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Effect of Yohimbine on the Development of Morphine Dependence in the Rat: Lack of Involvement of Cortical β-adrenoceptor Modifications

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AMBROSIO, E., V. IGLESIAS, C. GARCÍA–LECUMBERRI, L. ORENSANZ AND L. F. ALGUACIL. *Effect of yohimbine on the development of morphine dependence in the rat: lack of involvement of cortical β-adrenoceptor modifications.* PHARMACOL BIOCHEM BEHAV **56**(3) 487–491, 1997.—The alpha-2 receptor antagonist yohimbine has been previously shown to prevent the development of morphine dependence in a rat behavioral model. This study was directed to clarify the mechanism of this interaction, which is presently unknown. Since upregulation of cortical β-adrenoceptors has been suggested to be involved in morphine withdrawal, we have tested the possible correlation between receptor density and withdrawal behaviors in the presence of yohimbine. Sprague–Dawley male rats received a s.c. suspension of morphine (300 mg/kg) or the vehicle. Animals received saline or yohimbine (4 mg/kg, IP) 24, 28, 48 and 52 h after morphine and finally naloxone (1 mg/kg i.p) at 72 h; the subsequent signs of withdrawal (mainly wet-dog shakes and escape attempts) were recorded and the cerebral cortex dissected to study [^{3}H]-CGP 12177 binding. Morphine-treated animals displayed a marked withdrawal behavior together with β-adrenoceptor upregulation; nevertheless, these effects were not correlated. As expected, yohimbine prevented morphine withdrawal behavior but did not reverse the β-adrenoceptor upregulation induced by the opiate. These results confirm previous evidence against the involvement of β-adrenoceptor upregulation on morphine withdrawal behaviors and also permit to discard β-adrenoceptor regulation as the neurochemical basis of the antiwithdrawal effect of yohimbine. The possible contribution of some other neurochemical effects of yohimbine are discussed to explain the inhibition of morphine dependence by that drug.

Morphine withdrawal Morphine dependence β-adrenoceptor Yohimbine

THE involvement of central aminergic mechanisms in the pharmacological actions of opioids has been widely studied. When the effects of chronic opioid stimulation are considered, biological adaptations of opioid mechanisms emerge together with significant alterations of noradrenergic function; these variations consist of changes in noradrenaline utilization (18) as well as shifts in the number of different subtypes of noradrenergic receptors (20,26). Consequently, chronic opioids modify the sensitivity to noradrenergic drugs as shown by the analgesic cross-tolerance between opioid and noradrenergic agonists (15,27). Noradrenergic agents, by turn, interfere with the adaptations to prolonged opioid stimulation, as evidenced by the prevention of opioid tolerance provoked by noradrenergic receptor blockade (16). The signs of opioid withdrawal are also modified by drugs active on noradrenergic pathways, which has served as the basis for therapeutic actuations like the management of opioid abstinence with alpha-2 adrenergic agonists (11). Taken together, all these findings support the idea that modifications of noradrenergic function are among the most critical mechanisms involved both in the develop-

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ment and expression of opioid tolerance and dependence, as reviewed elsewhere (3).

Trying to explain how noradrenaline is engaged to opioid tolerance and dependence, Llorens et al. (20) established a hypothesis which related both phenomena with the noradrenergic function in the cerebral cortex. Their idea was that adaptational changes of cortical, postsynaptic β-adrenoceptors could be the final step in this interaction. They found that chronic morphine, by reducing noradrenergic release, leads to a compensatory increase of β-adrenoceptors; the postsynaptic hypersensitivity thus developed opposes the acute effect of morphine and then tolerance arises. If morphine is discontinued, noradrenaline release return to normal and provokes an exaggerated postsynaptic response due to β-adrenoceptor hypersensitivity: this fact could be the biochemical basis of opioid withdrawal signs. An increased number of cortical β-adrenoceptors has been also observed by other authors in the cerebral cortex of morphine-dependent rats (22,28). The possible correlation between this effect and the appearance of behavioral withdrawal signs has not been fully studied, although some evidence exists that this correlation is lacking (28).

