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Journal of Steroid Biochemistry & Molecular Biology 74 (2000) 25–32

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Antiestrogenic effect of opioid peptides in rat uterus

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Received 23 February 2000; accepted 14 April 2000

Abstract

The effects of a single injection or continuous infusion of opioid peptide, [D-Met^2,pro^5]enkephalinamide (ENK) on the hormone binding and transcriptional properties of estrogen receptors were investigated in estradiol (E_2) treated rat uterus. The level of estrogen- (ER) and progesterone receptor (PR) proteins, the hormone binding of E_2 receptors and the effects of single injection of ENK with or without naltrexone (NAL) on the E_2-induced changes in the level of Fos and Jun proteins and the binding of AP-1 proteins to DNA were studied. The receptor proteins levels were determined by Western blots and the binding of AP-1 to DNA by electrophoretic mobility shift assay. Both the ER and PR protein concentrations and the [^{3}H]Estradiol binding to the high affinity nuclear receptors decreased after ENK treatment during the first two days. At 72 h the PR concentration decreased further, while no significant changes were found in the level of ER, however, at this time the former competitive E_2 binding turned into positive cooperativity. The E_2-induced increase in the level of Fos proteins and the binding of AP-1 proteins to DNA was inhibited by a single injection of ENK. We conclude that the endogenous opioid peptides may interact with E_2 in the gene regulation of rat uterus. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Estrogen receptor; Estrogen binding; AP-1 complex; Opioid peptides; Rat uterus

1. Introduction

Estrogen influences the growth, differentiation and function of uterine cells by acting on the estrogen receptor, that is a member of nuclear receptor superfamily [1,2] and regulates the expression of target genes by attaching to the hormone-responsive element of the DNA.

Recently, an alternative pathway of ER action has been reported [3,4], where the receptor appears to be able to stimulate transcription by an AP-1 directed pathway. This alternate pathway of ER seems to be partly independent of the ER–DNA binding domain and it is believed to involve protein–protein, rather than protein–DNA contact [5].

All three members of the opioid peptide family as proenkephalin, prodynorphin, proopiomelanocortin and their receptors are expressed in the uterus [6–10], and their levels are affected by sex steroids [10,11]. Our previous results show that the opioid peptide agonist, [D-Met^2,pro^5]enkephalinamide (ENK) inhibits in vivo, and in vitro as well the E_2-induced cell proliferation in adult [12,13] and in developing [14] rat uterus and in human myometrial cell cultures [15]. The mechanism of this action is not clear.

According to our earlier studies an opioid peptide–estrogen antagonism can be observed at their receptor levels in invitro experiments [16] and the opioid peptide antagonist NAL or anti-met-enkephalin antiserum prevents the progesterone (P) antagonism of the E_2-induced cell proliferation [17]. All these data suggest that the E_2 action in the uterus may be influenced by opioid peptides.

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PII: S0960-0760(00)00085-6

To test our hypothesis, in this study we analyzed some parameters of E_2 action, the level of ER and PR, the transcription factors Jun and Fos, the [^{3}H]Estradiol binding, and the interaction of ER–AP-1 complex in the uterus of rats treated by single injection or continuos infusion of the opioid peptide agonist, [D-Met^2,pro^5]enkephalinamide.

2. Materials and methods

2.1. Chemicals

2-, 4-, 6-, 7-^{3}[H]-Estradiol (specific activity 3.4 TBq/mmol, MTA Budapest, Hungary) were used for the receptor ligand assay. [D-Met^2,Pro^5]enkephalinamide, a highly active enkephalin analogue was a generous gift by Dr. S. Bajusz (Institute for Drug Research, Budapest, Hungary). All other chemicals, unless stated otherwise, were purchased from SIGMA (St. Louis, MO, USA).

