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Opioids are non-competitive inhibitors of nitric oxide synthase in T47D human breast cancer cells

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Abstract

Opioids and nitric oxide (NO) interact functionally in different systems. NO-generating agents decrease the activity of opioid agonists, prevent opioid tolerance, and are used in opioid withdrawal syndromes. There exist, however, few reports indicating a direct interaction of the two systems. T47D human breast cancer cells in culture express opioid receptors, and opioid agonists inhibit their growth, while they release high amounts of the NO-related molecules NO_2^-/NO_3^- to the culture medium. We have used this system to assay a possible direct interaction of opiergic and nitric oxide systems. Our results show that δ- or μ-acting opioid agonists do not modify the release of NO_2^-/NO_3^-. In contrast, κ-acting opioid agonists (ethylketocyclazocine, and α_{S1}-casomorphine) decrease the release of NO_2^-/NO_3^-, in a time- and dose-dependent manner. The general opioid antagonist diprenorphine (10^{-6} M) produce a similar NO_2^-/NO_3^- release inhibition, indicating a possible non-opioid-receptor mediated phenomenon. In addition, ethylketocyclazocine, α_{S1}-casomorphin and diprenorphine directly inhibit NOS activity: agonists, interact with both calcium-dependent and independent NOS-isoforms, while the antagonist diprenorphine modifies only the activity of the calcium-dependent fraction of the enzyme. Analysis of this interaction revealed that opioids modify the dimeric active form of NOS, through binding to the reductase part of the molecule, acting as non-competitive inhibitors of the enzyme. This interaction opens interesting new possibilities for tumor biology and breast cancer therapy. *Cell Death and Differentiation* (2001) **8,** 943–952.

Keywords: nitric oxide; opioids; breast cancer

Abbreviations: EKC, ethylketocyclazocine; DSLET, [D-Ser[2], Leu[5]]-enkephalin-Thr[6]; DAGO, [D-Ala[2], N-Me-Phe[4], Gly[5]-ol]-enkephalin; NO, nitric oxide; NOS, nitric oxide synthase; PMA, phorbol 12-myristate, 13-acetate

Introduction

Opioids were found to derive, in all animal species, from three major propeptides (proenkephalin A and B and proopiomelanocortin) and to bind in three major classes of opioid receptors (δ, μ and κ) (see[1] for a recent review). Pharmacological evidences further indicate that multiple subtypes of each opioid receptor might exist (at least two for the δ, two for the μ and three for the κ opioid receptor). In addition to the classical opioid peptides (Met[5]- and Leu[5]-enkephalin, beta-endorphin, dynorphins) a number of opioid peptides, derived from the limited proteolysis of endogenous or food proteins, were identified, including casomorphins, derived from α- and β-caseins of animal and human origin (see[2] for a review). Recently, we have identified an opioid sequence from α_{S1}-casein, named alpha$_{S1}$-casomorphin, with a kappa-opioid receptor activity, which is very potent in inhibiting cell proliferation of a number of different cancer cell lines, *in vitro*.[2–5] In addition, a number of synthetic peptide analogs were reported as selective agonists of delta (DSLET), or mu (DAGO) opioid receptors. These peptides possess a greater stability to endo- and exopeptidases found in the receptor environment.[6]

Nitric oxide (NO) is a gaseous molecule. It is produced through the enzymatic deamination of arginin to citrulline, by the enzyme nitric oxide synthase (NOS). Nitric oxide synthase exists in three different isoforms in mammalian species: eNOS, membrane bound, nNOS bound to different cellular membrane elements, and the cytosolic iNOS. These three isoforms have different molecular masses (135, 150 and 130 kD respectively) and are products of different genes.[7,8] Calcium is necessary for the action of some forms of the enzyme, although no clear-cut distinction of the molecular form and calcium-dependency can be made. During the last decade, NO has been recognized as an important messenger molecule in mammalian species. It acts through modification of soluble guanylate cyclase activity[9] and cGMP intracellular levels. Nitric oxide is an important regulator of different functions, including vasodilation and neurotransmission, although high concentrations of this agent could be either beneficial (anti-bacterial, anti-parasitic or anti-viral) or detrimential, inducing cell death.[10] The mechanism of this dual action includes a sophisticated regulation of nucleat transcription factors, and regulation of a number of intracellular proteins.[10] The role of NO in cancer is also contradictory. Nitric oxide could oxidize nucleic acids and induce DNA damage, although it can

enhance the tumoricidal activity of the immune system. In addition, NO production by tumor cells could enhance the angiogenetic and metastatic potential of tumors (see[11] for a review).

A functional interaction between opiergic and nitric oxide (NO) systems in different organs has been reported so far. NO-related agents inhibit morphine action on testicular steroidogenesis, while nitric oxide synthase inhibitors reverse the action of opioid antagonists,[12] through separate intracellular pathways.[13] In addition, NO or arginin (a NO precursor) decrease the potency of morphine[14–16] through a modulation of intracellular concentrations of cGMP, without modifying the characteristics of opioid binding.[17,18] NO reverts the opioid-exerted inhibition on LHRH[19–21] and GnRH/CRH secretion.[22] In contrast, a direct stimulation of NO secretion at the cardiac atrium by morphine was reported,[23] while, in the vascular endothelium, a μ-opioid receptor was identified, acting, at least partially, through a functional coupling with c- and iNOS.[24–28]