It has been observed that pretreatment with the alpha-2 antagonist yohimbine is able to prevent the development of morphine dependence in vivo (12,29) and morphine tolerance in vitro (1). The mechanisms of this action have not been yet elucidated, but one could expect them to be closely related to noradrenergic function due to the pharmacological profile of yohimbine. Since one of the putative mechanisms could be the prevention of morphine-induced β-adrenoceptor upregulation, we have examined this possibility with the additional purpose of indirectly checking the correlation between these behavioral and biochemical markers of opioid dependence. A preliminary report of this study has been previously presented (13).

MATERIALS AND METHODS

Experimental Groups

Adult male Sprague–Dawley rats (IFFA CREDO, France) weighing 250–300 g were used for the experiments. They were housed in group cages under standard conditions (12-h light-dark cycle) with free access to food and water. At the beginning of the experiments they were isolated and half of them treated subcutaneously (3 ml/kg) with a suspension containing 1 g/ 10 ml morphine base (Spanish Health Authorities, Madrid, Spain); this treatment has been proven to induce opioid dependence within 72 h both in mice and rats (2,12). The remaining animals were administered with the vehicle of the suspension (50% saline, 42.5% paraffin oil and 7.5% Arlacel A). Bedding material was removed from the cages for 24 h to prevent deaths caused by dust inhalation in respiratory-depressed animals.

The rats were treated IP with saline or yohimbine (4 mg/kg; RBI, Natick, MA, USA) 24, 28, 48 and 52 h after the injection of morphine suspension; under this dose regime, yohimbine inhited the development of morphine dependence in previous studies (12). The experimental groups were then the following:

Placebo suspension + saline (CONTROL)
Placebo suspension + yohimbine (YOH)
Morphine suspension + saline (MOR)
Morphine suspension + yohimbine (MOR + YOH)

Morphine Withdrawal Behavior

Naloxone (1 mg/kg, IP) was injected to all the animals 72 h after the administration of the suspension or vehicle. The rats were immediately confined in plexiglass chambers (4) to record withdrawal behaviors during a 10-min observation period; this time interval coincided with the peak of the abstinent response as shown by previous studies (12). It must be pointed out that the lapse between the last injection of yohimbine and the administration of naloxone in the MOR + YOH treatment group was 20 h; this fact permits to affirm that in the present situation the possible modifications of opioid withdrawal by yohimbine would be mainly related to an interference with the development of dependence, rather than attributable to an acute effect of yohimbine on withdrawal expression.

The frequencies of wet-dog shakes (WDS) and escape attempts (EA) from the chamber were determined during the 10-min observation period. Other withdrawal signs (diarrhea, syalorrhea, rinorrhea, chromodachriorrhea, tachypnea, ejaculation, ptosis, teeth chattering, paw tremor, vocalization on touch and aggressive behavior on handling) were scored 1 if present and 0 if absent within this period; the sum of these values was termed global withdrawal score (GWS).

β-adrenoceptor Binding

Just after behavioral testing, the rats were decapitated, the cerebral cortex gently dissected into ice-cold saline, frozen on dry ice and stored at −80°C until the day of experiment. Binding parameters of cortical β-adrenoceptors were obtained from saturation experiments according essentially to Riva and Creese (25). Briefly, rat cerebral cortices were homogenized in 50 volumes of cold 50 mM Tris-HCl (pH 7.7 at 25°C) and centrifuged three times at 35000 *g* for 20 min. The final membrane pellet was resuspended in Tris-incubation buffer. The experiments were performed in borosilicate disposable tubes at 4°C. The final assay volume of 2 ml consisted of 100 μl of [^{3}H]-CGP 12177 (specific activity 51–53 Ci/mmol, Amersham International plc, Buckinghamshire, England), 100 μl of 10 fM alprenolol (Sigma) to measure the nonspecific binding or incubation buffer (50 mM Tris-HCl, pH 7.7 at 25°C), 800 μl of incubation buffer and 1 ml of membrane suspension (5.5 mg of wet weight/tube) added at the start of incubation.

