

The effect of angiotensin II receptor antagonists on diethylstilbestrol-induced vascular changes in the rat anterior pituitary gland: a quantitative evaluation

M. Pawlikowski¹, M. Grochal², A. Kulig³, K. Zieliński³, H. Stepień¹, J. Kunert-Radek¹ and S. Mucha¹

¹Institute of Endocrinology, Medical University of Lodz, ²Department of Neurosurgery and

³Department of Pathomorphology, Military School of Medicine, Lodz, Poland

Summary. The effects of diethylstilbestrol (DES) and of angiotensin II (Ang II) receptor antagonists, such as losartan (selective AT₁ receptor antagonist) or PD 123319 (selective AT₂ receptor antagonist) on the anterior pituitary microvasculature were studied by means of computer-assisted image analysis. The vascularization was visualized using Selye's method modified by Poely et al. (1964). It was found that DES induced a sharp increase in vessel area, mean vessel diameter and perimeter, whereas mean vessel number was reduced. These DES-induced changes were inhibited by simultaneous administration of losartan. On the other hand, PD 123319 was less effective. These findings suggest an involvement of Ang II, acting mainly via AT₁ receptors, in the mechanism of estrogen-induced vascular changes in the rat anterior pituitary gland.

Key words: Angiotensin II, Neovascularization, Estrogen, Pituitary tumors, Image analysis

Introduction

Estrogens are well known to induce anterior pituitary hyperplasia and tumorigenesis in rodents (Zondek, 1936; Mietkiewski, 1959). It has also been found that the estrogen-induced pituitary hyperplasia and the formation of pituitary tumor are accompanied by dramatic changes of the vasculature of the gland. Elias and Weiner (1984) have demonstrated an abundant neoarteriogenesis in the anterior pituitary gland of estrogen-treated Fischer 344 rats. This neovascularization can be relevant for pituitary hyperplasia and tumorigenesis, since newly formed arteries supply

the anterior lobe with a systemic blood poor in dopamine, instead of the blood coming from hypophyseal portal system. Dopamine is a well known inhibitor of prolactin secretion and lactotroph growth and its deficiency results in hyperprolactinemia and lactotroph hyperplasia. On the other hand, we have found recently that the estrogen-induced hyperprolactinemia and lactotroph hyperplasia could be attenuated by treatment with the angiotensin II (Ang II) synthesis inhibitors enalapril or enalaprilate (Mucha et al., 1993; Pawlikowski et al., 1995). This finding suggests the involvement of Ang II in estrogen-induced pituitary hyperplasia and hyperprolactinemia. It is worth recalling that Ang II has been found to stimulate vascular growth and can induce the neovascularization (Paquet et al., 1990; Re, 1991). The aim of the present study is to answer the question whether the blockade of Ang II receptors could prevent the estrogen-induced vascular changes in the rat anterior pituitary gland. As a procedure of induction of estrogen-dependent pituitary hyperplasia, a subcutaneous implantation of diethylstilbestrol (DES) was applied. This model of pituitary hyperplasia has been studied repeatedly in our and other laboratories (Wiklund et al., 1981; Kunert-Radek et al., 1994). The selective blockade of the two best known Ang II receptors AT₁ and AT₂ is now possible thanks to the introduction of specific blockers. Losartan (DuP 753) has been found to be a specific antagonist of AT₁ receptor (Duncia et al., 1992), whereas a compound called PD 123319 has been shown to block AT₂ sites (Blankley et al., 1991).

Materials and methods

Materials

Losartan (DuP 753) was kindly obtained from Du Pont Merck Pharmaceutical Company (Wilmington, DE, USA) and PD 123319 from Parke-Davis (Ann Arbor,

MI, USA). Both agents were stored at 4 °C and then dissolved in 0.9% NaCl solution.