NO-releasing agents can prevent μ receptor-mediated opioid tolerance but not dependence,[16,29–33] and they have been used experimentally for the treatment of opioid withdrawal syndromes,[34–42] while NOS inhibitors enhance the antinociceptive effects of opioids.[43,44] In opioid dependence, increased nNOS immunoreactivity was found.[45] Furthermore, after Met^5-enkephalin application in rat brain synaptosomes, an increase of membrane fluidity, NO and cGMP production, were observed.[46] The activation of NOS-cGMP pathway was proposed as the underlying mechanism for morphine-induced antinociception,[47–49] through two distinct isotypes of nNOS.[50] Mu-opioid receptors and nNOS were co-localized in the rat's nervous system.[51] In the canine gut, NOS inhibition decreased or abolished the action of μ- and δ-opioid agonists.[52] Finally, nociceptin was reported to act through an inhibition of tonic NO secretion.[53]

Previous works have shown that the proliferation of T47D human breast cancer cells is inhibited, in a dose-dependent and reversible manner, by opioid agonists, through an interaction with δ- and κ-opioid receptors.[2,54–56] In addition, these cells show a very high NO_2^-/NO_3^- release, and NOS activity, which is not further stimulated by the addition of mitogens.[57] It seemed therefore interesting to investigate a possible interaction of opioids on NO_2^-/NO_3^- release and NOS activity. Our results indicate that opioids can modify the release of NO_2^-/NO_3^-, and the enzymatic activity of NOS.

Results

Opioids modify NO release in the culture medium

Figure 1 presents the release of NO_2^-/NO_3^- in the culture medium of T47D cells, reflecting the release of NO (see Material and Methods). As shown, cells produce and release high concentrations of NO_2^-/NO_3^- (920 ± 19 μmoles/L as compared to the production and release of 30.5 ± 5.1 μmol l by MCF7 cells.[57] This high release was not further enhanced by the 24 h stimulation of cells by the general mitogen phorbol

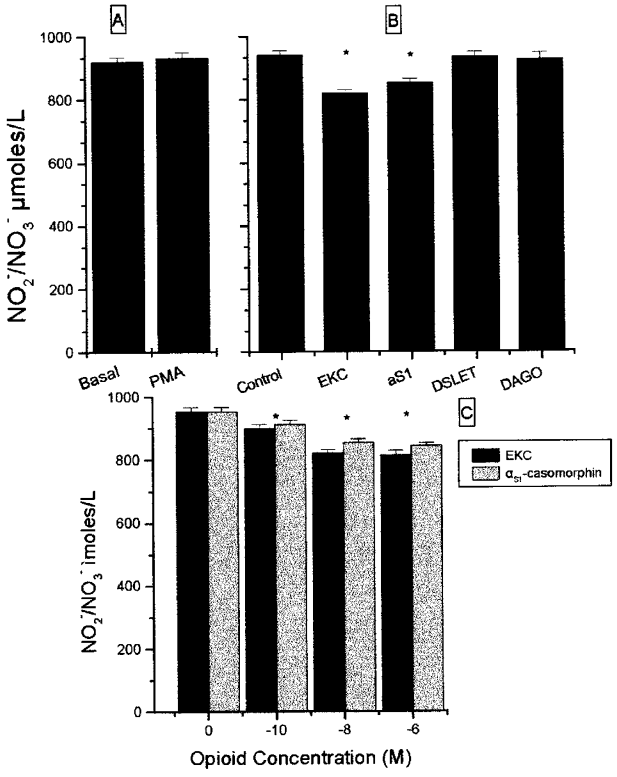

Figure 1 Release of NO_2^-/NO_3^- by T47D cells. (**A**) Basal and PMA-stimulated NO_2^-/NO_3^- release after 24 h incubation. 200 000 cells were incubated in 12-wells plates for 24 h. Thereafter, medium was replaced, and cells were incubated for another 24 h period, in the absence (basal) or in the presence of 50 ng/ml of phorbol 12-myristate, 13-acetate (PMA). Medium was collected and stored at -80°C until NO_2^-/NO_3^- determination, as described in the Material and Methods. (**B**) Basal NO_2^-/NO_3^- release in the presence of opioid agonists. 200 000 cells were incubated for 24 h after seeding. Thereafter, medium was replaced, and the indicated opioid drugs were added at a final concentration of 10^{-8} M. After 24 h incubation with the opioid drugs, medium was collected, centrifuged and NO_2^-/NO_3^- concentration was assayed. Mean $\pm$ S.E. of three experiments in triplicates. EKC: Ethylketocyclazocine; aS1: α_{S1}-casomorphin; DSLET: [D-Ser^2-Leu^5-enkephalin]-Thr^6; DAGO: [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin; *: $P<0.001$ (Student's one paired *t*-test), as compared to control. (**C**) Dose-effect of opioid agonists on the release of NO_2^-/NO_3^- in the culture medium. T47D cells were incubated for 24 h in the absence (0) or in the presence of the indicated concentrations of ethylketocyclazocine (EKC) or alpha$_{S1}$-casomorphin. Then, the medium was collected and assayed for NO_2^-/NO_3^-, as described in Material and Methods. Mean $\pm$ S.E. of three experiments in duplicate. No significant differences were observed between EKC and alpha$_{S1}$-casomorphin. *: $P<0.05$ at least (Student's one paired *t*-test), as compared to values in the absence of opioids

12-myristate, 13-acetate (PMA). The addition of 10^{-8} M of ethylketocyclazocine (EKC), acting on δ, μ and κ opioid receptors, or the κ-opioid agonist α_{S1}-casomorphine[2] decreased significantly the NO_2^-/NO_3^- released in the culture medium, while the addition of 10^{-8} M DSLET or DAGO (selective δ- and μ-opioid agonists respectively) had no effect, indicating that the action of EKC might be due to its κ-related activity. This opioid effect was equally not modified by the addition of PMA (not shown). The dose-dependence of the inhibition of NO_2^-/NO_3^- release is presented in Figure 1, lower panel (C). As shown, both opioid agonists inhibit the release of NO_2^-/NO_3^- after 24 h incubation in a dose-

dependent manner. At a concentration of 10^{-8} M both opioid agonists present a maximum effect on NO_2^-/NO_3^- release. Therefore, throughout this study, the effect of opioids on NO-producing system was assayed at that concentration (10^{-8} M). Interestingly, at this same concentration the maximum effect of opioid agonists on the arrest of cell growth was observed.[2,56]