The test tubes were incubated in triplicate for 35 min (5 min to temper and 30 min of incubation) at 37 °C, then immediately filtered under vacuum through Whatman GF/C filters, and washed three times with 8 ml of ice-cold Tris buffer, using a Brandel cell harvester (Brandel, Gaithersburg, MD, USA). Filters were placed in plastic scintillation vials and 8 ml of Aquasol (NEN, Boston, MA, USA) were added. Plastic scintillation vials were shaken for 60 min and then placed into a refrigerator for 120 min. Radiactivity into plastic scintillation vials was counted using a Beckman LS 5000 TD scintillation counter at an average efficiency of 46%. Saturation experiments of [^{3}H]-CGP 12177 were carried out at 8 concentrations in a range of 0.025 to 2.0 nM. At Kd concentration, the specific binding was higher than 90% in all the experiments. Saturation experiments were analyzed by using the INPLOT curve-fitting program (GraphPAD Software, San Diego, CA, USA). Protein determinations were performed by the method of Bradford (5). Results are expressed as femtomoles of [^{3}H]-CGP 12177 specifically bound per milligram of protein. Values given in the text are means ± SEM of the number of experiments shown in parentheses.

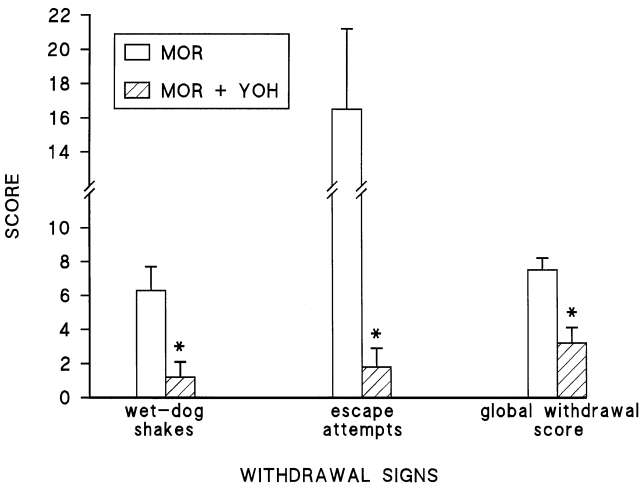

FIG. 1. Withdrawal signs elicited by naloxone administration (1 mg/kg IP) to animals pretreated 72 h before with a suspension of morphine (300 mg/kg s.c.). The scores reflect either the frequency of wet-dog shakes and escape attempts along the 10-min observation period or the total number of withdrawal signs included in the global withdrawal score which were present in the abstinent rats. The animals received yohimbine (4 mg/kg IP, $n = 6$; group MOR + YOH) or saline ($n = 6$; group MOR) 24, 28, 48 and 52 h after the administration of the morphine suspension. $^*P < 0.05$ vs. MOR.

Statistics

Withdrawal behaviors displayed by rats treated with morphine and morphine plus yohimbine were compared with Student *t*-test. Binding parameters of β-adrenoceptors were analyzed by one-way ANOVA followed by least significant differences post-hoc test. The analysis of correlations between behavioral and biochemical parameters was performed by calculation of Spearman coefficients. A probability of 0.05 or less was considered statistically significant.

RESULTS

The administration of naloxone to morphine-treated rats precipitated the appearance of typical opioid withdrawal signs along the 10 min-observation period; these signs were still present at the end of behavioral testing, when brain cortices were dissected for binding sudies. Among the counted signs, vigorous wet-dog shakes were prominent and presented a low dispersion, whilst the frequency of escape attempts was much more variable (Fig. 1). The marked presence of the remaining signs was reflected by the high global score obtained (Fig. 1).

The repeated administration of yohimbine provoked a significant decrease of behavioral withdrawal in morphine-treated rats upon naloxone challenge; this finding applied both to counted signs and the global score, which reflected only minimal manifestations of morphine dependence (Fig. 1).

High affinity binding of [^{3}H]-CGP 12177 to the cortical neural membranes was saturable and reversible by alprenolol 10^{-5} M. Computerized analysis of saturation binding experiments indicated a single population of binding sites for [^{3}H]-CGP 12177 with an apparent Kd of 0.22 ± 0.03 nM (controls, $n = 6$). The fits obtained with one ligand/one site model were significantly better than those obtained with one ligand/two binding sites model. The maximum number of specific binding sites (Bmax) for [^{3}H]-CGP 12177, as determined by the curve fitting program INPLOT, was 175 ± 9 fmol/mg protein in the cerebral cortices of control animals (Table 1). The analysis of variance revealed significant differences among treatment groups concerning β-adrenoceptor density [$F(3, 20) = 4.3$, $P < 0.02$]. Morphine-dependent rats showed a 27% increase of cortical β-adrenoceptor density with respect to controls; however, this significant increase in density of [^{3}H]-CGP 12177 binding sites occurred without a significant change in the affinity of the radioligand (Table 1). Spearman coefficients did not indicate significant correlations between any of the behavioral parameters of withdrawal and β-adrenoceptor density or receptor affinity (Table 2).