Animals and induction of prolactinoma

Young (4 weeks old) male Fischer 344 rats were obtained from Harlan Olac Limited (Bicester Oxon, England). They were housed in group cages, in controlled 12 h light/12 h dark environment and with free access to water and food. The animals underwent chronic estrogen treatment, using the following regime: capsules prepared from 5-mm-long Silastic tubes of 1.57 mm inner diameter and 2.4 mm outer diameter (Dow Corning Co., Midland, MI, USA), were filled with a saturated solution of diethylstilbestrol (DES; Stilboestrolum, Polfa, Poland) in 95% ethanol. After evaporation of alcohol, the capsules, containing 8-10 mg DES each, were sealed with Silastic medical adhesive (Dow Corning Co.), protected from light and stored at

4 °C. The capsule was then implanted s.c. under ether anaesthesia in the lumbar region of each rat. Such implants were estimated to release 18-45 µg/day of DES (Wiklund et al., 1981) and to induce massive hyperplasia of prolactin cells in the strain of rats used in the study. Empty Silastic capsules were implanted in intact rats treated as controls.

Experimental protocol

Four weeks after DES implantation animals were divided into four experimental groups, 11 rats in each, in the following manner: control group - including rats with empty implanted Silastic tubes; DES group - including animals with implanted Silastic tubes with DES. The animals in these groups received s.c. injections with 0.25 ml of 0.9% NaCl solution, which was the diluent for tested substances. The remaining two groups consisted of rats with implanted DES tubes, which received as follows: losartan at a dose of 10.0 mg/kg body weight/24 h in 0.25 ml volume or PD 123319 at a dose of 1.0 mg/kg body weight/24 h in 0.25 ml volume. The dosage of tested agents was selected on the basis of our earlier studies (Pawlikowski et al., 1995). At those doses losartan and PD 123319 were effective in inhibiting the Ang II-dependent prolactin secretion in rats. Injections were made once a day (at 8.00 a.m.), for the following 24 days. Four hours after the last injection all animals were decapitated. Pituitary glands were then carefully isolated, immersed in 5% formalin (diluted 7:1 with PBS) for six hours, and then embedded in paraffin using routine procedure. Serial sections of pituitary tissue (8 µm thick) were stained using Seyle method with Poely's modifications (Poely et al., 1964).

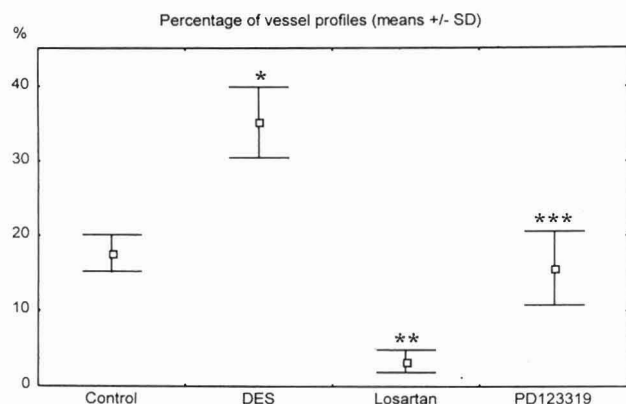


Fig. 1. Effects of losartan (10.0 mg/kg) and PD 123319 (1.0 mg/kg) on vessel area in anterior pituitary tissue. Results are means (rectangles) \pm SD (whiskers) of the percentage (n= 8). *: p<0.001 compared with control; **: p<0.001 compared with DES; ***: p<0.001 compared with DES.