2.2. Antibodies

Monoclonal antibodies against ER (clone: ER1D5) and PR (clone: PRI0A9) were purchased from Immunotech (Marseilles, France). For the detection of the transcription factors, anti-c-Jun protein rabbit polyclonal IgG (New England Biolabs, MA, USA), and anti-c-Fos protein rabbit polyclonal IgG (Santa Cruz Biotechnology, CA, USA) antibodies were used.

2.3. Animals and drug treatment

Two-month-old CFY strain female rats were used. They were ovariectomized under light ether anaesthesia 10 days before the experiments. The rats were housed in temperature-controlled animal quarters under a 12-h light–dark cycle and maintained on ad libitum food and water. For the ligand binding assay and Western blot analysis, [D-Met^2,Pro^5]enkephalinamide and/or naltrexone was administered subcutaneously as implantation of mini-osmotic pumps (ALCA Corporation, CA, USA) releasing the drugs at a dose of 5 μg/h. 17β-estradiol was given as an intraperitoneal injection 2 h before the sacrifice at a dose of 10 μg/100 g b.w. The rats were sacrificed by decapitation after 24, 48, 72 h of ENK implantation, and uteri were excised and trimmed of adhering fat. For electrophoretic mobility shift assay and Jun and Fos protein detection by Western blot, the ENK was administered as an intraperitoneal injection (100 μg/100 g b.w.) 15 min before the E_2 injection given 2 h before the sacrifice. To examine the effect of specific opiate antagonist, NAL was administered (100 μg/100 g b.w. i.p.) 20 min before the ENK injection.

2.4. Radioligand binding assay

The weighed uteri were homogenized in TE buffer (10 mM Tris, 1.5 mM EDTA) pH 7.4. Homogenates were centrifuged at 800 × *g* for 20 min. The resulting pellets were resuspended in TE buffer and centrifuged consecutively three times. DNA concentration was determined by Burton's method. [^{3}H]Estradiol binding was assessed by in vitro E_2-exchange assay. Samples (0.2 ml) in triplicates from the resuspended pellets were incubated at 30°C for 60 min with 0.5–16 nM [^{3}H]Estradiol with or without 1000-fold excess of diethylstilbestrol. After solubilization of binding sites (0°C, 16 h, 0.5 M NaSCN) free ligands were absorbed by dextran-coated charcoal at 0°C for 15 min. Radioactivity was measured in a Packard Tri-Carb 2100TR liquid scintillation analyzer. Binding parameters as described earlier were estimated by non-linear curve fitting in the least squares method aided by a computer program developed in our laboratory.

2.5. Western blot analysis

Uteri were immediately removed from rats after the sacrifice and homogenized by Ultra-Turrax homogenizer at 4°C with one burst in 2% sodium dodecyl sulfate (SDS) and 10 mM Tris at a buffer/tissue ratio of 2 ml/uterus. Samples were boiled for 5 min and centrifuged at 10 000 × *g* for 10 min. Aliquots of samples were taken for protein determination. The remaining samples were combined with an equal volume of SDS sample-storage buffer (4% SDS, 20% glycerol and 10% β-mercaptoethanol, in 0.125 M Tris–HCl, pH 6.8), boiled again for 5 min and stored at −20°C. Sample prepared from rat spleen was used as a negative control for ER and PR proteins.

Proteins (100 μg/sample) from uterus and spleen were separated by 10% SDS polyacrylamide gel electrophoresis (SDS–PAGE), (4% stacking gel and 12% separating gel, acrylamide/bisacrylamide = 37.5:1) and transferred to nitrocellulose membrane (Bio-Rad) by semi-dry electrophoretic blotting using the Trans-Blot SD cell (Bio-Rad) at 0.8 mA/cm^2 for 2 h in a buffer containing 25 mM Tris base, pH 8.5, 0.2 M glycine and 20% methanol. The nitrocellulose was blocked in TBS–T (1 × TBS and 0.1% Tween-20), 5% non-fat dry milk for overnight at 4°C. After washing with TBS–T, the nitrocellulose was incubated with the first antibody against ER, PR, Jun and Fos protein at 1:1000 dilution in TBS–T with 5% BSA at 4°C overnight. To ascertain specificity of the stained protein band, the first antibody was preincubated with excess antigen peptide (1.25 μg/ml) overnight before being exposed to the membrane. The second antibody buffer at 1:2000 dilution was incubated at room temperature for 1 h. The signals were visualized by an ECL system (Amersham, IL,

USA). The density of the ER, PR, Jun or Fos protein band was determined by densitometric scanning.