Opioids modify NO production

The above decrease of NO_2^-/NO_3^- release by opioids indicates a direct or functional interaction between the opioid and the NO systems. As a first attempt to investigate a possible direct effect of opioids on NO production, we have assayed the effect of opioid agonists and antagonists on the kinetics of NO production, by flow cytometry. As shown in Figure 2, T47D cells, under basal conditions, produce significant amounts of NO, a result reflected by the high release of NO_2^-/NO_3^- to the culture medium. After 1 h, about 95% of cells are positive for NO. This effect is decreased (but not abolished) by the addition of the NOS antagonist L-NAME (Nω-Nitro L-Arginin Methyl Ester), in a concentration-dependent manner (Figure 2, lower panel, insert).

The preincubation of cells with DSLET or DAGO (specific delta and μ opioid agonists respectively) for 10 min to 24 h, did not produce any significant modification of NO production (not shown), confirming the results presented in Figure 1 (absence of effect of δ and μ opioid agonists on the concentration of NO_2^-/NO_3^- to the culture medium). In contrast, when cells were incubated with 10^{-8} M ethylketocyclazocine, or α_{S1}-casomorphine for the same time periods (10 min to 24 h), a significant decrease of NO production was observed, varying from 25 to 60% of total fluorescence. For both opioid agonists this inhibition was very fast ($t_{1/2}$ ~3 min). In addition, when α_{S1}-casomorphin was used to inhibit NO production, a decline of its inhibitory effect was observed after 24 h, indicating a decline of the peptide potency, attributed to its partial degradation.

Diprenorphine is a general antagonist of opioid action. In T47D cells, this compound was found to inhibit the opioid receptor-mediated inhibition of cell proliferation.[56] The incubation of cells with diprenorphine alone (10^{-6} M) decreased the production of NO after short (Figure 3A) or long incubation times (Figure 3B). The opioid agonist EKC and the antagonist diprenorphine showed similar kinetic curves applied alone or in combination, indicating that the observed effect of opioids on the production of NO might not be mediated through membrane opioid receptors.

Opioids inhibit selectively the activity of NOS

The above results indicate a direct action of opioids on the production of NO by T47D cells. In order to further analyze this action, cells were preincubated for 24 h with different opioid agonists, and total NOS activity was assayed in a whole cell homogenate. The results are presented in Figure 4A. As shown, a significant (by almost 50%) inhibition of NOS activity

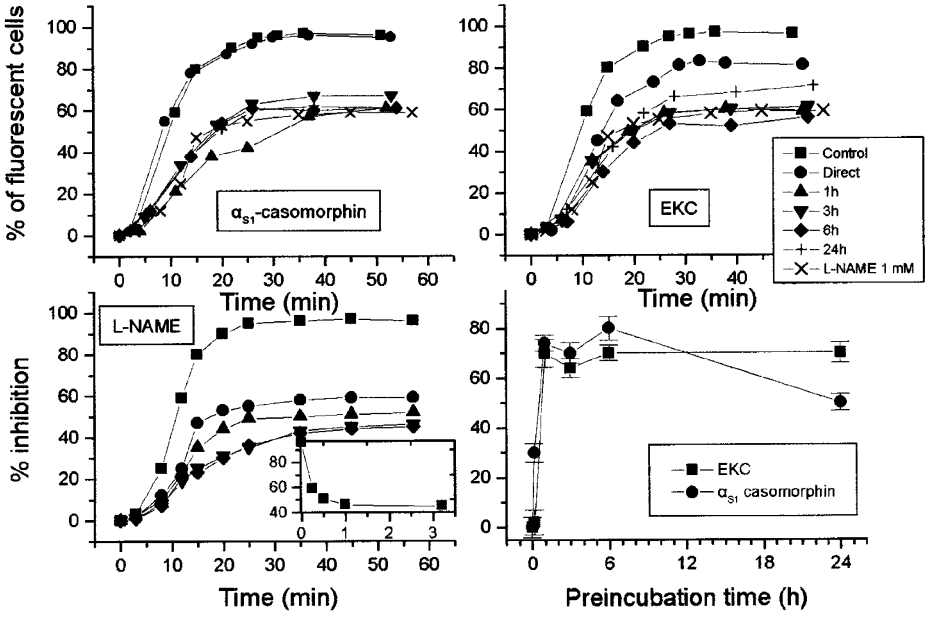

Figure 2 Kinetics of NO production. Upper panels: T47D cells were incubated for 24 h after seeding. Thereafter, medium was replaced and incubation was continued for the indicated time periods in the absence (control) or in the presence of 10^{-8} M of opioids. Then, the medium was discarded, cells were detached from dishes, washed with PBS, loaded with diaminofluorescein diacetate, and NO production was assayed by flow cytometry, for 60 min. Results of an experiment, which was repeated five times with similar results. Lower panels: left: Inhibition of DAF fluorescence by L-NAME. L-NAME was applied 10 min prior to the onset of measurements. Square: control; Circle: 0.25 mM; Up triangle: 0.5 mM; Down triangle: 1 mM; Diamond: 3.2 mM. Insert: Maximal inhibition of intracellular fluorescence, as a function of L-NAME concentration (mM). The final points of the kinetic determination are plotted as a function of L-Name concentrations. Means ± S.E. of three determinations. Right: Time dependence of opioid application on NO production. Data (final points of the kinetic experiments) are the means ± S.E. of five different experiments