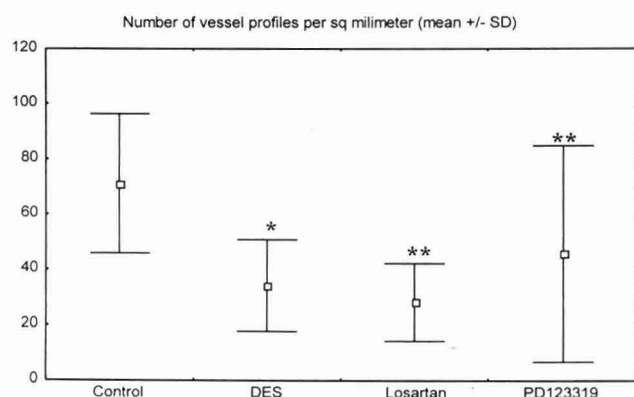


Fig. 2. Effects of losartan (10.0 mg/kg) and PD 123319 (1.0 mg/kg) on vessel profile number in anterior pituitary tissue. Results are means (rectangles) \pm SD (whiskers) (n=8). *: p<0.01 compared with control; **: p<0.01 compared with control; **: p<0.05 compared with DES; **: p>0.05 compared with DES.

Angiogenesis studies

To quantify the histological aspects of vascular

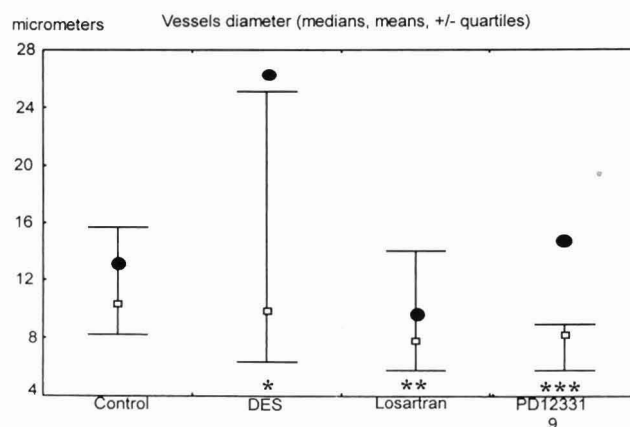


Fig. 3. Effects of losartan (10.0 mg/kg) and PD 123319 (1.0 mg/kg) on vessel diameter in anterior pituitary tissue. Results are medians (rectangles), means (filled circles) \pm quartiles (whiskers) (n=8). *: p<0.01 compared with control; **: p<0.05 compared with DES; ***: p>0.05 compared with DES.

Losartan and vasculature in the rat pituitary

proliferation in prolactinoma, a feasible and reproducible method was applied for computer-assisted image analysis of the visualized vasculature of the tumor tissue. Serial sections of pituitary tissue from control and treated animals were evaluated using Digital Image Analysis System IBAS 2000 (Kontron GmbH, Eching, Germany) attached to a black-white camera mounted on a light microscope (Wesseling et al., 1994). Fifty fields (1.31 mm^2 each) for every animal, were selected randomly and analysed from sections prepared from the tissue blocks. Microscopic image of a field seen in the camera was introduced into the computer using green and then red filter mounted in the optic system of the microscope. These procedures were used to distinguish vascular profiles (red erythrocytes) from surrounding tissue (green stroma). We assessed several vascular parameters: 1) vessel area - sum of areas of all vascular profiles in a field, expressed as percentage. 2) vessel number - mean number of vessel profiles in a field. 3) vessel diameter - mean vessel diameter (given in microns) of all individual vessel profiles. 4) vessel perimeter - mean vessel perimeter (given in μm) of all individual vessel profiles.

The values of the last three parameters concerned

only vessel profiles totally included in the measuring frame and not crossing the border of a measuring field. Mean vessel number was recalculated to one mm^2 of tissue surface. The first parameter informs about blood supply and the others about structure of the vascular network.

Data analysis

Statistical comparisons of data were carried out by one-way analysis of variance (ANOVA). The statistical unit for field parameter (vessel area) was one measuring field, and for individual parameters (i.e. mean vessel number, diameter and perimeter) was one vascular profile completely included into the frame. Differences between means in groups were assessed by the Student's t-test (if variances were equal: F-Snedecor test). Due to the fact that the distributive series of vascular parameters of individual profiles were not normal, these parameters were evaluated using the non-parametric Mann-Whitney test. A level of $p < 0.05$ was considered to be statistically significant. The mean value (mean), the median value (median) and the range of these parameters were assessed for each experimental group.