2.6. Preparation of nuclear protein extracts and electrophoretic mobility shift assay

Preparation of nuclear protein extracts from fresh tissues was carried out as previously described [18]. Briefly, dissected tissues were homogenized in a Polytron homogenizer with 2 vol of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml of pepstatin A, 10 μg/ml of leupeptin and 0.1 mM *p*-aminobenzidine). After centrifuging for 20 min at 4°C, the supernatants were removed and the nuclear pellets were further extracted in 1.5 vol of buffer B (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml of pepstatin A, 10 μg/ml of leupeptin, 0.1 mM *p*-aminobenzidine and 25% glycerol). The nuclear extracts were aliquoted, freezed and stored at −70°C until analysis. Protein concentration was quantitated by Bio-Rad protein assay. The DNA binding reaction was performed in a total volume of 30 μl containing 10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 4% glycerol, 5 mM DTT, 2 μg poly-(dI-dC), 10–20 μg of nuclear protein extracts and 0.2 ng of ^{32}P-labelled oligonucleotid probe, and incubated at room temperature for 30 min. The DNA–protein complexes were separated in 5% nondenaturing polyacrilamide gels. Gels were dried and quantitated by a Cyclone phosphor imager (Bio-Rad). A double-stranded AP-1 oligonucleotide probe (5′-GCAATTATGAGTCAGTTTGC-3′) containing the AP-1 site of the transin promoter and 5′-CAGACAGCGTGGGCTGTGGC-3′ containing the Zif268 binding site for competition studies were used in experiments.

Table 1
Effect of [D-Met^2,Pro^5]enkephalinamide implantation on nuclear [^{3}H]Estradiol binding in adult ovariectomized rat uterus[a]

Treatment	B_{max}	Kd × 10^{-9} M	Hill coefficient
Control	0.978 ± 0.12	1.231 ± 0.22	= 1
24 h	1.170 ± 0.36	0.960 ± 0.32	= 1
48 h	0.504 ± 0.15*	1.239 ± 0.34	= 1
72 h	0.914 ± 0.26	5.160 ± 0.86	> 1

[a] The animals were implanted with [D-Met^2,Pro^5]enkephalinamide (5 μg/h) at the indicated time before the sacrifice. Each group of rats received 17β-estradiol (10 μg/100 g b.w. i.p.) 2 h before the sacrifice. B_{max} are expressed as pmol/mg DNA. Values are means ± S.E.M. of three experiments.

* $P < 0.05$.

2.7. Statistics

The data are presented as mean ± S.E.M. from at least three experiments giving similar results. Group differences were analyzed by ANOVA followed by Student–Newman–Keul's multiple range test. Differences were considered to be statistically significant at $P < 0.05$ level.

3. Results

3.1. Estrogen receptors

Ovariectomized adult rats were used in this experiments. For synchronisation of the uterine ER levels, the animals were injected with E_2 at a dose of 10 μg/100 g b.w. 2 h before the sacrifice. We investigated the effect of continuously given ENK on the ER levels and the [^{3}H]Estradiol binding.