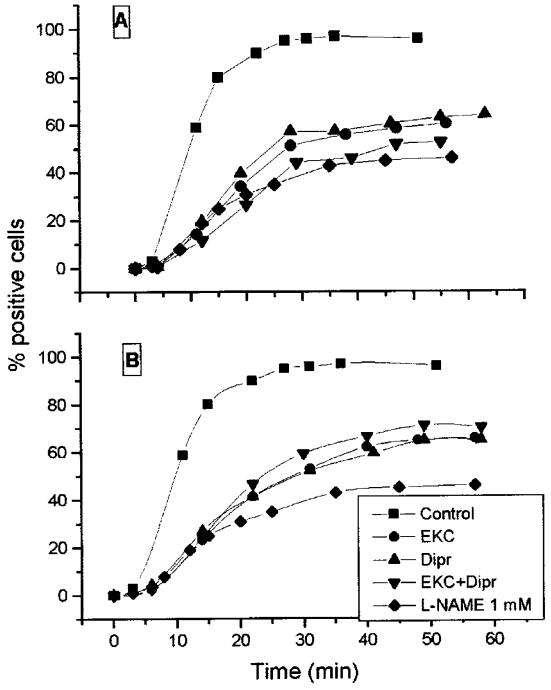

Figure 3 Effect of Diprenorphine on NO production in T47D cells after short (1 h, upper panel) or long (24 h, lower panel) incubation. T47 cells were incubated without (control) or with 10^{-8} M ethylketocyclazocine, in the absence, or in the presence of 10^{-6} M diprenorphine, for 1 h (**A**) or 24 h (**B**). Thereafter, they were detached with thrypsin-EDTA, loaded with DAF, and fluorescence was measured by flow cytometry, during 60 min. Results of a typical experiment. The above experiment was repeated four more times, with similar results. Intracellular fluorescence, in the presence of 1 mM L-NAME is presented, in order to evaluate the selective production of NO

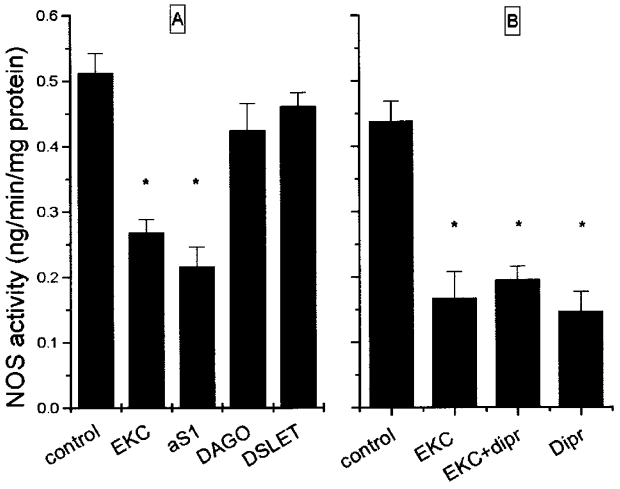

Figure 4 Effect of opioids on the activity of nitric oxide synthase. (**A**) Cells were preincubated for 24 h in the presence of 10^{-8} M of the indicated opioid agonists. Then, they were harvested, homogenized, centrifuged at $12\,000 \times g$ for 15 min, in order to eliminate nuclei and unbroken cells, and NOS activity was assayed in the supernatant as described in the Material and Methods section. (**B**) Cultured cells were harvested, the microsomal fraction was prepared as described above, and NOS activity was assayed, as described at the Material and Methods section, in the absence of opioid drugs (control) or in the presence of 10^{-8} M of EKC, and/or 10^{-6} M of the antagonist diprenorphine. Mean $\pm$ S.E. of three experiments in duplicate. *: $P<0.001$, Student's one paired *t*-test, as compared to the control

was observed only after the application of EKC or α_{S1}-casomorphine (10^{-8} M). In contrast, DSLET or DAGO did not produce any significant effect, indicating that the effect of opioids might be restricted to κ-acting opioids. Preincubation of cells with diprenorphin (10^{-6} M) produced the same effect as described for EKC (Figure 4B). The incubation of cells with a combination of EKC (10^{-8} M) and diprenorphine (10^{-6} M) did not have any additional effect on NOS activity, confirming that the observed direct effect of κ-opioids might not be mediated by opioid receptors.

Figure 5 presents the substrate-related enzyme velocity of NOS in T47D cells. As shown, increasing concentrations of arginin increase the enzyme velocity, which reaches a plateau at concentrations $>42\ \mu$M (see also Table 2). DSLET and DAGO did not show, as expected, any significant effect on enzyme velocity. In contrast, EKC and α_{S1}-casomorphine decreased significantly NOS activity, indicating a direct effect of opioids on the enzyme protein. Calculated K_M and V_{max} are presented in Table 2.

This opioid-related inhibition of NOS is dose-dependent. As shown in Figure 6 (upper panel), preincubation of cells with increasing concentrations of EKC or α_{S1}-casomorphin ($10^{-10}-10^{-6}$ M) decrease, in a dose-dependent manner, the activity of the enzyme, similar to the effect of opioids on the enzyme activity in whole cell homogenates (Figure 6, lower panel).

Effect of opioids on different isoforms of NOS

Nitric oxide synthase exists in three different isoforms in mammalian species: eNOS, membrane bound, nNOS bound to different cellular membrane elements, and the cytosolic iNOS. These three isoforms have different molecular masses (135, 150 and 130 kD respectively) and are products of different genes.[7] From another aspect, NOS molecules can be distinguished as calcium-dependent and calcium-independent, although no direct distinction of the three molecular isoforms could be made upon calcium dependence. Figure 7 presents the effect of κ-opioids on the activity of these Ca^{2+}-dependent and independent isoforms of NOS. As shown, both ethylketocyclazocine and α_{S1}-casomorphine interact with both forms of the enzyme (calcium-dependent and independent). In contrast, the antagonist diprenorphine inhibits only the calcium-dependent NOS activity, indicating a differential action of opioid agonists and antagonists.