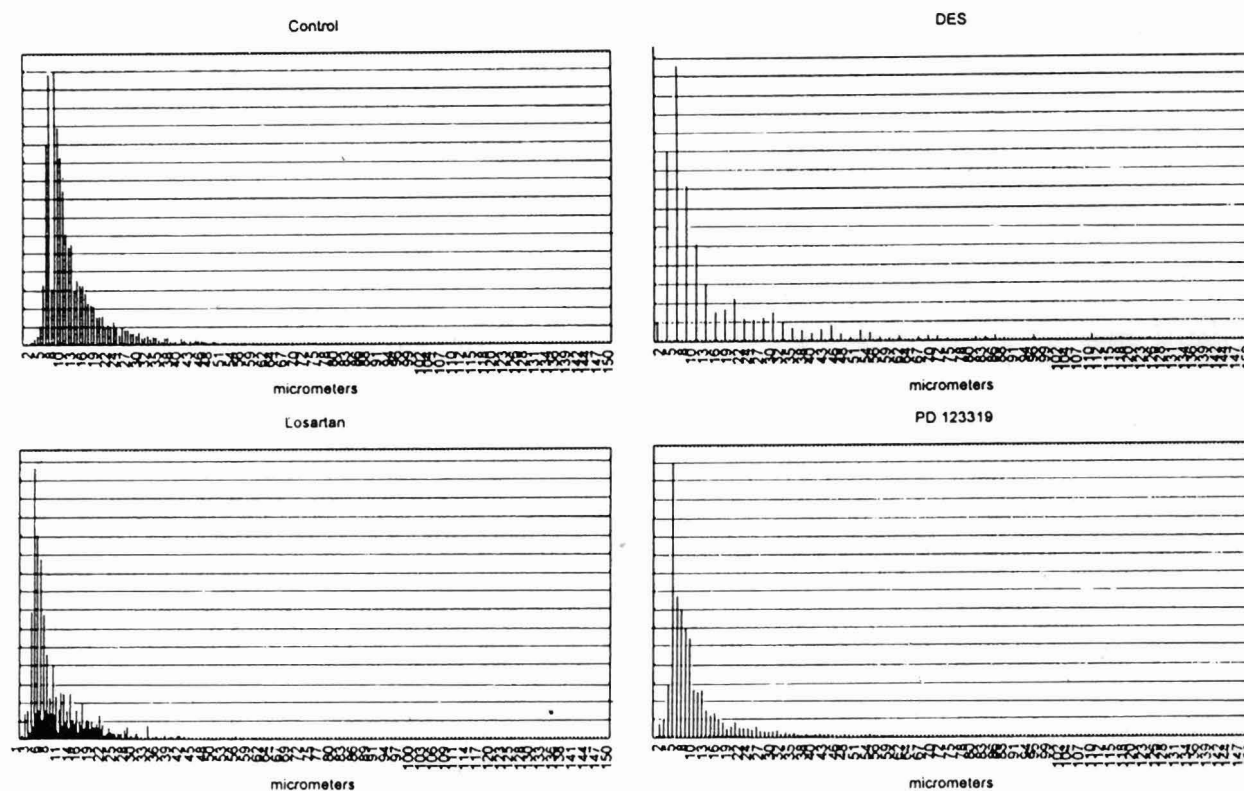


Fig. 4. Distributive series of vessel diameters in anterior pituitary tissue. The series are truncated at $150 \mu\text{m}$.

Results

The results of the quantitative analysis and the statistical evaluation of these results are presented in Figs. 1-6. Chronic treatment with DES resulted in an increase in percentage of vessel area (Figs. 1, 6). On the other hand, DES was found to diminish mean vessel number (Fig. 2). The co-administration of the AT₁ receptor antagonist losartan significantly decreased percentage of vessel area, even below control levels. Simultaneously, mean vessel number did not significantly change after treatment with the two tested agents. The mean for vessel perimeter and vessel diameter in losartan-treated rats were significantly smaller compared with DES-treated animals ($p < 0.05$). The administration of AT₂ receptor antagonist PD123319 was also effective in inhibiting estrogen-induced changes in the vasculature, but its action was much weaker and no normalization was observed (Figs. 1-6).