The [^{3}H]Estradiol-binding characteristics of the receptors were analyzed in the crude nuclear fraction of the uteri (Table 1). Saturation analysis at the applied concentration resulted in a single class of high affinity binding sites with Kd 1.231 ± 0.22 × 10^{-9} M. The linear transformation of data (Fig. 1A) indicated competitive binding (Hill coefficient ∼ 1). The concentration of ER in the uterus decreased by 40 ± 10% 24 h after ENK implantation (Fig. 2), however, no change in the [^{3}H]Estradiol binding was detected (Fig. 1B). The level of the ER decreased 48 h after the ENK treatment, but the decline during the second day was much slower than it was on the first 24 h (Fig. 2). At this point the [^{3}H]Estradiol binding was also affected, the binding capacity decreased by approx. 50% (Fig. 1C), which value is comparable with the rate of the loss of receptor. The pattern of the changes was different 72 h after implantation of ENK. The level of ER and the binding capacity are returned to the control levels, but the [^{3}H]Estradiol binding affinity was 5 times weaker, Kd 5.16 ± 0.86 × 10^{-9} M, and the profile of the binding turned to positive cooperativity, the Hill coefficient was > 1 (Fig. 1D, Fig. 2).

3.2. Progesterone receptors

The concentration of PR was also affected by the treatment and the changes were also dependent on time elapsed after ENK implantation (Fig. 3). No changes were observed at 24 h, but later a significant drop was detected at 48 and 72 h.

No changes were found throughout the experiments in the negligible amount of receptor proteins that were detected in spleen tissues.

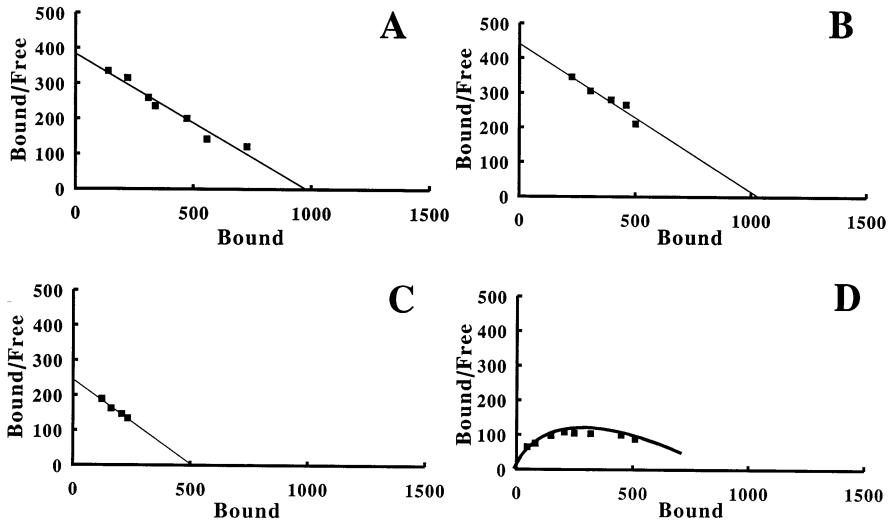

Fig. 1. Scatchard analysis of specific [^{3}H]Estradiol binding in the nuclear fraction of uterine homogenate. Adult OVX rats were treated with 17β-estradiol (10 μg/100 g b.w. i.p.) 2 h before the sacrifice. **A** Control (no ENK implantation) **B** 24 h, **C** 48 h, **D** 72 h after ENK (releasing 5 μg/h) implantation. Bounds are expressed as fmol/mg DNA.

3.3. AP-1 proteins and binding to DNA

Because both hormones E_2 and ENK can modulate transcriptional activation through the AP-1 response element, we looked for whether the ER and opioid receptors could influence each other's transcriptional effects at this site. We analyzed the AP-1–DNA binding activity by electrophoretic mobility shift assay (Fig. 4). The specificity of AP-1–DNA binding activity was evaluated by competition assay using the uterine nuclear extract. The addition of the specific cold AP-1 oligonucleotide, but not a non-specific oligonucleotide in the DNA binding reaction, resulted in elimination of the specific AP-1 complex with a minimal change in the non-specific complex (Fig. 4, Lanes s, ns).