TABLE 1

BINDING PARAMETERS FOR [^{3}H]-CGP 12177 TO β-ADRENOCEPTORS IN RAT CEREBRAL CORTEX MEMBRANES

Group	*n*	Kd (nM)	Bmax (fmol/mg prot)
CONTROL	6	0.22 ± 0.03	175 ± 9
YOH	6	0.23 ± 0.02	190 ± 10
MOR	6	0.39 ± 0.09	222 ± 12*
MOR + YOH	6	0.35 ± 0.07	207 ± 5*

Saturation experiments were performed in a concentration range of 0.025–2.0 nM of [^{3}H]-CGP 12177. Nonspecific binding was defined by 10 μM alprenolol. Binding parameters were obtained from the curve-fitting program INPLOT. The values of the table (mean ± SEM) are the result of 6 independent experiments done in triplicate (see methods for details). $^*P < 0.05$ vs. CONTROL.

Despite the fact that yohimbine inhibited the development of morphine dependence, this drug failed to affect β-adrenoceptor upregulation; therefore, the number of receptors in the MOR + YOH group remained significantly higher than that of controls (Table 1). Again, Spearman coefficients did not reveal any correlation between withdrawal behaviors and biochemical variables in this group. Even if all rats treated with morphine were considered together (regardless they were treated with yohimbine or saline), such correlations were absent (Table 2).

DISCUSSION

The results obtained indicate that chronic morphine treatment increases cortical β-adrenoceptor density in the rat cerebral cortex but do not modify Kd values. These findings are similar to those previously reported regarding both binding parameters, even though we used a more specific radioligand to measure cerebral β-adrenoceptors; thus, we found a 2.5-fold increase of fmol/mg protein in the control group compared to the mean value obtained by other authors who used [^{3}H]-dihydroalprenolol as radioligand (22). However, the morphine-induced changes of β-adrenoceptor binding parameters were qualitatively similar in the latter studies.

The emergence of behavioral signs of opioid withdrawal seemed to be independent of β-adrenoceptor upregulation, a conclusion that is supported by two findings. First, there was no correlation between β-adrenoceptor density and the abstinence signs exhibited by morphine-dependent animals; to accomplish this finding, we performed the experiments without pooling cerebral cortices, which enabled us to study behavioral withdrawal and binding parameters in parallel for each individual subject. Second, yohimbine-treated animals displayed

TABLE 2

ANALYSIS OF CORRELATIONS BETWEEN WITHDRAWAL SCORES AND β-ADRENOCEPTOR BINDING PARAMETERS

Group	*n*	Kd/WDS	Kd/EA	Kd/GWS	Bmax/WDS	Bmax/EA	Bmax/GWS
MOR	6	0.71	0.29	0.79	0.60	0.11	0.71
		($p = 0.11$)	($p = 0.58$)	($p = 0.06$)	($p = 0.21$)	($p = 0.83$)	($p = 0.12$)
MOR + YOH	6	0.43	0.27	0.09	0.09	0.58	0.09
		($p = 0.39$)	($p = 0.60$)	($p = 0.87$)	($p = 0.86$)	($p = 0.23$)	($p = 0.87$)
MOR, MOR + YOH	12	0.28	0.02	0.25	0.02	0.13	0.37
		($p = 0.38$)	($p = 0.96$)	($p = 0.43$)	($p = 0.96$)	($p = 0.96$)	($p = 0.24$)

Analysis of correlations between morphine wihtdrawal behaviors and binding parameters of cortical β-adrenoceptors was performed by calculation of Spearman R coefficients. WDS: wet-dog shakes. EA: escape attempts. GWS: global withdrawal score.

a negligible withdrawal behavior but similar β-adrenoceptor densities when compared to dependent rats not treated with the drug. Consequently, the adaptational meaning of β-adrenoceptor changes remains unknown but does not seem to be directly related to the emergence of typical signs of opioid withdrawal; it must be pointed out, however, that the evidence obtained in this study against the involvement of β-adrenoceptor regulation in morphine dependence is rather indirect, and therefore other experiments directly designed to address the issue are necessary.