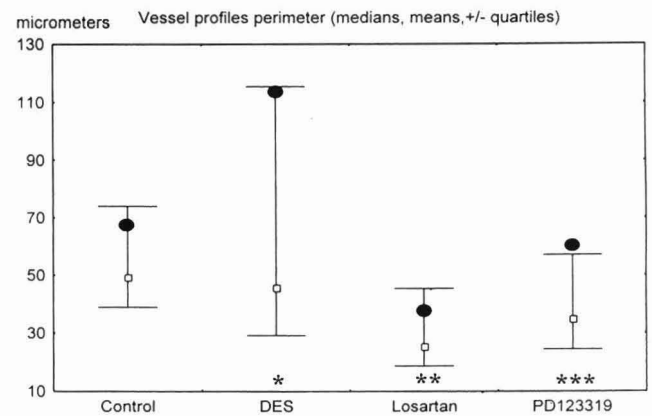


Fig. 5. Effects of losartan (10.0 mg/kg) and PD 123319 (1.0 mg/kg) on vessel perimeter in anterior pituitary tissue. Results are medians (rectangles), means (filled circles) \pm quartiles (whiskers) ($n=8$). *: $p < 0.01$ compared with control; **: $p < 0.05$ compared with DES; ***: $p > 0.05$ compared with DES.

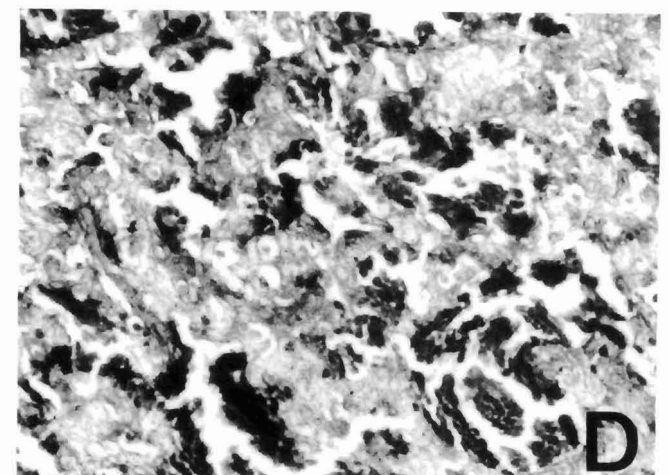
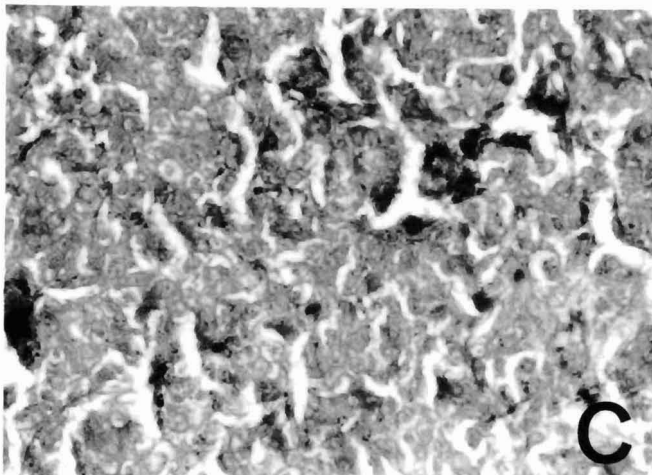
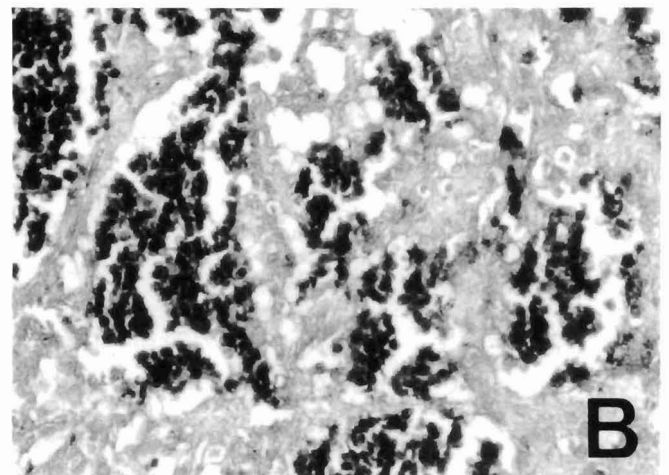
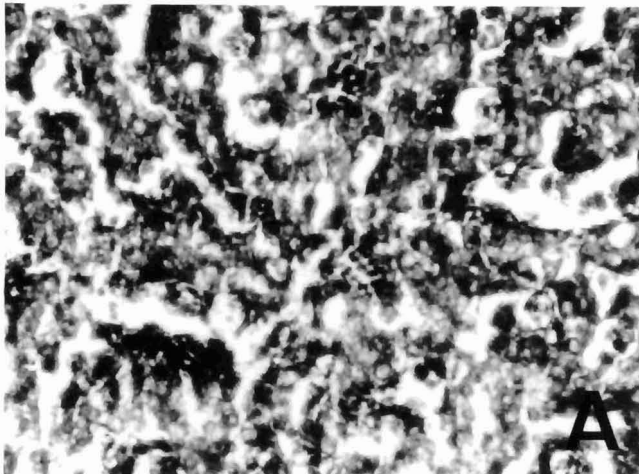