In this experiment, we applied the ENK in a single injection 15 min before E_2 administration given 2 h before the sacrifice. The E_2-induced AP-1–DNA binding (Fig. 4, Lane 2) significantly decreased in the animals received ENK injection 15 min before E_2 administration (Fig. 4, Lane 3). NAL given 20 min before ENK injection does not modify this effect (Fig. 4, Lane 4).

The Fos and Jun proteins are members of the AP-1 protein family. On the effect of E_2 treatment their levels suddenly increase paralleled by an increase in the AP-1–DNA binding activity. As Fig. 5 shows, in agreement with others [19], E_2 injection resulted in increased level of Fos proteins by 86 ± 14% (Fig. 5, Lane 2). In the animals where the E_2 treatment was preceded by ENK injection, no E_2-induced increase was observed (Fig. 5, Lane 3). The effect of ENK was prevented by NAL treatment (Fig. 5, Lane 4). By the same treatments detectable but not statistically significant changes were found in the Jun protein concentration (Fig. 6, Lanes 2, 3, 4) as well.

4. Discussion

As our results show, the ER and its hormone binding properties, the PR, and the estrogen-induced changes in the function of AP-1 complex changed in the ENK treated animals. The observed alterations showed time dependency by the ENK treatment.

Estrogens and the progesterone are crucial for regulation of growth and cell proliferation in the uterus. Specific receptor proteins and subsequent stimulation of

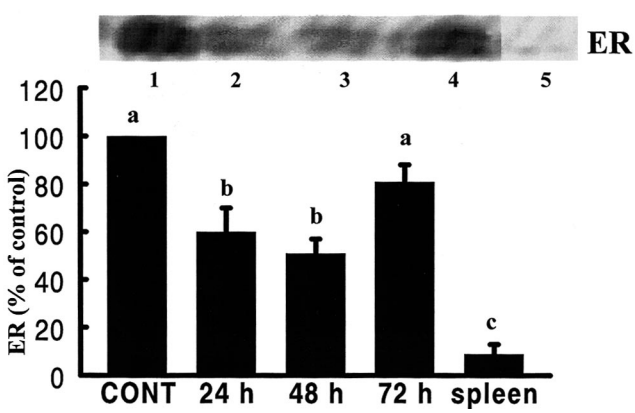

Fig. 2. Regulatory action of ENK implantation on ER protein in adult OVX rat uterus. The animals were treated with 17β-estradiol (10 μg/100 g b.w. i.p.) 2 h before the sacrifice. **Lane 1** Control (no ENK implantation), **Lane 2** 24 h, **Lane 3** 48 h, **Lane 4** 72 h after ENK (releasing 5 μg/h) implantation, **Lane 5** spleen (negative control). ER protein immunoblot was measured by densitometric scanning. The data are presented as percent of control (100%) ± S.E.M. of three experiments. The values marked by different letters are significantly different from each other at $P < 0.05$ level.

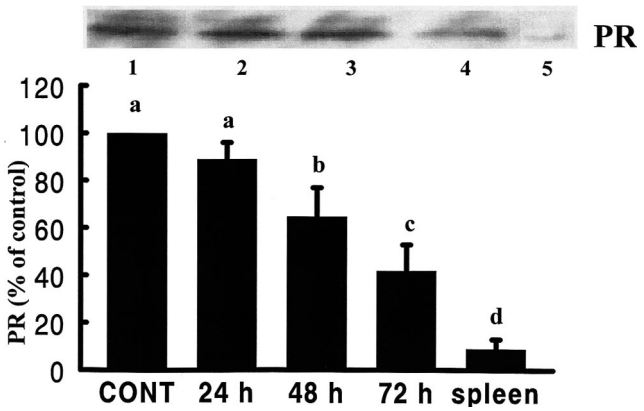

Fig. 3. Regulatory action of ENK implantation on PR protein in adult OVX rat uterus. For further details see the caption of Fig. 2. PR protein immunoblot was measured by densitometric scanning. The data are presented as percent of control (100%) ± S.E.M. of three experiments. The values marked by different letters are significantly different from each other at $P < 0.05$ level.