Effect of FAD on opioid-related enzyme inhibition

In the structure of NOS proteins two distinct domains can be identified: the reductase or FAD-FMN domain (towards the carboxylic end of the molecule) and the oxygenase or heme domain towards its NH_2 part.[58] An alignment of the three human types of NOS with the human κ opioid receptor revealed that the later fits better with the FAD-FMN reductase domain of NOS, indicating a possible binding of opioids at this part of the molecule. In order to confirm this interaction, we have performed assays of the enzyme activity, in the presence or the absence of 10^{-8} M ethylketocyclazocine,

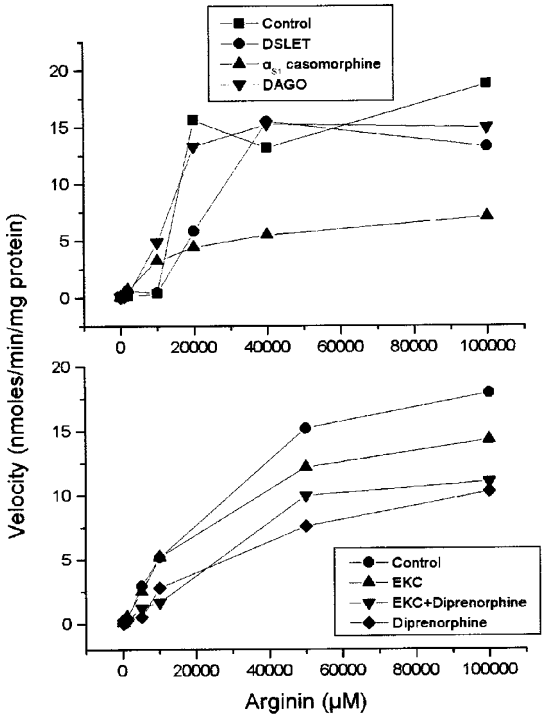

Figure 5 Effect of opioids on NOS reaction rate as a function of substrate (arginin) concentrations. T47D cells were harvested, nuclei and unbroken cells were eliminated as described in the legend of Figure 4, and NOS assay was performed as described in Material and Methods, in the presence of a constant concentration of labeled and increasing concentrations of unlabeled arginin, in the absence (control), or in the presence of 10^{-8} M of the different opioid agonists. The opioid antagonist diprenorphine was added at 10^{-6} M. Results of a typical experiment, repeated three times with similar results

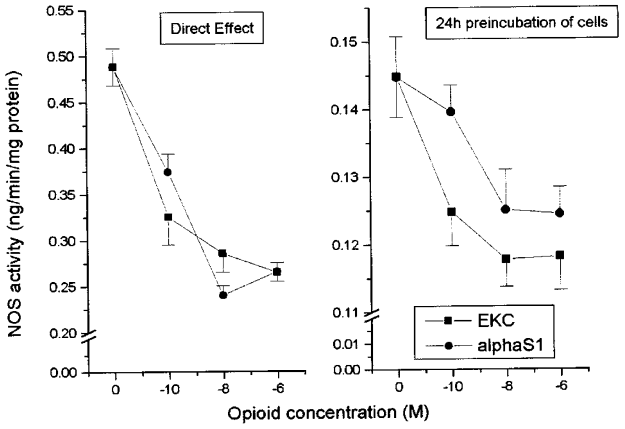

Figure 6 Dose-effect of opioids on NOS activity. Left panel: Whole cellular fraction of T47D cells was prepared and NOS activity was assayed as described in Material and Methods, in the absence (0) or in the presence of the indicated concentrations of ethylketocyclazocine (EKC) or alpha$_{S1}$-casomorphin (alphaS1). Mean ± S.E. of three experiments in duplicate. Right panel: T47D cells were incubated for 24 h in the absence (0) or in the presence of the indicated concentrations of ethylketocyclazocine (EKC) or alpha$_{S1}$-casomorphin (alphaS1). Then, the medium was aspirated, cells were washed twice with ice-cold phosphate buffered saline, homogenized with a dounce glass homogenizer, and NOS activity was assayed in a whole homogenate, after elimination of unbroken cells and nuclei. Mean ± S.E. of three experiments in duplicate

and varying concentrations of FAD, from 10^{-7} to 10^{-4} M (Figure 8). As shown, in the absence of the opioid agonist, the optimal concentration of FAD is 10^{-6} M. In contrast, when EKC is applied, the enzyme activity is decreased by about 40%. Increasing the concentration of FAD restores the activity of the enzyme.

Discussion

Previous investigations have shown a functional interaction between the opioid system and NO in different cell types.[12,14–16,19–22] In addition, NO-generating agents have been used as potential therapeutic drugs in opioid withdrawal syndromes, and NO was found to prevent morphine tolerance. Nevertheless, very few reports indicate a direct interaction between these two systems,[23–26] usually restricted to the modulation of cGMP and/or other signaling molecules.[17,18] The results of the present investigation indicate that, in T47D breast cancer cells, κ- but not δ- or μ-opioid agonists decrease NO_2^-/NO_3^- release, by a direct interaction on NOS.

