Roberts BB, Worden AC, Bajka A, Vickery BH. 1985. Long-term reversible suppression of oestrus in bitches with narfarelin acetate, a potent LHRH agonist. J Reprod Fertil 74:389–397.

Meldolesi J, Clementi E, Fasolato C, Zacchetti D, Pozzan T. 1991. Ca^{2+} influx following receptor activation. Trends Pharmacol Sci 12(8):289–292.

Montagnani Marelli M, Moretti RM, Dondi D, Limonta P, Motta M. 1997. Effects of LHRH agonists on the growth of human prostatic tumour cells: "in vitro" and "in vivo" studies. Arch Ital Urol Androl 69:257–263.

Morris JS, Dobson JM, Bostock DE, O'Farrel E. 1998. Effect of ovariohysterectomy in bitches with mammary neoplasms. Vet Rec 142:656–658.

Moulton JE. 1990. Tumours of mammary gland. In: Moulton JE, editor. Tumours in domestic animals. Berkeley: University of California Press.

Munson PJ, Rodbar D. 1980. LIGAND:a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239.

Neri C, Berthois Y, Schatz B, Drieu K, Martin PM. 1990. Compared effects of GnRH analogs and 4-hydroxytamoxifen on growth and steroid receptors in antiestrogen sensitive and resistant MCF-7 breast cancer cell sublines. Breast Cancer Res Treat 15:85–93.

Nicholson RI, McClelland RA, Gee JM, Manning DL, Cannon P, Robertson JF, Ellis IO, Blamey RW. 1994. Transforming growth factor-alpha and endocrine sensitivity in breast cancer. Cancer Res 54:1684–1689.

Owen LN. 1979. A comparative study of canine and human breast cancer. Invest Cell Pathol 2:257–275.

Robustelli della Cuna G. 1992. Principi di endocrinoterapia antitumorale. In: Bonadonna G, Robustelli della Cuna G, editors. Manuale di oncologia medica. Milan: Masson. p 311–333.

Sartin EA, Rahe CH, Wright JC, Sartin JL. 1995. Luteneinizing hormone releasing hormone, estrogen, and progesterone receptors in canine mammary lesions and tumor cell lines. Anticancer Res 15:2029–2032.

Scatchard G. 1949. The attraction of protein for small molecules and ions. Ann N Y Acad Sci 51:660.

Schneider R. 1970. Comparision of age, sex, and incidence rates in human and canine breast cancer. Cancer 26:49–426.

Schneider R, Dorn CR, Taylor DO. 1969. Factors influencing canine mammry cancer development and postsurgical survival. J Natl Cancer Inst 43:1249–1261.

Sica G, Iacopino F, Robustelli della Cuna G, Marchetti P, Marini L. 1994. Combined effects of estradiol, leuprorelin, tamoxifen and medroxyprogesterone acetate on cell growth and steroid hormone receptors in breast cancer cell. J. Cancer Res Clin Oncol 120:605–609.

Sica G, Schinzari G, Angelucci C, Lama G, Iacopino F. 2001. Direct effect of GnRH agonists in human hormone-sensitive endometrial cell. Mol Cell Endocrinol 176: 121–128.

Stewart AJ, Johnson MD, Westley FEB, Westley BR. 1990. Role of insuline-like growth factors and the type I insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells. J Biol Chem 265:21172–21178.

Szende B, Srkalovic G, Groot K, Schally AV. 1990. Growth inhibition of mouse MXT mammary tumour by the luteinizing hormone-releasing hormone antagonist SB-75. J Natl Cancer Inst 82:513–517.

Tateyama S, Furakawa H, Yamaguchi R, Nosaka D. 1990. Plate and collagen-gel cultures of normal canine mammary epithelial cells. Res Vet Sci 49:14–19.

Vickery BH, McRae GI, Goodpasture JC, Sanders LM. 1989. Use of a potent LHRH analogues for chronic contraception and pregnancy termination in dogs. J Reprod Fertil Suppl 39:175–187.

Vincze B, Palyi I, Daubner D, Krammer T, Szamel I, Bodrogi I, Sugar J, Seprodi J, Mezo I, Teplan I, Eckhardt S. 1991. Influence of luteinizing hormone-releasing hormone agonists on human mammary carcinoma cell lines and their xenografts. J Steroid Biochem Mol Biol 38:119–126.

Wagstaff SC, Bowler WB, Gallagher JA, Hipskind RA. 2000. Extracellular ATP activates multiple signalling pathways and potentiates growth factor-induced c-fos gene expression in MCF-7 breast cancer cells Carcinogenesis 21(12):2175–2181.

Wang J, Baimbridge KG, Leung PCK. 1989. Changes in cytosolic free calcium ion concentrations in individual rat granulose cells: effect of luteinizing hormone-releasing hormone. Endocrinology 124:1912–1917.

Wang DJ, Haung NN, Heppel LA. 1992. Extracellular ATP and ADP stimulate proliferation of porcine aortic smooth muscle cells. J Cell Physiol 153:221–233.

Wiltbank MC, Guthire PB, Mattson MP, Kater SB, Niswender GD. 1989. Hormonal regulation of free intracellular calcium concentrations in small and large ovine luteal cells. Biol Reprod 41:771–778.

Yamagami T, Kobayashi T, Takahashi K, Sugiyama M. 1996. Influence of ovariectomy at the time of mastectomy on the prognosis for canine malignant mammary tumours. J Small Anim Pract 37:462–464.