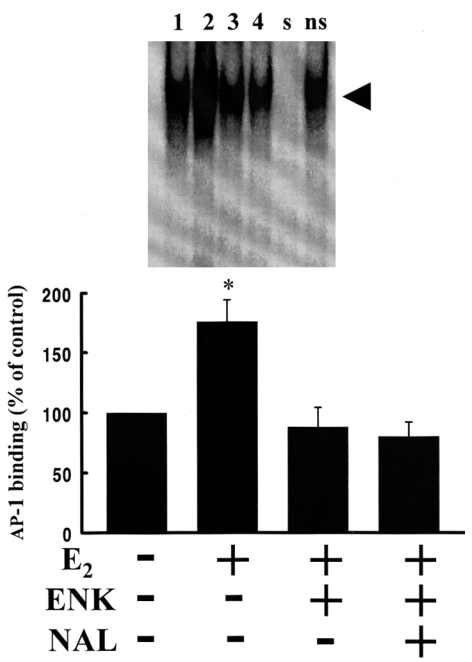

Fig. 4. The effect of ENK on the AP-1 binding activity of adult OVX rat uterine nuclear protein extracts analyzed by electrophoretic mobility shift assay. **Lane 1** OVX vehicle control, **Lane 2** OVX rats treated with 17β-estradiol (10 μg/100 g b.w. i.p.) 2 h before the sacrifice, **Lane 3** OVX rats treated with [D-Met^2,Pro^5]enkephalinamide (100 μg/100 g b.w. i.p.) 15 min before the E_2 administration, **Lane 4** OVX rats treated with NAL (100 μg/100 g b.w. i.p.) 20 min before the ENK injection, **s** specific control, **ns** non-specific control, *Arrowhead* indicates the specific AP-1 complexes. Graph shows the densitometric analysis of AP-1–DNA binding. The data are presented as percent of OVX vehicle control (100%) ± S.E.M. of three experiments, $*P < 0.05$.

gene transcription mediate these effects of steroid hormones. Recent results of this field [20] that have considerable attention are that estrogen controls growth and cell multiplication by regulating the synthesis of different growth factors and their receptors. Few data are available about hormones acting as inhibitor in growth and cell proliferation, however, as is the normal in biology each positive-acting factor or process is opposed by some system which operates in the opposite manner.

Ample evidences have been accumulated concerning the role of endogenous opioid peptides in the regulation of developmental events, cell growth and proliferation in different tissues [6,8,21]. The presence of opioid peptides and receptors in uterine tissue is well documented [11], however, relatively little is known on their effects in the uterus, though some scattered data suggest some functional role of these peptides [10,12].

Previously we described that E_2-induced DNA synthesis in adult, ovariectomized rat uterus in vivo as well as in vitro in cell culture can be inhibited by the opioid peptide, [D-Met^2,Pro^5]enkephalinamide [12,13]. The basal rate of cell proliferation in the adult rat uterus was not affected by opioid peptide. This observation led us to assume that the activated ER may contribute in the ENK effects. Previous studies demonstrated several potential points of interaction between the opioid peptides and gonadal steroid actions. The binding characteristics of hypothalamic μ-opioid receptors varied with the level of gonadal steroids in rat during estrous cycle and pregnancy [22,23]. In pregnant rats the number of μ-receptors in the uterus decreases as pregnancy progresses [10].