Morphine and κ-opioid agonists, but not β-endorphin were found to inhibit nNOS at the mM range.[59,60] Furthermore, δ-, μ-, or κ-opioids inhibit NO production in LPS-stimulated macrophages, in a concentration-dependent and reversible manner, indicating a functional coupling

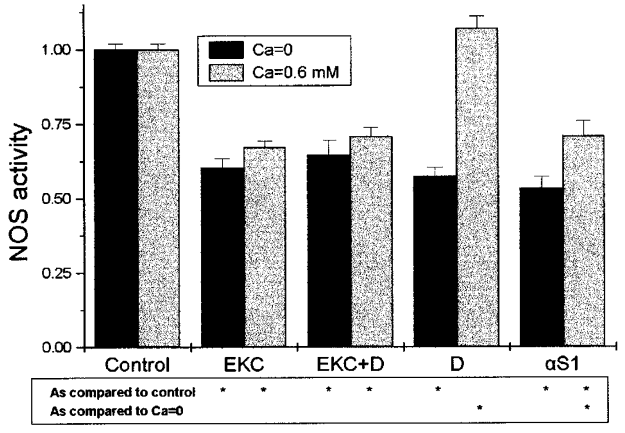

Figure 7 Effect of opioid agonists and antagonists on the calcium-dependent and independent NOS activity. Whole cellular fraction of T47D cells was prepared as described in Material and Methods. NOS assay, in the absence (control) or in the presence of the indicated opioid agonists, or antagonists (10^{-8} and 10^{-6} M respectively) was performed in the absence (black bars) or in the presence (grey bars) of 0.6 mM $CaCl_2$. Mean ± S.E. of two experiments in triplicate. EKC: ethylketocyclazocine; D: Diprenorphine; aS1: α_{S1}-casomorphin. *: $P<0.05$ at least (one tailed Student's *t*-test)

of the two systems,[61] possibly mediated through opioid receptors.[62] Increased NOS activity was found in the spinal cord of morphine-exposed animals.[63] In addition, it was reported that heroin administration reduces the expression of LPS-stimulated iNOS activity in rat liver, spleen and lung, through an opioid receptor-mediated mechanism.[64] Finally, U-50488H, a κ-opioid agonist,[59] inhibits nNOS activity *in vitro*, while NO blocks the opioid-induced tolerance *in vivo*.[65–67]

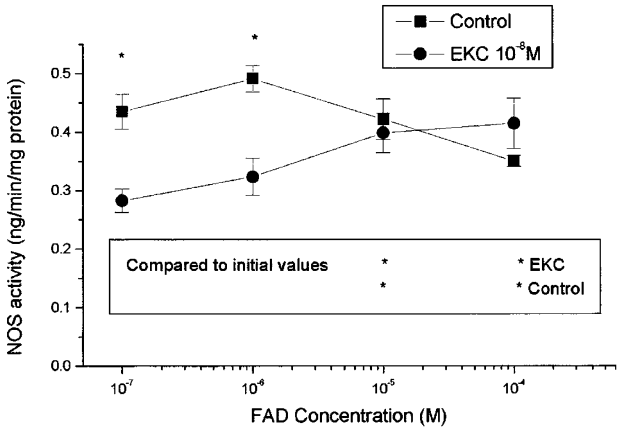

Figure 8 Effect of FAD concentration on NOS activity. Action of opioid agonists. Whole cellular fraction of T47D cells was prepared as indicated in the Material and Methods section. The activity of NOS was assayed in the absence (control) or in the presence of 10^{-8}M of the opioid agonist ethylketocyclazocine (EKC), and varying concentrations of FAD, ranging from 10^{-7} to 10^{-4}M. Results of three experiments, in duplicate. *: $P<0.05$ at least, one paired Student's *t*-test. In the box the statistical comparison of data as compared to their corresponding initial value are presented

Table 1 Kinetic characteristics of NO production in T47D cells: Effect of opioids

	Maximum (%)	k_1 min^{-1}
Control	96±3	0.1097±0.007
EKC	61±5*	0.0366±0.011*
α_{s1}-casomorphin	61±4**	0.0366±0.012*
DSLET	94±5	0.1113±0.005
DAGO	97±3	0.1064±0.008
EKC+Diprenorphine	63±4*	0.0357±0.009*
Diprenorphine	60±5*	0.0375±0.015*

Cells, cultured for 24 h in the absence (control) or in the presence of 10^{-8} M of the indicated opioids were loaded with diaminofluorescein diacetate, and the production of NO was measured by the increase of specific fluorescence. The general opioid antagonist diprenorphine was applied at a concentration of 10^{-6} M. Data were analyzed by the application of the first order kientic equation: Log (a/(a−x))=kt/2.303, in which a is the percentage of negative cells (100% at time 0), x is the percentage of positive cells at time t, and k is the kinetic rate constant, expressed as min^{-1} (83). Mean±S.E. of five different measurements. *:$P<0.05$, **: $P<0.01$ (two-tailed test)

Table 2 Calculation of the Michaelis constant (K_M) the maximal velocity (V_{max}) and the Hill coefficient of data presented in Figure 4, according to Dawes.[83] Mean±S.E. of three different experiments performed in duplicate

	K_M (μM)	V_{max} (nmoles/min/mg protein)	Hill coefficient
Control	20.7±2.1	18.8±2.0	1.59±0.2
DSLET	21.4±3.2	15.5±1.7	1.52±0.4
DAGO	22.2±2.7	15.2±1.3	1.68±0.5
α_{s1}-casomorphin	12.3±3.7*	7.1±0.8**	1.02±0.2*
EKC	22.2±2.5	16.1±0.9	1.21±0.2
Diprenorphine	39.9±3.4**	13.7±1.1*	1.25±0.3
EKC+Diprenorphine	22.2±2.8	11.5±1.0*	1.30±0.2

*:$P<0.05$, **: $P<0.01$ (one tail *t*-test)