Swann et al. (28) also claimed a lack of correlation between β-adrenoceptor density and morphine withdrawal behavior in the rat; however, they did not provide numeric data on the subject and the method used hardly permits to draw this conclusion. In fact, the tissues were prepared for binding in that study 40 min after completion of behavioral examinations, when the degree of precipitated abstinence could be expected to become low. The time we have chosen to obtain the samples matches the peak of withdrawal signs, therefore enabling an accurate study of the correlation between behavior and neurochemistry.

Trying to explain yohimbine antagonism of opioid dependence, it has been suggested that an interaction of alpha-2 and opioid mechanisms in the locus coeruleus (LC) could be involved. The activity of LC neurons seems to be critical for opioid withdrawal (17,21), which has been related with an increased noradrenaline turnover in brain areas innervated by the LC (23). Taylor et al. (29) proposed that an increased neuronal activity induced by yohimbine could oppose morphine inhibition of LC noradrenergic neurons, thus preventing the development of LC hyperactivity of opioid withdrawal; the authors proposed this mechanism to explain yohimbine reversal of morphine withdrawal behaviors. This hypothesis has not been tested and also has some drawbacks. First, alpha-2 adrenoceptor blockade has been shown to potentiate, rather than inhibit, the actions of mu-receptor agonists in the rat LC neurons (14). Second, some of the studies contributing to the idea that noradrenergic LC activity is related with opioid withdrawal behavior include significant shifts between behavioral and biochemical determinations: thus, naloxone-induced increase of noradrenaline turnover in brain areas innervated by the LC has been studied at time intervals too long to observe concomitant withdrawal reactions (8,19,31). This fact makes difficult to establish appropriate correlations. Third, there are several findings which do not support a parallelism of opioid withdrawal reactions and noradrenergic hyperfunction in brain areas innervated by the LC such as the rat brain cortex. Britton et al. (6) have shown that destruction of the dorsal noradrenergic bundle does not affect neither opioid withdrawal behaviors nor the inhibition of abstinence provided by clonidine. Furthermore, both clonidine and NMDA antagonists attenuate the behavioral signs of withdrawal without blocking withdrawal-induced increase of noradrenaline turnover in the rat brain cortex (9,24). The results that we report here are in the same direction, since we have observed that yohimbine prevents opioid dependence development but does not reverse the increase of β-adrenoceptors in the cortex of abstinent rats. According to the findings commented, the parallelism between yohimbine reversal of opioid dependence and inhibition of noradrenergic hyperactivity can be seriously questioned, at least regarding the ascending projections of LC neurons.

Despite the exact neuronal pathways involved in the effect of yohimbine, there is enough evidence to expect that the action of this drug on opioid dependence is related with a modulation of central alpha-2 adrenoceptors. Smith et al. (26) have shown that chronic morphine treatment decreases the number of alpha-2 adrenoceptors in several areas of the rat brain (hypothalamus, amygdala, brainstem, parietal cortex and caudate nucleus), an effect with could account for the lower pharmacological effects of alpha-2 agonists after chronic opioid administration (15,27). Other authors have also found a significant correlation of withdrawal severity and alpha-2 adrenoceptor density in brain areas of morphine-withdrawn rats, mainly the hypothalamus (10). These findings support the idea that alpha-2 adrenoceptor modulation is essential for the development and expression of opioid tolerance and dependence, thus rising the possibility that yohimbine could antagonize morphine dependence by interferring with opioid-induced adaptations of alpha-2 adrenoceptors. Other possible mechanisms involved could be related to the serotoninergic profile of yohimbine (30), since 5-hydroxytriptamine pathways have also been found to be involved in opioid dependence (7).

In summary, the main conclusion of this study is the lack of involvement of β-adrenoceptor modifications in morphine dependence prevention by yohimbine, which is in agreement with the idea that β-adrenoceptor regulation is of secondary importance in morphine dependence. Therefore, other pharmacological mechanisms such as those commented on before should be tested to explain the interaction observed.

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