Fig. 6. The vasculature of the anterior pituitary gland visualized by staining with Seyle method with Poely's modifications. A. Control. B. DES-treated rats. C. Losartan treatment. D. PD 123319 treatment. $\times 400$

Discussion

The data presented above indicate that hyperplasia and tumorigenesis of the anterior pituitary lobe induced by supraphysiological doses of estrogens is accompanied by marked alterations in the vasculature of the pituitary gland. We have observed a sharp increase in most morphometric parameters which describe the magnitude of the vascular wall of the particular blood vessel in estrogen-treated animals. It indicates an enlargement of blood vessels depending on their relaxation and/or growth of the vascular wall. Interestingly, this effect is totally blocked by a nonpeptide antagonist of AT₁ receptor, losartan, and, to a lesser degree, by AT₂ receptor antagonist PD 123319. These findings suggest that local or systemic renin-angiotensin systems are involved in the estrogen-induced vascular changes in the anterior pituitary gland. This observation is compatible with the numerous papers demonstrating the ability of Ang II to stimulate the vascular growth (Fernández et al., 1985; Paquet et al., 1990; Re, 1991). On the other hand, they are also in agreement with our earlier observations showing that the blockade of Ang II synthesis by angiotensin-converting enzyme inhibitors, enalapril or enalaprilate, prevents or diminishes estrogen-induced pituitary hyperplasia and hyperprolactinemia (Mucha et al., 1993; Pawlikowski et al., 1995).

The results of the present study suggest that the effects of Ang II on pituitary vascular bed are related to the AT₁ receptor. However, we cannot exclude the possible involvement of AT₂ receptors, since the dose of PD 123319 was lower than that of losartan. On the other hand, the dose in question has been previously found as effective in inhibiting prolactin secretion (Pawlikowski et al., 1995). Although the role for AT₂ receptor in the anterior pituitary has not been widely investigated, Moreau et al. (1994) confirmed an involvement only of AT₁ receptor in Ang II-induced PRL release. In conclusion, we suggest that the inhibitory effects of the renin-angiotensin system blockers on PRL secretion and tumorigenesis of estrogen-induced pituitary prolactinoma are mediated (at least in part) by the suppression of changes of the microvasculature.