In the T47D cell line, previous results have shown that low concentrations of κ-opioids decrease cell growth in a time-, dose-dependent and reversible manner.[2,56] We report here that opioid agonists, active on κ-opioid receptors decrease NO_2^-/NO_3^- release to the cell culture medium (Figure 1), and the activity of NOS (Figures 3A and 6) in a similar dose-dependent manner. In contrast, δ- and μ-acting opioid agonists (DSLET and DAGO respectively) had no effect (Figures 1, 4 and 5, Tables 1 and 2). The general opioid antagonist diprenorphine exhibits an inhibitory action on NOS too, indicating a non-opioid receptor-mediated phenomenon (Figures 3 and 4). In addition, diprenorphine action is restricted to the calcium-dependent fraction of NOS, while ethylketocyclazocine and α_{S1}-casomorphine act on both calcium dependent and independent fractions (Figure 7), indicating a differential action of opioid agonists and antagonists, possibly due to a different conformation of agonist and antagonist molecules. Previous results indicate that, in the vascular endothelium, opioid receptors could be coupled to cNOS,[24–26] although in this case opioid agonists produce an increase of the enzyme activity. In our hands, however, no effect of μ-acting opioids (DAGO) was found, a discrepancy possibly due to tissue differences in the two studies. Indeed, T47D cells express preferentially κ opioid sites, which are predominant,[56] while no μ- and rare δ-opioid sites have been identified, with μ-opioid agonists acting through somatostatin receptors.[54] Preliminary, unpublished, results from our laboratory indicate that somatostatin analogs cannot modify NOS activity. Finally, our results indicate that the observed opioid action is exerted directly on the enzyme molecule (Figures 3B, 4 and 6).

The mechanism of the direct inhibitory action of opioids on NOS is not clear. Our results give some hints on this interaction: Opioids that inhibit directly the enzyme activity (ethylketocyclazocine, α_{S1}-casomorphine and diprenorphine) decrease significantly the Hill coefficient (see Table 2), indicating a possible dissociation of the NOS dimer, which is considered as the only active form of the enzyme.[7] Furthermore, data presented in Table 2 indicate a non-competitive inhibition of enzyme activity.[68] The mechanistics of this type of inhibition indicate that opioids could bind to free and substrate-bound enzyme molecules, suggesting that they interact with a different site than that of arginin binding. Finally, alignment of the human κ-opioid receptor and the three human NOS sequences indicates that opioid agonists or antagonists might bind to reductase moiety of the NOS molecules, a result further indicated by the data presented in Figure 8, showing that increasing concentrations of FAD restore the enzymatic activity.

Our results show a very rapid inhibitory action of opioids on NO production (Figure 2), indicating that, perhaps, a receptor-mediated interaction could not be ruled out. Nevertheless, the fact that agonists and antagonists produce similar results indicates that, if a receptor-mediated action occurs, this might be mediated by another membrane receptor, different from opioid receptors. In addition, if the direct interaction of opioids on NOS molecules reported here, has a biological significance, opioid ligands might be present in the cytosol in an active form, after an extracellular application. A number of reports indicate that, after opioid treatment, the receptor-ligand complexes enter cells through

early then late endosomes and directed to lysosomes for degradation of either receptor and/or ligand. This intracellular trafficking of the receptor-ligand complex occurs minutes after ligand application, and reaches its maximum after 4 h.[69,70] Thereafter, if the ligand is applied for short periods (min) the receptor is redistributed to the plasma membrane, other ways it is directed to lysosomes for degradation.[69] The fate of opioid ligands is not well established. Alkaloids, being hydrophobic, could enter directly in the cytoplasm. In contrast, peptide ligands require an active transport through receptor-mediated endocytosis, and are either degraded, or they could exert specific intracellular effects. Indeed, beta-endorphin has been found to interact with intracellular binding sites, after its extracellular application, indicating internalization in an active form,[71] while encephalin analogs were found to recycle to the culture medium after endocytosis of the ligand-receptor complex, and lysosomal receptor degradation.[72] Human κ opioid receptors are internalized also rapidly, with $t_{1/2}$ of about 10 min, and about 40% of them are found in the cytoplasm after 20 min exposure to the ligand.[73] Interestingly, after agonist removal, receptors could recycle to the plasma membrane,[73] although other reports indicate that kappa selective peptides induce receptor down-regulation.[74] A recent report indicates that receptor degradation, involving down regulation, occurs by a combined action of lysosomes and proteasomes.[75] The above results indicate that receptor and/or ligand internalization is very rapid, occurring in minutes after receptor-ligand interaction, and could explain our results, of a very rapid opioid action on NOS activity (Figure 2).

The direct interaction of opioids with NOS, and the decrease of the enzyme activity reported here, opens new, interesting, possibilities for breast tumor biology. NO has been implied to tumor progression and metastasis,[11] while opioids, provided either by the general circulation, or locally produced, were found to decrease cell growth.[2,55,56,76] It is therefore tentative to relate the reported inhibition of NOS activity and NO production and release by opioids to tumor metastasis suppression, indicating a potential action of opioids in tumor biology and treatment.

Materials and Methods

Material and cell line

T47D cells were purchased from the European Collection of Cell Cultures (Salisbury, UK). They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), in a humidified atmosphere of 5% CO_2 in air. All culture media and sera were from Gibco BRL (Life Technologies, Paisley, UK). Medium, supplemented with the different compounds was changed every day.

DAGO and DSLET were from Sigma Chemical Co. (St Louis MO, USA). EKC was a gift from Sterling-Winthrop (Bayer Co., Leverkusen, Germany). Diprenorphine was from Reckit and Coleman Co.