Results of our previous experiments show that similar interaction exists at the level of E_2 and opioid receptors in rat uterus, E_2 treatment decreased [^{3}H]Naloxone binding in in vivo [11] and in in vitro experiments [24]. Competition between E_2 and opioid peptides at the receptor level in vitro has been also published [16,25]. Estrogens exert their physiological effects through two types of receptors, the classical is the alpha, and the new one is beta. Antibody used to detect ER recognizes the classical form of ER. The changes in ER concentration and [^{3}H]Estradiol binding found in the present experiments were dual. At 48 h following ENK treatment, a decrease was found in both the binding and the levels of ER. However, at 72 h after the implantation, the depression of ER concentration and function turned to opposite direction, the detected values were in the control range, but the characteristics of the binding parameters changed. At this period, a sharp decrease in the level of the PR was also detected, which might be a result of the ENK-induced impairment of ER function. Several data suggest, that the opioids regulate gene expression in neural cells, however, the precise mechanism is not clear. Among others, several pathways might be considered the cAMP-dependent mechanism, the AP-1–DNA binding, and the involvement of different post-translational modifications [26].

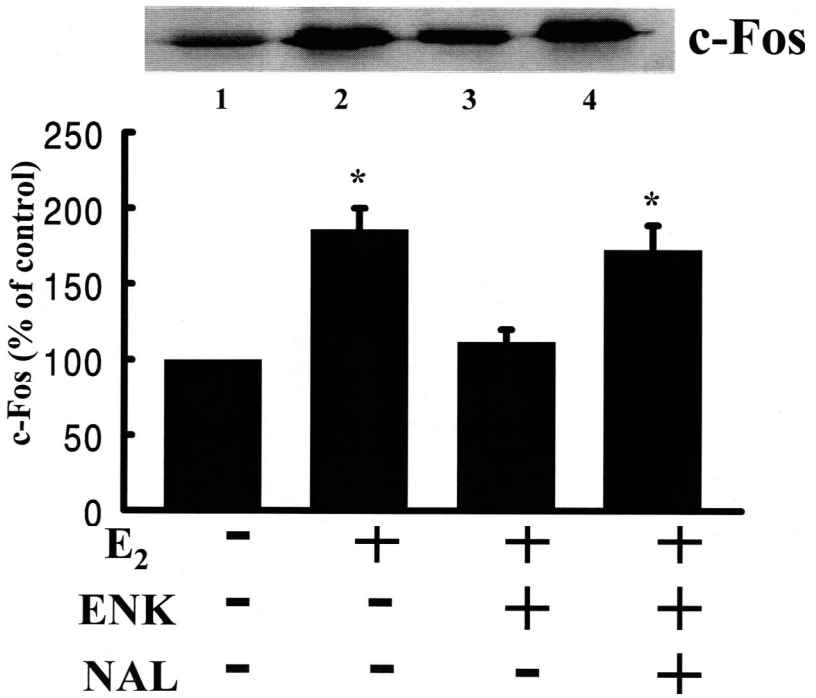

Fig. 5. The regulatory action of ENK treatment on c-Fos protein level in adult OVX rat uterus. **Lane 1** OVX vehicle control, **Lane 2** OVX rats treated with 17β-estradiol (10 μg/100 g b.w. i.p.) 2 h before the sacrifice, **Lane 3** OVX rats treated with [D-Met^2,Pro^5]enkephalinamide (100 μg/100 g b.w. i.p.) 15 min before the E_2 administration, **Lane 4** OVX rats treated with naltrexone (100 μg/100 g b.w. i.p.) 20 min before the ENK injection. c-Fos protein immunoblot was measured by densitometric scanning. The data are presented as percent of control (100%). The values represent the means ± S.E.M. of three experiments, $^*P < 0.05$.