Acknowledgements. This paper was supported by the Committee of Scientific Research of Poland grant No 6P20708904 to H.S.

References

Blankley C.J., Hodges J.C., Klutchko S.R., Himmelsbach R.J.,

- Chucholowski A.W., Connolly C.J., Neergaard S.J., VanNieuwenhze M., Sebastian A., Quin J. III, Essenburg A.D. and Cohen D.M. (1991). Synthesis and structure-activity relationships of a novel series of non-peptide angiotensin II receptor binding inhibitors specific for AT₂ subtype. *J. Med. Chem.* 34, 3248-3260.
- Duncia J.V., Carini D.J., Chiu A.T., Johnson A.L., Price W.A., Wong P.C., Wexler R.R. and Timmermans P.B.M.W.M. (1992). The discovery of DuP 753, a potent, orally active nonpeptide angiotensin II receptor antagonist. *Med. Res. Rev.* 12, 149-191.
- Elias K.A. and Weiner R.I. (1984). Direct arterial vascularization of estrogen-induced prolactin-secreting anterior pituitary tumors. *Proc. Natl. Acad. Sci. USA* 81, 4549-4553.
- Fernández L.A., Twickler J. and Mead A. (1985). Neovascularization produced by angiotensin II. *J. Lab. Clin. Med.* 105, 141-145.
- Kunert-Radek J., Stepień H. and Pawlikowski M. (1994). Inhibition of rat pituitary tumor cell proliferation by benzodiazepines in vitro. *Neuroendocrinology* 59, 92-96.
- Mietkiewski K. (1959). The influence of estrogens of hypophysis and genital system of the rat. *Folia Morphol.* 10, 9-27.
- Moreau C., Rasolonjanahary R., Audinot V., Kordon C. and Enjalbert A. (1994). Angiotensin II effects on second messengers involved in prolactin secretion are mediated by AT₁ receptor in anterior pituitary cells. *Mol. Cell. Neurosci.* 5, 597-603.
- Mucha S., Stepień H., Lachowicz-Ochedalska A. and Pawlikowski M. (1993). The effect of converting enzyme inhibition of estrogen-induced cell proliferation in the rat pituitary gland. *Neuroendocrinol. Lett.* 15, 369-376.
- Paquet I.L., Baudonin-Legros M., Brunelle I. and Meyer P. (1990). Angiotensin-II-induced proliferation of aortic urocytes in spontaneously hypertensive rats. *Hypertension* 8, 565-572.
- Pawlikowski M., Mucha S., Kunert-Radek J., Stepień H., Pisarek H. and Stawowy A. (1995). Is estrogen-induced pituitary hyperplasia and hyperprolactinemia mediated by angiotensin II? In: *Current concepts: tissue renin-angiotensin systems as local regulators in reproductive and endocrine organs.* Mukhopadhyay A.K. and Raizada M.K. (eds). Plenum Press. New York. pp 371-378.
- Poely R.W., Fobes C.D. and Hall M.J. (1964). Fuchsinophilia in early myocardial infarction. *Arch. Pathol.* 77, 325-329.
- Re R. (1991). Angiotensin and the regulation of cellular growth. *Am. J. Hypertens.* 4, 217S-219S.
- Wesseling P., Van der Laak J.A.W.M., De Leeuw H., Ruiter D.J. and Burger P.C. (1994). Quantitative immunohistological analysis of the microvasculature in untreated human glioblastoma multiforme. *J. Neurosurg.* 81, 902-909.
- Wiklund J., Wertz N. and Gorski J. (1981). A comparison of estrogen effects on uterine and pituitary growth and prolactin synthesis in F344 and Holtzman rats. *Endocrinology* 109, 1700-1707.
- Zondek B. (1936). Tumor of the pituitary induced with follicular hormone. *Lancet* I, 776-778.

Accepted April 21, 1996