Nitric oxide generation and assay

Cells were seeded in 12-well plates, at a density of 200 000 cells/well. Twenty-four hours later, the medium was replaced, and opioids or an equivalent volume of PBS (negative control) were added. After another 24-h incubation, media were collected, centrifuged and frozen at -80°C, for NO_2^-/NO_3^- determination.

NO is relatively unstable in the presence of molecular oxygen (O_2). Therefore, it is rapidly and spontaneously autooxidized, in aqueous (physiological) fluids to give the stable nitrite (NO_2^-), and (possibly through the action of certain oxyhemoproteins) nitrate (NO_3^-) ions. We have therefore measured these metabolites, as described by Grisham *et al*[77] and Granger *et al*.[78] Briefly, 100 μl of the culture medium were incubated with 0.1 U of nitrate reductase, from *Aspergillus*, for 30 min at 37°C, in 25 μl of 1 M HEPES buffer (pH 7.4), 25 μl of 0.1 mM FAD and 50 μl of 1 mM NADPH, in a total volume of 500 μl. This incubation transforms NO_3^- to NO_2^-. Thereafter, 5 μl of lactate dehydrogenase (1500 U/ml) and 50 μl of 100 mM pyruvate were added in each tube, in order to oxidize any unreacted NADPH, which inhibits strongly the Griess reaction. Samples were incubated at 37°C for 10 min. Subsequently, 1 ml of the Griess reagent (equal volumes of 0.2% (w/v) N-(1 naphtyl)-ethylenediamine, and 2% (w/v) sulfanilamide in 5% phosphoric acid, premixed shortly prior to use) was added, followed by 10 min incubation at room temperature. Absorbance was measured at 543 nm, and was linear with standard nitrite concentrations ranging from 2–60 μM. All reagents used were from Sigma (St Louis, MO, USA).

NO production by T47D cells was measured using the diaminofluorescein diacetate method[79,80] and flow cytometry. Briefly, cells, treated or not with opioids for the indicated time periods, were detached from dishes using a trypsin-EDTA solution (Gibco BRL, Life Technologies, Paisley, UK), adjusted to a concentration of 10^6 cells/ml, and diaminofluorescein diacetate (0.1 mM final concentration in a volume of 10 μl), obtained from Sigma (St Louis, MO, USA) was added. NO production was assayed by flow cytometry, using a Coulter Epics XL-MCL apparatus (Beckman-Coulter Inc., Foullerton, CA, USA), using an excitation wavelength of 485 nm (20-nm bandwidth) and an emission wavelength of 530 (25-nm bandwidth). The principle of the methods resides to the deacetylation of the dye by intracellular esterases to form diaminofluorescin (DAF), which, in turn, is transformed to the fluorescent dye 2′, 7′-diaminofluorescein under the action of NO. This dye remains trapped in the cell and can therefore be measured by flow cytometry. It was reported that DAF, in neutral solutions as the cellular environment, does not react with other oxidized forms of NO, such as NO_2 and NO_3, or other reactive nitrogen or oxygen species.[80] Nevertheless, in order to confirm these results, and to rule out other possible sources of fluorescence, we have considered as NO-related specific fluorescence the difference of the total fluorescent signal and that obtained in the presence 1 mM of the NOS inhibitor L-NAME. In addition, we have used as a blank in all our measurements a mixture of DAF (0.1 μM) and opioids (10^{-8} M), in the absence of cells.

Nitric oxide synthase assay

Nitric oxide synthase (NOS) activity was assayed, by the transformation of radioactive arginine to citrulline.[81,82] Briefly, cells were detached from dishes by Thrypsin-EDTA, washed with phosphate-buffered saline (PBS), harvested in PBS-1 mM EDTA, and homogenized with repeated pipetting with 250 μl homogenization buffer (250 mM Tris-EDTA, 10 mM EDTA, 10 mM EGTA). Nuclei and unbroken cells were separated by centrifugation at 12 000 $\times$ *g* for 15 min, and discarded, while the supernatant was used for the assay of NOS. The concentration of proteins was adjusted at 10 μg/ml. A reaction mixture (sufficient for 10 data points) is prepared, with 250 μl

of 50 mM Tris -HCl pH 7.4 containing 6 μM tetrahydrobiopterin, 2 μM flavin adenine dinucleotide and 2 μM flavin adenine mononucleotide, 50 μl of 10 mM NADPH, 10 μl [^{3}H] Arginine (Amersham, Buckinghamshire, UK), 50 μl of 6 mM $CaCl_2$ and 40 μl distilled water. Fourty μl of this reaction mixture were mixed with 10 μl of the protein extract and incubated for 1 h at 37°C. During this incubation time [^{3}H]-arginine is converted by NOS to [^{3}H]-citrulline. The reaction was stopped with 400 μl of ice-cold 50 mM HEPES (pH 5.5)-5 mM EDTA. Non-reacted arginine was eliminated by resin absorption (AG 50Wx*, BioRad Laboratories, Hercules, CA, USA). The eluate was mixed with scintillation fluid (SigmaFluor, Sigma, St Louis, MO, USA) and the radioactivity was measured in a liquid scintillation counter (Tricarb, Packard, Instrument Co., Meriden, CT, USA), with 60% efficiency for tritium. For the detection of calcium-independent NOS isoforms, $CaCl_2$ was replaced by water. In order to assay the effect of FAD on the enzyme activity, different concentrations of FAD, ranging from 10^{-7}–10^{-4} M, were introduced in the reaction mixture, in the presence or the absence of 10^{-8} M EKC.

Calculations

All calculations were performed using the Origin v 5.0 microcomputer program (Northampton, MA, USA). Statistical analysis (ANOVA, Student's *t*-test), were made by the use of Systat v 9.0 (SPSS Science, Chicago IL, USA) microcomputer program.

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