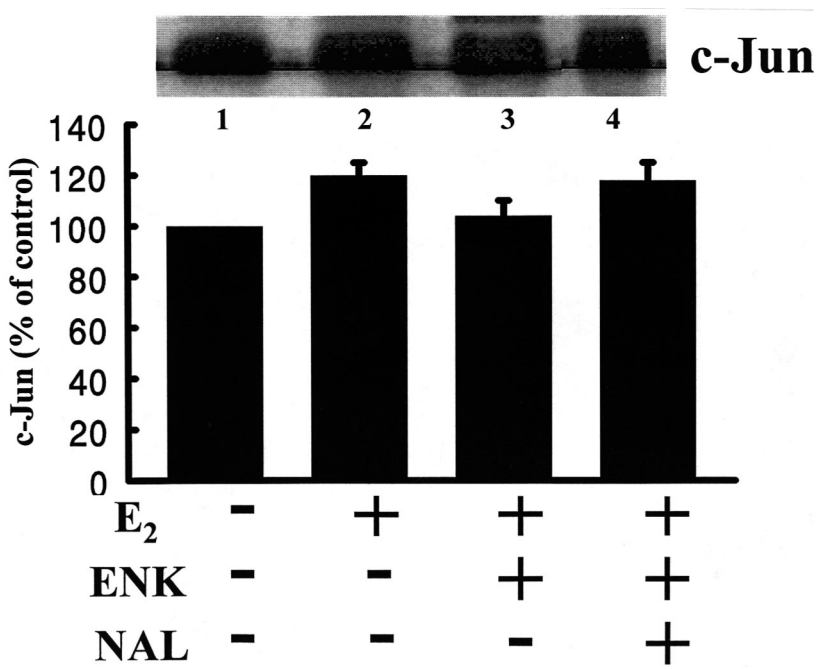

Fig. 6. Regulatory action of ENK treatment on c-Jun protein level in adult OVX rat uterus. For further details, see the caption of Fig. 5. c-Jun protein immunoblot was measured by densitometric scanning. The data are presented as percent of control (100%). The values represent the means ± S.E.M. of three experiments.

Steroids act by binding to cognate receptors. The steroid–receptor complex then binds to the DNA at a hormone-responsive element and activates gene expression. These interactions are specific, dictated by the DNA binding region of ER and the sequence of its cognate response element [27], thus interaction between steroid and opioid peptides at this level is not likely.

It has been shown recently that the ER could interact in an indirect manner through protein–protein contacts with AP-1 transcription factors [5,28] as well. Increasing number of evidences suggest that the AP-1 transcription factors are involved in the effects of opioid peptides [26,29] too. The increase of the quantity of AP-1 transcription factors is usually followed by the rise of the DNA binding activity [30]. The activity of this transcription factor is believed to reflect cell proliferation in many tissues [31] and it is modulated by growth factors, cytokines, activated protein kinase C [30]. The AP-1 response element seems to be one of the integrator of the transcriptional effect of different hormones [31–33]. The AP-1 transcription factors are groups of proteins that recognise and bind to specific AP-1–DNA sequences of the genes. The family consists of two groups namely the Fos- and Jun related proteins. These proteins are usually expressed at low basal level and they could be induced by several factors in the different cells. Estrogen stimulates *c-fos* expression in immature rat uterus [19]; brain region specific expression of the *fos* gene was also published [34]. In our present experiments, E_2 induced a considerable increase in the level of Fos protein. This effect was abolished by ENK administration. Similar tendencies were found in the analysis of Jun protein, but the changes were less expressed.

To discuss these results is difficult due to lack of relevant data concerning the effect of opioid peptides in the uterus. From the data presented here, the following conclusions can be drown. First, the results demonstrate that the effect of E_2 in the uterus could be modified by the changes of the level of opioid peptides, which are expressed in the uterine cells. Second, the AP-1 driven transcription may be involved in the functional interaction between the two hormones. And finally, this predominantly antagonistic interaction could be one of the responsible factors in the inhibitory effect of opioid peptide on the estrogen induced cell proliferation in rat uterus.

Acknowledgements

This work was supported by the National Science Research Fund (OTKA-T-16316, OTKA-T-29267, by the Ministry of Education (FKFP 0494/1999) and by the Ministry of Health (ETT 093/1999) of Hungary. We are indebted to Dr. S. Bajusz (Institute for Drug Research, Budapest, Hungary) for the generous gift of [D-Met^2,Pro^5]enkephalinamide. We thank Prof. J. Szeberényi (Department of Biology, University Medical School, Pécs, Hungary) for discussions and comments on the manuscript.

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